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Highlights	・ Rifampicin (RFP) has strong mucoviscosity-suppressing activity against hypervirulent *Klebsiella pneumoniae* (hvKP). ・RFP inhibits capsular polysaccharide biosynthesis, reducing capsular thickness. ・RFP exerts its effect through inhibition of *rmpA* gene transcription. ・RFP may serve as a potential antivirulence agent for hvKP infection
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Discovery of anti-mucoviscous activity of rifampicin and its potential as a candidate anti-virulence agent against hypervirulent *Klebsiella pneumoniae*

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Abstract

A recent increase in the incidence of hypervirulent *Klebsiella pneumoniae* (hvKP) infections, especially those caused by a sublineage of the clonal group CG23 (CG23-I), is raising serious health concerns worldwide. The high virulence of hvKP is, at least in part, attributed to the overproduction of capsular polysaccharide (CPS), which is triggered by a positive regulator of capsular polysaccharide synthesis (*cps*) genes, named RmpA. Although extensive research has been conducted on the mechanisms of hvKP virulence, no study has focused on the development of anti-virulence therapeutics. Here, we attempted to identify and validate an antimicrobial agent able to suppress hvKP hypermucoviscosity. We tested 18 commercially available agents including β–lactams, quinolones, and aminoglycosides. We found that rifampicin (RFP) has a strong anti-mucoviscous activity against CG23-I hvKP that is exerted even at subinhibitory concentrations. Polysaccharide extracts from hvKP showed substantially lowered viscosity when cells were grown with RFP. Moreover, microscopic observations demonstrated that RFP treatment results in a drastic reduction in the thickness of the CPS layer around hvKP cells. RFP treatment decreased transcript levels of *rmpA* and *rmpA*-regulated *cps* genes, indicating that RFP suppresses mucoviscosity of hvKP through inhibition of *rmpA* transcription. Our data suggest that RFP may serve

as a potential anti-virulence agent for refractory infection by hvKP.

Keywords: hypervirulent *Klebsiella pneumoniae*, hypermucoviscous, capsular polysaccharide, rifampicin, anti-mucoviscous, anti-virulence agent.

[1]

[1] **Abbreviations**

hypervirulent *Klebsiella pneumoniae* (hvKP); capsular polysaccharide (CPS); capsular polysaccharide synthesis genes (*cps* genes); mucoviscosity-associated gene A (*magA*); regulator of mucoid phenotype A (*rmpA*); *rmpA* homolog (*rmpA2*); clonal group 23 (CG23); Mueller Hinton II (M-H II); minimum inhibitory concentrations (MICs); extracellular polysaccharide (EPS); quantitative real-time PCR (qRT-PCR); complementary DNA (cDNA); sequence type 23 (ST23); RNA polymerase β subunit (RpoB); methicillin-resistant *Staphylococcus aureus* (MRSA); meropenem (MEPM); metronidazole (MNZ); ofloxacin (OFLX); azithromycin (AZM); clarithromycin (CAM); tetracycline (TC); chloramphenicol (CP); rifampicin (RFP); rounds per minute (RPM)

1. Introduction

Klebsiella pneumoniae is a common gram-negative pathogen in community-acquired and nosocomial infections [1]. *K. pneumoniae* has long been regarded as an opportunistic pathogen that causes pneumonia and urinary tract infection to immunocompromised people. However, a new type called hypervirulent *K. pneumoniae* (hvKP) has emerged in East and Southeast Asia over the past two decades [2,3]. It was first recognized in the 1980s in a Taiwanese patient with community-acquired primary liver abscess that involved hvKP [4]. Many reports primarily in Taiwan and Korea have followed. *K. pneumoniae* strains associated with primary liver abscesses characteristically express a distinct sticky phenotype when grown on agar [5] and are therefore called hypermucoviscous *K. pneumoniae*. Hypermucoviscous and hypervirulent phenotypes do not necessarily coincide; i.e., some hypermucoviscous strains are not hypervirulent. Similarly, some hypervirulent strains give negative results in a positive string test [6]. While hvKP infections, originally, were limited to parts of Asia, they have spread to other geographic areas and are now recognized as a serious clinical issue worldwide with a recent increase in incidence [7,8]. HvKP has the ability to cause life-threating community-acquired infections even in young, healthy hosts. Its high tendency to cause bacteremia, primary liver abscess, and metastatic infections such

as endophthalmitis, meningitis, and pneumonia, which are associated with the remarkably high mortality is a major characteristic of hvKP that distinguishes it from normal *K. pneumoniae* strains [3]. We reported two severe cases of hvKP infection that caused multiple organ abscesses and endophthalmitis [9].

Research efforts have yielded significant progress in the epidemiology of hvKP. Researchers first realized that hvKP virulence and the serologic type of their capsular polysaccharide (CPS) are strongly related [10]. Among the 78 *K. pneumoniae* capsular serotypes described to date, only eight have been found in hvKP [3]. The majority of hvKP isolates are serotypes K1 and K2, which account for 2/3 or more of all isolates. Genome-based studies have further revealed that the most prevalent lineages belong to the clonal group 23 (CG23) [11,12]. Strikingly, whereas K2-type hvKPs show a relatively high degree of divergence, almost all K1 hvKPs fall into a highly homogenous clade (CG23) in phylogenetic analyses, suggesting their recent emergence from a single common ancestor and the following rapid worldwide dissemination. Moreover, according to a recent report [13], the CG23-I sublineage, which was estimated to have emerged in the 1920s after acquisition of an integrative conjugative element named ICE*Kp10*, occupies the majority of the CG23 clinical isolates. ICE*Kp10* carries several virulence determinants including synthetic genes for colibactin, a

genotoxin that causes DNA damage in eukaryotic cells [14]. Since CG23-I is the only sublineage in CG23 possessing the colibactin gene cluster, it can be used as a genetic marker for identifying CG23-I.

The correlation between hypermucoviscosity and hypervirulence has been intensively studied. Overproduction of capsular material creates hypermucoviscosity [15,16], which directly correlates with high serum resistance and the clinical characteristics of hvKP [17,18]. Consistent with the non-hypervirulent strains [19], loss of capsule synthesis reduces virulence by reducing the antiphagocytic effect against macrophages and neutrophils [20,21]. Genetic studies have revealed the functions of several virulence genes associated with hypermucoviscosity, including the mucoviscosity-associated gene A (*magA*) and the regulator of mucoid phenotype A (*rmpA*) gene [17,22]. The chromosomal *magA* gene, also named *wzy*_K1, encodes a K1-specific capsular polysaccharide polymerase that is essential for the production of hypermucoviscous CPS for serotype K1 hvKP [18,20]. Many other genes, involved in capsular polysaccharide synthesis, transportation, and cell surface assembly, are encoded on the K1 capsular polysaccharide synthesis (*cps*) gene cluster, together with putative promoters at upstream regions of *galF*, *wzi*, and *manC* [23]. The *rmpA* gene is a regulator of CPS synthesis encoded on a large virulence plasmid carried by most hvKP

strains [12,16]. There is also a *rmpA* homolog (*rmpA2*) located on the plasmid; however, *rmpA2* may not be functional in CG23 because of truncation of the structural gene resulting from frame shifts [12]. RmpA and RmpA2 are positive regulators that directly control the expression of *cps* genes [15,16,24]. Both *magA* and *rmpA* are strongly associated with abscess formation [17,18]. In addition to the hypermucoviscous capsule, siderophore is known to be an important hvKP virulence factor. Strains of hvKP typically possess biosynthetic and transport genes for aerobactin (*iucABCD* and *iutA*) and salmochelin (*iroBCDN*) on the virulence plasmid [12]. Aerobactin has been shown to be a critical virulence factor under some circumstances [25], leading to aerobactin synthetase (IucA) being thoroughly investigated [26]. Russo et al. recently demonstrated that aerobactin is a key marker that distinguishes hvKP from non-hvKP strains [27].

Many efforts have been directed toward revealing the mechanisms of hvKP virulence. However, no studies have focused on how to combat this difficult-to-treat infection in clinical settings. We hypothesized that inhibiting the production of critical virulence factors would result in more effective treatment of the infections. In this study, we aimed to identify an antimicrobial agent that could be used as a mucoviscosity-suppressing agent against hvKP CG23-I. Our study is the first step

toward development of a new treatment strategy for hvKP infection.

2. Materials and methods

2.1. Strains, media, and culture conditions

Four hvKP strains were used in this study (OCU_hvKP1, OCU_hvKP2, OCU_hvKP3, and OCU_hvKP4), and all were isolated from patients with liver and/or prostate abscesses from the Osaka City University Hospital. Strains OCU_hvKP1 and OCU_hvKP2, both of which are serotype K1 *K. pneumoniae* sequence type 23 (ST23) strains, are original isolates described previously (OCU_hvKP1 and OCU_hvKP2 correspond to Case 1 and Case 2, respectively) [9]. OCU_hvKP3 and OCU_hvKP4 were *magA*-positive (data not shown) and thus considered to be K1 serotypes. *K. pneumoniae* ATCC 700603, which is a K6 serotype possessing neither *magA* nor *rmpA*, was used as a non-hvKP control strain. The strains were routinely grown in cation-adjusted Mueller Hinton II broth (M-H II; Becton Dickinson Company, Franklin Lakes, New Jersey, USA) at 37°C with shaking at 130 rounds per minute (RPM). Growth was monitored by measuring optical density at 600 nm (OD_{600}) with a spectrophotometer (U-1500, Hitachi, Tokyo, Japan).

