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Subinhibitory Doses of Aminoglycoside Antibiotics Induce Changes in the Phenotype of *Mycobacterium abscessus*

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Subinhibitory doses of antibiotics have been shown to cause changes in bacterial morphology, adherence ability, and resistance to antibiotics. In this study, the effects of subinhibitory doses of aminoglycoside antibiotics on *Mycobacterium abscessus* were investigated. The treatment of *M. abscessus* cells with subinhibitory doses of amikacin was found to change their colony from a smooth to a rough morphotype and increase their ability to adhere to a polyvinylchloride plate, aggregate in culture, and resist phagocytosis and killing by macrophages. *M. abscessus* cells treated with a subinhibitory dose of amikacin also became more potent in Toll-like receptor 2 (TLR-2) stimulation, leading to increased tumor necrosis factor alpha (TNF-α) production by macrophages. The MAB_3508c gene was shown to play a role in mediating these phenotypic changes, as its expression in *M. abscessus* cells was increased when they were treated with a subinhibitory dose of amikacin. In addition, overexpression of MAB_3508c in *M. abscessus* cells caused changes similar to those induced by subinhibitory doses of amikacin, including a switch from smooth to rough colony morphology, increased ability to aggregate in liquid culture, decreased motility, and increased resistance to killing by macrophages. These findings suggest the importance of using sufficient doses of antibiotics for the treatment of *M. abscessus* infections.

Antibiotics have been widely used to treat infectious diseases. However, at subinhibitory concentrations, antibiotics may act as signal molecules and modulate bacterial phenotypes such as virulence, colony morphology, and biofilm formation (1, 2). Transcriptomic and proteomic analyses reveal that subinhibitory doses of antibiotics can cause significant changes in gene expression profiles in *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (1, 3–5).

Mycobacterium abscessus is a rapid-growing nontuberculosis mycobacterium. It is a common cause of infections in patients after tympanostomy tube placement and in those with surgical wounds or cystic fibrosis (6–10). Prolonged antibiotic therapy is generally required for treatment of infections caused by *M. abscessus* because of its intrinsic and acquired resistance to multiple antibiotics. Commonly used antibiotics for *M. abscessus* infections include clarithromycin, cefoxitin, tigecycline, and amikacin (AMK) (11).

Amikacin is an aminoglycoside and is the most effective bactericidal antibiotic for *M. abscessus*. We have found that subinhibitory doses of amikacin can convert colonies of *M. abscessus* from a smooth to a rough morphotype. Because colony morphology is related to virulence, we investigated the effects of subinhibitory doses of amikacin on various activities of *M. abscessus*.

MATERIALS AND METHODS

Bacteria and culture media. The *M. abscessus* cs1c-S strain used in this study is an attenuated variant derived from the clinical isolate cs1c-R (12). *M. abscessus* cells were grown at 37°C on Middlebrook 7H11 (Difco, USA) agar plates supplemented with 10% oleic acid-bovine albumin-dextrose-catalase (OADC) (Becton Dickinson, USA) or in Middlebrook 7H9 broth (Difco) containing 10% OADC, 0.2% glycerol, and 0.05% Tween 80. The colony morphology was examined by light microscopy.

Sliding motility assay. One colony of *M. abscessus* was inoculated in the center of a 7H9 plate with 0.3% agar; the plate was incubated at 37°C for 5 days. The sliding distance of the inoculated *M. abscessus* cells was measured in millimeters.

Aggregation assay. *M. abscessus* cells were adjusted to an optical density at 600 nm (OD_{600}) of 0.1, and 100 μl of this cell suspension was inoculated into 5 ml of 7H9 broth in a tube. The culture was incubated with shaking at 37°C for 3 days. After standing still at room temperature for 10 min, the upper portion of the culture containing dispersed cells was removed, and its OD_{600} value was determined. The OD_{600} value of the bottom portion of culture was measured after the aggregated cells had been completely suspended by vortexing with glass beads of 4.5 mm in diameter (Biospec, USA) as described previously (13–15). The aggregation index of each culture was calculated as the ratio of the OD_{600} value of aggregated cells to that of dispersed cells.

Adherence assay. *M. abscessus* cells were adjusted to an OD_{600} of 0.1 in 7H9 broth, and 100 μl of this cell suspension was placed in each well of a sterile 96-well polyvinylchloride (PVC) plate (Becton Dickinson). Following incubation for 6 days, the medium in each well was discarded, and the wells were washed with phosphate-buffered saline (PBS) to remove planktonic cells. The adhered cells in each well were then stained with 0.1% (wt/vol) crystal violet for 1 h. After the cells were washed with PBS, the crystal violet associated with the adhered cells in each well was dissolved in 95% ethanol, and the OD_{595} value of the resulting solution was determined.

Cord formation. Examination of cord formation was performed by inoculating *M. abscessus* cells into 7H9 broth at an OD_{600} of 0.1. After incubation at 37°C for 5 days, *M. abscessus* cells were collected, stained

Received 14 May 2015 Returned for modification 24 June 2015
Accepted 16 July 2015
Accepted manuscript posted online 20 July 2015
Citation Tsai S-H, Lai H-C, Hu S-T. 2015. Subinhibitory doses of aminoglycoside antibiotics induce changes in the phenotype of *Mycobacterium abscessus*. Antimicrob Agents Chemother 59:6161–6169. doi:10.1128/AAC.01132-15.
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doi:10.1128/AAC.01132-15

