

1 **Title**

2 Immunomodulatory effect of linezolid on MRSA supernatant-induced MUC5AC
3 overexpression in human airway epithelial cells

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5 **Running title**

6 Effect of linezolid on MUC5AC overexpression

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1 **Abstract**

2 Linezolid is the first member of the oxazolidinones and is active against drug-resistant
3 gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA).
4 Additionally, linezolid showed an immunomodulatory effect, such as an inhibition of the
5 inflammatory cytokines production. In this study, we examined the effect of linezolid on
6 MRSA-induced MUC5AC overexpression in airway epithelial cells. In this study, an MRSA
7 supernatant was used to avoid the direct effect of linezolid on MRSA. MUC5AC protein
8 production was significantly increased with a 40-fold dilution of MRSA supernatant. At the
9 messenger RNA (mRNA) level, MUC5AC gene expression was significantly increased at 6
10 and 9 hours after stimulation. In an inhibition study, linezolid significantly reduced
11 MRSA-induced MUC5AC protein and mRNA overexpression at concentrations of 5 and 20
12 µg/mL, which were same as the trough and peak concentrations in human epithelial lining
13 fluid. In an analysis of cell signaling, among the mitogen-activated protein kinase inhibitors,
14 only the extracellular signal-regulated protein kinase (ERK1/2) inhibitor reduced the
15 MUC5AC protein production to the same level as that of the control; on Western blot
16 analysis, only ERK1/2 was phosphorylated by the MRSA supernatant. In addition, the
17 ERK1/2 phosphorylation was inhibited by linezolid. MUC5AC as well as MUC5B is the
18 major barrier that traps inhaled microbial organisms, particulates, and foreign irritants.
19 However, in patients with chronic respiratory diseases, pathogen-induced MUC5AC
20 overexpression causes many problems, and control of the overexpression is important. Thus,
21 this study revealed that linezolid showed the direct immunomodulatory effect in airway
22 epithelial cells.

23

24 **Key words:** linezolid, MRSA, mucin, MUC5AC, airway epithelial cell

1 ***Introduction***

2 Linezolid is the first member of the oxazolidinones, a new class of antimicrobial
3 agents. It acts by inhibiting the initiation of bacterial protein biosynthesis and is active against
4 drug-resistant gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus*
5 (MRSA) and vancomycin-resistant enterococci, and it is one of the recommended antibiotics
6 for patients with MRSA pneumonia in the guidelines for the management of
7 hospital-acquired pneumonia (1) Linezolid also reduced a production of bacterial toxin (2-4).
8 In addition, linezolid has been shown the direct immunomodulatory effects on inflammatory
9 cells to inhibit the production of inflammatory cytokines such as interleukin (IL)-1 beta, IL-6,
10 IL-8, and tumor necrosis factor alpha (5-7). The direct immunomodulatory effects of
11 antimicrobial agents have been well elucidated with macrolides. Macrolides affect host cells
12 by down-regulating inflammation, reducing production of reactive oxygen, inhibiting
13 neutrophil activation and mobilization, accelerating neutrophil apoptosis, and blocking
14 activation of nuclear transcription factors (8). Then, one of the immunomodulatory effects of
15 the macrolides on human airway epithelial cells is the inhibition of pathogen-induced
16 MUC5AC overexpression (9-14).

17 MUC5AC and MUC5B are gel-forming mucins that are strongly expressed in the
18 lung (15). The Mucin is the major barrier that traps inhaled microbial organisms, particulates,
19 and foreign irritants in airway epithelium. In particular, MUC5B may play important roles in
20 an airway defense, because loss of *Muc5b* gene reduced survival by causing bacterial
21 infection in a murine model (16). In contrast,, MUC5AC overexpression was observed in
22 patients with chronic respiratory diseases such as, diffuse panbronchiolitis and asthma (17),
23 (18). The overexpression was also observed in patients with ventilator-associated pneumonia
24 (VAP) (19). Since mucin overexpression causes airway obstruction, atelectasis, reduction of

1 oxygenation, and reduction of antibiotic permeability, inhibition of MUC5AC overexpression
2 seemed to be useful.

3 MUC5AC overexpression is induced by various pathogens, such as *Pseudomonas*
4 *aeruginosa*, *Escherichia coli*, *Haemophilus influenzae*, *Fusobacterium nucleatum*,
5 *Chlamydophila pneumoniae*, and *Legionella pneumophila* (9-14, 20, 21). MUC5AC
6 overexpression also induced by peptidoglycan from *S. aureus* was reported (22). Although *S.*
7 *aureus*, especially MRSA, is an important pathogen in patients with chronic respiratory
8 diseases and VAP, there has been no study that has reported the effect of anti-MRSA
9 antibiotics on MUC5AC overexpression. In addition, there was no report about direct
10 immunomodulatory effect of linezolid on human airway epithelial cells. The purpose of this
11 study was to reveal the direct immunomodulatory effect of linezolid by inhibition of
12 MRSA-induced MUC5AC overexpression.

13

1 **Materials and Methods**

2 **Materials**

3 Linezolid (Pfizer, Groton, CT, USA) was dissolved in distilled water. The mouse
4 anti-MUC5AC monoclonal antibody (clone 45M1) was purchased from MONOSAN (Uden,
5 the Netherlands). The goat anti-mouse horseradish peroxidase–conjugated secondary
6 antibody was obtained from Bio-Rad (Hercules, CA, USA). The extracellular signal–
7 regulated protein kinase (ERK) inhibitor (U0126) was purchased from Promega (Madison,
8 WI, USA). The p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580) and
9 c-Jun N-terminal kinase (JNK) inhibitor II were purchased from Calbiochem (San Diego, CA,
10 USA). The anti-ERK, anti–phospho-ERK1/2, anti-p38 MAPK, anti–phospho-p38 MAPK,
11 anti-JNK, anti–phospho-JNK, anti-I-kappa B alpha (IkBa), and anti-phospho-IkBa antibodies
12 were purchased from Cell Signaling Technology (Danvers, MA, USA). The ERK1/2 control
13 cell extracts, p38 MAPK control cell extracts, JNK control cell extracts, and NF-kappa B
14 (NF-kB) control cell extracts also purchased from Cell Signaling Technology (Danvers, MA,
15 USA).