2.2. Genetic characterization of hvKP isolates

Multilocus sequence typing (MLST) was performed using the Institut Pasteur MLST scheme (https://bigsdb.pasteur.fr/klebsiella/primers_used.html), which uses internal fragments of the following seven housekeeping genes: *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*. The presence of colibactin cluster was confirmed by PCR amplification of *clbB* and *clbN* using the following primers [28]: clbBF (GATTTGGATACTGGCGATAACCG), clbBR (CCATTTCCCGTTTGAGCACAC), clbNF (GTTTTGCTCGCCAGATAGTCATTC), and clbNR (CAGTTCGGGTATGTGTGGAAGG).

2.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of antimicrobial agents were determined using a broth microdilution method according to the 2016 Clinical Laboratory Standards Institute guidelines. M-H II broth (cation-adjusted) (Becton Dickinson Company) was used as growth medium. The following 18 antimicrobial agents were purchased from commercial sources and used in the testing: benzylpenicillin, ampicillin, meropenem (MEPM), metronidazole (MNZ), streptomycin, kanamycin, gentamycin, amikacin, ciprofloxacin, ofloxacin (OFLX), azithromycin (AZM), clarithromycin (CAM),

tetracycline (TC), chloramphenicol (CP), rifampicin (RFP), vancomycin, polymyxin B, and colistin.

2.4. Evaluation of anti-mucoviscous activity of antimicrobial agents

We subjected all the 18 antimicrobial agents described above to an assessment of anti-mucoviscosity activity against hvKP. We used OCU_hvKP1 for screening the candidate antimicrobials and confirmed the effect of RFP with three other hvKP isolates. Mucoviscosity of each strain under a given condition was evaluated by measuring the viscosity of extracellular polysaccharide (EPS) extracts prepared as follows (also see Supplemental Fig. S1). The bacterial strains, in two separate batches, were grown in the absence or presence of a target antimicrobial agent with shaking at 130 RPM overnight (16–20 h) in 6 ml of M-H II using 14-ml round-bottom tubes. EPS was extracted from the cells by incubating at 50°C for 30 min with 1.2 ml of 100 mM citric acid (pH 2.0) containing 1% Zwittergent 3-14 detergent (Sigma-Aldrich, St. Louis, Missouri, USA). After centrifugation at 5800 × *g* for 15 min, the supernatant was filtered through a 0.45-μm PVDF filter (Millex-HV, Merck Millipore, Burlington, Massachusetts, USA). The filtrate (6 ml) was evaluated with an Ostwald viscometer (capillary internal diameter 0.5 mm) (size 1; Sibata Scientific Technology Ltd, Saitama, Japan).

Measurements were performed according to the manufacturer's instruction, except for being done at room temperature without a water bath. The specific viscosity value (η_{sp}) was calculated using the following equation:

$$\eta_{sp} = (T_{test} - T_{Water})/T_{Water}$$

where T_{test} and T_{Water} represent the time for a certain volume (approximately 3 ml) of the test sample and pure water, respectively, to flow through the capillary. Because of minimal differences in sample density, the solution density parameter was omitted from the calculations.

2.5. Microscopic observation of capsule

Overnight cultures were mixed with equal volumes of India ink and 2-μl samples of the mixtures placed on microscope slides and covered with a cover slip. The samples were evaluated by light microscopy using an Olympus BX50 microscope (Olympus, Tokyo, Japan) with a 100× objective lens. Images were captured using a Canon EOS Kiss X5 digital camera (Canon, Tokyo, Japan). The scheme is illustrated in Supplemental Fig. S1.

For determining time-dependent changes in capsule thickness, OCU_hvKP1 was grown overnight in M-H II broth, with or without RFP (8 μg/ml), and then subcultured

in fresh media starting at 0.05 OD_{600} units. At the indicated times post inoculation, a sample of the culture was diluted to 0.1 OD_{600} in fresh M-H II media and mixed with an equal volume of India ink. For each sample, four slides were prepared and microscopically evaluated. The shortest diameter of the clear zone around each bacterial cell was recorded as the capsule thickness. All cells observed in four randomly selected visual fields were included when calculating the average capsule thickness.

2.6. Quantitative real-time polymerase chain reaction

rmpA, magA, galF, wzi, manC, iucA, and *iroN* expression levels were determined by quantitative real-time PCR (qRT-PCR). OCU_hvKP1 was grown in M-H II media with or without RFP (8 μg/ml) for 6 h. The inocula (1%) were overnight cultures grown without RFP. Total RNA was extracted using a hot phenol method [29]. Briefly, 1-ml samples of cultured bacteria were harvested by centrifugation and suspended in 400 μl of buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% SDS). The samples were extracted with equal volumes citrate buffer (pH 4.2)-saturated phenol (Acid Phenol, Nippon Gene, Tokyo, Japan) at 65°C for 45 min then cooled on ice for 10 min. After centrifugation, the supernatants were subjected to acid-phenol extraction followed by chloroform extraction. RNA was ethanol precipitated and suspended in 100 μl of

RNase-free water. Residual DNA was digested with 10 units of RNase-free recombinant DNase I (TaKaRa, Shiga, Japan) at 37°C for 10 min. Samples were acid-phenol and chloroform extracted, the RNA was precipitated, and samples were suspended in 100 μl of RNA-free water. The quantity and quality of the RNAs were assessed using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Complementary DNA (cDNA) was synthesized from the RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). The cDNA was evaluated by qRT-PCR using Applied Biosystems PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Primers used for qRT-PCR are listed in Table 1. Transcript levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method [30]. Since RFP targets the RNA polymerase β subunit (RpoB), it could affect the expression of any gene, including housekeeping genes. Therefore, in a series of preliminary experiments we evaluated the use of *rpoB*, *recA*, and 16S and 23S rRNA genes as reference genes to determine data reliability. There were no major differences in the results between experiments using the different housekeeping genes; no matter which reference gene was used. Data showed that the addition of RFP caused a drastic decrease of *rmpA* and *magA* expression levels without affecting the expression of *iucA* (data not shown). We

thus chose to use the 16S rRNA gene as a reference in subsequent experiments.

2.7. Statistical analyses

Mann-Whitney *U* tests were performed using EZR (version 1.37; Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical interface for R (version 3.4.1; The R Foundation for Statistical Computing, Vienna, Austria). The tests were used for analyzing data presented in Figs. 2, 3, and 4. *P*-values < 0.05 indicate that the difference in values for two given groups is statistically significant.

3. Results

We first characterized the four hvKP strains used in this study. MLST analysis revealed that all the hvKP isolates were ST23, which is the major sequence type of CG23. PCR detection of a colibactin cluster further revealed that all the isolates belonged to the CG23-I sublineage. The MICs of 18 agents against each of the strains, determined using a broth microdilution method, are shown in Table 2. These data demonstrate that the antimicrobial susceptibility patterns of our strains were similar to that of typical non-hvKP strains, thus showing good susceptibility to β-lactam antimicrobials except benzylpenicillin and ampicillin.

When grown in the absence of antimicrobials, the specific viscosity of EPS extracts divided by the optical density of the bacterial culture (η_{sp}/OD_{600}) from four different hvKP strains (OCU_hvKP1, OCU_hvKP2, OCU_hvKP3, and OCU_hvKP4) was about ten times higher compared to that from a non-hvKP control strain (0.44, 0.23, 0.54, and 0.38 vs. 0.04, respectively). We then compared the mucoviscosity of OCU_hvKP1 in the presence and absence of each antimicrobial agent at one-fourth of the MIC (Figure 1A). We chose this concentration to find an agent that can exert an anti-mucoviscosity effect through a mechanism different from ordinary growth inhibitory mechanisms. Notably, the η_{sp}/OD_{600} value in the presence of RFP (0.06) was markedly lower compared to that in the absence of the antimicrobial. We noted that at the RFP concentration used in this experiment (8 μg/ml) had only a small effect on the growth of the bacteria. We also tested the effect of RFP on the other three hvKP strains and obtained similar results. The η_{sp}/OD_{600} values for OCU_hvKP2, OCU_hvKP3, and OCU_hvKP4 in the absence of RFP treatment were 0.23, 0.54, and 0.38, respectively. These values all decreased drastically to 0.04 when the bacteria were treated with RFP, which was similar to the level observed for the non-hvKP control strain. On the other hand, the η_{sp}/OD_{600} value for the non-hvKP control strain did not change at all by RFP treatment.

We then determined the lowest concentration of RFP that could exert the mucoviscosity-suppressing activity. The η_{sp} value began to decrease slightly when the bacteria were treated with RFP at 0.5 μg/ml and started to show a dramatic effect once the RFP concentration reached 4 μg/ml (Figure 1B). These findings clearly demonstrated that RFP treatment had a strong mucoviscosity-suppressing activity against hvKP strains that was observed at even subinhibitory concentrations.