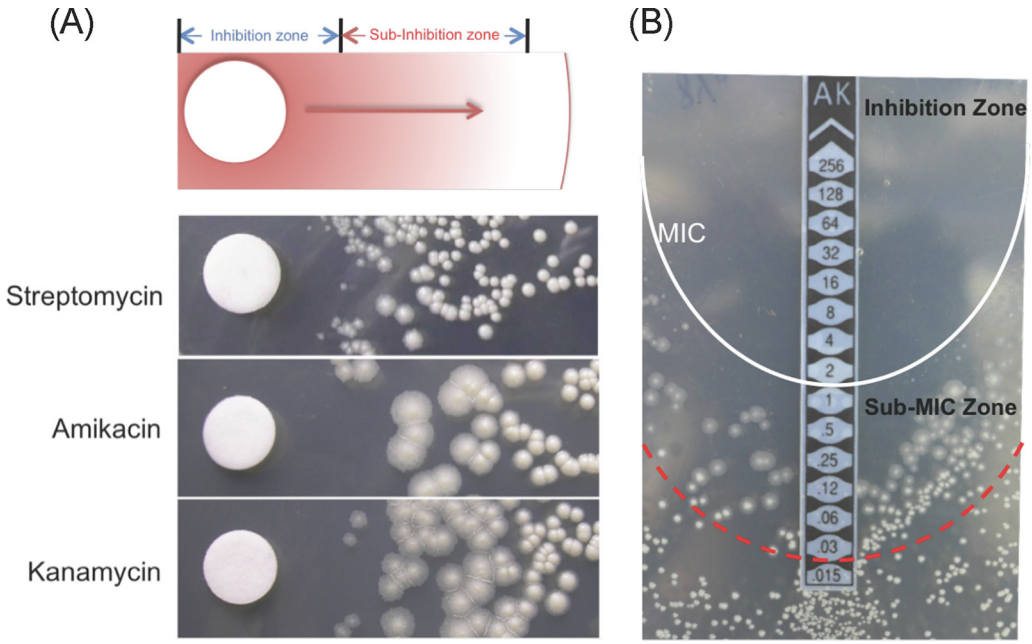

FIG 1 *M. abscessus* colony morphology switching induced by aminoglycoside antibiotics. (A) Antibiotic disc diffusion assay. Cells of *M. abscessus* cs1c-S strain were plated at 200 to 500 CFU on a 7H11 agar plate. A disc containing streptomycin (100 μg), amikacin (20 μg), or kanamycin (20 μg) was then placed on the plate. Three-day-old *M. abscessus* colonies were examined for colony morphology. (B) Etest assay. An amikacin Etest strip was placed on a 7H11 plate inoculated with *M. abscessus*. The plate was incubated at 37°C for 3 days. The amikacin MIC and sub-MIC for *M. abscessus* are as indicated. The long arrow indicates the antibiotic diffusion direction.

with crystal violet, and examined at ×400 magnification under a light microscope as previously described (16).

Treatment of *M. abscessus* cells with subinhibitory concentrations of amikacin. *M. abscessus* cells were grown to stationary phase in 7H9 broth at 37°C; 100 μl of this cell suspension was inoculated into 5 ml of 7H9 broth with or without a subinhibitory concentration (SIC) (1 μg/ml) of amikacin and incubated at 37°C for 24 h. The bacteria were then washed and resuspended in PBS at a concentration of 4×10^8 CFU/ml.

Survival in macrophages. THP-1 cells, derived from a human acute monocytic leukemia cell line, were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in a humidified CO_2 incubator. THP-1 cells were differentiated into adherent macrophages by adding phorbol-12-myristate-13-acetate (PMA) (Sigma, USA) to the culture to a final concentration of 500 ng/ml. For investigation of tumor necrosis factor alpha (TNF-α) release, THP-1-derived macrophages were cocultured with *M. abscessus* cells at a multiplicity of infection (MOI) of 10 2 days after PMA addition. Twenty-four hours after *M. abscessus* infection, TNF-α levels in culture supernatants were measured using the Duo-Set enzyme-linked immunosorbent assay (ELISA) development system kit (R&D Systems, USA). Some THP-1 cells were treated with anti-Toll-like receptor 2 (anti-TLR-2) antibody or isotype control antibody (eBioscience, USA) for 30 min before infection with *M. abscessus*. ELISA plates were analyzed using an Emax microplate reader (Molecular Devices, USA) at 450 nm. To examine macrophage-mediated killing, THP-1-derived macrophages were infected with *M. abscessus* at an MOI of 0.1 at 37°C. At 1, 2, and 3 days after infection, THP-1 macrophages were lysed with 1% Triton X-100. Each cell lysate was plated on 7H11 agar plates to determine the CFU of the surviving bacteria.

Phagocytosis assay. Quantitative measurement of phagocytosis was performed by flow cytometry. *M. abscessus* cells were stained with the green fluorescent dye SYTO 9 (Molecular Probes, USA). The stained *M. abscessus* cells were incubated at an MOI of 10 with THP-1 macrophage cells for 1 h. The infected THP-1 cells were washed extensively with PBS to remove extracellular bacteria and then stained with trypan blue to quench the fluorescence of membrane-bound bacteria. Green fluorescence was measured at 525 nm after being excited with 488 nm light. Data acquisition and analysis were performed with BD CellQuest (BD Biosciences, USA).

Determination of mRNA levels by real-time RT-PCR and RNA-seq. *M. abscessus* cells were suspended in TRIzol reagent (Invitrogen, USA) and lysed with the aid of mechanical grinding using 0.1-mm silica-zirconium beads in a Mini-Beadbeater (Biospec, USA). Total RNA was then isolated using an RNA isolation kit (Bioman Scientific Co. Ltd., Taipei, Taiwan) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript III reverse transcription kit (Invitrogen, USA). Real-time reverse transcriptase PCR (RT-PCR) (quantitative PCR [qPCR]) was performed using Kapa SYBR FAST 2× qPCR master mix (Kapa Biosystems, USA). Primers used in this PCR included RT-MAB_3508c-F (5′-CCTGCTCAAGAATCTCACC-3′), RT-MAB_3508c-R (5′-CTGTGGTTCGCGGAAAC-3′), RT-16S rRNA-F (5′-GGACCACACACTTCATGGTG-3′), and RT-16S rRNA-R (5′-GAGTCT GGGCCGTATCTCAG-3′). The relative MAB_3508c mRNA level was determined as the ratio of the qPCR cycle threshold (C_T) value of MAB_3508c mRNA to that of the 16S rRNA of each sample. For RNA-sequencing (RNA-seq), the libraries were prepared using a TruSeq Stranded mRNA sample preparation kit (Illumina Inc., USA). The libraries were sequenced on a MiSeq instrument (Illumina Inc.) to produce 75-bp paired-end reads. The sequences were analyzed by CLC Genomics Workbench v8.0 (CLC Bio).

