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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Mechanism of Macrolide-Induced Inhibition of Pneumolysin Release Involves Impairment of Autolysin Release in Macrolide-Resistant *Streptococcus pneumoniae*

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ABSTRACT *Streptococcus pneumoniae* is a leading cause of community-acquired pneumonia. Over the past 2 decades, macrolide resistance among *S. pneumoniae* organisms has been increasing steadily and has escalated at an alarming rate worldwide. However, the use of macrolides in the treatment of community-acquired pneumonia has been reported to be effective regardless of the antibiotic susceptibility of the causative pneumococci. Although previous studies suggested that sub-MICs of macrolides inhibit the production of the pneumococcal pore-forming toxin pneumolysin by macrolide-resistant *S. pneumoniae* (MRSP), the underlying mechanisms of the inhibitory effect have not been fully elucidated. Here, we show that the release of pneumococcal autolysin, which promotes cell lysis and the release of pneumolysin, was inhibited by treatment with azithromycin and erythromycin, whereas replenishing with recombinant autolysin restored the release of pneumolysin from MRSP. Additionally, macrolides significantly downregulated *ply* transcription followed by a slight decrease of the intracellular pneumolysin level. These findings suggest the mechanisms involved in the inhibition of pneumolysin in MRSP, which may provide an additional explanation for the benefits of macrolides on the outcome of treatment for pneumococcal diseases.

KEYWORDS autolysin, azithromycin, community-acquired pneumonia, erythromycin, macrolide-resistant *Streptococcus pneumoniae*, pneumolysin

Streptococcus pneumoniae is a common cause of community-acquired pneumonia (CAP), otitis media, and meningitis. Invasive infections by this bacterium continue to be a principal cause of morbidity and mortality worldwide (1, 2). In the early 2000s, several guidelines strongly recommended the use of macrolides for the empirical treatment of outpatients with CAP (3–5). Indeed, a meta-analysis of 28 observational studies showed that the use of macrolide for CAP patients was associated with a significant 18% relative reduction in mortality compared with that of nonmacrolide therapies (6). However, current guidelines recommend the use of amoxicillin (1st choice) or doxycycline (2nd choice) rather than macrolides (7, 8). The reason for this difference is an increased risk of infection with macrolide-resistant *S. pneumoniae*

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(MRSP), which is a result of the widespread use of macrolides (9). The resistance to macrolides is mainly mediated by two mechanisms via transposons (10, 11): (i) the modification of the ribosomal target by methylation, which is secondary to the acquisition of the *ermB* gene, and (ii) active drug efflux via a physiological pump on the cytoplasmic membrane, which is encoded by the *mefA* gene. Reportedly, the prevalence of MRSP is 27.9% in the United States. Among them, 29.1% and 80.9% of MRSP strains harbored *ermB* and *mefA* genes, respectively (12).

Although some studies have linked the increased prevalence of MRSP with treatment failure in CAP (13, 14), other studies have reported that the resistance against macrolides did not worsen the outcomes in patients hospitalized for pneumococcal pneumonia (15, 16). Additionally, macrolides have been reported to be effective in the treatment of CAP, even in patients with MRSP pneumonia (17, 18). One of the molecular mechanisms responsible for treatment success in these cases is thought to be the reduction of pneumolysin (PLY) production and release by sub-MICs of macrolides (19, 20).

S. pneumoniae possesses several virulence factors, including PLY and LytA autolysin, which contribute to the development of pneumococcal diseases (21). PLY is a potent intracellular toxin which induces ring-shaped pores in cholesterol-containing membranes and mediates cell death (22). In this regard, we previously reported that PLY induced neutrophilic cell lysis and the release of neutrophil elastase, which eventually disrupted pulmonary immune defenses (23). PLY may also interact with Toll-like receptor 4 and induce proinflammatory cytokines (24). LytA is responsible for the characteristic autolytic behavior associated with pneumococci, which subsequently contributes to the release of intracellular proteins (25). Therefore, the release of PLY into the extracellular space is highly dependent on LytA-induced autolysis in *S. pneumoniae* (23).

In this study, we investigated the *in vitro* effects of azithromycin (AZM) and erythromycin (ERY) against MRSP on the production and release of both PLY and LytA. We also analyzed the effect of these macrolides on the proliferation of MRSP.

RESULTS

Macrolides significantly decreased hemolytic activity in the supernatant of macrolide-treated MRSP. We first investigated the antimicrobial activity of macrolides against pneumococcal strains. Both AZM and ERY inhibited the growth of *S. pneumoniae* D39, with an MIC of $<$0.4 μg/ml. In contrast, AZM and ERY did not completely prevent the growth of *S. pneumoniae* NU4471, and the MICs of these antibiotics against *S. pneumoniae* NU4471 were $>$1,000 μg/ml, indicating that this strain is highly resistant to macrolides. It has been reported that PLY is responsible for more than 99.9% of the pneumococcal hemolytic activity (26). Therefore, to determine the inhibitory effect of macrolides on the production of PLY, we next investigated the hemolytic activity of the supernatant from AZM- or ERY-treated *S. pneumoniae* NU4471 against sheep erythrocytes. Consistent with the findings from other studies (19, 20), sub-MICs of AZM and ERY significantly decreased the hemolytic activity in the supernatant from *S. pneumoniae* NU4471 (Fig. 1).