We applied negative staining with Indian ink and light microscopy (Supplemental Fig. S1) to the hvKP strains and a non-hvKP strain to confirm and compare the thickness of the bacterial capsule. In contrast to the thin capsule layer around non-hvKP cells, a thick capsule was observed around hvKP cells (Figure 2A). Remarkably, when hvKP was grown in the presence of RFP (8 μg/ml), the capsular thickness reduced to the same level as that of the non-hvKP strain (Figure 2A). This indicated that RFP suppressed hvKP mucoviscosity by inhibiting CPS synthesis. We then monitored the change in capsule thickness over time following the addition or removal of RFP. The thickness of the hvKP capsule gradually decreased after the addition of RFP until a minimum thickness was reached after six hours (Figure 2B). Conversely, when RFP was removed from an overnight culture of hvKP, the capsule slowly regained its thickness over several hours (Figure 2C). These results demonstrated that the effect of RFP on the

hvKP capsule was reversible. The fact that the loss and gain of capsule thickness occurred over several hours was consistent with the mechanism of action involving changes in the expression level of the genes that synthesize CPS.

We hypothesized that RFP repressed *cps* genes by repressing *rmpA*. Thus, we examined the expression levels of *rmpA*, *magA*, *galF*, *wzi*, and *manC* in hvKP grown with or without RFP. We also measured the expression levels of *iucA* and *iroN*, genes involved in aerobactin and salmochelin siderophore systems, respectively, to confirm the effect of RFP on virulence genes other than capsule-related genes. As expected, the level of transcripts for all the capsule-related genes were markedly lower when hvKP was grown in the presence of RFP compared to that in the absence of RFP (Figure 3). In contrast, RFP showed no inhibitory effect on either *iucA* or *iroN*. These observations are consistent with our hypothesis on the anti-mucoviscous mechanism of RFP. Overall, these data suggested that RFP inhibited the transcription of *rmpA* with a certain level of selectivity; i.e., *rmpA* was more strongly affected than other genes such as *iucA*, *iroN*, *rpoB*, *recA*, and 16S and 23S rRNA genes.

Finally, we monitored the temporal change in the level of transcript for *rmpA* after inoculation into fresh media (Figure 4). We also monitored the change in the transcription level of *magA* as a representative *cps* gene. When hvKP was grown

without RFP, the level of *rmpA* transcripts were low at two hours post-inoculation but dramatically increased over a period between two and six hours. Strikingly, no increase in the *rmpA* transcript levels was observed in the presence of RFP. The time course of the transcript levels of *magA* exhibited a different pattern. When the bacteria were grown in the absence of RFP, a 3-fold increase in the level of transcripts was detected between two and four hours, after which it remained constant for at least six hours. However, in the presence of RFP a decrease in the amounts of transcripts was detected between two and four hours. The reason the transcript level was higher at two hours in the presence of RFP compared to that in the absence of RFP is unknown. It may reflect a different ratio between the transcription levels of the 16S rRNA gene and *magA* in the presence of RFP. Alternatively, the level of *magA* transcription may have temporarily increased by an unknown mechanism.

4. Discussion

The aim of the current study was to identify an agent that could suppress the mucoviscosity of hvKP strains without affecting hvKP growth. To achieve our goal, we first attempted to establish a method to evaluate the mucoviscosity of hvKP under controlled growth conditions. Until now, the string test, OD_{600} measurement after

low-speed centrifugation of bacterial liquid culture, and quantification of glucuronic acid in CPS extracts have been primarily used as ways to evaluate hvKP mucoviscosity [31,32]. Of these methods, the string test is merely a quantitative method and not applicable for our study. Similarly, the limitations regarding quantitation and reproducibility of OD_{600} measurements would not allow us to distinguish subtle differences on hvKP that may be caused by the test antimicrobials. As for the measurement of glucuronic acid, we avoided this method since we were unsure if this sugar accurately reflected the mucoviscosity of CPS. Therefore, instead of using the existing methods, we developed our own using an Ostwald viscometer to directly evaluate EPS viscosity of bacteria grown in the presence of each antimicrobial drug. This method physically measured bacterial EPS viscosity, including the capsular material, independent of the polysaccharide composition included. In the first screening of candidate antimicrobials, we used η_{sp} values divided by OD_{600} for comparison of their anti-mucoviscosity effect. However, one should be cautious in using OD_{600} for normalization because OD_{600} is not necessarily proportional to cell density/number when comparing cells under different growth conditions. One might also need to pay attention to the concentration-dependent nature of η_{sp}, especially when the cell density has large deviations. Although there is room for discussion on the details of the

methodology, the performance was successful in terms of quantitation and reproducibility, sufficiently reflecting the mucoviscosity of hvKP. Overall, our method was ideal for evaluating the anti-mucoviscosity of the antimicrobial compounds and, as a result, we succeeded in identifying the mucoviscosity-suppressing effect of RFP against hvKP.

It appeared that several other agents, such as MEPM, OFLX, and CP also demonstrated some activity to suppress mucoviscosity, while others showed mucoviscosity-promoting activity. These observations may be reflective of the actual activity of these agents to affect the bacterial CPS production. There may also have been some contribution from errors associated solely with the methodology. We noted that bacteriostatic agents with protein synthesis-inhibitory activity (i.e. AZM, CAM, TC, and CP) tended to cause relatively large deviations of η_{sp}/OD_{600} values. In the presence of these agents, we observed severe growth inhibition even at one-fourth of the MIC. In the presence of AZM, CAM, TC, and CP, the cell densities only reached OD_{600} of 2.4, 2,9, 3.0, and 1.4, respectively, after overnight cultivation, when it should have reached around 5.0 without an antimicrobial agent. In these cases, the antimicrobials might have altered the overall pattern of protein synthesis, resulting in apparent promotion or inhibition of CPS synthesis. On the other hand, MEPM, MNZ, and OFLX caused a

decrease of the η_{sp}/OD_{600} value without inhibiting the bacterial growth. We assume that these agents might indeed have some anti-mucoviscosity activity, although their specificity seems to be much lower than that of RFP.

In addition to viscosity measurements, we also used India ink staining to evaluate the effect of RFP on the mucoviscosity-related phenotype of hvKP. Negative staining with India ink has long been used as a method for microscopically demonstrating the presence of capsule [33]. There are also similar methods using other dyes, such as nigrosine, instead of India ink. By using these methods, the bacterial capsule can be observed as a clear halo around the cells against a black background. Despite their usefulness, our search of the literature revealed no study using a negative staining method to compare the capsule thickness of hvKP and non-hvKP strains. Our results successfully demonstrated the presence of atypically thick capsules around hvKP cells, consistent with a previous study that showed thick capsules for hvKP serotype K2 using a fluorescent-antibody staining method [34]. If the thick capsule correlates with the high virulence of hvKP, staining with India ink may be a good method for distinguishing hvKP isolates from non-hvKP isolates.

It has been established that *K. pneumoniae cps* cluster typically contains at least three promoters, located at the upstream regions of *galF* and *wzi*, and downstream of *gnd*

(which, in many cases, corresponds to upstream of *manC*) [23]. The transcriptional regulator RmpA upregulates the activities of all these promoters [15]. We hypothesized that the effect of RFP on hvKP may be exerted via the repression of *rmpA*. If this is the case, all the *cps* genes are expected to be repressed by RFP treatment. We thus examined the influence of RFP on the expression levels of *rmpA*, *magA*, *galF*, *wzi*, and *manC*. As expected, qRT-PCR revealed reduced transcription level of *rmpA* in hvKP grown in the presence of RFP. Moreover, all the *cps* genes tested were strongly repressed by the RFP treatment, supporting our hypothesis.

Based on these observations, we suggest a model for the mechanism of action of RFP on the mucoviscosity of hvKP. According to the model, RFP inhibits the transcription of *rmpA*, which in turn causes a reduction of the expression level of *cps* genes. The exact way RFP causes the reduction in the level of *rmpA* transcription is unclear, but two possible mechanisms exist. One mechanism may involve some unknown target to which RFP binds, causing modification of the transcription level of *rmpA*. For the other mechanism, RFP may partially bind and inhibit RpoB. This may not affect bacterial growth but could modulate the overall gene-expression pattern. With the latter mechanism, we assume that some non-essential genes may be more strongly affected by the partial inhibition of RpoB activity than were the essential housekeeping genes. As

an example, *rmpA* would be one of such strongly affected genes.

The capsule of *K. pneumoniae* confers an increased resistance to phagocytosis and intracellular killing by neutrophils and bactericidal complement [7]. Overproduction of CPS is strongly related to abscess formation and metastatic complications by this bacterium [17,18]. Based on these facts, we hypothesize that hvKP treatment would be more efficient if the mucoviscosity of hvKP could be reduced by the administration of RFP. *K. pneumoniae* is generally not susceptible to RFP, as exemplified by our antimicrobial susceptibility test on the hvKP isolates (Table 2). In addition, our study demonstrated that the anti-mucoviscosity effect of RFP on hvKP cells is reversible. We therefore would not expect that administration of RFP alone is sufficient as a therapy for hvKP infection. Instead, we assume the usage of RFP as a concomitant drug in a combination therapy with other bactericidal agents. Various types of bacterial infections including tuberculosis are commonly treated with RFP. It is sometimes used in combination with anti-methicillin-resistant *Staphylococcus aureus* (MRSA) agents in the treatment of MRSA infection, especially for refractory infections such as osteomyelitis and prosthetic joint infections [35]. Considering these successful uses in various clinical settings, we believe that the use of RFP for hvKP infection may be a realistic strategy.