Lipid extraction and analysis. *M. abscessus* cells were inactivated by heating at 60°C for 1.5 h. After washing, lipids were extracted by treating 500 mg of bacteria (wet weight) with 2 ml of chloroform-methanol (2:1, vol/vol) at 56°C for 1 h. The organic phase of the extraction was collected and washed with water. The solvent was evaporated, and the dried lipid was weighed. The lipids were then dissolved in chloroform and subjected to thin-layer chromatography (TLC) on a Silica Gel_{60} TLC plate (Merck, Germany) using a solvent containing chloroform, methanol, and water at

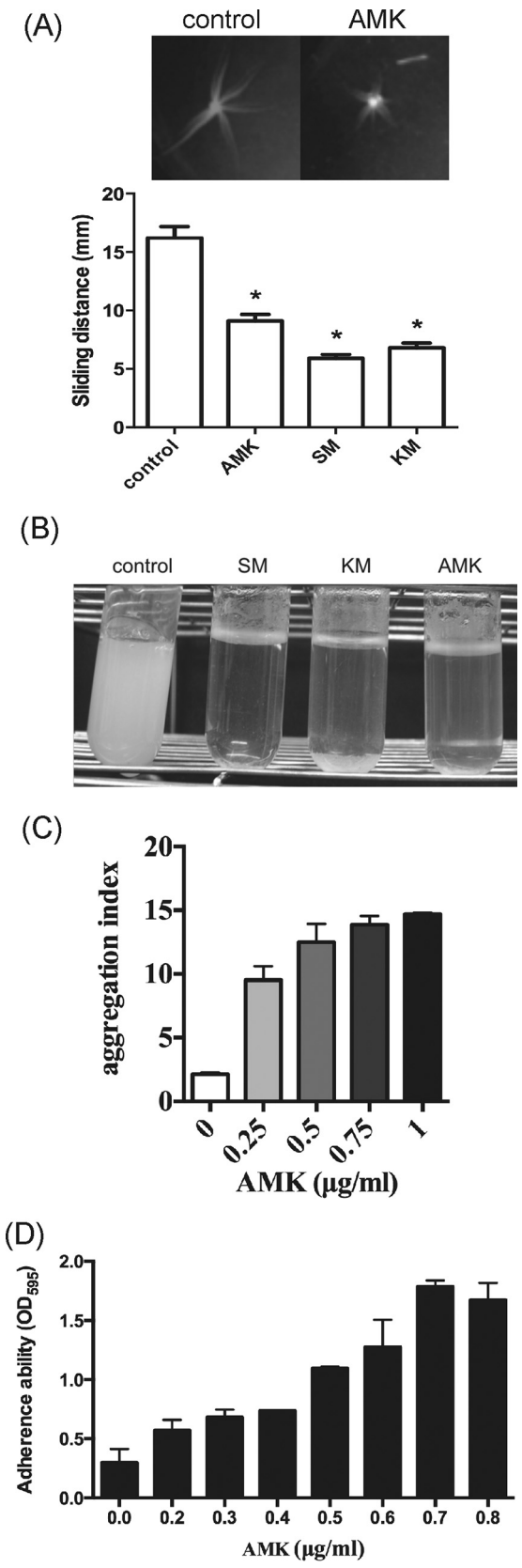

FIG 2 Alteration of *M. abscessus* cell surface-associated phenotypes by aminoglycoside antibiotics. (A) Sliding motility. *M. abscessus* cells were inoculated in the center of a 7H9 0.3% agar plate containing the MIC_{50}s of the indicated antibiotics (control, no antibiotic; amikacin [AMK], 1 μg/ml; streptomycin [SM], 10 μg/ml; kanamycin [KM], 1 μg/ml). The sliding distance was measured in millimeters and plotted. (B) Bacterial aggregation ability. *M. abscessus* cells were cultured with low doses of antibiotics (SM, 10 μg/ml; KM, 1 μg/ml; AMK, 1 μg/ml) for 36 h at 37°C with shaking. Pictures of cultures were taken after the cultures were allowed to stand still for 10 min. (C) *M. abscessus* cells were cultured with various concentrations (0 to 1 μg/ml) of amikacin for 36 h at 37°C with shaking. The aggregation index of each culture was determined and plotted. (D) Adherence ability. Cultures of *M. abscessus* were grown in wells of a 96-well PVC plate containing the indicated concentrations (0 to 0.8 μg/ml) of amikacin for 6 days. The adhered bacteria in each well were washed and then stained with crystal violet. Optical density readings of the stain are presented as means ± standard deviations for three independent experiments. Data were analyzed by the Student *t* test. *, significant difference ($P < 0.05$).

a ratio of 100:14:0.8 as previously described (16). To visualize lipids, the plate was sprayed with 10% H_2SO_4 and then charred with hot air until spots with hues characteristic of different lipid classes appeared. The mass of each lipid species was determined by matrix-assisted laser desorption ionization−time of flight mass spectrometry (MALDI-TOF MS) with a pulse laser emitting at 337 nm as described previously (17). Samples were mixed with 2,5-dihydroxybenzoic acid as the matrix and analyzed in reflectron mode with an accelerating voltage of 25 kV.

Plasmid construction. The DNA fragment containing the coding region of MAB_3508c was amplified by PCR using TaKaRa *Ex Taq* DNA polymerase (TaKaRa Corp., Shiga, Japan) and the primer pair MAB_3508c-F (5′-ATGGATCCATGATGACCGTTGAAGTGGA-3′) and MAB_3508c-R (5′-ATAAGCTTTCATGCCGCGGCGGTGTCGG-3′), containing BamHI and HindIII sites (underlined), respectively. The resulting MAB_3508c DNA fragment was cloned into pQE80L-p*ptr* to generate pQE80L-p*ptr*-MAB_3508c so that the MAB_3508c gene is driven by the *ptr* promoter (18). The DNA fragment containing the *ptr* promoter and MAB_3508c was cloned into the mycobacterial shuttle vector pMV261 (19), generating the plasmid pMN-p*ptr*-MAB_3508c.

Statistical analysis. All experiments were performed at least three times. Results are presented as means ± standard deviations. Statistical comparisons were performed using the Student *t* test.

