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Discovery of anti-mucoviscous activity of rifampicin and its potential as a candidate antivirulence 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 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* (regulator of mucoid phenotype A). Although extensive research has been conducted on the mechanisms of hvKP virulence, no study has focused on the development of antivirulence therapeutics. This study attempted to identify and validate an antimicrobial agent able to suppress hvKP hypermucoviscosity. A total of 18 commercially available antimicrobial agents, including β-lactams, quinolones and aminoglycosides, were tested. Rifampicin (RFP) was found to have strong anti-mucoviscous activity against CG23-I hvKP 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. These data suggest that RFP may serve as a potential antivirulence agent for refractory hvKP infection.

1. Introduction

Klebsiella pneumoniae is a common Gram-negative pathogen in community-acquired and nosocomial infections [1]. *Klebsiella pneumoniae* has long been regarded as an opportunistic pathogen causing pneumonia and urinary tract infections in 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 recognised 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. *Klebsiella pneumoniae* strains associated with primary liver abscesses characteristically express a distinct sticky phenotype when grown on agar [5] and are therefore called hypermucoviscous. Hypermucoviscous and hypervirulent phenotypes do not necessarily coincide, i.e. some hypermucoviscous strains are not hypervirulent and, similarly, some hypervirulent strains give negative results in the string test [6]. Whilst hvKP infections were originally limited to parts of Asia, they have spread to other geographic areas and are now recognised as a serious clinical issue worldwide with a recent increase in incidence [7,8]. hvKP has the

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ability to cause life-threating community-acquired infections even in young, healthy hosts. Its high tendency to cause bacteraemia, primary liver abscess, and metastatic infections such as endophthalmitis, meningitis and pneumonia, which are associated with remarkably high mortality, is a major characteristic of hvKP that distinguishes it from normal *K. pneumoniae* strains [3]. We previously 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 realised that hvKP virulence and the serological type of their capsular polysaccharide (CPS) are strongly related [10]. Among the 78 *K. pneumoniae* capsular serotypes described to date, only 8 have been found in hvKP [3]. The majority of hvKP isolates are serotypes K1 and K2, which account for at least two-thirds of all isolates. Genome-based studies have further revealed that the most prevalent lineages belong to clonal group 23 (CG23) [11,12]. Strikingly, whereas K2-type hvKP shows a relatively high degree of divergence, almost all K1 hvKP fall into a highly homogenous clade (CG23) in phylogenetic analyses, suggesting their recent emergence from a single common ancestor and subsequent rapid worldwide dissemination. Moreover, according to a recent report [13], the CG23-I sublineage, which was estimated to have emerged in the 1920s following acquisition of an integrative conjugative element named ICE*Kp10*, comprising the majority of 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 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 CPS polymerase that is essential for the production of hypermucoviscous CPS for serotype K1 hvKP [18,20]. Many other genes involved in CPS 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 an *rmpA* homolog (*rmpA2*) located on a plasmid; however, *rmpA2* may not be functional in CG23 because of truncation of the structural gene resulting from frameshifts [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, siderophores are known to be important hvKP virulence factors. 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 at revealing the mechanisms of hvKP virulence. However, no studies have focused on how to combat these difficult-to-treat infections in clinical settings. We hypothesised that inhibiting the production of critical virulence factors would result in more effective treatment of these infections. The aim of this study was to identify an antimicrobial agent that could be used as a mucoviscosity-suppressing agent against hvKP CG23-I. This study is the first step towards development of a new treatment strategy for hvKP infection.

2. Materials and methods

2.1. Strains, medium and culture conditions

Four hvKP strains were used in this study (OCU_hvKP1, OCU_hvKP2, OCU_hvKP3 and OCU_hvKP4). All four strains were isolated from patients with liver and/or prostate abscess from Osaka City University Hospital (Osaka, Japan). 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. *Klebsiella 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 (M-H II) broth (Becton Dickinson & Co., Franklin Lakes, NJ) at 37 °C with shaking at 130 rpm. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a U-1500 spectrophotometer (Hitachi, Tokyo, Japan).

2.2. Genetic characterisation of hypervirulent Klebsiella pneumoniae 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 seven housekeeping genes *rpoB, gapA, mdh, pgi, phoE, infB* and *tonB*. Presence of the 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 by the broth microdilution method according to the 2016 Clinical and Laboratory Standards Institute (CLSI) guidelines. Cation-adjusted M-H II broth was used as growth medium. The following 18 antimicrobial agents were purchased from commercial sources and were used for antimicrobial susceptibility testing: benzylpenicillin; ampicillin; meropenem; metronidazole; streptomycin; kanamycin; gentamicin; amikacin; ciprofloxacin; ofloxacin; azithromycin; clarithromycin; tetracycline; chloramphenicol; rifampicin (RFP); vancomycin; polymyxin B; and colistin.