There are still many uncertainties to be addressed before moving this methodology to clinical settings. For example, the mechanism of action of RFP has not been completely elucidated. There is a need to clarify this so that the potential limitations and possible pitfalls of this agent in treating hvKP infections can be discussed. Experiments using molecular techniques such as RNA-Seq analysis are necessary to fully reveal the mechanism. Second, we confirmed only the effect of RFP against hvKP *in vitro*. *In vivo* experiments using animal models of infection are necessary to confirm the effect of RFP against hvKP infection.

In conclusion, RFP potently inhibited the transcription of *rmpA* and *rmpA*-regulated *cps* genes and reduced the production of CPS, thus showing strong anti-mucoviscosity activity against CG23-I hvKP. We suggest that RFP may serve as a potential anti-virulence agent for this problematic pathogen. The development of a new treatment strategy using RFP as an anti-virulence agent will require molecular-based experiments to reveal the mechanisms of the RFP activity and *in vivo* experiments using animal models to verify its efficacy.

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Declarations of interest: None.

Ethical approval: Not required.

References

[1] Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 1998;11:589–603.

[2] Chang FY, Chou MY, Fan RL, Shaio MF. A clinical study of *Klebsiella* liver abscess. Taiwan Yi Xue Hui Za Zhi 1988;87:282–7.

[3] Shon AS, Bajwa RP, Russo TA. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: a new and dangerous breed. Virulence 2013;4:107–18.

[4] Liu YC, Cheng DL, Lin CL. *Klebsiella pneumoniae* liver abscess associated with septic endophthalmitis. Arch Intern Med 1986;146:1913–6.

[5] Yu VL, Hansen DS, Ko WC, Sagnimeni A, Klugman KP, von Gottberg A, et al. Virulence characteristics of *Klebsiella* and clinical manifestations of *K. pneumoniae* bloodstream infections. Emerg Infect Dis 2007;13:986–93.

[6] Catalán-Nájera JC, Garza-Ramos U, Barrios-Camacho H. Hypervirulence and hypermucoviscosity: two different but complementary *Klebsiella* spp. phenotypes? Virulence 2017;8:1111-23.

[7] Siu LK, Yeh KM, Lin JC, Fung CP, Chang FY. *Klebsiella pneumoniae* liver abscess: a new invasive syndrome. Lancet Infect Dis 2012;12:881–7.

[8] Sellick JA, Russo TA. Getting hypervirulent *Klebsiella pneumoniae* on the radar screen. Curr Opin Infect Dis 2018;31:341-6.

[9] Namikawa H, Yamada K, Fujimoto H, Oinuma KI, Tochino Y, Takemoto Y, et al. Two unusual cases of successful treatment of hypermucoviscous *Klebsiella pneumoniae* invasive syndrome. BMC Infect Dis 2016;16:680.

[10] Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: Going on the offense with a strong defense. Microbiol Mol Biol Rev 2016;80:629–61.

[11] Bialek-Davenet S, Criscuolo A, Ailloud F, Passet V, Jones L, Delannoy-Vieillard AS, et al. Genomic definition of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* clonal groups. Emerg Infect Dis 2014;20:1812-20.

[12] Struve C, Roe CC, Stegger M, Stahlhut SG, Hansen DS, Engelthaler DM, et al. Mapping the evolution of hypervirulent *Klebsiella pneumoniae*. mBio 2015;6:e00630-15.

[13] Lam MMC, Wyres KL, Duchêne S, Wick RR, Judd LM, Gan YH, et al. Population genomics of hypervirulent *Klebsiella pneumoniae* clonal-group 23 reveals early emergence and rapid global dissemination. Nat Commun 2018;9:2703.

[14] Faïs T, Delmas J, Barnich N, Bonnet R, Dalmasso G. Colibactin: More Than

a New Bacterial Toxin. Toxins (Basel) 2018;10:E151.

[15] Cheng HY, Chen YS, Wu CY, Chang HY, Lai YC, Peng HL. RmpA regulation of capsular polysaccharide biosynthesis in *Klebsiella pneumoniae* CG43. J Bacteriol 2010;192:3144–58.

[16] Hsu CR, Lin TL, Chen YC, Chou HC, Wang JT. The role of *Klebsiella pneumoniae rmpA* in capsular polysaccharide synthesis and virulence revisited. Microbiology 2011;157:3446–57.

[17] Yu WL, Ko WC, Cheng KC, Lee HC, Ke DS, Lee CC, et al. Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. Clin Infect Dis 2006;42:1351–8.

[18] Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. J Exp Med 2004;199:697–705.

[19] Lawlor MS, Hsu J, Rick PD, Miller VL. Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model. Mol Microbiol 2005;58:1054–73.

[20] Yeh KM, Lin JC, Yin FY, Fung CP, Hung HC, Siu LK, et al. Revisiting the importance of virulence determinant *magA* and its surrounding genes in *Klebsiella pneumoniae* causing pyogenic liver abscesses: exact role in serotype K1 capsule

formation. J Infect Dis 2010;201:1259–67.

[21] Fung CP, Chang FY, Lin JC, Ho DM, Chen CT, Chen JH, et al. Immune response and pathophysiological features of *Klebsiella pneumoniae* liver abscesses in an animal model. Lab Invest 2011;91:1029–39.

[22] Kawai T. Hypermucoviscosity: an extremely sticky phenotype of *Klebsiella pneumoniae* associated with emerging destructive tissue abscess syndrome. Clin Infect Dis 2006;42:1359–61.

[23] Shu HY, Fung CP, Liu YM, Wu KM, Chen YT, Li LH, et al. Genetic diversity of capsular polysaccharide biosynthesis in *Klebsiella pneumoniae* clinical isolates. Microbiology 2009;155:4170-83.

[24] Lai YC, Peng HL, Chang HY. RmpA2, an activator of capsule biosynthesis in *Klebsiella pneumoniae* CG43, regulates K2 *cps* gene expression at the transcriptional level. J Bacteriol 2003;185:788–800.

[25] Russo TA, Olson R, Macdonald U, Metzger D, Maltese LM, Drake EJ, et al. Aerobactin mediates virulence and accounts for increased siderophore production under iron-limiting conditions by hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*. Infect Immunol 2014;82:2356–67.

[26] Bailey DC, Drake EJ, Grant TD, Gulick AM. Structural and functional

characterization of aerobactin synthetase *iucA* from a hypervirulent pathotype of *Klebsiella pneumoniae*. Biochemistry 2016;55:3559–70.

[27] Russo TA, Olson R, Fang CT, Stoesser N, Miller M, MacDonald U, et al. Identification of biomarkers for differentiation of hypervirulent *Klebsiella pneumoniae* from classical *K. pneumoniae*. J Clin Microbiol 2018;56:e00776-18.

[28] Lai YC, Lin AC, Chiang MK, Dai YH, Hsu CC, Lu MC, et al. Genotoxic *Klebsiella pneumoniae* in Taiwan. PLoS One 2014;9:e96292.

[29] Hanaoka N, Takano Y, Shibuya K, Fugo H, Uehara Y, Nimi M. Identification of the putative protein phosphatase gene *PTC1* as a virulence-related gene using a silkworm model of *Candida albicans* infection. Eukaryot Cell 2008;7:1640–8.

[30] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.

[31] Choi MJ, Ko KS. Loss of hypermucoviscosity and increased fitness cost in colistin-resistant *Klebsiella pneumoniae* sequence type 23 strains. Antimicrob Agents Chemother 2015;59:6763–73.

[32] Ares MA, Fernández-Vázquez JL, Rosales-Reyes R, Jarillo-Quijada MD, von Bargen K, Torres J, et al. H-NS nucleoid protein controls virulence features of

Klebsiella pneumoniae by regulating the expression of type 3 pili and the capsule polysaccharide. Front Cell Infect Microbiol 2016;6:13.

[33] Hoogerheide JC. Studies on Capsule Formation: I. The conditions under which *Klebsiella pneumoniae* (Friedländer's bacterium) forms capsules. J Bacteriol 1939;38:367–89.

[34] Wacharotayankun R, Arakawa Y, Ohta M, Tanaka K, Akashi T, Mori M, et al. Enhancement of extracapsular polysaccharide synthesis in *Klebsiella pneumoniae* by RmpA2, which shows homology to NtrC and FixJ. Infect Immun 1993;61:3164–74.

[35] Darley ES, MacGowan AP. Antibiotic treatment of gram-positive bone and joint infections. J Antimicrob Chemother 2004;53:928–35.

[36] Choi MJ, Kim S, Ko KS. Pathways regulating the *pbgP* operon and colistin resistance in *Klebsiella pneumoniae* strains. J Microbiol Biotechnol. 2016;26(9):1620-8.

[37] Gomes AÉI, Stuchi LP, Siqueira NMG, Henrique JB, Vicentini R, Ribeiro ML, et al. Selection and validation of reference genes for gene expression studies in *Klebsiella pneumoniae* using Reverse Transcription Quantitative real-time PCR. Sci Rep. 2018;8(1):9001.