RESULTS

***M. abscessus* colony morphotype changes induced by subinhibitory doses of aminoglycoside antibiotics.** To investigate changes in colony morphology in response to antibiotics, disc diffusion assays were performed. *M. abscessus* cells were inoculated at 200 to 500 CFU to form colonies on agar plates. As shown in Fig. 1A, the colonies of *M. abscessus* cs1c-S (smooth morphotype) exhibited a rough morphology near the disc containing the aminoglycoside antibiotic streptomycin, amikacin, or kanamycin, but a smooth morphology in areas distal to the disc. This colony morphology change was more profound in *M. abscessus* cells exposed to amikacin and kanamycin. An amikacin Etest showed that *M. abscessus* cs1c-S cells had a MIC of 2 μg/ml; the same MIC result was obtained with the microdilution assay (data not shown). The concentrations of amikacin that triggered a colony morphotype switch were 0.03 to 1 μg/ml. To investigate whether the morphotype switch caused by aminoglycoside antibiotics is reversible, the morphotype-switched *M. abscessus* cells were inoculated on another agar plate containing no antibiotics, and the colony morphology of these cells was found to switch from a rough back to a smooth form (data not shown).

Reduced sliding ability and increased aggregation and adherence by subinhibitory doses of amikacin. Because the colony morphology of mycobacteria is related to their sliding, aggregation, and adherence abilities, we investigated the effects of amin-

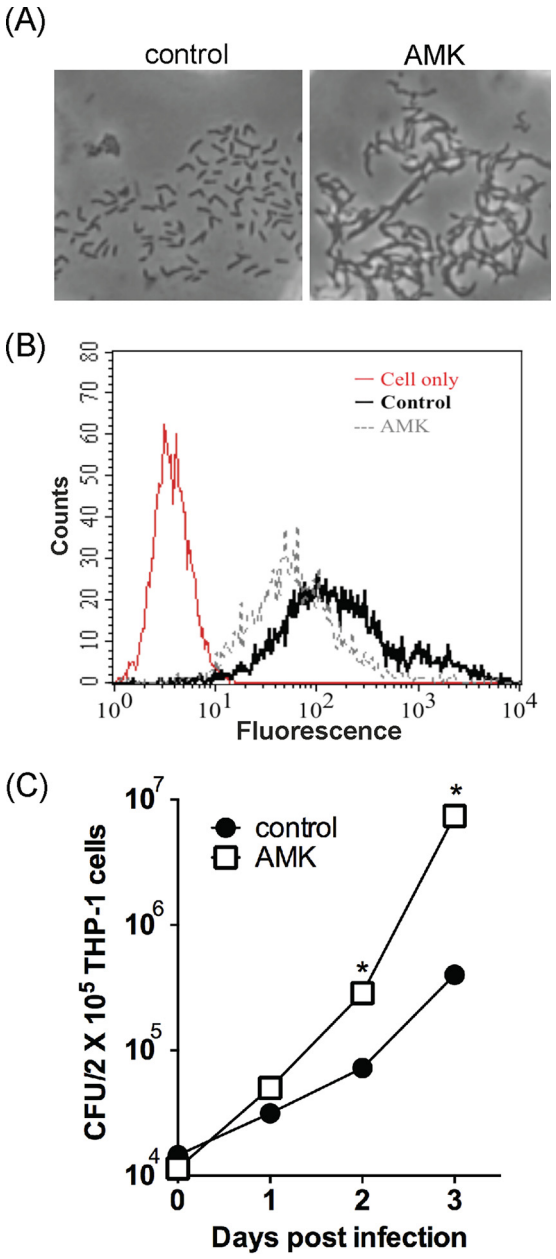

FIG 3 Virulence of the *M. abscessus* smooth variant enhanced by subinhibitory concentrations of amikacin. (A) Cord formation. *M. abscessus* cells were incubated in RPMI 1640 medium with or without a MIC_{50} (1 μg/ml) of amikacin for 2 days. The cells were collected, stained with crystal violet, and examined by microscopy. (B) Antiphagocytosis ability. *M. abscessus* cells were pretreated with a MIC_{50} of amikacin for 24 h, labeled with SYTO 9, and then cocultured with THP-1 macrophages at an MOI of 1 for 2 h. Bacterial uptake by the macrophages was determined by flow cytometry, and the fluorescence intensity of phagocytosed amikacin-treated *M. abscessus* cells was compared to that of phagocytosed non-amikacin-treated *M. abscessus* cells (control) and THP cells only (red curve). (C) Resistance against macrophage-mediated killing. *M. abscessus* cells were treated with or without a MIC_{50} of amikacin and then cocultured with THP-1 macrophages at an MOI of 1 for 1 h. THP-1 cells were lysed at the indicated time points after extensive washing with PBS. The cell lysates were plated for CFU determination. Data were analyzed by the Student *t* test. *, significant difference ($P < 0.05$).

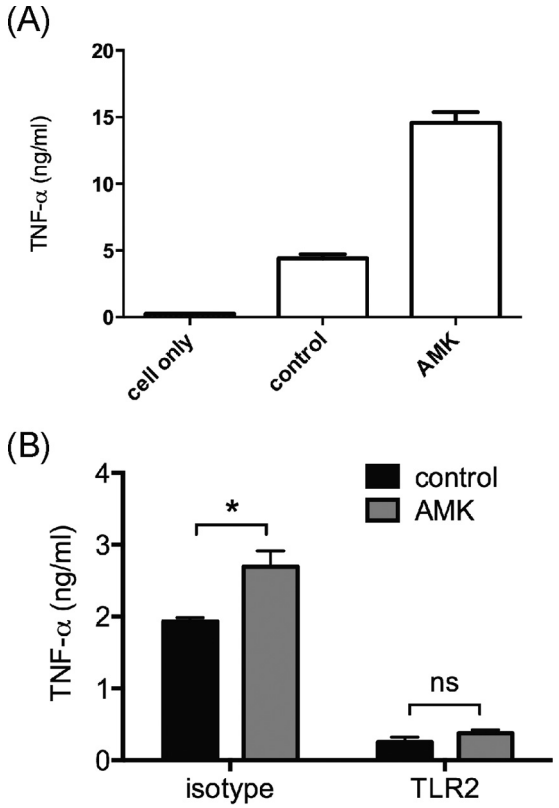

FIG 4 Enhanced TNF-α release through TLR-2 signaling by amikacin. (A) THP-1 macrophages were infected with untreated (control) *M. abscessus* cells or with *M. abscessus* cells treated with a MIC_{50} (1 μg/ml) of amikacin (AMK) at an MOI of 10 for 16 h. The levels of TNF-α in culture supernatants were determined by ELISA. (B) THP-1 macrophages were treated with anti-TLR-2 or isotype antibody for 30 min and then cocultured with untreated (control) or amikacin-treated *M. abscessus* cells for 24 h. The levels of TNF-α in culture supernatants were determined by ELISA. Data were analyzed by the Student *t* test. *, significant difference ($P < 0.05$); ns, no significant difference ($P > 0.05$).