Sub-MICs of macrolides retarded the growth of MRSP. Although 24-h incubation of MRSP with up to 250 μg/ml of macrolides did not affect the optical density at 600 nm (OD_{600}) of the bacterial culture, we considered the possibility that sub-MICs of macrolides exhibit inhibitory effects on the bacterial growth. Figure 2 shows that both AZM and ERY significantly extended the lag phase of MRSP compared with that of the untreated control, but all groups showed comparable growth rates. Additionally, treatment with 4 μg/ml of ERY induced a significantly prolonged lag phase compared with that of AZM-treated groups. These findings suggest that macrolides upregulate *ermA* or *mefA* gene transcription in the lag phase of MRSP followed by bacterial proliferation. Indeed, our data show that the *ermB* transcription level was significantly increased by AZM treatment in the early log phase (OD_{600} of 0.2) of MRSP, followed by a decrease in transcription (see Fig. S1A in the supplemental material). On the other

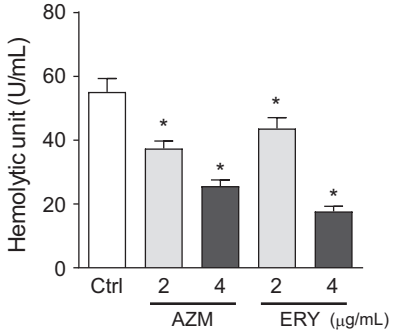

FIG 1 AZM and ERY treatment decreased hemolytic activity in the supernatants from macrolide-resistant *S. pneumoniae* (MRSP). MRSP strain NU4471 was inoculated in TS broth and cultured with various concentrations of AZM or ERY for 12 h at 37°C. The hemolytic activity in each cell-free supernatant sample was determined using sheep erythrocytes. Data represent the means ± SDs from triplicate experiments and were evaluated using one-way analysis of variance with Dunnett's multiple-comparison test. *, $P < 0.05$ versus Ctrl; AZM, azithromycin; Ctrl, control; ERY, erythromycin; SD, standard deviation; TS, tryptic soy.

hand, the *mefA* transcription level was significantly increased by both AZM- and ERY-treatment during the entire log phase (Fig. S1B and C).

Macrolides inhibited the release of PLY after excluding the influence of inhibitory effects on bacterial growth. These results suggested the possibility that the macrolide-induced prolonged lag phase results in the decrease of PLY protein level and hemolytic activity. To exclude macrolide-induced inhibition of bacterial growth, MRSP bacteria were incubated in the presence or absence of macrolides until they reached stationary phase (OD_{600} of 0.6 at incubation periods of 7, 8, 9, and 11 h in the untreated control group, the 2 to 4 μg/ml AZM group, the 2 μg/ml ERY group, and the 4 μg/ml ERY group, respectively), and then the supernatant samples were collected. Figure 3A shows that 4 μg/ml of AZM or ERY significantly decreased the hemolytic activity in the bacterial supernatant, whereas 2 μg/ml of AZM or ERY did not, suggesting that higher concentrations of macrolides decreased the hemolytic activity independently of the inhibitory effect on bacterial growth. Consistent with this, a downregulation of PLY was observed in the supernatant from macrolide-treated MRSP by Western blotting (Fig. 3B and C).

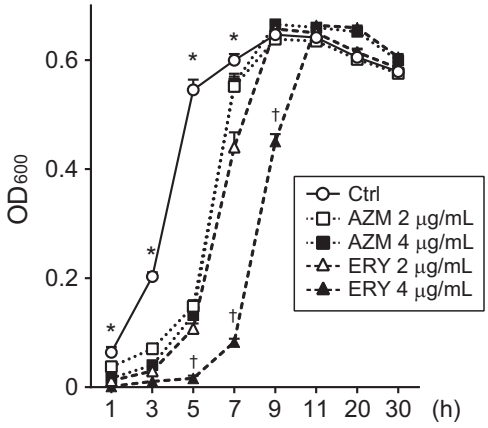

FIG 2 AZM and ERY treatment retarded the growth of MRSP. MRSP strain NU4471 was inoculated into TS broth and cultured with two concentrations of AZM or ERY at 37°C. The OD_{600} of each sample was then measured at each time point. Data represent the means ± SDs from quadruplicate experiments and were evaluated using a two-way analysis of variance with Tukey's multiple-comparison test. *, $P < 0.05$ versus all other macrolide-treated groups; †, $P < 0.05$ versus Ctrl and other macrolide-treated groups; AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD_{600}, optical density at 600 nm; SD, standard deviation; TS, tryptic soy.

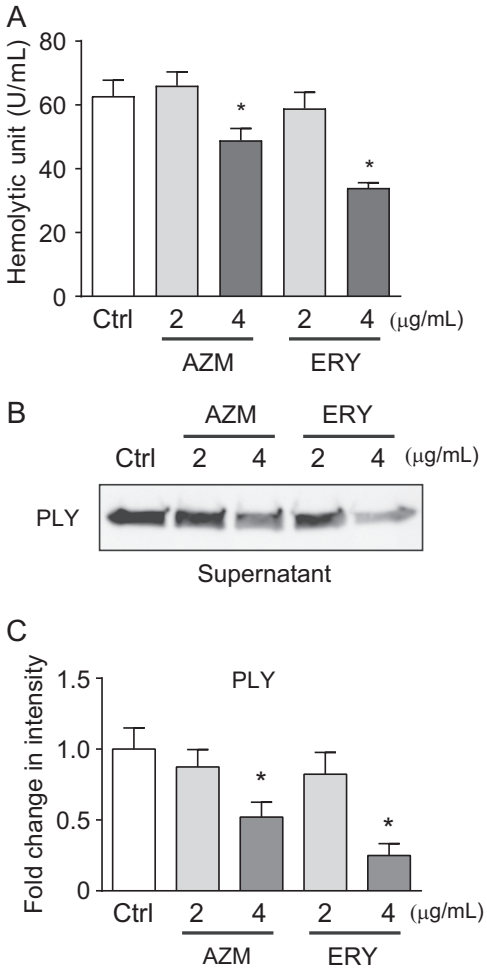

FIG 3 AZM and ERY inhibited the release of PLY and reduced the hemolytic activity in pneumococcal supernatants after excluding the influence of the inhibitory effects on bacterial growth. MRSP NU4471 cells were incubated in the presence or absence of AZM or ERY until they reached stationary phase (OD_{600} of 0.6 for incubation periods of 7, 8, 9, and 11 h for the untreated control group, the 2 to 4 μg/ml AZM group, the 2 μg/ml ERY group, and the 4 μg/ml ERY group, respectively). (A) The hemolytic activity in each cell-free supernatant sample was determined. (B) PLY protein levels in the culture supernatants from macrolide-treated pneumococci were determined by Western blotting with an anti-PLY antibody. (C) The intensity of Western blotting signals of PLY was quantified by densitometry software. Data represent the means ± SDs from triplicate experiments and were evaluated using a one-way analysis of variance with Dunnett's multiple-comparison test. *, $P < 0.05$ versus Ctrl; AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD_{600}, optical density at 600 nm; PLY, pneumolysin; SD, standard deviation.