[38] Lin CT, Wu CC, Chen YS, Lai YC, Chi C, Lin JC, et al. Fur regulation of the capsular polysaccharide biosynthesis and iron-acquisition systems in *Klebsiella pneumoniae* CG43. Microbiology. 2011;157:419-29.

[39] Clifford RJ, Milillo M, Prestwood J, Quintero R, Zurawski DV, Kwak YI, et al. Detection of bacterial 16S rRNA and identification of four clinically important bacteria by real-time PCR. PLoS One. 2012;7:e48558.

[40] Hartman LJ, Selby EB, Whitehouse CA, Coyne SR, Jaissle JG, Twenhafel NA, et al. Rapid real-time PCR assays for detection of *Klebsiella pneumoniae* with the *rmpA* or *magA* genes associated with the hypermucoviscosity phenotype: screening of nonhuman primates. J Mol Diagn. 2009;11:464-71.

[41] Ares MA, Fernández-Vázquez JL, Rosales-Reyes R, Jarillo-Quijada MD, von Bargen K, Torres J, et al. H-NS nucleoid protein controls virulence features of *Klebsiella pneumoniae* by regulating the expression of type 3 pili and the capsule polysaccharide. Front Cell Infect Microbiol. 2016;6:13.

Tables

Table 1. Primers used for quantitative real-time PCR

Primer	Target gene	Sequence (*5′→3′*)	Reference
rpoB-F	*rpoB*	CGCGTATGTCCGATCGAAA	[36]
rpoB-R	*rpoB*	GCGTCTCAAGGAAGCCATATTC	[36]
recA-F	*recA*	TTAAACAGGCCGAATTCCAG	[37]
recA-R	*recA*	CCGCTTTCTCAATCAGCTTC	[37]
23S-F	23S rRNA gene	GGTAGGGGAGCGTTCTGTAA	[38]
23S-R	23S rRNA gene	TCAGCATTCGCACTTCTGAT	[38]
16S-F	16S rRNA gene	ACTCCTACGGGAGGCAGCAGT	[39]
16S-R	16S rRNA gene	TATTACCGCGGCTGCTGGC	[39]
magA-F	*magA*	CGAAAGTGAACGAATTGATGCT	[40]

magA-R	*magA*	GTTTCTGCTGCAGATTCGAAGA	[40]
rmpA-F	*rmpA*	AGAGTATTGGTTGACTGCAGGATTT	[40]
rmpA-R	*rmpA*	AAACATCAAGCCATATCCATTGG	[40]
galF-F	*galF*	CAAAGGCAATTCCAAAGGAG	[41]
galF-R	*galF*	TGCGTCACCAGAACAATCTC	[41]
wzi-F	*wzi*	CAGGGGTTTGGTCAGACACA	[41]
wzi-R	*wzi*	CGTTGAAGCGTGATCCGTTG	[41]
manC-F	*manC*	AGCGGCATGTTTATGTTCCG	[41]
manC-R	*manC*	AAATGTCATGCGGGATGCTG	[41]
iucA-F	*iucA*	TCTCCCGGCTTATTGTTGATA	[38]
iucA-R	*iucA*	GGAAGGTTTCGCAACTGGT	[38]
iroN-F	*iroN*	ACCGGGATATTCGCCTGAA	[37]

iroN-R	*iroN*	GGCCAGGCTCATTGTAGGT	[37]

Table 2. Minimum inhibitory concentrations of 18 antimicrobials against four *Klebsiella pneumoniae* (hvKP) strains

	MIC (μg/ml)			
Antimicrobial agent	OCU_hvKP1	OCU_hvKP2	OCU_hvKP3	OCU_hvKP4
Benzylpenicillin	64	64	128	64
Ampicillin	64	64	128	128
Meropenem	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Metronidazole	≥ 128	≥ 128	≥ 128	≥ 128
Streptomycin	4	4	4	4
Kanamycin	2	2	2	4
Gentamycin	0.25	0.5	0.5	0.5

Amikacin	1	2	4	2
Ciprofloxacin	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Ofloxacin	≤ 0.125	≤ 0.125	0.25	≤ 0.125
Azithromycin	8	8	8	8
Clarithromycin	128	128	128	128
Tetracycline	4	4	8	8
Chloramphenicol	8	4	2	4
Rifampicin	32	32	32	32
Vancomycin	≥ 128	≥ 128	≥ 128	≥ 128
Polymyxin B	0.5	1	1	1
Colistin	0.5	1	0.5	0.5

MIC, minimum inhibitory concentration.

Figure legends

Figure 1: Effects of antimicrobial agents on the mucoviscosity of hypervirulent *Klebsiella pneumoniae* (hvKP)

(A) Effect of 18 antimicrobial agents on the mucoviscosity of hvKP.

The dashed line indicates the mucoviscosity level of hvKP grown without an antimicrobial agent. (B) Concentration dependence of RFP activity on the growth inhibition and anti-mucoviscosity against hvKP. Values presented are means of results from three independent experiments. Bars indicate standard deviations.

PCG, benzylpenicillin; ABPC, ampicillin; MEPM, meropenem; MNZ, metronidazole; SM, streptomycin; KM, kanamycin; GM, gentamycin; AMK, amikacin; CPFX, ciprofloxacin; OFLX, ofloxacin; AZM, azithromycin; CAM, clarithromycin; TC, tetracycline; CP, chloramphenicol; RFP, rifampicin; VCM, vancomycin; PL-B, polymyxin B; CL, colistin.

