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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

KKL-35 Exhibits Potent Antibiotic Activity against *Legionella* Species Independently of *trans*-Translation Inhibition

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ABSTRACT *trans*-Translation is a ribosome-rescue system that is ubiquitous in bacteria. Small molecules defining a new family of oxadiazole compounds that inhibit *trans*-translation have been found to have broad-spectrum antibiotic activity. We sought to determine the activity of KKL-35, a potent member of the oxadiazole family, against the human pathogen *Legionella pneumophila* and other related species that can also cause Legionnaires' disease (LD). Consistent with the essential nature of *trans*-translation in *L. pneumophila*, KKL-35 inhibited the growth of all tested strains at submicromolar concentrations. KKL-35 was also active against other LD-causing *Legionella* species. KKL-35 remained equally active against *L. pneumophila* mutants that have evolved resistance to macrolides. KKL-35 inhibited the multiplication of *L. pneumophila* in human macrophages at several stages of infection. No resistant mutants could be obtained, even during extended and chronic exposure. Surprisingly, KKL-35 was not synergistic with other ribosome-targeting antibiotics and did not induce the filamentation phenotype observed in cells defective for *trans*-translation. Importantly, KKL-35 remained active against *L. pneumophila* mutants expressing an alternate ribosome-rescue system and lacking transfer-messenger RNA, the essential component of *trans*-translation. These results indicate that the antibiotic activity of KKL-35 is not related to the specific inhibition of *trans*-translation and its mode of action remains to be identified. In conclusion, KKL-35 is an effective antibacterial agent against the intracellular pathogen *L. pneumophila* with no detectable resistance development. However, further studies are needed to better understand its mechanism of action and to assess further the potential of oxadiazoles in treatment.

KEYWORDS *Legionella*, *trans*-translation

Legionella pneumophila is a ubiquitous freshwater bacterium that infects a wide spectrum of environmental protozoans. Human-made systems, such as sanitary water networks and air-cooling towers, can disseminate contaminated water through aerosolization. The breathing of microscopic droplets contaminated with *L. pneumophila* can lead to infection of alveolar macrophages and development of a life-threatening pneumonia called Legionnaires' disease (LD) or legionellosis. LD remains an important cause of both morbidity and mortality in Europe, with over 6,900 cases being reported in 2014 (1). Guidelines for the management of LD recommend the use of macrolides (with a preference for azithromycin) or fluoroquinolones (levofloxacin or moxifloxacin) to treat the infection (2, 3). Despite a rapid diagnosis and the correct administration of antibiotics, the death rate among those with LD is over 10% (4). *L.*

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pneumophila isolates are considered susceptible to macrolides and fluoroquinolones (5), but mutants resistant to both antibiotic families can easily be obtained *in vitro*, suggesting that resistant strains may emerge during treatment (6–8). Indeed, the acquisition of resistance to fluoroquinolones during the course of fluoroquinolone therapy has recently been reported (9, 10). New compounds that are active against *L. pneumophila* strains resistant to fluoroquinolones and macrolides or that could potentiate these existing treatments may improve the outcome of the disease.

trans-Translation has recently been proposed to be a novel target for the development of a new class of antibiotics (11). *trans*-Translation is the primary bacterial mechanism used to resolve ribosome stalling in bacteria (12–14). Ribosome stalling can be induced by translation of an mRNA lacking a stop codon (non-stop mRNA) or when ribosomes pause before the stop codon is read (i.e., due to ribosome-targeting antibiotics, rare sense codon stretches, a lack of necessary tRNAs, etc). Ribosome stalling is a life-threatening issue in metabolically active bacteria (15, 16). *trans*-Translation is operated by a highly conserved nucleoprotein complex (17) encoded by two genes: *ssrA*, encoding a highly expressed and structured RNA called transfer-messenger RNA (tmRNA) (18, 19), and *smpB*, encoding a small protein involved in the specific recognition and loading of tmRNA in stalled ribosomes (20–22). Once the complex is loaded into the free A site of the stalled ribosome, translation resumes using the coding section of the tmRNA as the template. This messenger section of tmRNA encodes a degradation tag that is appended to the unfinished polypeptide, targeting it to different proteases (23–25). The coding section of tmRNA ends with a stop codon, allowing the normal termination of translation and dissociation of the ribosomal subunits. In addition, the tmRNA-SmpB complex interacts with RNase R to degrade the faulty mRNA (26, 27). Thus, in addition to resolving ribosome stalling, the *trans*-translation system prevents the rise of further problems by promoting the degradation of both the problematic mRNA and the aborted polypeptide (28).