16

17 **Bacterial strain**

18 The MRSA strain used in this study was NUMR101, which was a clinical isolate
19 obtained from the blood sample of a patient at the Nagasaki University Hospital (23). The
20 bacteria were stored at –80°C in a Microbank bead-based preservation system (Pro-Lab
21 Diagnostics, Ontario, CA, USA) until use. The genetic characteristic of NUMR101 was
22 identified by real-time polymerase chain reaction (PCR) using the same method as described
23 in a previous report (24); the staphylococcal cassette chromosome *mec* (SCC*mec*) was type II,
24 and the strain carried virulence genes such as *sec* and *tsst* but did not carry *etb* and *pvl* genes.

25

1 ***Preparation of MRSA supernatant***

2 To avoid the direct effect of linezolid on MRSA, we used an MRSA supernatant. The
3 MRSA supernatant was prepared using a method modified described in our previous report
4 (12). The NUMR101 strain was cultured on Mueller Hinton II agar (Becton, Dickinson and
5 Company, Sparks, MD, USA) at 37°C with 5% CO₂ in fully humidified air. After overnight
6 incubation, the NUMR101 strain was harvested and incubated in 10 mL of Luria-Bertani
7 (LB) broth (MO BIO Laboratories, Carlsbad, CA, USA) at 37°C with shaking at 250 rpm for
8 72 hours. After incubation, the bacteria were centrifuged at 10,000g for 10 minutes at 4°C,
9 and the supernatant was filtered using a 0.22-µm Millex-GP filter (Millipore Corporation,
10 Billerica, MA, USA). The MRSA supernatant was stored at –80°C until use.

11

12 ***Cell Culture***

13 The NCI-H292 (human airway epithelial) cell line was cultured in RPMI 1640
14 medium supplemented with 10% fetal bovine serum (FBS). The cells were grown at 37°C
15 with 5% CO₂ in fully humidified air. For the MUC5AC production studies, the cells were
16 cultured in RPMI 1640 medium supplemented without FBS for 24 hours. After serum
17 starvation, the cells were stimulated with the MRSA supernatant for enzyme-linked
18 immunosorbent assay (ELISA) or reverse-transcription (RT)-PCR. The cells were treated
19 with linezolid simultaneously with stimulation using the MRSA supernatant in the inhibition
20 studies. In reference to the previous study, which reported the trough and peak concentrations
21 of linezolid in the epithelial lining fluid (ELF) of patients with VAP (25), linezolid was used
22 at 2 concentrations (5 and 20 µg/mL). Since there is possibility that LB broth induce
23 MUC5AC overexpression, the controls were incubated with the same amount of LB broth as
24 the MRSA supernatant. The cells were also pretreated with signal transduction inhibitors at

1 concentrations of 10 μ M for 30 minutes before stimulation and the cells in the controls were
2 incubated with only the medium and the same amount of dimethyl sulfoxide as the inhibitors.

3 ***ELISA***

4 The MUC5AC protein level was measured using an enzyme-linked immunosorbent
5 assay (ELISA) (10). The cells were cultured in 24-well plates, and after stimulation with the
6 MRSA extracts for 24 hours, the culture medium was collected as the cell supernatant. The
7 supernatant was then incubated at 40°C in a 96-well plate until dry. After incubation, the
8 plates were blocked with 2% FBS for 1 hour at room temperature and then incubated with the
9 anti-MUC5AC antibody diluted in phosphate-buffered saline containing 0.05% Tween 20 for
10 1 hour. Horseradish peroxidase–conjugated anti-goat immunoglobulin G was then dispensed
11 into each well. After 1 hour, the color developed using a 3,3',5,5'-tetramethylbenzidine
12 peroxidase solution, and the reaction was stopped by the addition of 1-N H₂SO₄. The
13 absorbance was measured at 450 nm.

14

15 ***RNA extraction and real-time quantitative RT-PCR***

16 Total RNA was extracted from the NCI-H292 cells cultured in 6-well plates using
17 QuickGene Mini-80 and QuickGene RNA cultured cell kits (Kurabo Industries, Osaka,
18 Japan) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse
19 transcribed into complementary DNA using oligo(dT) primers and SuperScript III reverse
20 transcriptase (Invitrogen, Carlsbad, CA, USA) and then treated with RNase H. To quantify
21 the expression of the MUC5AC gene, PCR primers and TaqMan probes were designed and
22 used as reported previously (forward primer, 5'-CAGCCACGTCCCCTTCAATA-3'; reverse
23 primer, 5'-ACCGCATTTGGGCATCC-3'; TaqMan probe,
24 5'-6-FAM-CCACCTCCGAGCCCGTCACTGAG-TAMRA-3') (11). The MUC5AC was
25 amplified for 40 cycles (15 s at 95°C and 30 s at 60°C) using a LightCycler system (Roche

1 Diagnostics, Basel, Switzerland). To normalize the MUC5AC expression, human
2 porphobilinogen deaminase was also measured using specific PCR primers and TaqMan
3 probes (forward primer, 5'-AACCAGCTCCCTGCGAAGA-3'; reverse primer,
4 5'-CCAGGATGATGGCACTGAACT-3'; TaqMan probe,
5 5'-FAM-ACTCCTGAACTCCAGATGCGGGAACT-TAMRA-3') (26).

6

7 ***Western blot analysis***

8 The cells were harvested at 0, 30, 60, and 90 min after MRSA stimulation and then
9 washed and homogenized at 4°C in lysis buffer (0.1% sodium dodecyl sulfate, 1% Igepal
10 CA-630, and 0.5% sodium deoxycholate). The cell lysates (20–50 µg) were resolved by
11 electrophoresis on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride
12 membrane. After blocking the membrane using 10% FBS and 0.1% Tween 20 in
13 Tris-buffered saline for 1 hour at room temperature, the blots were hybridized overnight at
14 4°C with primary antibodies. Hybridization with secondary antibodies was performed and the
15 immunocomplexes were visualized using an ECL Western blotting detection reagent (GE
16 Healthcare, Chalfont St. Giles, United Kingdom).