Fig. 1A

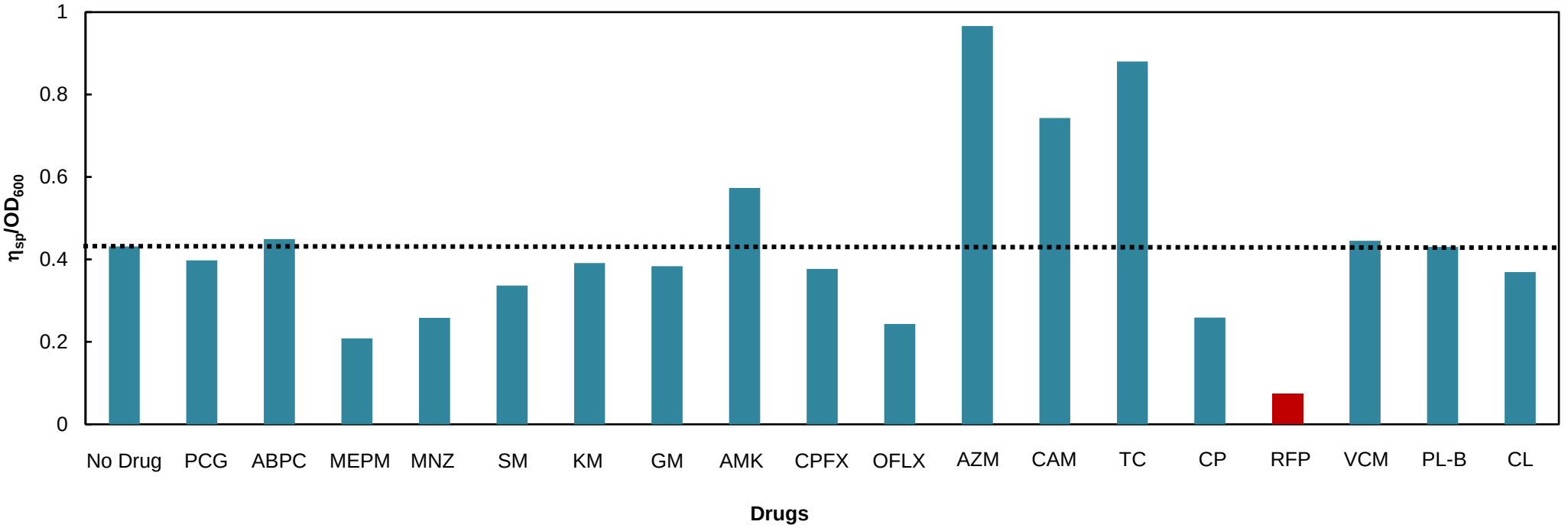

1
0.8
0.6
0.4
0.2
0
η_{sp}/OD_{600}
No Drug
PCG
ABPC
MEPM
MNZ
SM
KM
GM
AMK
CPFX
OFLX
AZM
CAM
TC
CP
RFP
VCM
PL-B
CL
Drugs

Fig. 1B

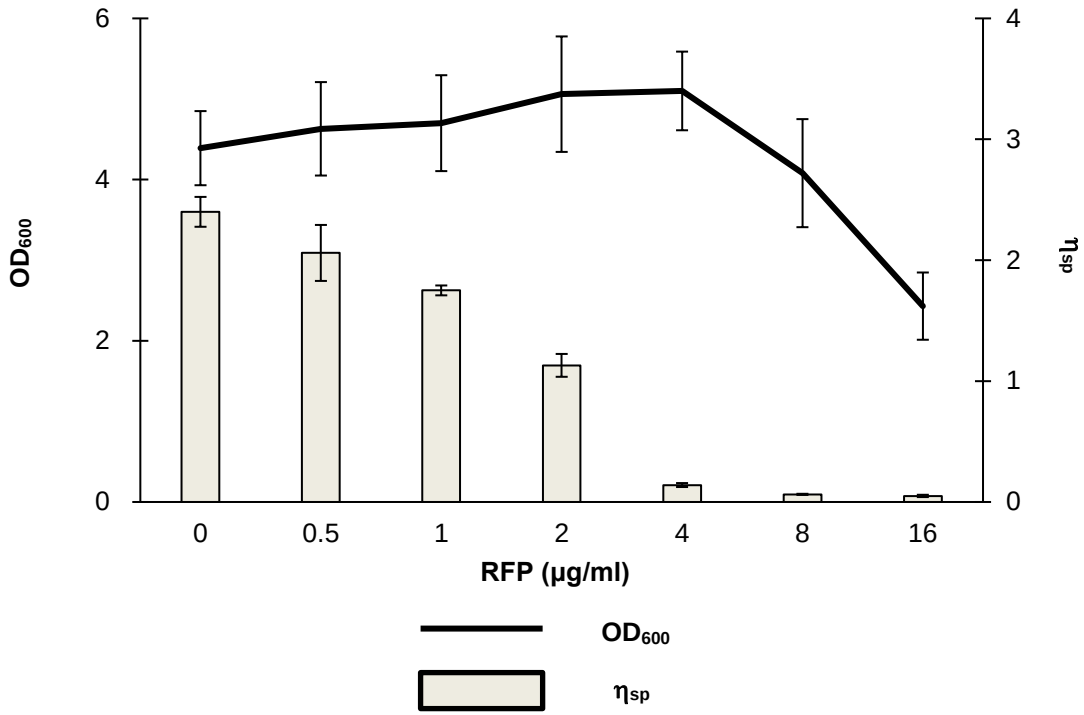


Figure 2: Effect of rifampicin (RFP) on the thickness of the hypervirulent *Klebsiella pneumoniae* (hvKP) capsule

(A) Microscopic evaluation of the capsule visualized using India ink staining. Representative images are shown for a non-hvKP control strain grown in the absence of RFP (Left), OCU_hvKP1 grown without RFP (Center), and OCU_hvKP1 grown in the presence of 8 μg/ml RFP (Right). Bars indicate 10 μm. (B) Temporal changes in the capsule thickness under growth with or without RFP. At least 50 cells were measured at each time point for the calculations. Bars indicate standard deviations. (C) Temporal changes of the capsule thickness after removal of RFP. At least 50 cells were measured at each time point. Bars indicate standard deviations. *, $P < 0.001$ (Mann-Whitney *U* test)

Fig. 2A

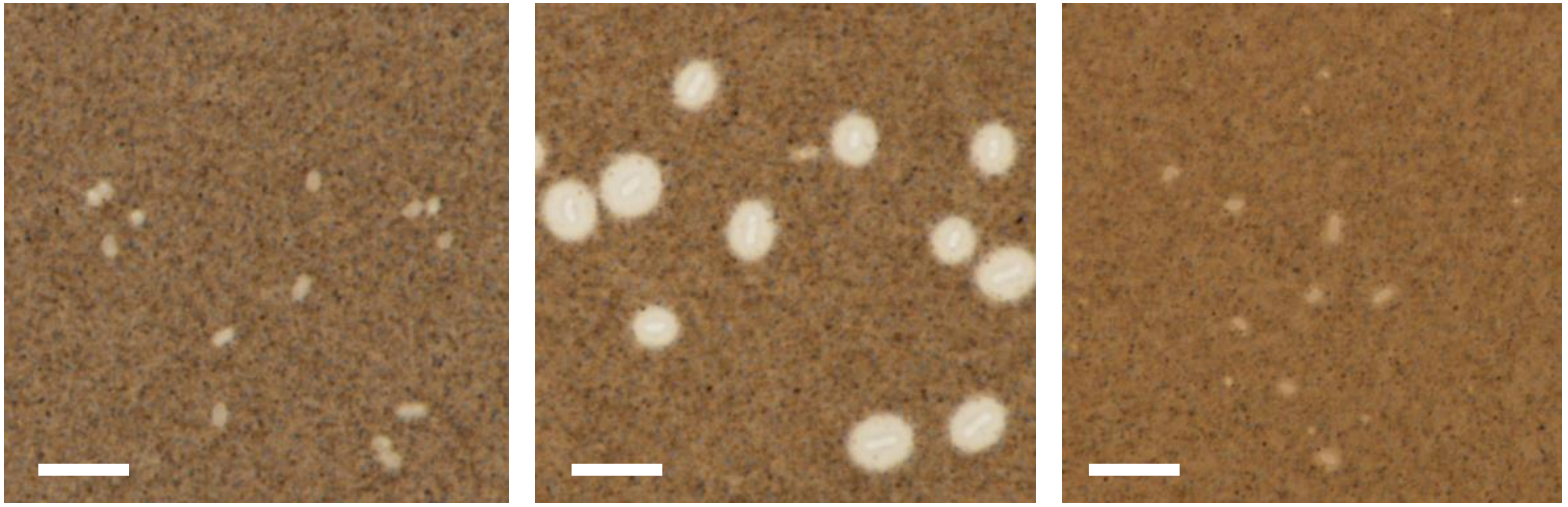


Fig. 2B

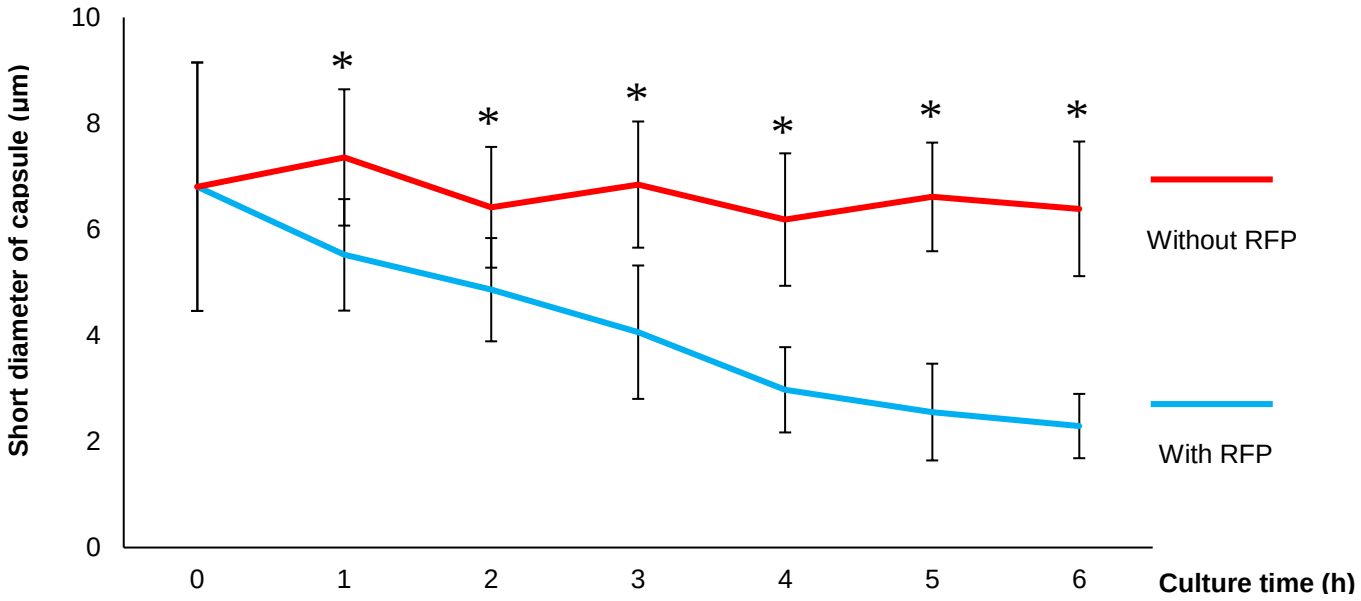

Short diameter of capsule (μm)
10
8
6
4
2
0
0
1
2
3
4
5
6
Culture time (h)
*
*
*
*
*
*
Without RFP
With RFP