Alternative ribosome-rescue systems have been identified in *Escherichia coli* and named ArfA and ArfB (alternative rescue factors A and B, respectively) (29–31). Both ArfA and ArfB can partially complement the loss of *trans*-translation by promoting the dissociation of the stalled ribosome but lack the mechanisms to trigger degradation of the aborted polypeptide and faulty mRNA (12). These appear to be less conserved than the tmRNA-SmpB system (15). *trans*-Translation is essential in species lacking alternative mechanisms (16). In agreement with these observations, alternative ribosome-rescue systems are absent in members of the *Legionellaceae* family, and we indeed found that *trans*-translation is essential for *L. pneumophila* growth and infection of its cellular host (32). In *L. pneumophila*, expression of the alternate rescue factor ArfA from *E. coli* can compensate for the loss of *trans*-translation activity, indicating that the ribosome-dissociating activity of the *trans*-translation system is the sole function required for viability (32). Because it is essential for viability in multiple pathogens, the *trans*-translation system has been proposed to be a valid yet unexplored target for a new class of antibiotics (11).

A high-throughput screen using an *in vivo* assay of *trans*-translation recently identified a family of small molecules able to inhibit *trans*-translation at micromolar concentrations (33). One of the most active compounds, KKL-35, was found to exhibit bactericidal activity against several pathogenic bacterial species in which *trans*-translation was known to be essential (33). KKL-35 and two related compounds, KKL-10 and KKL-40, displayed antibiotic activity against the intracellular pathogen *Francisella tularensis* during infection of its host (34). However, the specificity of action of the molecules has not been confirmed in this species. The present study assessed the activity of KKL-35 against the intracellular pathogen *L. pneumophila*. We report that KKL-35 exhibits potent antibiotic activity against *L. pneumophila* at very low concentrations and is able to stop bacterial multiplication in a model of infection of human macrophages, yet multiple pieces of evidence indicate that KKL-35 does not target *trans*-translation, and as such, its true target(s) in *L. pneumophila* remains to be identified.

TABLE 1 MICs of KKL-35 for several *Legionella* species *in vitro*

Strain	MIC[a] (mg/liter)
L. pneumophila Paris	0.04 ± 0
L. pneumophila Lens	0.04 ± 0
L. pneumophila Lorraine	0.04 ± 0
L. pneumophila Philadelphia-1	0.067 ± 0.062
L. pneumophila 130b	0.04 ± 0
L. longbeachae ATCC 33484	0.08 ± 0
L. micdadei ATCC 33218	0.08 ± 0
L. dumoffii ATCC 35280	0.08 ± 0

[a]The values are the averages ± standard deviations from three independent determinations.

RESULTS

KKL-35 inhibits *Legionella* growth *in vitro*. The MICs of KKL-35 for five *L. pneumophila* strains and three non-*L. pneumophila* species causing LD were determined *in vitro* using the broth microdilution method (Table 1). KKL-35 strongly inhibited the growth of all tested species and was particularly potent against the species *Legionella pneumophila*, with all tested strains exhibiting a MIC of about 0.04 mg/liter. A time-kill assay with *L. pneumophila* strain Paris showed the bactericidal activity of KKL-35, with a decrease in viability being seen at 24 h after addition of KKL-35 at concentrations equal to or higher than the MIC (Fig. 1A). At 72 h following addition of KKL-35 at the MIC, the viable count was reduced by 4 orders of magnitude. Exposure to KKL-35 at half the MIC led to transient bacteriostatic activity for 48 h, but then this was followed by growth, suggesting that KKL-35 degrades and loses activity under those conditions. We also tested the activity of KKL-35 against 12 *L. pneumophila* mutants that were evolved from the Paris strain to become highly resistant to erythromycin and azithromycin (4,000-fold increases in the MICs) (8). The MIC of KKL-35 for these mutants was identical to that for the parent strain (0.04 mg/liter) and was thus unaffected by ribosomal mutations involved in macrolide resistance (23S rRNA and L4 and L22 protein mutations). Interestingly, KKL-35 was poorly active in conventional charcoal-yeast extract (CYE) solid medium for *L. pneumophila* isolates with MICs of >10 mg/ml. A paper disk containing 100 μg of KKL-35 produced an inhibition zone of 7 to 8 mm in diameter (Fig. 1B). To test the possibility that the agar-charcoal gelling base of CYE plates