We next examined the effects of tetracycline (TET) and linezolid (LZD), nonmacrolide protein synthesis inhibitors, on *S. pneumoniae* NU4471. TET and LZD inhibited the growth of *S. pneumoniae* NU4471 with MICs of 20 and 0.2 μg/ml, respectively. Consistent with the treatment with macrolides, treatment with sub-MICs of TET (2 μg/ml) and LZD (0.1 μg/ml) significantly extended the lag phase of MRSP (see Fig. S2A). However, TET and LZD did not decrease the hemolytic activity in the supernatant (Fig. S2B), indicating that these antibiotics do not inhibit PLY release from MRSP.

Macrolides inhibited the release of LytA and downregulated the leakage of PLY. Reportedly, LytA causes autolysis of pneumococci, which results in the leakage of other virulence components, such as PLY (27). Therefore, we further examined whether macrolides inhibit the release of LytA by Western blotting. Interestingly, LytA protein levels were significantly downregulated in the supernatant from MRSP treated with 4 μg/ml of AZM or ERY (Fig. 4A and B). The treatment with recombinant LytA significantly increased the hemolytic activity in the supernatant from macrolide-treated MRSP

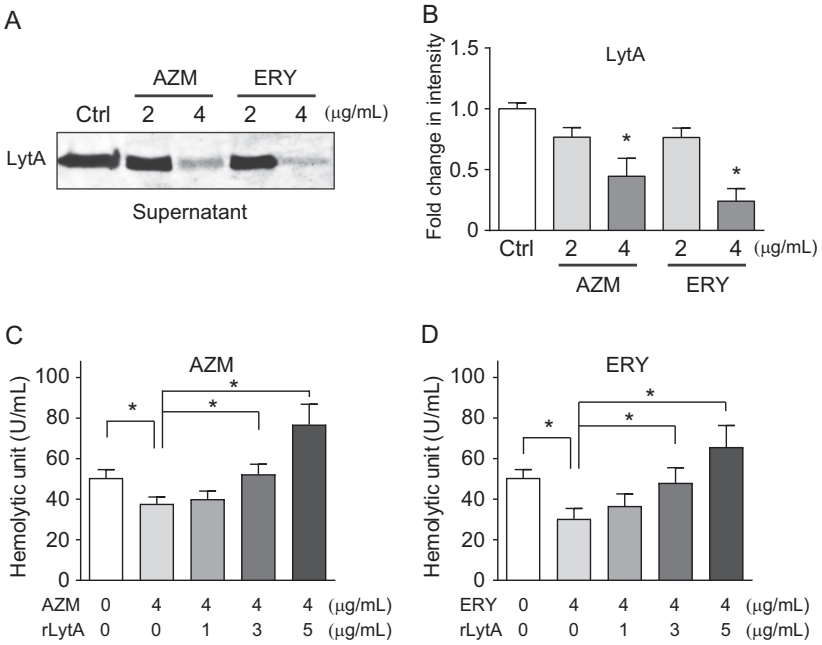

FIG 4 AZM and ERY reduced the release of PLY through the inhibition of LytA release. MRSP strain NU4471 was incubated in the presence or absence of AZM or ERY until stationary phase was reached (OD_{600} of 0.6). (A) LytA protein levels in the culture supernatants from macrolide-treated pneumococci were determined by Western blot analysis. (B) The intensity of Western blotting signals of LytA was quantified by densitometry. Data represent the means ± SDs from triplicate experiments and were evaluated using a one-way analysis of variance with Dunnett's multiple-comparison test. *, $P < 0.05$ versus Ctrl. MRSP strain NU4471 was incubated with 4 μg/ml of AZM (C) or 4 μg/ml of ERY (D) in the presence of various concentrations of recombinant LytA followed by the evaluation of hemolytic activity in the supernatants. Data represent the means ± SDs from quadruplicate experiments and were evaluated using one-way analyses of variance with Dunnett's multiple-comparison tests. *, $P < 0.05$ versus the macrolide-treated group in the absence of recombinant LytA; AZM, azithromycin; Ctrl, control; ERY, erythromycin; OD_{600}, optical density at 600 nm; PLY, pneumolysin; SD, standard deviation.

(Fig. 4C and D), suggesting that the inhibition of the extracellular release of PLY is caused by the downregulation of LytA-dependent autolysis. Additionally, 4 μg/ml AZM and ERY slightly but significantly decreased the intracellular PLY protein level in MRSP (Fig. 5A and B), which was due to a downregulation of *ply* gene transcription (Fig. 6A). However, AZM and ERY did not affect the intracellular LytA protein level or *lytA* gene transcription (Fig. 5A and C and 6B).

DISCUSSION

The results of this study provide intriguing insights into how macrolides mediate the downregulation of PLY in MRSP. Sub-MICs of macrolides reduce the leakage of PLY by two mechanisms: (i) an impairment of LytA release, which leads to the inhibition of autolysis, and (ii) a downregulation of *ply* gene transcription and subsequent protein production.

PLY is a member of the cholesterol-dependent cytolysins and is reportedly produced by all clinical isolates of *S. pneumoniae* (28). The binding to cholesterol-containing membranes results in oligomerization and membrane pore formation, and the subsequent cytolytic effects induce lung injury. It has also been reported that PLY activates the complement via the classical pathway. Both the cytolytic and complement-activating activities of PLY contribute to the pathogenesis of pneumococcal infections (29). An animal study has shown that an *S. pneumoniae* strain deficient in PLY shows impaired bacterial clearance (30). Together with our findings, a macrolide-dependent downregulation of PLY release may result in the reduced virulence of pneumococci. Consistent with this, it has been reported that macrolides reduced the PLY protein level in MRSP-infected murine lungs and improved the survival rates compared with those