Fig. 2C

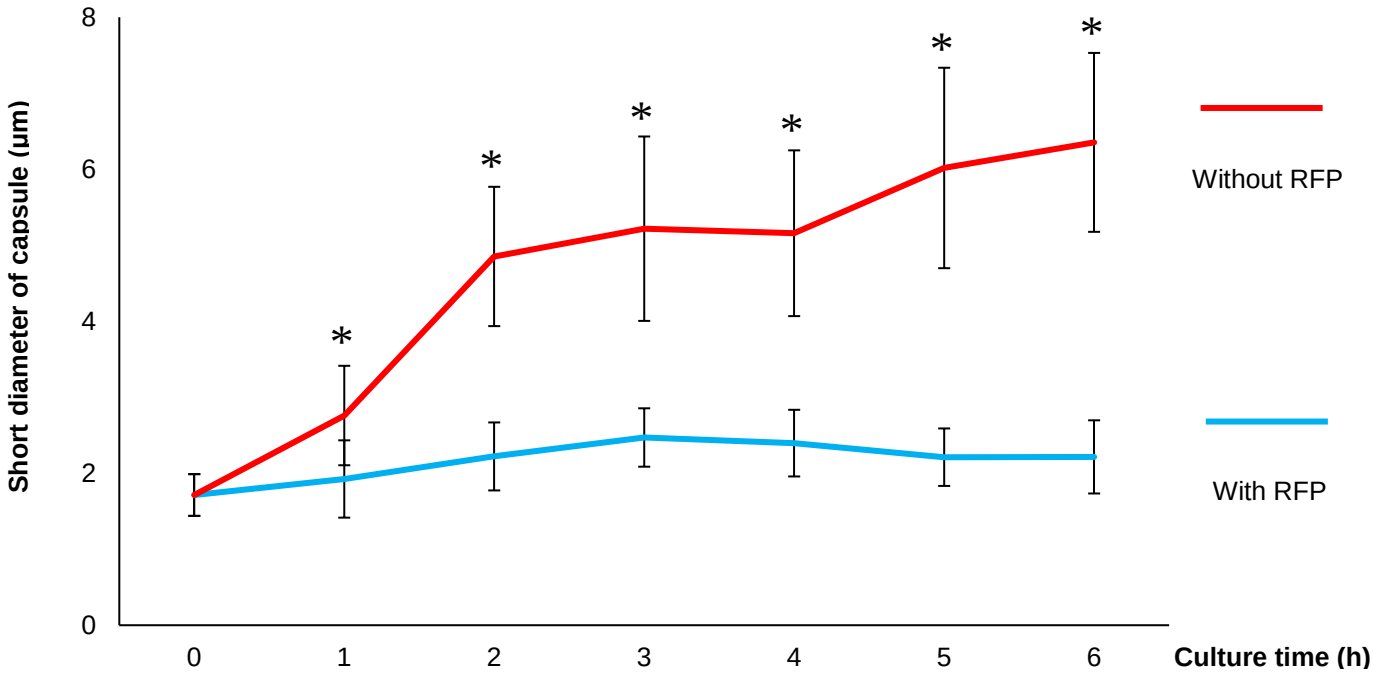

Short diameter of capsule (μm)
8
6
4
2
0
0
1
2
3
4
5
6
Culture time (h)
*
*
*
*
*
*
Without RFP
With RFP

Figure 3: Effect of rifampicin (RFP) on the expression levels of capsule- and siderophore- related genes

The expression levels in hvKP grown without RFP were set to 1.0 for each gene. The values presented are mean relative mRNA levels from four (*magA*) or six (*rmpA* and *iucA*) or five (*galF*, *wzi*, *manC*, and *iroN*) independent experiments. Bars indicate standard deviations. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney *U* test)

Fig. 3

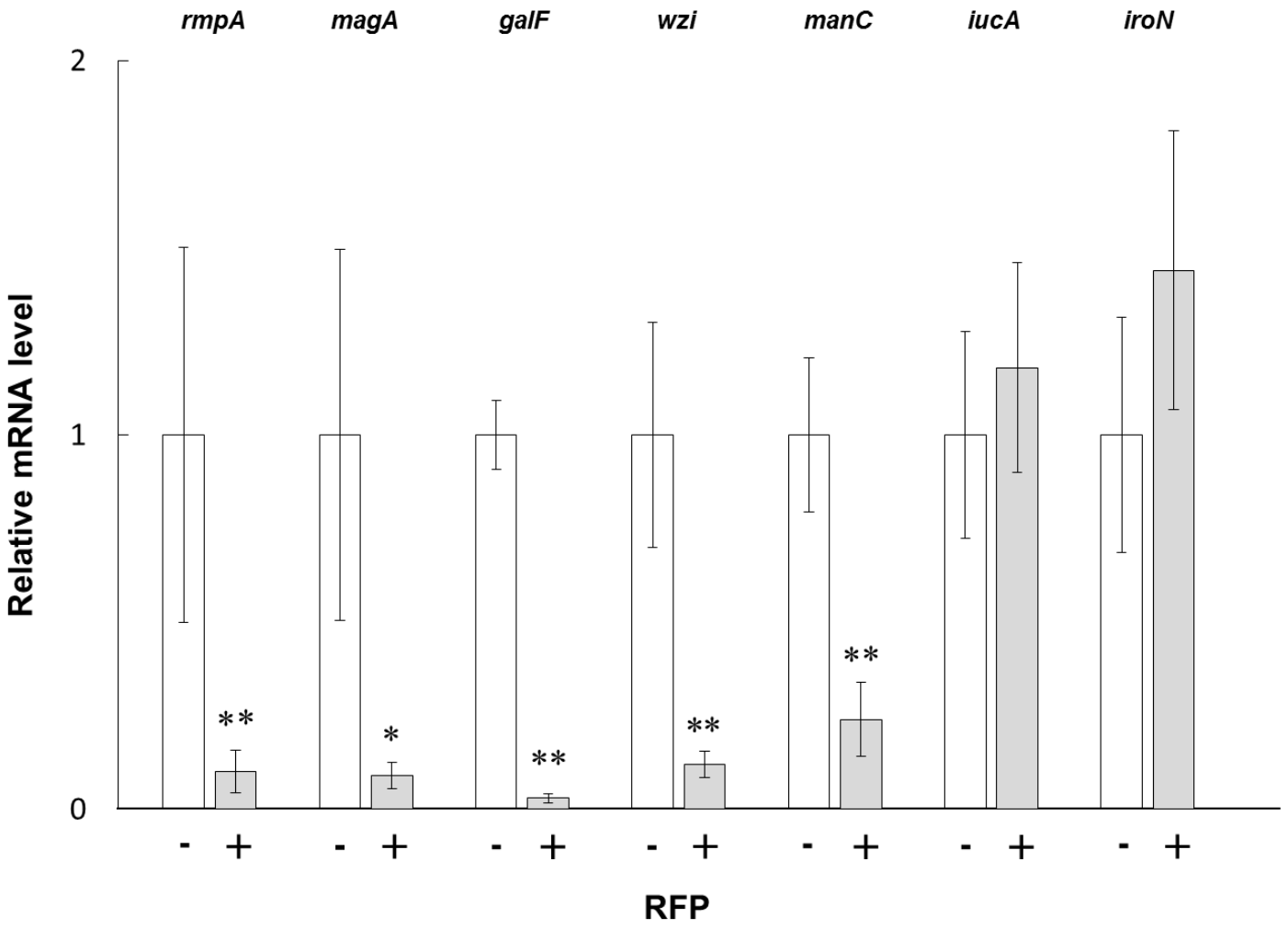

rmpA
magA
galF
wzi
manC
iucA
iroN
2
1
0
Relative mRNA level
**
*
**
**
**
- + - + - + - + - + - + - +
RFP

Figure 4: Time-dependent change of the transcript levels of *rmpA* (A) and *magA* (B) under growth with or without rifampicin (RFP)

The expression levels at two hours of growth without RFP were set to 1.0 for each gene. Presented values are mean relative mRNA levels from four or five independent experiments. Bars indicate standard deviations. *, P <0.05; **, P <0.01 (Mann-Whitney *U* test