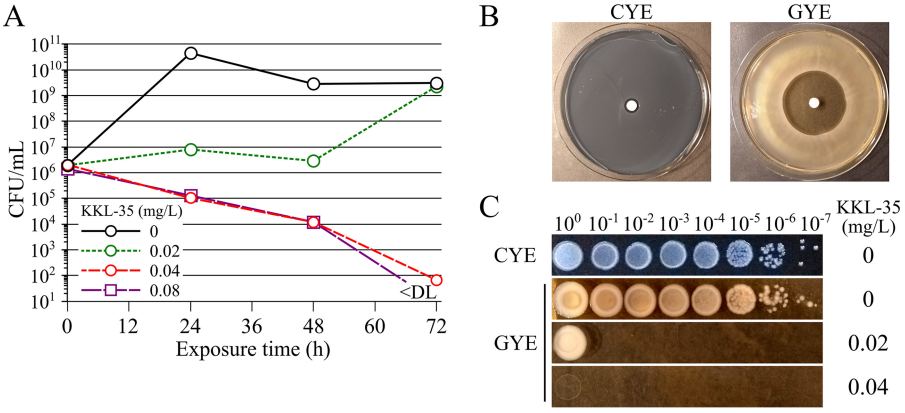

FIG 1 Antibiotic activity of KKL-35 against *L. pneumophila* in liquid and solid media. (A) Time-kill analysis of the activity of KKL-35 against *L. pneumophila* in AYE liquid medium. *L. pneumophila* strain Paris was resuspended in AYE medium at 3 × 10^6 CFU/ml with a range of 2-fold dilutions of KKL-35. The tubes were then incubated at 37°C. Every 24 h, serial dilutions were plated on CYE agar and the numbers of CFU were counted. The data presented are averages for triplicate samples. The data presented are representative of those from three experiments performed independently. (B) Antibiotic activity of KKL-35 in a solid medium disk diffusion assay. A paper disk containing 100 μg of KKL-35 was placed at the center of a CYE or GYE plate, which was inoculated by flooding with a suspension of *L. pneumophila*. (C) Determination of the MIC of KKL-35 on GYE plates. Serial 10-fold dilutions of a culture of *L. pneumophila* in stationary phase (~5 × 10^9 CFU/ml) were spotted (10 μl) on CYE (no KKL-35) and GYE plates containing increasing concentrations of KKL-35.