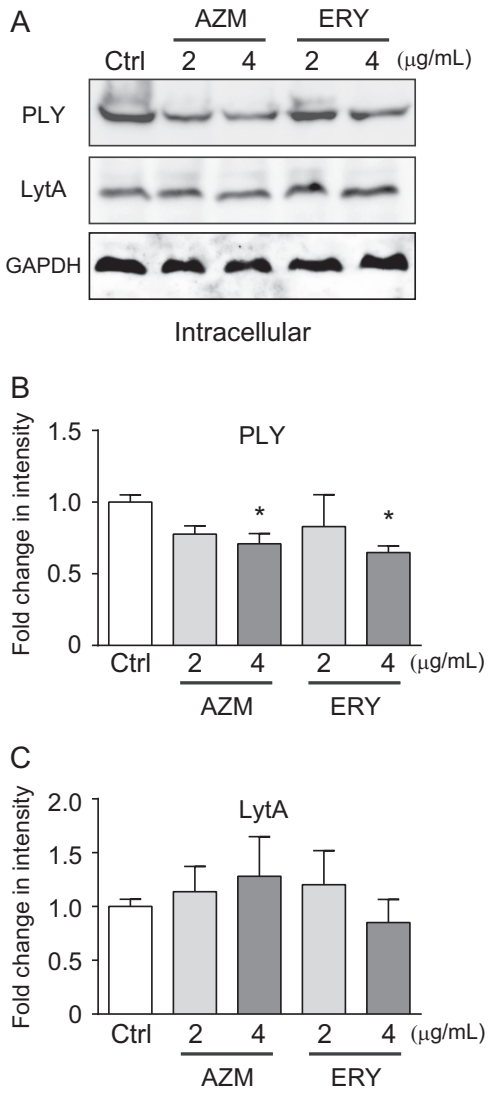

FIG 5 AZM and ERY slightly decreased the intracellular PLY level without affecting LytA level in MRSP. MRSP strain NU4471 was incubated in the presence or absence of AZM or ERY until the stationary phase. (A) Intracellular PLY and LytA protein levels in macrolide-treated pneumococcal cells were determined by Western blotting. The intensity of Western blotting signals of PLY (B) or LytA (C) was quantified by densitometry. Data represent the means ± SDs from triplicate experiments and were evaluated using one-way analyses of variance with Dunnett's multiple-comparison tests. *, $P < 0.05$ versus Ctrl; AZM, azithromycin; Ctrl, control; ERY, erythromycin; PLY, pneumolysin; SD, standard deviation.

of the control mice (19). Unlike other cholesterol-dependent cytolysins, PLY lacks an N-terminal secretion signal sequence, indicating that LytA-induced autolysis is required to release PLY. Therefore, we hypothesized that the macrolide-dependent downregulation of PLY release is caused by reduced LytA release.

LytA is a member of a widely distributed group of enzymes that degrade the peptidoglycan backbones of bacterial organisms. The action of LytA promotes cell lysis, which leads to pneumococcal cell death (31). In contrast, it has also been reported that encapsulated pneumococci survive in the presence of antimicrobial peptides by removing the capsule from the cell surface in a process dependent on LytA (32). Additionally, LytA plays a role in a variety of physiological cell functions associated with cell wall growth, its turnover, and cell separation in pneumococci (28). In addition to the release of PLY, LytA contributes to the release of intracellular virulence factors, which induce the production of proinflammatory cytokines in macrophages via Toll-like receptor 4 (33). Mutations of the *lytA* gene in *S. pneumoniae* lead to a significantly

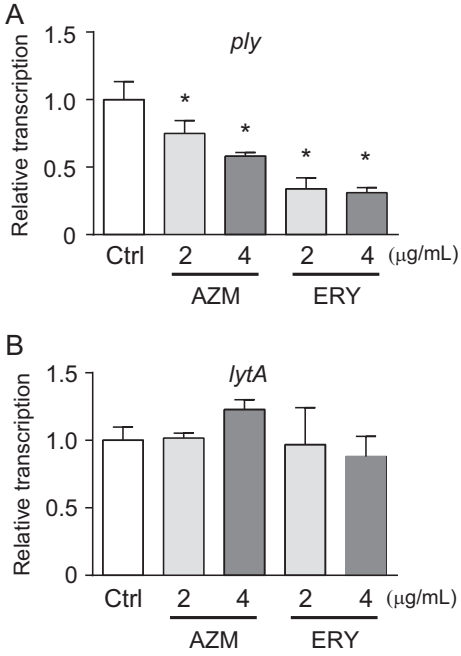

FIG 6 AZM and ERY decreased *ply* gene transcription without affecting *lytA* gene transcription in MRSP. MRSP strain NU4471 was incubated with 2 and 4 μg/ml of AZM or ERY for 2 h at 37°C. Real-time PCR was performed to quantify transcription levels of *ply* (A) and *lytA* (B) in the strain. The relative quantities of these genes were normalized to the relative quantity of 16S rRNA. Data represent the means ± SDs from triplicate experiments and were evaluated using one-way analyses of variance with Dunnett's multiple-comparison tests. *, $P < 0.05$ versus Ctrl; AZM, azithromycin; Ctrl, control; ERY, erythromycin; SD, standard deviation.

decreased virulence of this organism compared to that of the wild-type strain in a mouse intraperitoneal infection model (34). Although it has been reported that the combination treatment of multidrug-resistant pneumococcus with both levofloxacin and ceftriaxone induces a slight reduction in *lytA* gene transcription (35), this is, to our knowledge, the first report to identify the inhibitory effect of macrolides on the release of LytA protein, leading to the inhibition of PLY release. Additional effects resulting from the inhibition of LytA release should be addressed in future studies.

Different types of macrolide resistance are reported in streptococcal strains: (i) an inducible resistant type, which manifests as immediate logarithmic growth in medium containing a high concentration of macrolides only after previous exposure of the organisms to sub-MICs of macrolides, and (ii) the constitutive resistant type, which demonstrates continued logarithmic growth in medium containing a high concentration of macrolides without prior drug exposure (36). Subsequent studies have shown that the expression of *ermB* is inducible by common 14- and 15-member macrolides, such as AZM and ERY (37). Additionally, Li et al. demonstrated that macrolides increase ermB protein expression in *Streptococcus gallolyticus* subsp. *pasteurianus*, which confers high macrolide resistance (38). Furthermore, macrolides also upregulate *mef* gene transcription in *S. pneumoniae*, resulting in increased resistance to macrolides (39, 40). These findings suggest that the initial exposure to sub-MICs of macrolides increases the expression of antimicrobial resistance genes in the inducible resistant type of streptococci, which results in bacterial proliferation in media containing high concentrations of the drug. In this study, we demonstrated that sub-MICs of macrolides upregulated the transcription of macrolide resistance genes and retarded the growth of MRSP followed by pneumococcal regrowth, indicating that *S. pneumoniae* NU4471 is of the inducible resistant type. On the other hand, AZM treatment significantly increased both *ermB* and *mefA* transcription levels, whereas ERY treatment only increased *mefA* transcription in *S. pneumoniae* NU4471. These results suggest the mechanism by which the

ERY-induced prolonged lag phase of *S. pneumoniae* NU4471 involves a reduced inducibility of *ermB* transcription compared to that by AZM treatment. Further research is thus needed to elucidate the relationship between the induction of these resistance genes by macrolides and bacterial proliferation in culture medium supplemented with the drug.