oglycoside antibiotics on these phenotypes. The sliding motility assay was performed on agar plates with and without the presence of a subinhibitory dose (MIC_{50} [one-half of the MIC]) of antibiotics. As shown in Fig. 2A, *M. abscessus* cells were highly motile (16.2 ± 2.2 mm) on agar plates containing no antibiotics, but their motility was greatly decreased in the presence of a MIC_{50} of amikacin (9.1 ± 1.2 mm), streptomycin (5.9 ± 0.7 mm), or kanamycin (6.8 ± 0.9 mm).

To investigate the effect of subinhibitory doses of aminoglycosides on aggregation properties, *M. abscessus* cells were grown in liquid 7H9 medium with or without antibiotics at 37°C with shaking for 3 days. Results showed that *M. abscessus* cells exhibited a homogenously dispersed culture in the broth containing no antibiotics. In the presence of low concentrations (MIC_{50}) of amikacin, streptomycin, or kanamycin, the cultures showed a clear supernatant with bacterial cells clumped in the bottom of the culture tubes (Fig. 2B). To quantify the aggregation ability, *M. abscessus* cells were grown in liquid 7H9 medium with various concentrations of amikacin (0, 0.25, 0.5, 0.75, and 1 μg/ml). The cultures were incubated at 37°C for 3 days with shaking and then allowed to stand still for 10 min so that the bacterial cells settled to the bottom of the culture tubes. The OD_{600} values of the supernatant

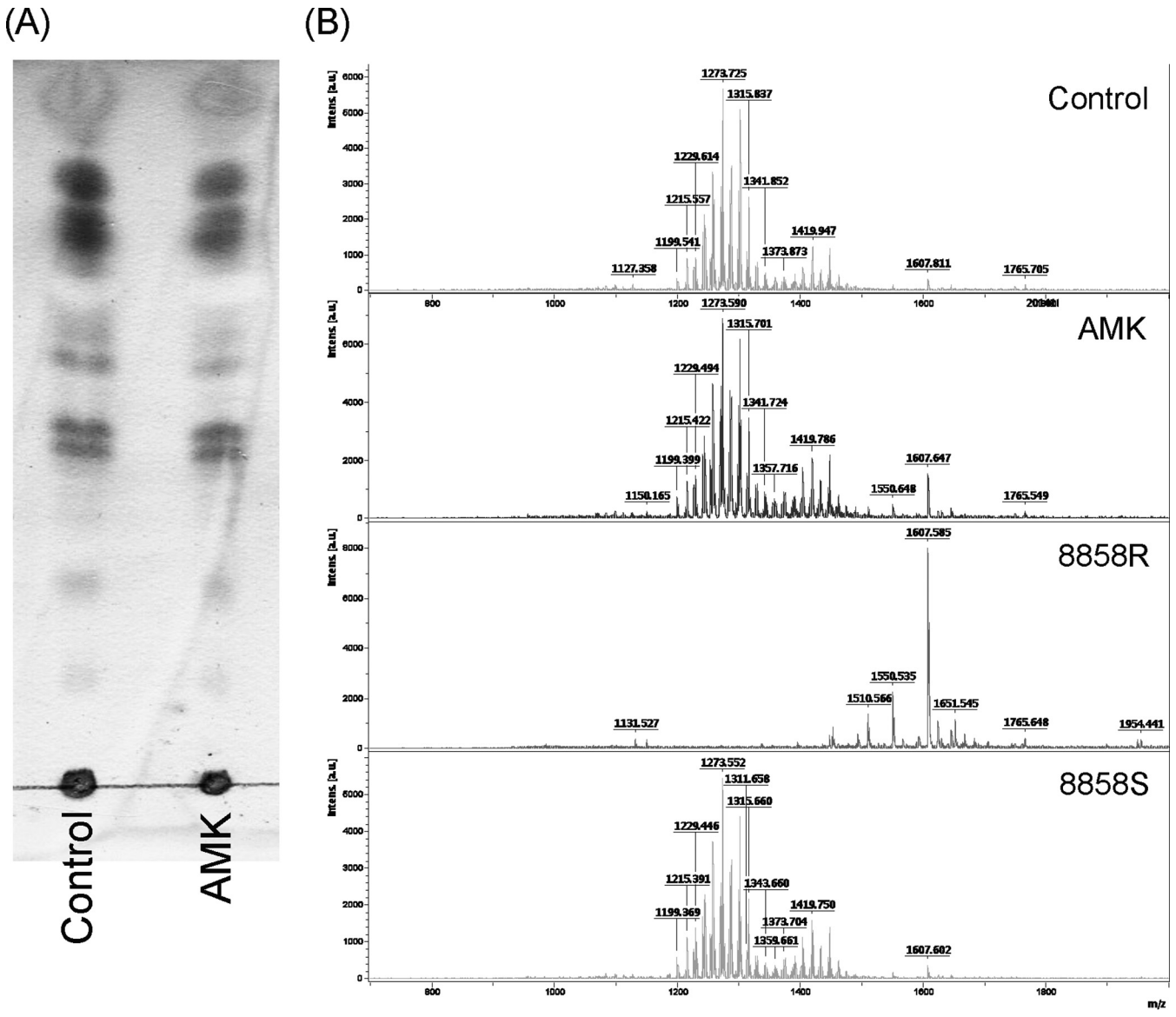

FIG 5 GPL contents of *M. abscessus* cs1c-S cells with or without treatment with a subinhibitory dose of amikacin. (A) TLC analysis. Equal weights of total lipid extracts from *M. abscessus* cs1c-S cells with or without amikacin treatment were resolved by thin-layer chromatography with $CHCl_3$-CH_3OH-H_2O (100:14:0.8) and visualized with 1% 1-naphthol. (B) MALDI-TOF mass spectrometry of total lipids from *M. abscessus* cs1c-S cells with or without amikacin treatment and control strains 8858R (GPL-deficient rough type) and 8858S (GPL-abundant smooth type).