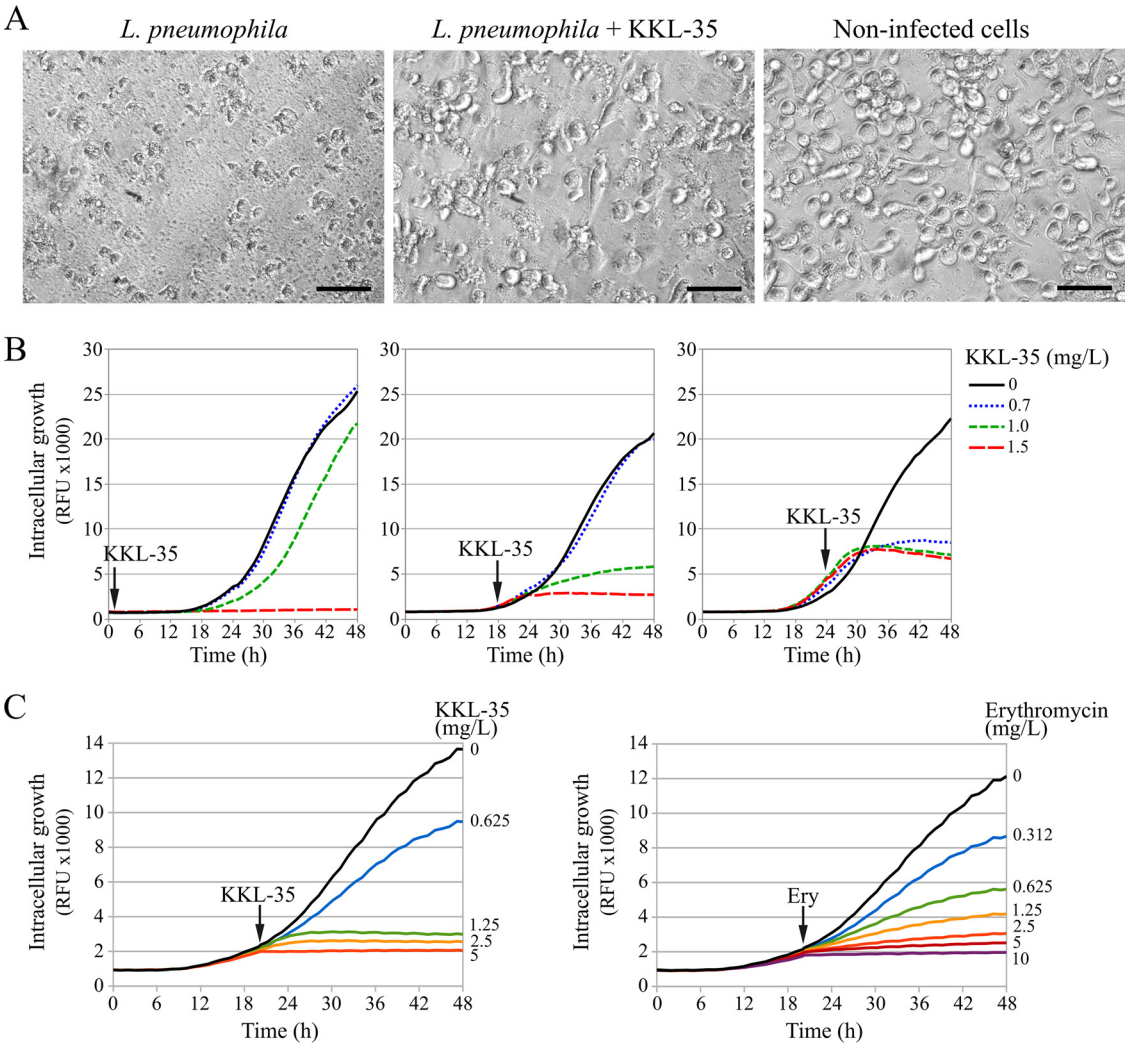

FIG 2 Activity of KKL-35 against *L. pneumophila* in an intracellular replication model. (A) Bright-field microscopy imaging of U937-derived macrophages infected with *L. pneumophila* (MOI = 10) for 72 h in the presence of absence of KKL-35 at 10 mg/liter. Bars, 50 μm. (B) Live monitoring of intracellular replication of GFP-producing *L. pneumophila* strain Paris carrying plasmid pX5 in U937-derived macrophages. KKL-35 was added at 1 h, 18 h, or 24 h postinfection. GFP fluorescence levels were automatically monitored every hour for 48 h. RFU, relative fluorescence units. Data are averages for three wells and are representative of those from an experiment performed three times independently. (C) Comparison of the activity of KKL-35 and erythromycin on the intracellular replication of *L. pneumophila*. KKL-35 and erythromycin were added at 20 h postinfection. Data are averages for three wells and are representative of those from an experiment performed twice independently.

reduced the activity of KKL-35, we replaced it with the guar gum gelling agent. On guar gum-yeast extract (GYE) plates, *L. pneumophila* formed colonies exactly like those on CYE plates (Fig. 1C), but a disk of 100 μg of KKL-35 produced an inhibition zone of 40 mm in diameter (Fig. 1B). KKL-35 at 0.02 g/liter could already inhibit the growth of the inoculum with less than 10^7 CFU, and at 0.04 g/liter (the MIC in broth), no growth could be observed even with the highest inoculum ($\sim 10^8$ CFU) (Fig. 1C). Thus, KKL-35 at a low concentration inhibited *L. pneumophila* growth both in liquid medium and in solid medium.

KKL-35 inhibits intracellular growth of *L. pneumophila*. *L. pneumophila* can infect human macrophages and replicate extensively within a membrane-bound compartment until cell lysis. Two molecules, KKL-10 and KKL-40, structurally related to KKL-35 were found to be nontoxic to macrophages at concentrations up to 19 mg/liter (34). Indeed, we found that KKL-35 was not toxic at 10 mg/liter and even protected monocyte-derived macrophages from killing by *L. pneumophila* at a multiplicity of

infection (MOI) of 10 (Fig. 2A). In order to better characterize the inhibitory activity of KKL-35, we used a green fluorescent protein (GFP)-based time-resolved assay to follow the replication of GFP-expressing *L. pneumophila* in monocyte-derived macrophages (35). Within minutes of forced contact with macrophages, *L. pneumophila* is internalized in a vacuolar compartment that escapes fusion with lysosomes (36, 37). Addition of KKL-35 at 1 h after infection, when bacteria are intracellular but not yet multiplying, prevented *L. pneumophila* replication at concentrations above 1 mg/liter (Fig. 2B). Moreover, when added at later time points (18 or 24 h), when multiplication is ongoing, KKL-35 could inhibit replication at even lower concentrations (0.7 mg/liter) (Fig. 2B). This may indicate either that KKL-35 is more active against actively dividing cells or that the active fraction of KKL-35 gradually decreases over time. The ability of KKL-35 to completely halt replication was then compared to the activity of the macrolide erythromycin, a recommended treatment for LD. When added to actively replicating *L. pneumophila*, erythromycin began to inhibit replication at 0.31 mg/liter (Fig. 2C). Each 2-fold increase in concentration further inhibited replication. A nearly complete and immediate inhibition of replication was obtained at a concentration 32 times higher than the first inhibitory concentration (10 mg/liter). While KKL-35 began to inhibit replication at a concentration of 0.62 mg/liter, it completely stopped replication at a concentration only 8 times higher (5 mg/liter) (Fig. 2C). This indicates that KKL-35 may be more bactericidal than erythromycin. Altogether, the data show that KKL-35 inhibits the replication of *L. pneumophila* within macrophages.