17

18 ***Statistical analysis***

19 A statistical software package (StatMate IV for Windows; ATMS Co., Ltd., Tokyo,
20 Japan) was used for all statistical comparisons. All data are expressed as mean and standard
21 deviation (SD). One-way analysis of variance was used to determine the statistically
22 significant differences between the groups. The Tukey test was used for pairwise comparison.
23 All tests of significance were 2 tailed. The alpha level for denoting statistical significance
24 was set at < 0.05.

25

1 **Results**

2 ***MRSA supernatant-induced MUC5AC protein production and gene overexpression***

3 Of the several concentrations of MRSA supernatant, only the 1/40-fold dilute solution
4 of MRSA supernatant significantly increased the protein level of MUC5AC after 24 hours of
5 stimulation ($334.1 \pm 150.3\%$ greater than control, $P < 0.05$ vs. control) (Fig. 1A). To make
6 sure that 72 hours incubation in the preparation of MURSA supernatant was most increased
7 protein level of MUC5AC, 6 hours (mid-log phase) and 72 hours incubation were compared.
8 The protein level of MUC5AC was 6.6 times higher in MRSA supernatant obtained from 72
9 hours incubation than that from 6 hours incubation. Based on this result, the 1/40-fold dilute
10 solution of MRSA supernatant obtained from 72 hours incubation was used for further
11 studies. Subsequently, the influence of the MRSA supernatant on MUC5AC messenger RNA
12 (mRNA) expression was evaluated. The cells were stimulated for 3, 6, and 9 hours, and the
13 mRNA level of MUC5AC was assayed by RT-PCR. The MRSA supernatant significantly
14 increased the mRNA level at 6 and 9 hours to approximately 4 times than that of the control
15 ($P < 0.01$) (Fig. 1B).

16

17 ***Linezolid inhibited MRSA supernatant-induced MUC5AC protein production and gene*** 18 ***expression***

19 We then examined the effect of linezolid on MRSA supernatant-induced MUC5AC
20 protein production and gene expression. Compared with the stimulation group, linezolid
21 significantly reduced MRSA supernatant-induced MUC5AC protein production at
22 concentrations of 5 and 20 $\mu\text{g/mL}$ ($373.6 \pm 77.8\%$ greater than control vs. $14.0 \pm 68.8\%$
23 greater than control [$P < 0.001$] and $373.6 \pm 77.8\%$ greater than control vs. $12.8 \pm 13.8\%$
24 greater than control [$P < 0.001$], respectively). However, this did not occur in a
25 dose-dependent manner. At the mRNA level, linezolid significantly reduced MRSA

1 supernatant-induced MUC5AC mRNA expression at both concentrations compared with the
2 stimulation group (16.1 ± 8.2 vs. 6.9 ± 1.8 [$P < 0.001$] and 16.1 ± 8.2 vs. 8.6 ± 3.2 [$P <$
3 0.001], respectively) (Fig. 2B).

4

5 ***Effect of MAPK inhibitors on MRSA supernatant-induced MUC5AC protein production***

6 To reveal MRSA-induced activation of cell signaling, we examined the MAPK
7 pathway that concerned MUC5AC protein production. The cells were treated with or without
8 MAPK inhibitors (ERK inhibitor, p38 MAPK inhibitor, or JNK inhibitor), and the protein
9 level of MUC5AC was evaluated by ELISA. Compared with the stimulation group, the ERK
10 inhibitor significantly reduced MRSA-induced protein production to the same protein level as
11 in the control at concentration of $10\mu\text{M}$ ($88.7 \pm 22.9\%$ greater than control vs. $3.5 \pm 37.1\%$
12 greater than control, $P < 0.01$) (Fig. 3). The effect of ERK inhibitor was observed at
13 concentration of $2\mu\text{M}$, but not at $0.4\mu\text{M}$ ($141.9 \pm 11.2\%$ greater than control [stimulation
14 group] vs. $-1.2 \pm 5.3\%$ greater than control [$P < 0.01$] and $159.3 \pm 4.0\%$ greater than control
15 [not significant difference], respectively). In contrast, the p38 MAPK inhibitor and JNK
16 inhibitor did not reduce protein production at concentration of $10\mu\text{M}$ (Fig. 3).

17

18 ***Linezolid inhibited the phosphorylation of ERK in MRSA supernatant-activated***

19 ***NCI-H292 cells***

20 To demonstrate the effect of linezolid on the MAPK pathway, we examined the
21 phosphorylation of MAPKs. As shown in Fig. 4A, the MRSA supernatant increased the
22 phosphorylation of ERK1/2, which was inhibited by linezolid at a concentration of $5\mu\text{g/mL}$
23 (Fig. 4A). In contrast, there was no significant change in the expression of p38 MAPK and
24 JNK. The inhibitory effect of linezolid on the phosphorylation of ERK1/2 was also observed
25 at a concentration of $20\mu\text{g/mL}$ (Fig. 4B). Additionally, we also examined the activation of

1 MAPK pathway at 360 and 540 minutes in which MUC5AC mRNA overexpression was
2 observed. At these times, only phosphorylation of ERK1/2 was observed (Fig. 5A). The anti-
3 phospho-p38 MAPK and anti-phospho-JNK antibodies worked with positive control cell
4 extracts (Fig. 5B). Then, an influence of MRSA supernatant on NF-kB pathway was
5 examined. As shown in Fig. 6, phosphorylation of I κ B α was not observed in the western blot
6 analysis.