Consistent with our current results, several studies have shown that macrolides inhibit PLY protein expression in MRSP. Lagrou et al. observed that ERY significantly reduced the adherence of a highly macrolide-resistant *S. pneumoniae* against human respiratory epithelial cells in cell culture medium, which was accompanied by an almost complete prevention of PLY release (41). Also, Anderson et al. reported that sub-MICs of clarithromycin inhibit the production of PLY not only in MRSP strains, including *mefA*-positive and *ermB*-negative strains and *mefA*-negative and *ermB*-positive strains, but also in macrolide-susceptible strains (20). Additionally, some clinical studies have demonstrated the remarkable clinical efficacy of macrolides in the treatment of CAP regardless of the *in vitro* susceptibility of the causative pneumococci (17, 18). However, another clinical study reported a relationship between AZM therapy and lower clinical cure rates in patients with acute bacterial sinusitis or acute otitis media caused by MRSP, whereas no difference in the clinical cure rates was observed between CAP patients infected with AZM-susceptible (94.2%) and AZM-resistant (92.6%) *S. pneumoniae* (42). To discuss the clinical effectiveness of macrolides on MRSP infections, the immunomodulatory effects of macrolides, which include the modulation of inflammatory cell function, the modulation of epithelial cell function, improved mucociliary clearance, and an attenuation of the inflammatory response through the nitric oxide pathway (43), may also be taken into consideration, because these antibiotics are effective against noninfectious autoimmune conditions, such as diffuse panbronchiolitis (44). Further clinical studies are needed to evaluate the efficacy of macrolides on MRSP infection.

It was largely assumed that macrolides indiscriminately inhibit the production of all cellular polypeptides by preventing the nascent chain egress through the ribosomal exit tunnel, thereby blocking translation (45). However, two recent studies demonstrated that 100- to 200-fold MICs of macrolide selectively induce translation arrest of certain proteins in *Escherichia coli* and *Staphylococcus aureus* (46, 47). The prevalent motif at the sites of ERY-induced translation arrest conformed to the consensus [R/K]X[R/K] and XP. Although macrolides downregulated *ply* gene transcription and decreased the PLY protein level in MRSP cells in this study, it is also possible that macrolides specifically inhibit the translation of the *ply* gene at an early stage due to the $RK_{49}K$ motif. As for the *lytA* gene, the treatment of *S. pneumoniae* with ERY may inhibit the translation of the gene at an intermediate stage at the $KD_{187}K$ motif. Reportedly, the C-terminal choline-binding domain of LytA, consisting of residues V_{188} to K_{318} (48), enables the enzyme to bind the pneumococcal cell wall, which is required for autolysis (49). In this study, macrolide treatment inhibited the release of LytA from MRSP without affecting intracellular LytA protein levels. Macrolide-induced translation arrest of the *lytA* gene might thus result in the loss of LytA activity. Interestingly, Kannan et al. showed that the translation of some genes responded differently to macrolide presence in living *E. coli* cells or in the cell-free translation system (46). This indicates that additional factors may stimulate or interfere with the action of macrolides. Therefore, it is possible that macrolide-targeting proteins vary among different bacterial species. Currently, no study has investigated whether sub-MICs of macrolides induce site-specific arrest of translation. To clarify these issues, a ribosome profiling analysis in *S. pneumoniae* treated with sub-MICs of macrolides needs to be performed.

In conclusion, our study has demonstrated that sub-MICs of AZM and ERY reduce the release of PLY in a highly macrolide-resistant *S. pneumoniae* through mechanisms that include the inhibition of autolysis and the downregulation of *ply* gene transcription, which may provide an additional explanation for the benefits of macrolides on the outcome of treatment for pneumococcal diseases.

TABLE 1 Primer sequences for real-time PCR

Target	Primer direction	Sequence (5′→3′)
ply	Forward	AGCGATAGCTTTCTCCAAGTGG
	Reverse	CTTAGCCAACAAATCGTTTACCG
lytA	Forward	AGTTTAAGCATGATATTGAGAAC
	Reverse	TTCGTTGAAATAGTACCACTTAT
16S rRNA	Forward	TGAGGTAACCGTAAGGAGCCA
	Reverse	TCACCCCAATCATCTATCCCA

MATERIALS AND METHODS

Bacterial culture and reagents. A clinical isolate of MRSP, NU4471 (serotype 19), which harbors both *ermB* and *mefA* genes (19), or macrolide-susceptible *S. pneumoniae* strain D39 (NCTC 7466) was grown at 37°C in tryptic soy (TS) broth (Becton Dickinson, Franklin Lakes, NJ, USA) under aerobic conditions. Recombinant LytA protein was kindly provided by Yuuki Sakaue (Niigata University, Niigata, Japan). AZM and ERY were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Each macrolide was dissolved in ethanol and diluted further with sterile water. Antibodies against pneumococcal LytA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were generated by Eurofins Genomics K.K. (Tokyo, Japan) as described previously (31).

MICs of macrolides against MRSP. The inhibitory activities of macrolides against bacterial growth were examined using 96-well plates (Corning, Corning, NY, USA). Two-microliter aliquots of *S. pneumoniae* NU4471 grown to the exponential phase were inoculated in 200 μl of TS broth. AZM and ERY were separately added to these bacterial cultures and incubated at 37°C for 24 h. The inhibitory activity against bacterial growth was measured at a wavelength of 620 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Effect of macrolides on the growth of MRSP. Fifty-microliter aliquots of *S. pneumoniae* NU4471 grown to the exponential phase were inoculated in 5 ml of TS broth. Thereafter, ethanol (as a control) or 2 and 4 μg/ml of AZM and ERY were separately added to these bacterial cultures and incubated at 37°C. At each time point, bacterial growth was measured at a wavelength of 600 nm using a Mini Photo 518R (Taitec, Tokyo, Japan).