KKL-35 does not induce phenotypes associated with a loss of *trans*-translation. A lack of *trans*-translation increases the sensitivity to ribosome-targeting antibiotics in *E. coli* (38, 39) and in *L. pneumophila* (32). The *L. pneumophila ssrA*^{ind} mutant strain, carrying an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible allele of the tmRNA-encoding gene *ssrA*, is unable to grow if IPTG is not supplied in the medium (32). Low levels of IPTG allow growth with artificially reduced levels of tmRNA, resulting in increased susceptibility to erythromycin and chloramphenicol (32). A complete lack of *trans*-translation may further increase the sensitivity of *L. pneumophila* to these antibiotics. Thus, we anticipated that KKL-35 could be synergistic with erythromycin and chloramphenicol. To determine a potential synergy, we performed a checkerboard analysis (40). Interestingly, the MICs of erythromycin (0.125 mg/liter) and chloramphenicol (1 mg/liter) were not affected by KKL-35, indicating the absence of synergy (fractional inhibitory concentration index [FICI] = 2). Thus, unlike the genetic alteration of *trans*-translation, KKL-35 does not potentiate the activity of ribosome-targeting antibiotics. Another phenotype of *L. pneumophila* cells genetically deprived of tmRNA is extended filamentation, indicating that *trans*-translation is required for cell division (32). In contrast to *L. pneumophila* cells defective for *trans*-translation, *L. pneumophila* cells treated with KKL-35 at concentrations at, below, or above the MIC still displayed a normal morphology (Fig. 3A). The inability of KKL-35 to reproduce the phenotypes associated with a loss of *trans*-translation suggests that its potent antibiotic activity is not primarily linked to inhibition of *trans*-translation.

KKL-35 is equally active against *L. pneumophila* lacking a *trans*-translation ability. To test whether the antibiotic activity of KKL-35 was linked to the inhibition of *trans*-translation, we tested the activity of KKL-35 against the *L. pneumophila ssrA*^{ind} mutant strain. When IPTG was supplied at high concentrations, tmRNA was expressed at nearly normal levels, and the strain grew like the wild-type strain. As expected, in the presence of IPTG this strain was as sensitive to KKL-35 as the wild-type strain (MIC = 0.04 mg/liter) (Fig. 3B). In the absence of IPTG, the growth of this strain was strongly impaired, yet despite its low levels of tmRNA, the strain was not more sensitive to KKL-35. Ectopic expression of the alternate ribosome-rescue system ArfA from *E. coli* could restore the growth of the *ssrA*^{ind} strain in the absence of IPTG. Under these conditions, the strain does not produce tmRNA and is therefore deficient in *trans*-translation (32). Even though it did not require *trans*-translation for growth, the MIC of KKL-35 for this strain remained identical to that for the wild-type strain (Fig. 3B).