7

1 **Discussion**

2 In this study, the MRSA-induced MUC5AC overexpression was inhibited by
3 linezolid. The inhibitory effect on pathogen-induced MUC5AC overexpression has been
4 reported as one of the immunomodulatory effects of macrolides (9-12, 14). MUC5AC as well
5 as MUC5B is gel-forming mucin that is strongly expressed in the lung (15). They are the
6 major barrier that traps inhaled microbial organisms, particulates, and foreign irritants in
7 airway epithelium. In particular, MUC5B may play important roles in an airway defense,
8 because a loss of *Muc5b* gene reduced survival by causing bacterial infection in a murine
9 model (16). In contrast, the survival of mice was unaffected by a loss of *Muc5ac* gene.
10 However, MUC5AC overexpression was observed in the *Muc5b*^{-/-} mice, and it caused
11 abnormal breathing and hypoxemia by impaired mucociliary clearance (16). In patients with
12 chronic respiratory diseases such as, diffuse panbronchiolitis and asthma, MUC5AC
13 overexpression was also observed (17, 18). In these diseases, mucin overexpression would
14 cause airway obstruction, atelectasis, reduction of oxygenation, and reduction of antibiotic
15 permeability. The overexpression is induced by various pathogens, such as *Pseudomonas*
16 *aeruginosa*, *Escherichia coli*, *Haemophilus influenzae*, *Fusobacterium nucleatum*,
17 *Chlamydomphila pneumoniae*, and *Legionella pneumophila* (9-14, 20, 21). Thereby, it is very
18 important to control pathogens-induced MUC5AC overexpression in patients with respiratory
19 infectious diseases, and the inhibition of the overexpression was considered as the
20 immunomodulatory effect of linezolid.

21 The immunomodulatory effect of linezolid was reported in several studies. Some
22 studies have shown that linezolid reduces the level of lipopolysaccharide-induced production
23 of inflammatory cytokines in whole blood (5-7). Additionally, in the previous studies with
24 methicillin-sensitive *S. aureus* and MRSA, linezolid has been shown to have an inhibitory
25 effect on the pathogens-induced production of inflammatory cytokines (2-4). However, in

1 these studies, bacteria were co-cultured with linezolid, and linezolid also reduced the levels
2 of toxin production (2-4). As a result, it was concluded that the inhibitory effect of linezolid
3 on the production of cytokines is associated with a reduction in toxin production. In this study,
4 we used a supernatant of MRSA culture without linezolid to avoid the direct effect of
5 linezolid on MRSA, including the effect on toxin production. Accordingly, our results
6 showed the direct immunomodulatory effect of linezolid on airway epithelial cells.

7 Although *S. aureus* is one of the important pathogens causing respiratory tract
8 infection or pneumonia, there have been few reports regarding the influence of *S. aureus* on
9 MUC5AC production; one study used an *S. aureus* supernatant (20), and another used
10 peptidoglycan from *S. aureus* (22). In this study, we revealed the mechanism of the
11 overexpression. The mechanisms of MUC5AC overexpression in airway epithelial cell lines
12 due to activation of MAPK pathway such as p38 MAPK and ERK1/2 has been reported at
13 various stimulates (27), and the previous study using peptidoglycan from *S. aureus* reported
14 that peptidoglycan-induced MUC5AC expression was activated the ERK1/2 pathway (22).
15 This finding was similar to our results; in this study, only the ERK1/2 inhibitor reduced
16 MUC5AC overexpression at the protein level. In western blot analysis, ERK1/2 was
17 phosphorylated and p38 MAPK and JNK were not phosphorylated. Thus, activation of the
18 ERK1/2 pathway seems to be specific to *S. aureus*-induced MUC5AC overexpression.
19 Furthermore, we showed that the mechanism of the effect of linezolid: linezolid inhibited the
20 phosphorylation of ERK1/2 in western blot analysis. The upper stream factors of ERK1/2,
21 which was affected by MRSA and linezolid, was not investigated in this study, but the
22 findings about the inhibitory effect of linezolid in MAPK pathway could suggest the direct
23 effect of linezolid on airway epithelial cells.

24 There are some limitations in this study. We used only 1 MRSA strain that is a
25 clinical isolate obtained from our hospital, and there is some possibility that the MUC5AC

1 overexpression was strain specific. We did not compare the effect of linezolid with the other
2 anti-MRSA agents, and it was not recognized whether the effect is a specific superior
3 characteristics. However, we think that our results showed a possibility that the
4 immunomodulatory effect of linezolid helps a treatment in the critically ill patients. In this
5 study, linezolid showed the inhibition effect at a trough concentration in the ELF of patients
6 with VAP (25). In patients with VAP, MRSA is the most common pathogen, and the
7 mortality rate is as high as 32.2% (28). Additionally, mucin overexpression was also
8 observed in the patients, and the overexpression contributes to impending mucociliary
9 clearance and favor the colonization of *Pseudomonas aeruginosa* that is also the common
10 pathogen in VAP (19). Consequently, the control of mucin overexpression seemed to be
11 important in such patients, and linezolid might have a potential to inhibit the overexpression.

12 In conclusion, our study showed that the MRSA supernatant-induced MUC5AC
13 expression via activation of the ERK1/2 pathway and that linezolid inhibits MUC5AC
14 overexpression. The inhibition effect was considered as a direct immunomodulatory effect on
15 airway epithelial cells, and the effect has a possibility to help a treatment in patients with
16 VAP caused by MRSA.

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7

1 **Figure legends**

2 **Fig. 1. Influence of the MRSA supernatant on MUC5AC protein production and gene**
3 **expression.**

4 The NCI-H292 cells were stimulated with 1/80-, 1/40-, and 1/20-fold diluted
5 solution of the MRSA supernatant. In the control, cells were stimulated with the same amount
6 of LB broth as the supernatant. (A) After 24 hours of stimulation, the protein levels were
7 measured by ELISA and provided in terms of percentage greater than control (n = 3). Of the
8 3 concentrations, only the 1/40-fold dilute solution significantly increased the protein level of
9 MUC5AC. (B) Cells were stimulated with the 1/40-fold dilute solution of the MRSA
10 supernatant for 3, 6, and 9 hours. The mRNA level of MUC5AC was assayed by RT-PCR.
11 The MRSA supernatant significantly increased MUC5AC mRNA expression at 6 and 9 hours.
12 Results are expressed as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$.

13 ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA,
14 methicillin-resistant *Staphylococcus aureus*; RT-PCR, reverse-transcription polymerase chain
15 reaction

16

17 **Fig. 2. Effect of linezolid on MRSA supernatant-induced MUC5AC protein and gene**
18 **overexpression.**

19 The NCI-H292 cells were treated with linezolid (5 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$). In the
20 control, the cells were treated with culture medium only. (A) After 24 hours of treatment, the
21 protein levels were measured by ELISA and provided in terms of percentage greater than
22 control (n = 3). Linezolid significantly reduced the MRSA supernatant-induced protein
23 production at both concentrations. (B) The cells were treated with linezolid for 6 hours, and
24 the mRNA level of MUC5AC was assayed by RT-PCR. Linezolid inhibited MRSA
25 supernatant-induced MUC5AC mRNA expression at both concentrations. Results are

1 expressed as the mean \pm SD. * P < 0.001 compared with the stimulation group.