Fig. 4A

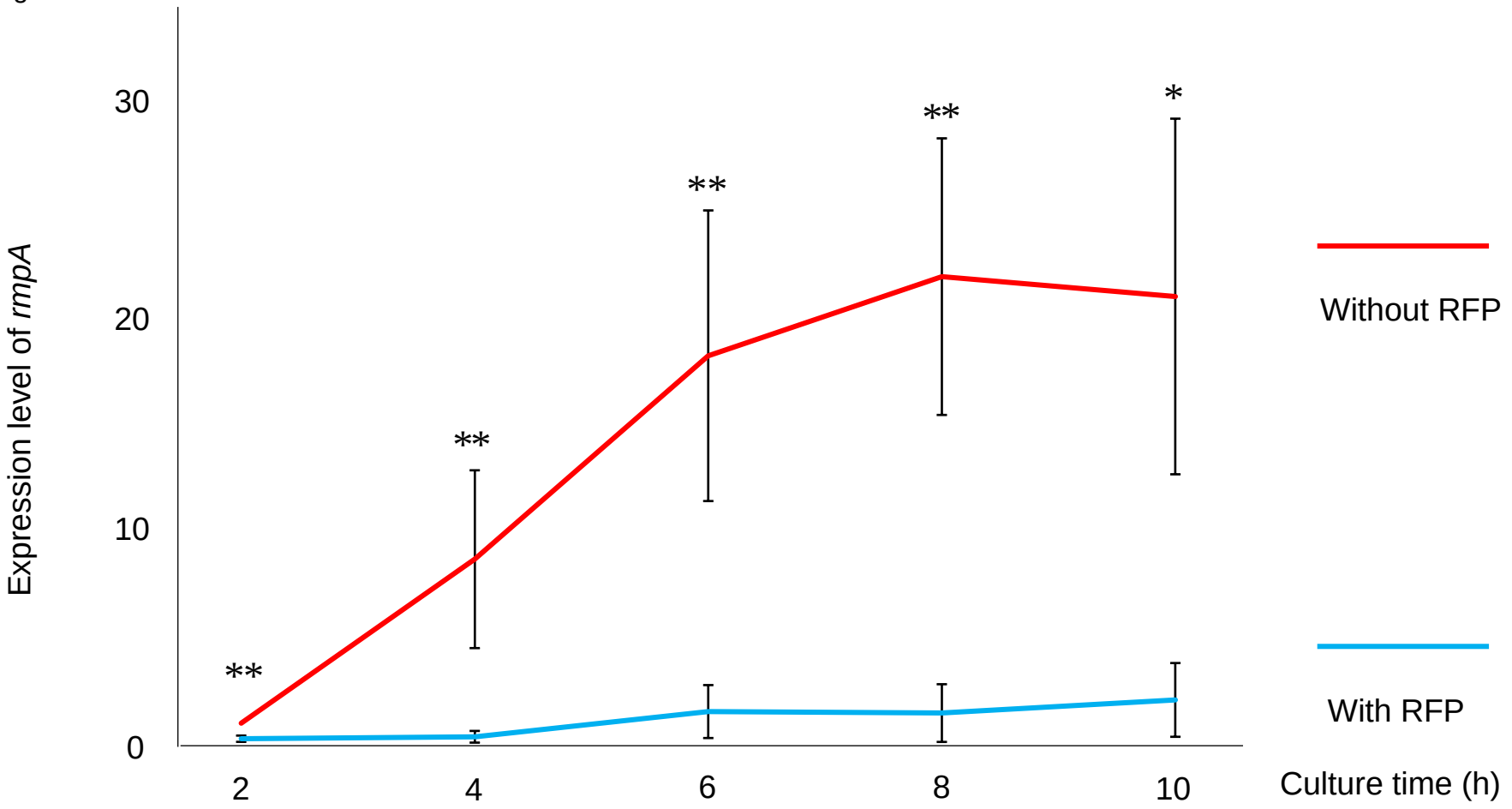
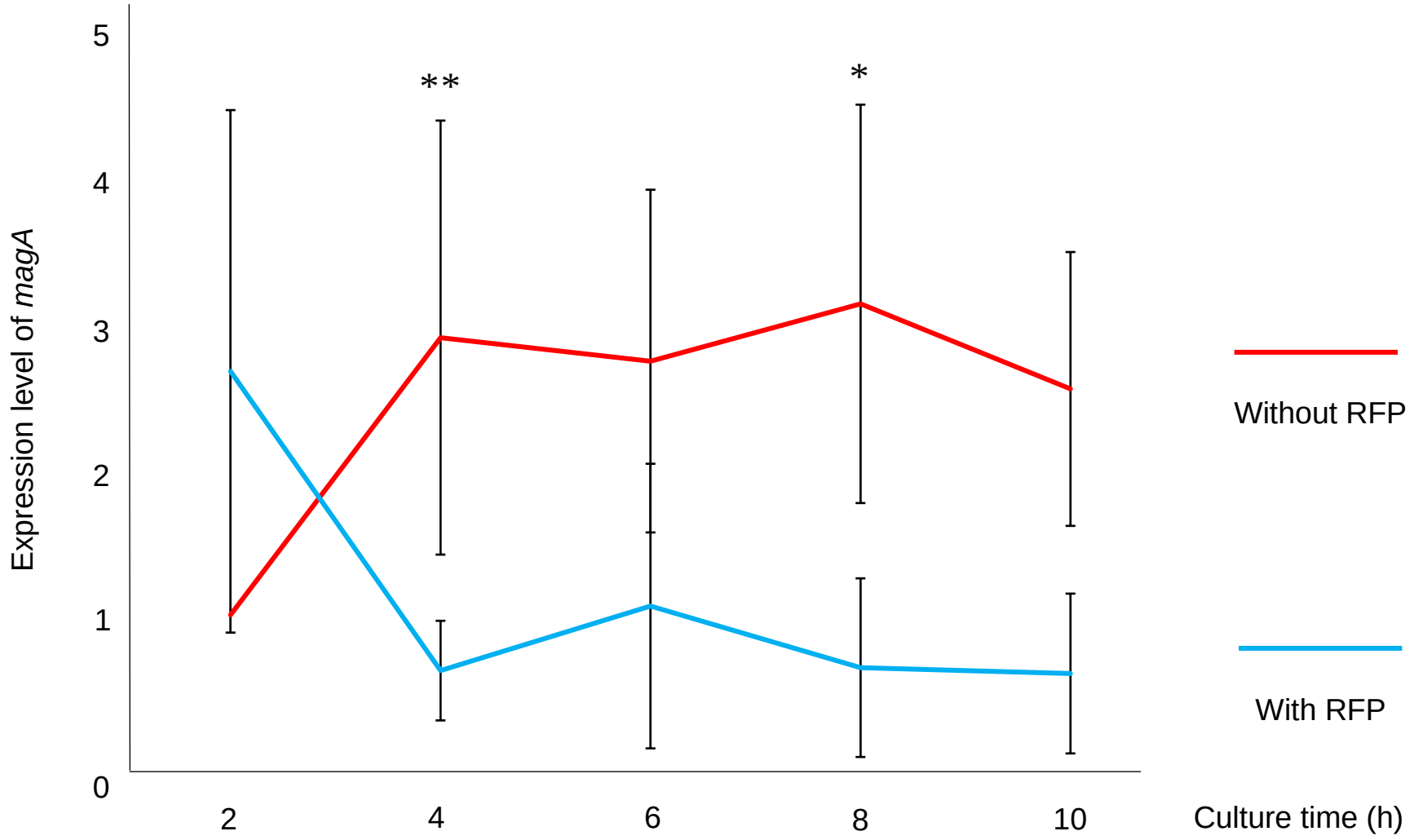

Expression level of rmpA
30
20
10
0
**
**
**
**
*
2
4
6
8
10
Culture time (h)
Without RFP
With RFP

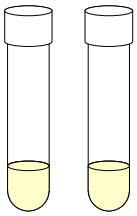
Fig. 4B


Expression level of magA
5
4
3
2
1
0
**
*
2
4
6
8
10
Culture time (h)
Without RFP
With RFP

Supplemental Figure Legend

Fig. S1. Illustration of two methods for evaluating the mucoviscous phenotype of hypervirulent *Klebsiella pneumoniae* used in this study. (A) Viscosity measurement of extracellular polysaccharide extract. (B) Evaluation of capsular thickness using India ink staining. M-H II, Mueller Hinton II; EPS, extracellular polysaccharide; ID, internal diameter.

A



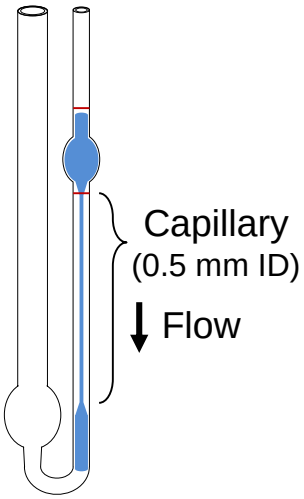
Cultivation
(M-H II, 37°C)

Add citrate buffer (pH 2)
with Zwittergent 3-14

Extract EPS by
heating at 50°C

Remove cells by centri-
fugation and filtration

Viscosity measurement
with Ostwald viscometer

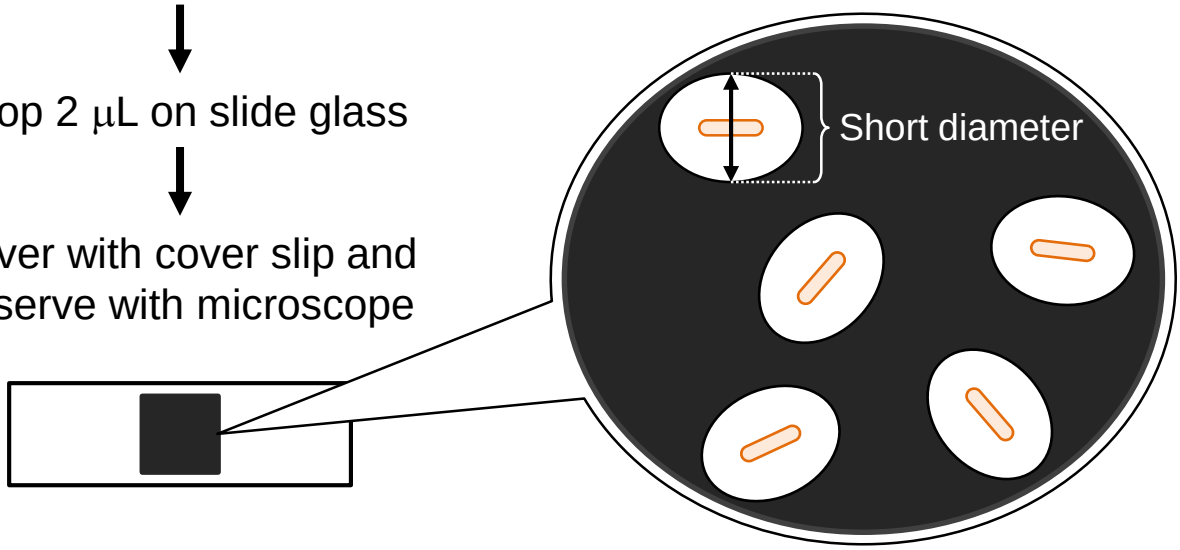


B

Mix bacterial culture
with India ink (1:1 ratio)

Drop 2 μL on slide glass

Cover with cover slip and
observe with microscope



Measure short diameter of
clear zone around cells