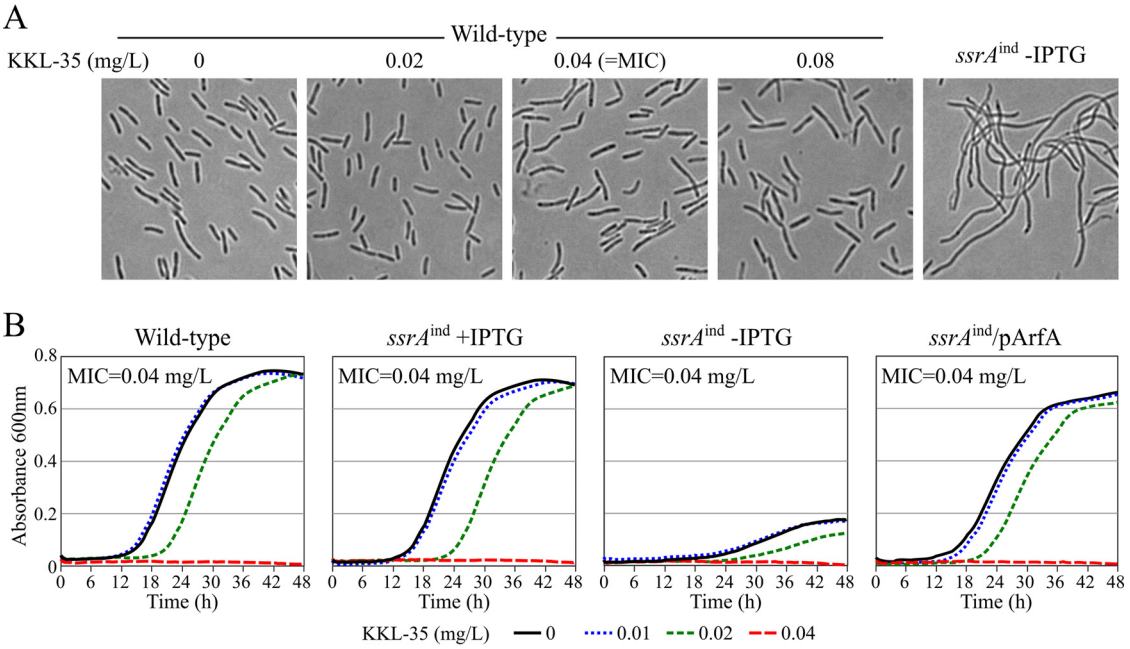

FIG 3 KKL-35 does not primarily target *trans*-translation in *L. pneumophila*. (A) Phase-contrast light microscopy of wild-type *L. pneumophila* treated with KKL-35 for 24 h and of the *trans*-translation-deficient *ssrA*^{ind} mutant deprived of IPTG for 24 h. (B) Activity of KKL-35 against *L. pneumophila* strains deficient for *trans*-translation. Representative growth curves of the wild-type strain, the *ssrA*^{ind} mutant in the presence or absence of IPTG, and the *ssrA*^{ind} mutant rescued by expression of *E. coli* ArfA are shown. MICs were determined three times independently on the basis of the absorbance reading.

***L. pneumophila* does not acquire resistance to KKL-35.** *In vitro* selection of resistance is a common way to identify and characterize potential resistance determinants. Plating of a large number of bacteria on solid medium containing an antibiotic at concentrations above the MIC (e.g., rifampin, streptomycin) often allows the isolation of resistant mutants when resistance is conferred by a single mutation. Consistent with published data on *E. coli* (33), this strategy failed to produce mutants resistant to KKL-35 even when up to 10^{10} *L. pneumophila* cells were plated on GYE plates containing KKL-35 at concentrations of 2 to 8 times the MIC (0.08 to 032 g/liter). Occasionally, colonies could be obtained on GYE plates with KKL-35 at the MIC (0.04 g/liter), but the colonies could not grow again on freshly prepared GYE plates with the same concentration of KKL-35 (data not shown). These colonies likely emerged because KKL-35 degrades over time. Continuous culture of a bacterial population in the presence of increasing concentrations of antibiotics represents an alternate approach when several mutations are required to confer resistance. In *L. pneumophila*, this method has been used to characterize the mutational path to resistance to fluoroquinolones and macrolides (7, 8). In agreement with previous reports, in two independent experiments, we observed here a 500-fold increase in the MIC of norfloxacin in only six passages (about 30 generations) (Fig. 4). In contrast, no significant increase in the MIC of KKL-35 was obtained even after 10 passages (over 60 generations) (Fig. 4). Thus, in the experimental setup tested, *L. pneumophila* could not acquire resistance to KKL-35.

DISCUSSION

We found KKL-35 to display potent antibiotic activity against *L. pneumophila* with a MIC of 0.04 mg/liter (0.125 μM). KKL-35 showed significant bactericidal activity and was found to retain normal activity against the different strains of erythromycin-resistant *L. pneumophila* tested. In addition, and in contrast to the findings for fluoroquinolones and macrolides, *L. pneumophila* did not develop resistance *in vitro*. Supporting its potential use for the treatment of LD, KKL-35 could stop *L. pneumophila* from multiplying within monocyte-derived human macrophages. This indicates that KKL-35 is able

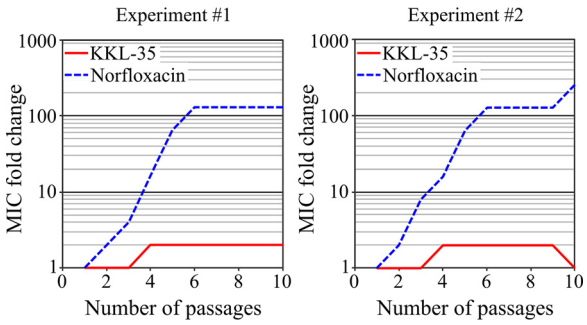

FIG 4 *L. pneumophila* does not acquire resistance to KKL-35. In two different experiments (several weeks apart), two different lineages were founded from *L. pneumophila* strain Paris and propagated by serial passages in the presence of KKL-35 or norfloxacin. The MIC was determined at each passage and is presented relative to the initial MIC (norfloxacin, 0.25 mg/liter; KKL-35, 0.04 mg/liter).

to cross the biological membranes of the macrophage to reach intracellular *L. pneumophila*.