2 ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA,
3 methicillin-resistant *Staphylococcus aureus*; RT-PCR, reverse-transcription polymerase chain
4 reaction

5

6 **Fig. 3. Effect of MAPK inhibitors on MUC5AC protein overexpression.**

7 The cells were treated with an ERK inhibitor, p38 MAPK inhibitor, or JNK
8 inhibitor at concentration of 10 μ M. After 24 hours, the protein levels were evaluated by
9 ELISA. Only the ERK inhibitor significantly reduced MRSA-induced MUC5AC protein
10 production compared with the stimulation group. Results are expressed as the mean \pm SD. * P
11 < 0.01 compared with the stimulation group.

12 ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated protein
13 kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA,
14 methicillin-resistant *Staphylococcus aureus*.

15

16 **Fig. 4. Effect of linezolid on the MAPK pathway.**

17 The NCI-H292 cells were treated with or without linezolid for 0, 30, 60, or 90
18 minutes, and the cells were harvested after treatment and evaluated by western blotting. (A)
19 The MRSA supernatant increased only phosphorylation of ERK1/2. The phosphorylation of
20 ERK1/2 was inhibited by linezolid at a concentration of 5 μ g/mL. There were no significant
21 changes in other MAPKs, such as p38 MAPK and JNK. (B) The inhibitory effect of linezolid
22 on the phosphorylation of ERK1/2 was observed at concentrations of 5 μ g/mL and 20 μ g/mL
23 at 60 and 90 minutes. ERK, extracellular signal-regulated protein kinase; JNK, c-Jun
24 N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA, methicillin-resistant
25 *Staphylococcus aureus*.

1

2 Fig. 5. Influence of MRSA supernatant on the MAPK pathway.

3 The NCI-H292 cells were stimulated with MRSA supernatant for 0, 180, 360, or 540
4 minutes, and the cells were harvested after treatment and evaluated by western blotting. (A)
5 The MRSA supernatant increased only phosphorylation of ERK1/2 at 180, 360, and 540
6 minutes. In contrast, there were no significant changes in p38 MAPK and JNK. (B) The anti-
7 phospho-p38 MAPK and anti-phospho-JNK antibodies worked with positive control cell
8 extracts.

9 ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK,
10 mitogen-activated protein kinase; MRSA, methicillin-resistant *Staphylococcus aureus*.

11

12 Fig. 6. Influence of MRSA supernatant on the NF- κ B.

13 The NCI-H292 cells were stimulated with MRSA supernatant, and the cells were
14 harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant did
15 not activate the NF- κ B pathway at 30, 60, and 90 minutes. (B) The MRSA supernatant did
16 not activate the NF- κ B pathway at 30, 60, and 90 minutes. (C) The anti-phospho-I κ B α
17 MAPK antibody worked with positive control cell extracts.

18 NF- κ B, NF-kappa-B; I κ B α , I-kappa-B alpha; MRSA, methicillin-resistant *Staphylococcus*
19 *aureus*.

20

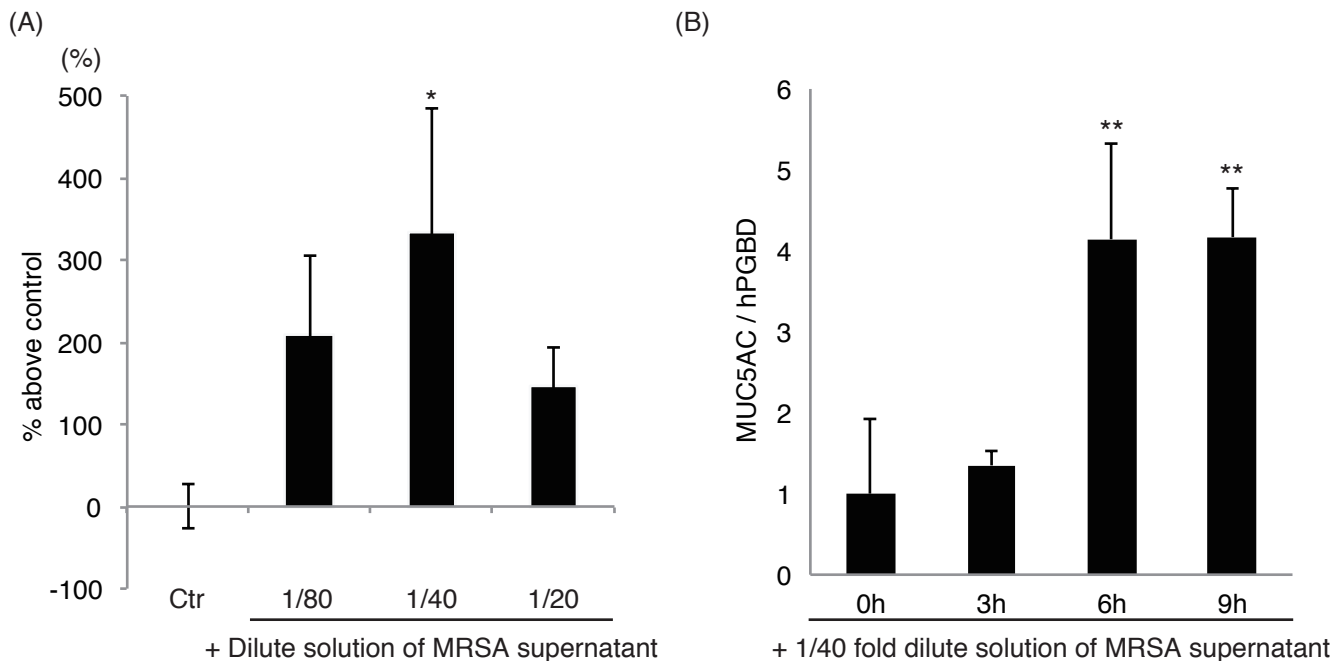


Fig. 1. Influence of the MRSA supernatant on MUC5AC protein production and gene expression.