and the resuspended cell pellet were then measured to determine the aggregation index of each culture. As shown in Fig. 2C, the aggregation index of the culture without amikacin was approximately 2 but was increased to 10 to 15 in a dose-dependent manner in the presence of 0.25 to 1 μg/ml of amikacin. This result indicates that subinhibitory doses of aminoglycosides enhanced the aggregation property of *M. abscessus* cells.

The effect of subinhibitory doses of amikacin on adherence ability was then examined. *M. abscessus* cells were grown in wells of a 96-well PVC plate for 6 days. The adhered bacteria in each well were stained, and the intensity of the staining, which reflects the level of the adherence ability, was determined by measuring the OD_{595} of the dissolved stain. As shown in Fig. 2D, the adherence ability was increased by amikacin (0.2 to 0.7 μg/ml) in a dose-dependent manner.

Enhanced *M. abscessus* cord formation and antiphagocytosis ability by subinhibitory doses of amikacin. Cord formation usually occurs in rough *M. abscessus* variants (16, 20). It is a mechanism of evading phagocytosis (21). Therefore, the possibility that subinhibitory doses of amikacin can trigger cord formation of *M. abscessus* cells and enhance their resistance to phagocytosis by macrophages was examined. As shown in Fig. 3A, microscopic examination of *M. abscessus* cs1c-S suspension cultures revealed no cord formation in the absence of amikacin. However, in the presence of a low dose (MIC_{50}, 1 μg/ml) of amikacin, the cells formed cord-like structures. To perform the phagocytosis assay, *M. abscessus* cells were grown with or without a low dose (1 μg/ml) of amikacin to exponential phase, stained with SYTO 9, and then cocultured with THP-1 macrophages. The fluorescence intensity of the THP-1 cells containing phagocytosed SYTO 9-stained *M.*

TABLE 1 Ratios of mRNA levels of genes involved in the GPL biosynthesis of *M. abscessus* cells treated with subinhibitory doses of amikacin versus those of untreated (control) cells

GPL gene	Gene name	AMK[a]/control
MAB_0934	*gap*-like	1.08
MAB_0935c	*fadD23*	0.78
MAB_0936c	*pe*	0.85
MAB_0937c	*mmpL10*	0.77
MAB_0938c	*papA3*	0.81
MAB_0939	*pks*	0.87
MAB_4098c	*mps2*	1.13
MAB_4099c	*mps1*	0.89
MAB_4100c	*mbtH*	0.82
MAB_4103c	*fmt*	1.21
MAB_4104	*gtf*	1.53
MAB_4105c	*rmt3*	1.43
MAB_4110c	*rmt2*	1.29
MAB_4111c	*rmlB*	1.19
MAB_4112c	*gtf3*	1.14
MAB_4113	*rmlA*	1.06
MAB_4114	*Rv1174*	0.86
MAB_4115c	*mmpL4b*	0.75
MAB_4116c	*mmpL4a*	0.90
MAB_4117c	*mmpS4*	1.25
MAB_4454c	*sap*	0.98
MAB_4459c	*ecf*	1.09
MAB_4633	*Rv0926*	1.04

[a] AMK, amikacin.

abscessus cells was determined. As shown in Fig. 3B, the fluorescence intensity of the THP-1 cells that phagocytosed amikacin-treated *M. abscessus* cells was significantly lower than that of those that phagocytosed untreated *M. abscessus* cells. These results indicate that *M. abscessus* cells gained antiphagocytosis ability after treatment with a subinhibitory dose of amikacin.

Experiments were then performed to compare the tolerance of macrophage-mediated killing between untreated and amikacin-treated *M. abscessus* cells. THP-1-derived macrophages were infected with untreated or amikacin-treated *M. abscessus* cells. At 1, 2, and 3 days postinfection, the THP-1 macrophages were lysed, and the cell lysates were plated on agar plates. Results showed that the sample from THP-1 cells infected with amikacin-treated *M. abscessus* had a CFU of approximately 1×10^7, but that from those infected with untreated *M. abscessus* had a CFU of 5×10^5 (Fig. 3C). This result suggests that amikacin treatment increased the ability of *M. abscessus* to persist and replicate in THP-1 cells (Fig. 3C).

Increased TNF-α secretion by macrophages stimulated with amikacin-treated *M. abscessus* cells. Since TNF-α is a potent proinflammatory cytokine and promotes aggregation of monocytes after *Mycobacterium tuberculosis* (22) and *M. abscessus* infection (16, 23), experiments were performed to determine whether, after amikacin treatment, *M. abscessus* cells induce more TNF-α release by macrophages. After pretreatment with a low dose (MIC_{50}) of amikacin for 24 h, *M. abscessus* cells were cocultured with THP-1 macrophages for 1 day. As shown in Fig. 4A, TNF-α release was significantly higher by the THP-1 cells that were cocultured with amikacin-treated *M. abscessus* cells than those cocultured with untreated *M. abscessus* cells. To investigate whether Toll-like receptor 2 (TLR-2) plays a role in *M. abscessus*-simulated TNF-α secretion (24), THP-1 cells were treated with anti-TLR-2 antibody for 30 min and then were incubated with *M. abscessus* cells that were treated with or without a subinhibitory dose (1 μg/ml) of amikacin. Results showed that treatment with anti-TLR-2 antibody abrogated the ability of THP-1 cells to release TNF-α upon stimulation with amikacin-treated or untreated *M. abscessus* cells. However, THP-1 cells that were treated with control (isotype) antibody released approximately 30% more TNF-α when they were stimulated with amikacin-treated *M. abscessus* cells than those stimulated with untreated *M. abscessus* cells (Fig. 4B).