Hemolytic assay. Bacterial cultures, as described above, were incubated at 37°C for 12 h or until they reached early stationary phase (OD_{600} of 0.6). Thereafter, bacterial supernatants were collected by centrifugation at 3,000 $\times g$ for 10 min and subsequently filtered (0.22-μm filter; Merck Millipore, Billerica, MA, USA). The hemolytic unit was then determined using fresh sheep erythrocytes as described previously (50). A hemolytic unit is defined as the dilution that causes 50% lysis of 1% sheep erythrocyte suspension after 30 min at 37°C (51).

Western blot analysis. Ethanol (as a control) and 2 and 4 μg/ml of AZM and ERY were separately added to pneumococcal cultures and incubated until they reached early stationary phase. Thereafter, bacterial supernatants were collected as described above. The bacterial pellets were resuspended in 2% SDS sample buffer and homogenized with a MagNA Lyser instrument (Roche Diagnostics, Basel, Switzerland) using 0.1-mm silica beads in a 2-ml tube (MP Biomedicals, Santa Ana, CA, USA). The whole-cell lysates or pneumococcal supernatants mixed with SDS sample buffer were heated at 95°C for 3 min, separated by SDS-PAGE using 12% gels (Bio-Rad Laboratories, Hercules, CA, USA), and transferred to polyvinylidene difluoride membranes (Merck Millipore). The membranes were incubated with blocking reagent (Nacalai Tesque, Kyoto, Japan) to block nonspecific binding and probed with the anti-PLY antibody (Abcam, Cambridge, UK), anti-LytA antibody, and anti-GAPDH antibody diluted in Tris-buffered saline containing 0.05% Tween 20 (TaKaRa, Shiga, Japan). The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) in Tris-buffered saline containing 0.05% Tween 20. The membrane was treated with HRP substrates (GE Healthcare, Little Chalfont, UK) and analyzed by a chemiluminescence detector (Fujifilm, Tokyo, Japan). The intensity of the signal was quantified using Image Studio software (LI-COR Bioscience, Lincoln, NE, USA).

Quantification of *ply* and *lytA* gene transcription by real-time PCR. *S. pneumoniae* NU4471 was inoculated into TS broth and cultured until it reached exponential growth phase (OD_{600} of 0.3) followed by incubation with 2 and 4 μg/ml of AZM or ERY for 2 h at 37°C. Gene transcription in the pneumococcal strain was quantified using quantitative real-time PCR. Briefly, the bacterial pellet was resuspended in TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and homogenized with a MagNA Lyser instrument using 0.1-mm silica beads in a 2-ml tube followed by RNA isolation using a Direct-zol RNA kit (Zymo Research, Irvine, CA, USA). The RNA was reverse transcribed using SuperScript VILO master mix (Thermo Fisher Scientific), and quantitative real-time PCR with cDNA was performed with the StepOnePlus real-time PCR system (Thermo Fisher Scientific) with the use of SYBR green detection protocol according to the manufacturer's instructions. The primers used for real-time PCR are shown in Table 1.

Statistical analysis. Data were analyzed statistically by analyses of variance with Dunnett's or Tukey's multiple-comparison tests using Graph Pad Prism software version 6.05 (GraphPad Software, Inc., La Jolla, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00161-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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The authors declare no competing financial interests.