The NCI-H292 cells were stimulated with 1/80-, 1/40-, and 1/20-fold diluted solution of the MRSA supernatant. In the control, cells were stimulated with the same amount of LB broth as the supernatant. (A) After 24 hours of stimulation, the protein levels were measured by ELISA and provided in terms of percentage greater than control (n = 3). Of the 3 concentrations, only the 1/40-fold dilute solution significantly increased the protein level of MUC5AC. (B) Cells were stimulated with the 1/40-fold dilute solution of the MRSA supernatant for 3, 6, and 9 hours. The mRNA level of MUC5AC was assayed by RT-PCR. The MRSA supernatant significantly increased MUC5AC mRNA expression at 6 and 9 hours. Results are expressed as the mean \pm SD. *P < 0.05, **P < 0.01. ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA, methicillin-resistant *Staphylococcus aureus*; RT-PCR, reverse-transcription polymerase chain reaction

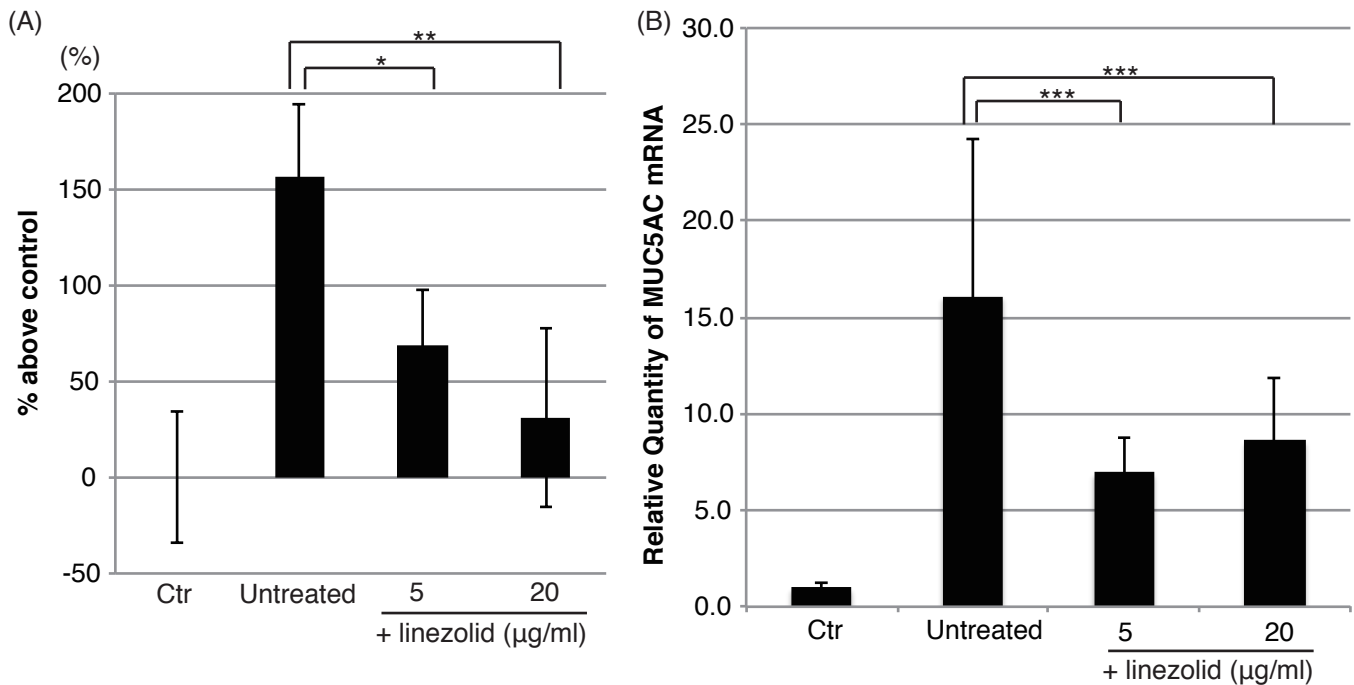


Fig. 2. Effect of linezolid on MRSA supernatant-induced MUC5AC protein and gene overexpression.

The NCI-H292 cells were treated with linezolid (5 µg/mL and 20 µg/mL). In the control, the cells were treated with culture medium only. (A) After 24 hours of treatment, the protein levels were measured by ELISA and provided in terms of percentage greater than control (n = 3). Linezolid significantly reduced the MRSA supernatant-induced protein production at both concentrations. (B) The cells were treated with linezolid for 6 hours, and the mRNA level of MUC5AC was assayed by RT-PCR. Linezolid inhibited MRSA supernatant-induced MUC5AC mRNA expression at both concentrations. Results are expressed as the mean \pm SD. *P < 0.001 compared with the stimulation group.

ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA, methicillin-resistant *Staphylococcus aureus*; RT-PCR, reverse-transcription polymerase chain reaction

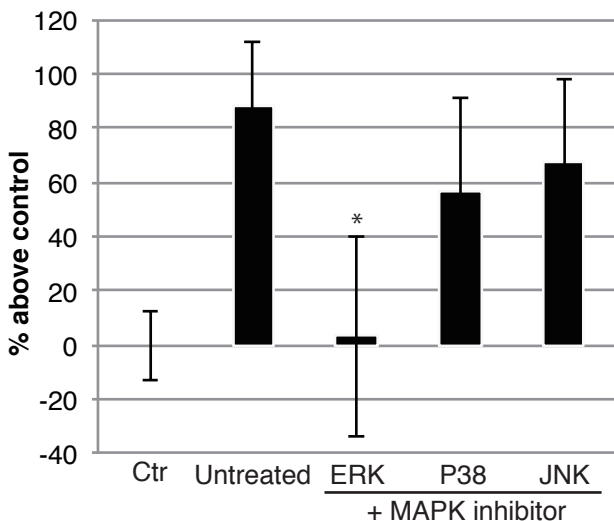


Fig. 3. Effect of MAPK inhibitors on MUC5AC protein overexpression.