No effect on glycopeptidolipid production of *M. abscessus* by subinhibitory amikacin treatment. In previous studies, *M. abscessus* rough colony morphology was attributed to the lack of glycopeptidolipid (GPL) production (16, 25–27). To determine whether the smooth to rough colony morphotype change induced by amikacin was due to reduced GPL production, the GPL contents of *M. abscessus* cells with or without the treatment of subinhibitory doses of amikacin were analyzed. After pretreatment with the MIC_{50} of amikacin for 24 h, total lipids of *M. abscessus* cells were examined by TLC. Results showed that the GPL profile of amikacin-treated *M. abscessus* cells was similar to that of untreated control cells (Fig. 5A). Results of MALDI-TOF spectrometry revealed that the GPL profile of both untreated (control) and amikacin-treated *M. abscessus* cells was very similar to that of strain 8858S, which is a normal GPL producer (Fig. 5B).

To confirm that GPL production was not affected by treatment with subinhibitory doses of amikacin, the mRNA levels of genes involved in GPL production (28) were determined by RNA-seq. The ratios for mRNA levels of various GPL-associated genes of *M. abscessus* cells treated with subinhibitory doses of amikacin (AMK) versus untreated cells are listed in Table 1. These results showed that the expression of GPL-associated genes in *M. abscessus* cells was not significantly affected by subinhibitory doses of amikacin.

Characterization of MAB_3508c responsible for pleiotropic phenotype switch of *M. abscessus*. WhiB7 is a transcription factor that activates the intrinsic antibiotic resistance system of mycobacteria (29). We have found that the MAB_3508c gene of *M. abscessus* shares 55% amino acid sequence identity with *whiB7* (our unpublished data). To determine whether amikacin has any effect on the expression of MAB_3508c, *M. abscessus* cells were treated with or without the MIC_{50} of amikacin and then examined for MAB_3508c mRNA levels. The results showed that amikacin treatment caused a 5-fold increase in MAB_3508c mRNA levels (Fig. 6A), compared to that of untreated cells.

To investigate whether MAB_3508c plays a role in aminoglycoside-induced phenotype changes, the MAB_3508c gene was cloned and placed under the control of the constitutive promoter *ptr* from *Streptomyces coelicolor* for expression (18). The resulting construct was then introduced into *M. abscessus* cells. The transformed cells (designated p*ptr*-MAB_3508c cells) were found to have a rough morphology, whereas *M. abscessus* cells containing the vector (pMV261) had a smooth morphology (Fig. 6B). Phenotype analyses showed that p*ptr*-MAB_3508c cells had increased aggregation ability (Fig. 6C) and reduced sliding motility (Fig. 6D) than cells containing the vector. The sliding distance of *M. abscessus* cells transformed with the vector was 9.3 ± 2.6 mm and that of p*ptr*-MAB_3508c cells was 5.4 ± 1.6 mm. The p*ptr*-MAB_3508c cells were also found to be more resistant to phagocytosis by THP-1 cells as *M. abscessus* colony counts of THP-1 cells infected

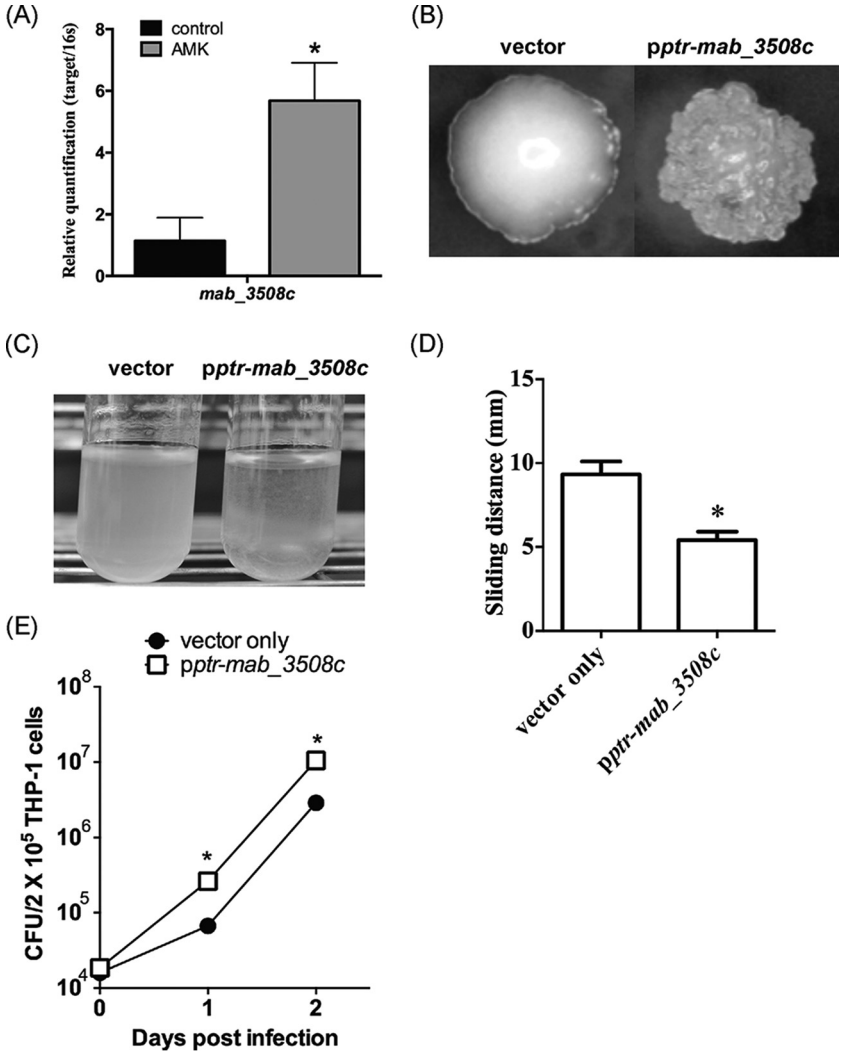

FIG 6 Role of *M. abscessus* MAB_3508c in amikacin-induced phenotype alterations. (A) MAB_3508c mRNA levels in untreated (control) and amikacin (1 μg/ml)-treated *M. abscessus*. (B to E) Colony morphology (B), aggregation in broth culture (C), sliding motility (D), and survival (E) of THP-1 macrophages of *M. abscessus* cs1c-s cells transformed with pMV-p*ptr*-MAB_3508c or with the vector pMV261. Data were analyzed by the Student *t* test. *, significant difference ($P < 0.05$).

with p*ptr*-MAB_3508c cells were higher ($2.63 \times 10^5 \pm 0.63 \times 10^5$ at day 1 and $1.06 \times 10^7 \pm 0.13 \times 10^7$ CFU at day 2) than those of cells infected with *M. abscessus* containing the vector pMV261 ($6.68 \times 10^4 \pm 1.55 \times 10^4$ at day 1 and $2.89 \times 10^6 \pm 0.58 \times 10^6$ CFU at day 2) (Fig. 6E).