REFERENCES

1. Kalin M, Örtqvist Å Almela M, Aufwerber E, Dwyer R, Henriques B, Jorup C, Julander I, Marrie TJ, Mufson MA, Riquelme R, Thalme A, Torres A, Woodhead MA. 2000. Prospective study of prognostic factors in community-acquired bacteremic pneumococcal disease in 5 countries. J Infect Dis 182:840–847. https://doi.org/10.1086/315760.
2. Stanek RJ, Mufson MA. 1999. A 20-year epidemiological study of pneumococcal meningitis. Clin Infect Dis 28:1265–1272. https://doi.org/10.1086/514777.
3. Niederman MS, Mandell LA, Anzueto A, Bass JB, Broughton WA, Campbell GD, Dean N, File T, Fine MJ, Gross PA, Martinez F, Marrie TJ, Plouffe JF, Ramirez J, Sarosi GA, Torres A, Wilson R, Yu VL, American Thoracic Society. 2001. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. Am J Respir Crit Care Med 163:1730–1754.
4. Bartlett JG, Dowell SF, Mandell LA, File TM, Jr, Musher DM, Fine MJ. 2000. Practice guidelines for the management of community-acquired pneumonia in adults. Infectious Diseases Society of America. Clin Infect Dis 31:347–382. https://doi.org/10.1086/313954.
5. Heffelfinger JD, Dowell SF, Jorgensen JH, Klugman KP, Mabry LR, Musher DM, Plouffe JF, Rakowsky A, Schuchat A, Whitney CG. 2000. Management of community-acquired pneumonia in the era of pneumococcal resistance: a report from the Drug-Resistant *Streptococcus pneumoniae* Therapeutic Working Group. Arch Intern Med 160:1399–1408. https://doi.org/10.1001/archinte.160.10.1399.
6. Sligl WI, Asadi L, Eurich DT, Tjosvold L, Marrie TJ, Majumdar SR. 2014. Macrolides and mortality in critically ill patients with community-acquired pneumonia: a systematic review and meta-analysis. Crit Care Med 42:420–432. https://doi.org/10.1097/CCM.0b013e3182a66b9b.
7. Wiersinga WJ, Bonten MJ, Boersma WG, Jonkers RE, Aleva RM, Kullberg BJ, Schouten JA, Degener JE, Janknegt R, Verheij TJ, Sachs AP, Prins JM, Dutch Working Party on Antibiotic Policy, Dutch Association of Chest Physicians. 2012. SWAB/NVALT (Dutch Working Party on Antibiotic Policy and Dutch Association of Chest Physicians) guidelines on the management of community-acquired pneumonia in adults. Neth J Med 70:90–101.
8. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le Jeune I, Macfarlane JT, Read RC, Roberts HJ, Levy ML, Wani M, Woodhead MA, Pneumonia Guidelines Committee of the BTS Standards of Care Committee. 2009. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax 64 Suppl 3:iii1–iii55. https://doi.org/10.1136/thx.2009.121434.
9. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, Dowell SF, File TM, Jr, Musher DM, Niederman MS, Torres A, Whitney CG, Infectious Diseases Society of America, American Thoracic Society. 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 44 Suppl 2:S27–S72. https://doi.org/10.1086/511159.
10. Lynch JP, III, Martinez FJ. 2002. Clinical relevance of macrolide-resistant *Streptococcus pneumoniae* for community-acquired pneumonia. Clin Infect Dis 34 Suppl 1:S27–S46. https://doi.org/10.1086/324527.
11. Schroeder MR, Stephens DS. 2016. Macrolide resistance in *Streptococcus pneumoniae*. Front Cell Infect Microbiol 6:98. https://doi.org/10.3389/fcimb.2016.00098.
12. Farrell DJ, Jenkins SG. 2004. Distribution across the USA of macrolide resistance and macrolide resistance mechanisms among *Streptococcus pneumoniae* isolates collected from patients with respiratory tract infections: PROTEKT US 2001–2002. J Antimicrob Chemother 54 Suppl 1:i17–i22. https://doi.org/10.1093/jac/dkh312.
13. Lonks JR, Garau J, Gomez L, Xercavins M, Ochoa de Echagüen A, Gareen IF, Reiss PT, Medeiros AA. 2002. Failure of macrolide antibiotic treatment in patients with bacteremia due to erythromycin-resistant *Streptococcus pneumoniae*. Clin Infect Dis 35:556–564. https://doi.org/10.1086/341978.
14. Fogarty C, Goldschmidt R, Bush K. 2000. Bacteremic pneumonia due to multidrug-resistant pneumococci in 3 patients treated unsuccessfully with azithromycin and successfully with levofloxacin. Clin Infect Dis 31:613–615. https://doi.org/10.1086/313976.
15. Cilloniz C, Albert RK, Liapikou A, Gabarrus A, Rangel E, Bello S, Marco F, Mensa J, Torres A. 2015. The effect of macrolide resistance on the presentation and outcome of patients hospitalized for *Streptococcus pneumoniae* pneumonia. Am J Respir Crit Care Med 191:1265–1272. https://doi.org/10.1164/rccm.201502-0212OC.
16. Ewig S, Ruiz M, Torres A, Marco F, Martinez JA, Sanchez M, Mensa J. 1999. Pneumonia acquired in the community through drug-resistant *Streptococcus pneumoniae*. Am J Respir Crit Care Med 159:1835–1842. https://doi.org/10.1164/ajrccm.159.6.9808049.
17. Yanagihara K, Izumikawa K, Higa F, Tateyama M, Tokimatsu I, Hiramatsu K, Fujita J, Kadota J, Kohno S. 2009. Efficacy of azithromycin in the treatment of community-acquired pneumonia, including patients with macrolide-resistant *Streptococcus pneumoniae* infection. Intern Med 48:527–535. https://doi.org/10.2169/internalmedicine.48.1482.
18. Kohno S, Tateda K, Kadota J, Fujita J, Niki Y, Watanabe A, Nagashima M. 2014. Contradiction between *in vitro* and clinical outcome: intravenous followed by oral azithromycin therapy demonstrated clinical efficacy in macrolide-resistant pneumococcal pneumonia. J Infect Chemother 20:199–207. https://doi.org/10.1016/j.jiac.2013.10.010.
19. Fukuda Y, Yanagihara K, Higashiyama Y, Miyazaki Y, Hirakata Y, Mukae H, Tomono K, Mizuta Y, Tsukamoto K, Kohno S. 2006. Effects of macrolides on pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. Eur Respir J 27:1020–1025. https://doi.org/10.1183/09031936.06.00116805.
20. Anderson R, Steel HC, Cockeran R, Smith AM, von Gottberg A, de Gouveia L, Brink A, Klugman KP, Mitchell TJ, Feldman C. 2007. Clarithromycin alone and in combination with ceftriaxone inhibits the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of *Streptococcus pneumoniae*. J Antimicrob Chemother 59:224–229. https://doi.org/10.1093/jac/dkl479.
21. Morales M, Martín-Galiano AJ, Domenech M, García E. 2015. Insights into the evolutionary relationships of LytA autolysin and Ply pneumolysin-like genes in *Streptococcus pneumoniae* and related streptococci. Genome Biol Evol 7:2747–2761. https://doi.org/10.1093/gbe/evv178.
22. Tilley SJ, Orlova EV, Gilbert RJ, Andrew PW, Saibil HR. 2005. Structural basis of pore formation by the bacterial toxin pneumolysin. Cell 121:247–256. https://doi.org/10.1016/j.cell.2005.02.033.
23. Domon H, Oda M, Maekawa T, Nagai K, Takeda W, Terao Y. 2016. *Streptococcus pneumoniae* disrupts pulmonary immune defence via elastase release following pneumolysin-dependent neutrophil lysis. Sci Rep 6:38013. https://doi.org/10.1038/srep38013.
24. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. 2003. Recognition of

pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci U S A 100:1966–1971. https://doi.org/10.1073/pnas.0435928100.
25. Balachandran P, Hollingshead SK, Paton JC, Briles DE. 2001. The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. J Bacteriol 183:3108–3116. https://doi.org/10.1128/JB.183.10.3108-3116.2001.
26. Berry AM, Alexander JE, Mitchell TJ, Andrew PW, Hansman D, Paton JC. 1995. Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*. Infect Immun 63:1969–1974.
27. Mitchell TJ, Alexander JE, Morgan PJ, Andrew PW. 1997. Molecular analysis of virulence factors of *Streptococcus pneumoniae*. J Appl Microbiol 83:62S–71S. https://doi.org/10.1046/j.1365-2672.83.s1.7.x.
28. Cockeran R, Anderson R, Feldman C. 2003. Pneumolysin in the immunopathogenesis and treatment of pneumococcal disease. Expert Rev Anti Infect Ther 1:231–239. https://doi.org/10.1586/14787210.1.2.231.
29. Marriott HM, Mitchell TJ, Dockrell DH. 2008. Pneumolysin: a double-edged sword during the host-pathogen interaction. Curr Mol Med 8:497–509. https://doi.org/10.2174/156652408785747924.
30. Hirst RA, Gosai B, Rutman A, Guerin CJ, Nicotera P, Andrew PW, O'Callaghan C. 2008. *Streptococcus pneumoniae* deficient in pneumolysin or autolysin has reduced virulence in meningitis. J Infect Dis 197:744–751. https://doi.org/10.1086/527322.
31. Jedrzejas MJ. 2001. Pneumococcal virulence factors: structure and function. Microbiol Mol Biol Rev 65:187–207. https://doi.org/10.1128/MMBR.65.2.187-207.2001.
32. Kietzman CC, Gao G, Mann B, Myers L, Tuomanen EI. 2016. Dynamic capsule restructuring by the main pneumococcal autolysin LytA in response to the epithelium. Nat Commun 7:10859. https://doi.org/10.1038/ncomms10859.
33. Nagai K, Domon H, Maekawa T, Oda M, Hiyoshi T, Tamura H, Yonezawa D, Arai Y, Yokoji M, Tabeta K, Habuka R, Saitoh A, Yamaguchi M, Kawabata S, Terao Y. 2018. Pneumococcal DNA-binding proteins released through autolysis induce the production of proinflammatory cytokines via Toll-like receptor 4. Cell Immunol 325:14–22. https://doi.org/10.1016/j.cellimm.2018.01.006.
34. Berry AM, Paton JC. 2000. Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. Infect Immun 68:133–140. https://doi.org/10.1128/IAI.68.1.133-140.2000.
35. Majhi A, Adhikary R, Bhattacharyya A, Mahanti S, Bishayi B. 2014. Levofloxacin-ceftriaxone combination attenuates lung inflammation in a mouse model of bacteremic pneumonia caused by multidrug-resistant *Streptococcus pneumoniae* via inhibition of cytolytic activities of pneumolysin and autolysin. Antimicrob Agents Chemother 58:5164–5180. https://doi.org/10.1128/AAC.03245-14.
36. Hyder SL, Streitfeld MM. 1973. Inducible and constitutive resistance to macrolide antibiotics and lincomycin in clinically isolated strains of *Streptococcus pyogenes*. Antimicrob Agents Chemother 4:327–331. https://doi.org/10.1128/AAC.4.3.327.
37. Leclercq R, Courvalin P. 1991. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob Agents Chemother 35:1267–1272. https://doi.org/10.1128/AAC.35.7.1267.
38. Li M, Cai C, Chen J, Cheng C, Cheng G, Hu X, Liu C. 2016. Inducible expression of both *ermB* and *ermT* conferred high macrolide resistance in *Streptococcus gallolyticus* subsp. *pasteurianus* isolates in China. Int J Mol Sci 17:1599. https://doi.org/10.3390/ijms17101599.
39. Wierzbowski AK, Boyd D, Mulvey M, Hoban DJ, Zhanel GG. 2005. Expression of the *mef*(E) gene encoding the macrolide efflux pump protein increases in *Streptococcus pneumoniae* with increasing resistance to macrolides. Antimicrob Agents Chemother 49:4635–4640. https://doi.org/10.1128/AAC.49.11.4635-4640.2005.
40. Ambrose KD, Nisbet R, Stephens DS. 2005. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. Antimicrob Agents Chemother 49:4203–4209. https://doi.org/10.1128/AAC.49.10.4203-4209.2005.
41. Lagrou K, Peetermans WE, Jorissen M, Verhaegen J, Van Damme J, Van Eldere J. 2000. Subinhibitory concentrations of erythromycin reduce pneumococcal adherence to respiratory epithelial cells *in vitro*. J Antimicrob Chemother 46:717–723. https://doi.org/10.1093/jac/46.5.717.
42. Zhanel GG, Wolter KD, Calciu C, Hogan P, Low DE, Weiss K, Karlowsky JA. 2014. Clinical cure rates in subjects treated with azithromycin for community-acquired respiratory tract infections caused by azithromycin-susceptible or azithromycin-resistant *Streptococcus pneumoniae*: analysis of phase 3 clinical trial data. J Antimicrob Chemother 69:2835–2840. https://doi.org/10.1093/jac/dku207.
43. Emmet O'Brien M, Restrepo MI, Martin-Loeches I. 2015. Update on the combination effect of macrolide antibiotics in community-acquired pneumonia. Respir Investig 53:201–209. https://doi.org/10.1016/j.resinv.2015.05.003.
44. Kudoh S, Azuma A, Yamamoto M, Izumi T, Ando M. 1998. Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. Am J Respir Crit Care Med 157:1829–1832. https://doi.org/10.1164/ajrccm.157.6.9710075.
45. Vázquez D. 1979. Inhibitors of protein biosynthesis. Molecular biology biochemistry and biophysics, vol 30. Springer-Verlag, Berlin, Germany.
46. Kannan K, Kanabar P, Schryer D, Florin T, Oh E, Bahroos N, Tenson T, Weissman JS, Mankin AS. 2014. The general mode of translation inhibition by macrolide antibiotics. Proc Natl Acad Sci U S A 111:15958–15963. https://doi.org/10.1073/pnas.1417334111.
47. Davis AR, Gohara DW, Yap MN. 2014. Sequence selectivity of macrolide-induced translational attenuation. Proc Natl Acad Sci U S A 111:15379–15384. https://doi.org/10.1073/pnas.1410356111.
48. Fernández-Tornero C, López R, García E, Giménez-Gallego G, Romero A. 2001. A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. Nat Struct Biol 8:1020–1024. https://doi.org/10.1038/nsb724.
49. Giudicelli S, Tomasz A. 1984. Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. J Bacteriol 158:1188–1190.
50. Paton JC, Lock RA, Hansman DJ. 1983. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. Infect Immun 40:548–552.
51. Cheung AL, Chien YT, Bayer AS. 1999. Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*. Infect Immun 67:1331–1337.