KKL-35, along with other oxadiazoles (KKL-10 and KKL-40), was identified in a high-throughput screen for inhibitors of *trans*-translation activity *in vitro* and was initially found to display antibiotic activity against several species. Oxadiazoles have since shown potent antibiotic activity against additional pathogens, such as *Francisella tularensis*, *Bacillus anthracis*, or *Mycobacterium tuberculosis* (34, 41, 42). The antibiotic activity of oxadiazoles against these pathogens was assumed to derive from inhibition of *trans*-translation but was not demonstrated. For *L. pneumophila*, several results led us to question the link between the inhibition of *trans*-translation and antibiotic activity. First, no synergy between KKL-35 and ribosome-targeting antibiotics was found, whereas we previously found that a reduction in tmRNA levels led to an increased susceptibility of *L. pneumophila* to such antibiotics (32). Second, *L. pneumophila* cells treated with KKL-35 did not display the filamentation phenotype observed in cells lacking tmRNA. Most importantly, the MICs were identical when KKL-35 was tested with a wild-type strain, with a growth-affected mutant expressing low levels of tmRNA, or with a mutant that does not require *trans*-translation because tmRNA was replaced by an alternative ribosome-rescue system (ArfA from *E. coli*). Taken together, our data indicate that *trans*-translation is not the primary target of KKL-35 in *L. pneumophila* and probably also in other bacteria. Indeed, similar results have been obtained with *E. coli*, in which KKL-35 is equally effective against strains deficient for *trans*-translation (43). A novel double-fluorescent reporter system for the simultaneous and specific detection of *trans*-translation and proteolysis activities showed that KKL-35 has no direct effect on *trans*-translation (43). Altogether, the data challenge the initial report that KKL-35 is a *trans*-translation inhibitor and that *trans*-translation is a promising antibiotic target (33). The molecular target of KKL-35 has not yet been identified, but the inability to obtain resistant mutants indicates that this target is not prone to support viable mutations. While this is a valuable property for an antibacterial agent, this hampered our efforts to identify the true target of KKL-35 in *L. pneumophila*. In conclusion, KKL-35 is an effective, broad-spectrum antibacterial agent active against the intracellular pathogen *L. pneumophila*. However, further studies are needed to better understand its mechanism of action and to assess further the potential of oxadiazoles in treatment.

MATERIALS AND METHODS

Strains, growth media, and antibiotics used. The strains used in this study included *L. pneumophila* clinical isolates Paris (CIP 107629), Lens (CIP 108286), Philadelphia-1, Lorraine (CIP 108729), and 130b, as well as *L. longbeachae* ATCC 33484, *L. dumoffii* ATCC 35280, and *L. micdadei* ATCC 33218. *L. pneumophila* strain Paris, which is resistant to erythromycin and azithromycin, was obtained from a previous work (8). *L. pneumophila* Paris was transformed with the plasmid pX5, a pMMB207C derivative harboring the *gfp*$^+$ gene under the control of a strong constitutive promoter, and was used for live monitoring of intracellular multiplication by reading of the fluorescence. The *ssrA*^{ind} and *ssrA*^{ind}/pArfA tmRNA mutant strains were previously described (32). ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-yeast extract

(AYE) broth medium was prepared with 10 g/liter ACES, 12 g/liter yeast extract, 0.3 g/liter iron(III) pyrophosphate, and 0.5 g/liter L-cysteine. The pH was adjusted to 6.9 with KOH, and the solution was filter sterilized and kept away from light and at 4°C. ACES-buffered charcoal-yeast extract (CYE) plates were prepared by combining a 2-fold concentrate of ACES and yeast extract (20 g/liter each in the concentrate) with the same volume of an autoclaved solution of 30 g/liter agar and 4 g/liter charcoal (final concentrations, 15 g/liter agar and 2 g/liter charcoal). The medium was then complemented with 0.25 g/liter filtered iron(III) nitrate and 0.4 g/liter L-cysteine, and the mixture was poured to produce CYE plates. Alternatively, the agar-charcoal solution was replaced by an autoclaved guar gum solution in distilled water (2× concentrate at 10 g/liter; final concentration, 5 g/liter) to produced guar gum-yeast extract (GYE) plates. Unless indicated otherwise, cultures on CYE (or GYE) were incubated for 72 h at 37°C in air and then patched onto CYE again for 24 h to obtain fresh cultures before the experiments were performed. When appropriate, chloramphenicol (5 μg/ml) was added to the medium. A stock solution of KKL-35 (Ambinter, Orléans, France) was prepared at 10 mM (3.2 g/liter) in dimethyl sulfoxide (DMSO) and stored at −20°C. The highest concentration of KKL-35 used was 10 mg/liter (Fig. 2A) with U937 cells or 1.5 mg/liter with *L. pneumophila*, corresponding to final concentrations of DMSO of 0.3% and 0.05%, respectively.