DISCUSSION

In this study, we showed that subinhibitory concentrations of aminoglycoside antibiotics, such as amikacin, induced *M. abscessus* to change its colony morphology from a smooth to a rough morphotype. This change was associated with increased ability of *M. abscessus* cells to adhere to PVC, to aggregate in culture, and to resist phagocytosis and killing by macrophages. *M. abscessus* cells treated with a subinhibitory dose (1 μg/ml) of amikacin also became more potent in TLR-2 stimulation, leading to increased TNF-α production by macrophages and severity of lung inflammation.

The morphotype of *M. abscessus* cells is closely related to their glycopeptidolipid (GPL) biosynthesis (16). Previous studies showed that variations in the production of GPL by *M. abscessus* affect its colony morphology (23), and most rough *M. abscessus* strains were found to produce less GPL than smooth strains (16, 25–27). Deletion of the *mmpl4b* gene, which is involved in GPL biosynthesis, switched *M. abscessus* from a smooth to a rough morphotype (23). A genome-wide analysis of *M. abscessus* clinical isolates has identified a number of genetic mutations or deletions associated with the decrease in GPL production and the smooth to rough morphotype switching (25). Although GPL production is an important factor in the colony morphotype of *M. abscessus*, it may not be the sole determinant, as some smooth and rough strains of *Mycobacterium smegmatis* show no differences in GPL production (30). In the present study, we examined the surface lipids of smooth and antibiotic-induced rough *M. abscessus* strains by TLC and MALDI-TOF MS and found no differences in lipid profiles (Fig. 5). We also performed RNA-seq and found no significant differences in the mRNA levels of various genes involved

in GPL biosynthesis (Table 1). Therefore, the antibiotic-induced morphotype switching may not be completely dependent on GPL biosynthesis. Further investigation is required to clarify this possibility.

We also found that treatment of *M. abscessus* cells with a subinhibitory concentration of amikacin increased the expression of MAB_3508c (Fig. 6A). Furthermore, overexpression of MAB_3508c in *M. abscessus* cells caused a switch from smooth to rough colony morphology, increased ability to aggregate in culture, decreased motility, and increased resistance to killing by macrophages (Fig. 6B to E). All of these properties of MAB_3508c-overexpressed *M. abscessus* cells are the same as those induced by a subinhibitory concentration of amikacin, suggesting that the effects of subinhibitory doses of aminoglycoside antibiotics on *M. abscessus* phenotype changes are mediated by MAB_3508c.

The MAB_3508c gene is homologous to the *whiB7* gene of *M. tuberculosis*. WhiB7 has been shown to play a major role in the intrinsic antibiotic resistance of *M. tuberculosis* by activating the expression of a multidrug transporter (*tap*, *Rv1258c*) (31) and a ribosomal methyltransferase (*erm*, *Rv1988*) (32); *whiB7*-null mutants of *M. tuberculosis* are hypersusceptible to antibiotics. The expression of *whiB7* in *M. tuberculosis* is inducible by antibiotics such as erythromycin, streptomycin, and tetracycline (33). WhiB7 also regulates the expression of *eis* (enhanced intracellular survival; *Rv2416c*), which has been shown to reduce the host immune response (34) and enhance mycobacterial survival in macrophages (33). The functions of MAB_3508c have not been characterized. The results of this study suggest that it regulates the phenotypic changes of *M. abscessus* cells when they are exposed to subinhibitory concentrations of amikacin. We are in the process of knocking out the MAB_3508c gene to confirm its function.

M. abscessus is the second most common cause of nontuberculosis mycobacterial infections in patients (6, 7). Only a limited number of antibiotics are effective for treatment of *M. abscessus* infections, including clarithromycin, kanamycin, amikacin, cefoxitin, and tigecycline (35, 36). Since most strains of *M. abscessus* are natively resistant or readily acquire resistance to multiple antibiotics, the doses of antibiotics used for prophylaxis or treatment of *M. abscessus* infections may be subinhibitory. This may make *M. abscessus* more virulent by the aforementioned mechanisms.

Subinhibitory doses of antibiotics may cause a wide variety of effects, such as increased mutation frequency, enhanced adhesion, and decreased protein secretion (1). Aminoglycosides are known to cause misreading errors during translation, leading to reduced growth rates (37), elongated cell morphology (38), and enhanced virulence in various bacteria. It has been shown that subinhibitory concentrations of tobramycin (an aminoglycoside) induce biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* (13). In *P. aeruginosa*, this effect is mediated by the aminoglycoside response regulator (*arr*), which regulates the levels of the second messenger cyclic di-GMP (c-di-GMP).

The results of this study might provide a guide for physicians in the treatment of *M. abscessus* infections. Sufficient concentrations of aminoglycoside antibiotics should be used for both prophylaxis and treatment. Investigations to determine whether subinhibitory concentrations of other nonaminoglycoside antibiotics also exacerbate the virulence of *M. abscessus* should be conducted, so that appropriate measures can be taken when these antibiotics are used.

ACKNOWLEDGMENTS

We thank Chao-Hung Lee for discussions and critical editing of the manuscript.

This work was supported by grants from the Ministry of Science and Technology (NSC102-2320-B-010-020-MY3) and the Ministry of Education (Aiming for the Top University Plan), Republic of China, to S.-T. Hu.

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