The cells were treated with an ERK inhibitor, p38 MAPK inhibitor, or JNK inhibitor at concentration of 10 μ M . After 24 hours, the protein levels were evaluated by ELISA. Only the ERK inhibitor significantly reduced MRSA-induced MUC5AC protein production compared with the stimulation group. Results are expressed as the mean \pm SD.

*P < 0.01 compared with the stimulation group.

ELISA, enzyme-linked immunosorbent assay;

ERK, extracellular signal-regulated protein kinase;

JNK, c-Jun N-terminal kinase;

MAPK, mitogen-activated protein kinase;

MRSA, methicillin-resistant *Staphylococcus aureus*.

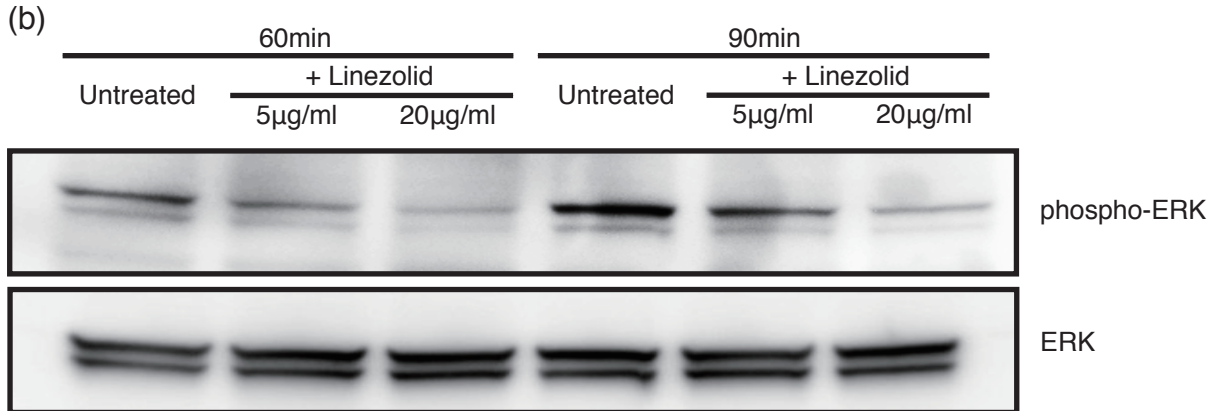
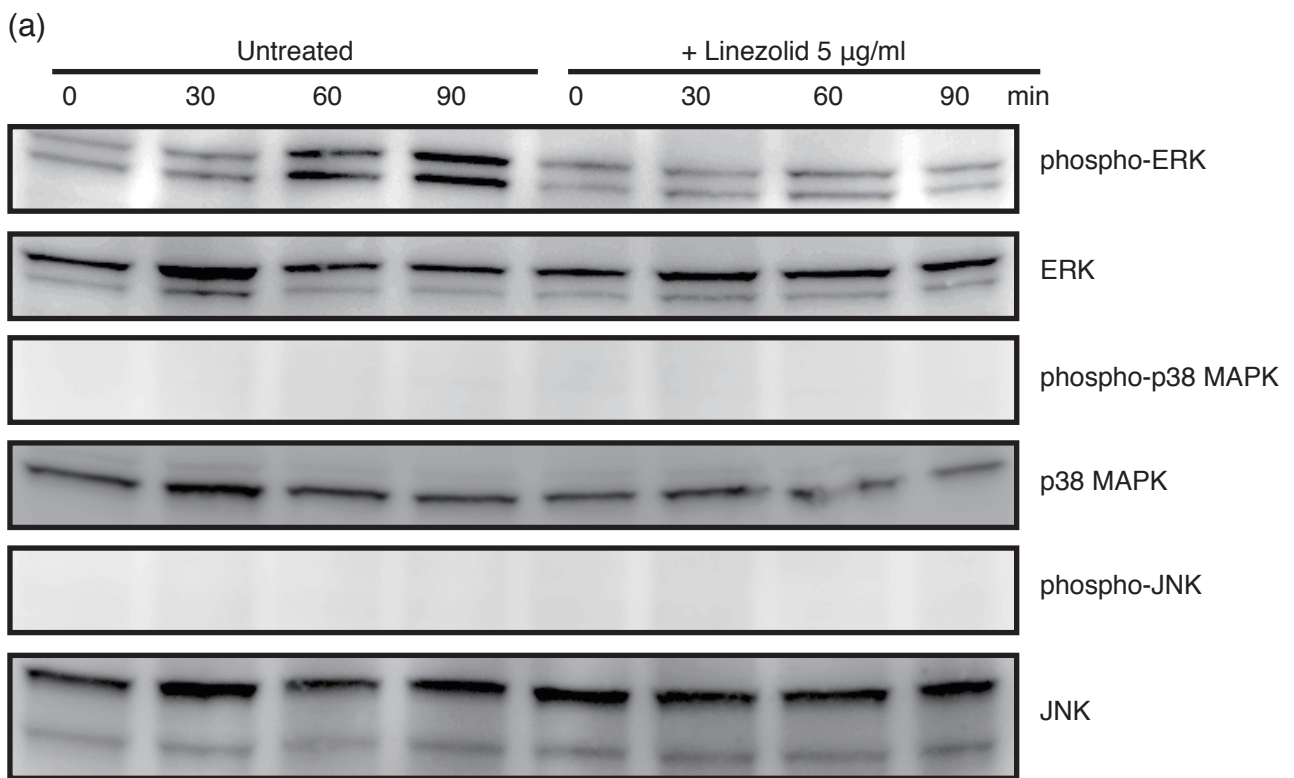


Fig. 4. Effect of linezolid on the MAPK pathway.

The NCI-H292 cells were treated with or without linezolid for 0, 30, 60, or 90 minutes, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant increased only phosphorylation of ERK1/2. The phosphorylation of ERK1/2 was inhibited by linezolid at a concentration of 5 $\mu\text{g/mL}$. There were no significant changes in other MAPKs, such as p38 MAPK and JNK. (B) The inhibitory effect of linezolid on the phosphorylation of ERK1/2 was observed at concentrations of 5 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ at 60 and 90 minutes.

ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA, methicillin-resistant *Staphylococcus aureus*.

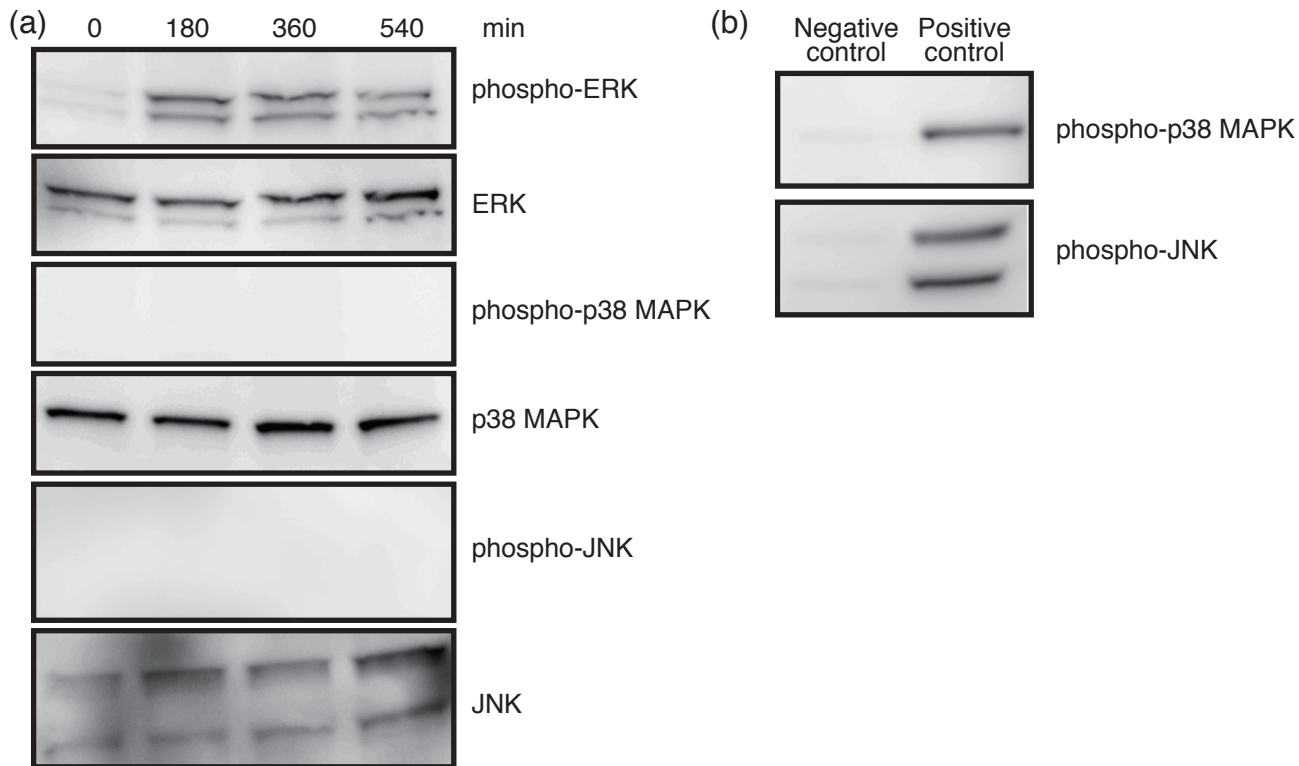


Fig. 5. Influence of MRSA supernatant on the MAPK pathway.

The NCI-H292 cells were stimulated with MRSA supernatant for 0, 180, 360, or 540 minutes, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant increased only phosphorylation of ERK1/2 at 180, 360, and 540 minutes. In contrast, there were no significant changes in p38 MAPK and JNK. (B) The anti-phospho-p38 MAPK and anti-phospho-JNK antibodies worked with positive control cell extracts.

ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA, methicillin-resistant *Staphylococcus aureus*.

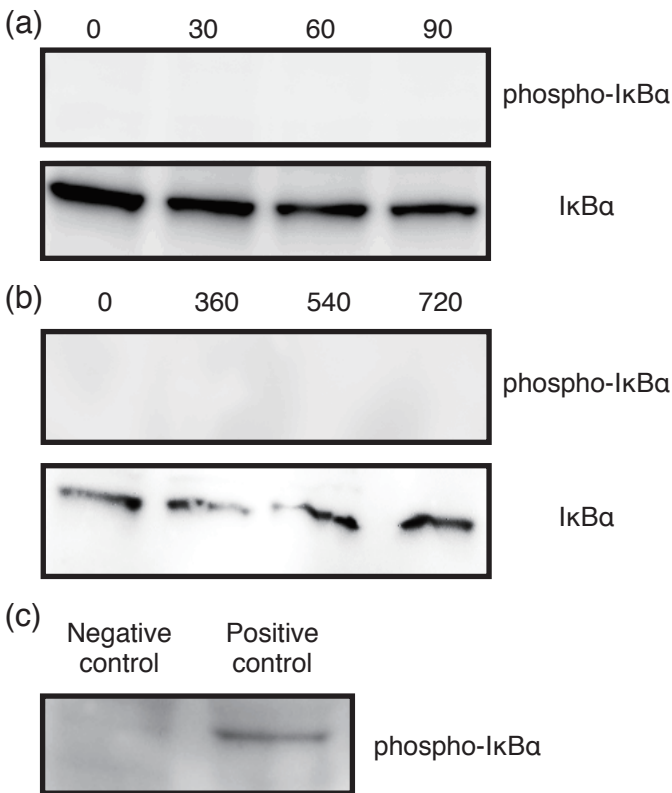


Fig. 6. Influence of MRSA supernatant on NF-κB. The NCI-H292 cells were stimulated with MRSA supernatant, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant did not activate the NF-κB pathway at 30, 60, and 90 minutes. (B) The MRSA supernatant did not activate the NF-κB pathway at 30, 60, and 90 minutes. (C) The anti-phospho-IκBα MAPK antibody worked with positive control cell extracts. NF-κB, NF-kappa-B; IκBα, I-kappa-B alpha; MRSA, methicillin-resistant *Staphylococcus aureus*.