Time-kill assay and determination of MICs. For the time-kill assay, *L. pneumophila* strain Paris was resuspended in AYE medium at 3×10^6 CFU/ml with a range of 2-fold dilutions of KKL-35. The tubes were then incubated at 37°C in air with shaking. Every 24 h, serial dilutions were plated on CYE agar and the numbers of CFU were counted. For MIC determination, no CLSI guidelines are available for testing the antibiotic susceptibility of *Legionella* strains. EUCAST guidelines were recently published but are based on the gradient strip test. KKL-35 strip tests are not commercially available, and we found the charcoal of CYE medium to seriously impede KKL-35 activity. Therefore, we used the previously described AYE broth microdilution method for MIC determination (44). Briefly, strains were resuspended in AYE medium and placed into the wells of a 96-well polystyrene plate, and a range of 2-fold dilutions of KKL-35 was added to the cultures. The inoculum (10^6 CFU/ml) was verified by plating and counting of serial dilutions of the cultures at the beginning of the experiment. The 96-well plate was sealed with a Breathe-Easy membrane (Sigma-Aldrich) to prevent evaporation and was incubated for 48 h at 37°C in air with no agitation. At 48 h, the MICs were determined visually as the lowest concentrations inhibiting bacterial growth.

Evaluation of synergistic activity. The checkerboard broth microdilution method was used to evaluate possible synergistic activity between KKL-35 and chloramphenicol or erythromycin against *L. pneumophila* strain Paris. The bacteria were inoculated in AYE medium in a 96-well polystyrene plate containing a 2-fold range of concentrations of KKL-35 in columns crossing a range of concentrations of another antibiotic in rows. The plate was then incubated for 48 h in a Tecan Infinite M200Pro reader at 37°C with both agitation and reading of the absorbance at 600 nm every 10 min. The growth value was defined as the highest absorbance reading recorded during growth kinetics. Compared to the classic qualitative evaluation of growth by visual observation, this method allowed a quantitative measure of growth to be obtained. Growth inhibition was defined as a maximal absorbance value that was $<$10% of the value for the positive control. The fractional inhibitory concentration index (FICI) was interpreted in the following way: an FICI of $\leq$0.5 indicated synergy, an FICI of $>$4.0 indicated antagonism, and an FICI of $>$0.5 to 4 indicated no interaction (40).

Activity of KKL-35 on intracellular growth. U937 cells grown in RPMI 1640 containing 10% fetal calf serum (FCS) were differentiated into human macrophages by addition of phorbol 12-myristate 13-acetate (PMA) at 100 ng/ml and then seeded into 96-well polystyrene plates for 3 days (10^6 cells/well). At 4 h before infection, the medium was replaced with fresh medium with 10% FCS. *L. pneumophila* strain Paris was plated from a glycerol stock at −80°C onto CYE plates, incubated at 37°C in air for 72 h, and then plated again onto CYE plates for 24 h to obtain a fresh culture. At 4 h before infection, the bacteria were resuspended in RPMI 1640 and incubated at 37°C. Infection of macrophages was performed by replacing their medium with RPMI 1640 and 2% FCS containing *L. pneumophila* at a multiplicity of infection of 10. The plates were centrifuged for 10 min at 1,000 × *g* and then incubated at 37°C with 5% CO_2 for 72 h. Micrographs were taken with an inverted microscope (Nikon Eclipse TS100). Live monitoring of infection of U937 macrophages was performed as described above, except that GFP-producing *L. pneumophila* strain Paris/pX5 was used and the infection was performed in CO_2-independent medium after differentiation and was monitored by use of a Tecan Infinite M200Pro plate reader. The plate was incubated in the reader at 37°C, and GFP fluorescence levels were automatically monitored every hour for 72 h at an excitation wavelength of 470 nm and an emission wavelength of 520 nm.

Selection of resistant mutants by serial passages. Two different lineages were founded from *L. pneumophila* strain Paris and propagated by serial passages in the presence of KKL-35 or norfloxacin, as previously described (7, 8). Briefly, a suspension of *L. pneumophila* strain Paris in AYE was added to a concentration of 10^8 CFU/ml in a 24-well polystyrene plate with 2-fold KKL-35 or norfloxacin concentrations ranging from 0.5 time to 8 times the MIC that was determined for the parental strain (norfloxacin, 0.25 mg/liter; KKL-35, 0.04 mg/liter). The plates were sealed with a Breathe-Easy membrane (Sigma-Aldrich) and incubated for 4 days at 37°C in air without agitation, after which the MIC was noted for each antibiotic. A 1:40 dilution of the bacteria from the well with the highest antibiotic concentration in which growth was observable was transferred to a new plate containing 2-fold KKL-35 or norfloxacin concentrations ranging from 0.5 time to 8 times the MIC of the previous cycle. Serial passages were repeated 10 times, and the experiment was performed twice independently.

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