2.4. Evaluation of the anti-mucoviscous activity of antimicrobial agents

The anti-mucoviscosity activity of all 18 antimicrobial agents was assessed against the hvKP strains. OCU_hvKP1 was used for screening the candidate antimicrobials, and the effect of RFP was confirmed using the 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 (see also Supplementary 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 broth using 14-mL round-bottom

Table 1
Primers used for quantitative real-time PCR.

Primer	Target gene	Sequence (5′→3′)	Reference
rpoB-F	*rpoB*	CGCGTATGTCCGATCGAAA	[30]
rpoB-R		GCGTCTCAAGGAAGCCATATTC	
recA-F	*recA*	TTAAACAGGCCGAATTCCAG	[31]
recA-R		CCGCTTTCTCAATCAGCTTC	
23S-F	23S rRNA gene	GGTAGGGGAGCGTTCTGTAA	[32]
23S-R		TCAGCATTCGCACTTCTGAT	
16S-F	16S rRNA gene	ACTCCTACGGGAGGCAGCAGT	[33]
16S-R		TATTACCGCGGCTGCTGGC	
magA-F	*magA*	CGAAAGTGAACGAATTGATGCT	[34]
magA-R		GTTTCTGCTGCAGATTCGAAGA	
rmpA-F	*rmpA*	AGAGTATTGGTTGACTGCAGGATTT	[34]
rmpA-R		AAACATCAAGCCATATCCATTGG	
galF-F	*galF*	CAAAGGCAATTCCAAAGGAG	[37]
galF-R		TGCGTCACCAGAACAATCTC	
wzi-F	*wzi*	CAGGGGTTTGGTCAGACACA	[37]
wzi-R		CGTTGAAGCGTGATCCGTTG	
manC-F	*manC*	AGCGGCATGTTTATGTTCCG	[37]
manC-R		AAATGTCATGCGGGATGCTG	
iucA-F	*iucA*	TCTCCCGGCTTATTGTTGATA	[32]
iucA-R		GGAAGGTTTCGCAACTGGT	
iroN-F	*iroN*	ACCGGGATATTCGCCTGAA	[31]
iroN-R		GGCCAGGCTCATTGTAGGT	

Table 2
Minimum inhibitory concentrations (MICs) of 18 antimicrobial agents against four hypervirulent *Klebsiella pneumoniae* (hvKP) strains.

Antimicrobial agent	MIC (μg/mL)			
	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
Gentamicin	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

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, MO). Following centrifugation at 5800 × *g* for 15 min, the supernatant was filtered through a 0.45-μm PVDF filter (Millex-HV; Merck Millipore, Burlington, MA) and 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 instructions, 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 then 2 μL samples of the mixtures were placed on microscope slides and covered with a cover slip. 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 Supplementary Fig. S1.

To determine time-dependent changes in capsule thickness, OCU_hvKP1 was grown overnight in M-H II broth with or without RFP (8 μg/mL) and was then subcultured in fresh medium 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 medium and was mixed with an equal volume of India ink. For each sample, four slides were prepared and were evaluated microscopically. 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 PCR (qPCR)

Expression levels of *rmpA, magA, galF, wzi, manC, iucA* and *iroN* were determined by qPCR. OCU_hvKP1 was grown in M-H II medium with or without RFP (8 μg/mL) for 6 h. 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 were suspended in 400 μL of buffer [50 mM Tris–HCl (pH 8.0), 10 mM ethylene diamine tetra-acetic acid (EDTA) and 1% sodium dodecyl sulfate (SDS)]. Samples were extracted with equal volumes of citrate buffer (pH 4.2)–saturated phenol (Acid Phenol; Nippon Gene, Tokyo, Japan) at 65 °C for 45 min and were then cooled on ice for 10 min. Following centrifugation, the supernatants were subjected to acid-phenol extraction followed by chloroform extraction. RNA was ethanol precipitated and was suspended in 100 μL of RNase-free water. Residual DNA was digested with 10 U 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 RNA was assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesised from the RNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). The cDNA was evaluated by qPCR using an Applied Biosystems PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The primers used for qPCR are listed in Table 1. Transcript levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method [35]. 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 the use of rpoB, recA, and 16S and 23S rRNA genes as reference genes was evaluated 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.

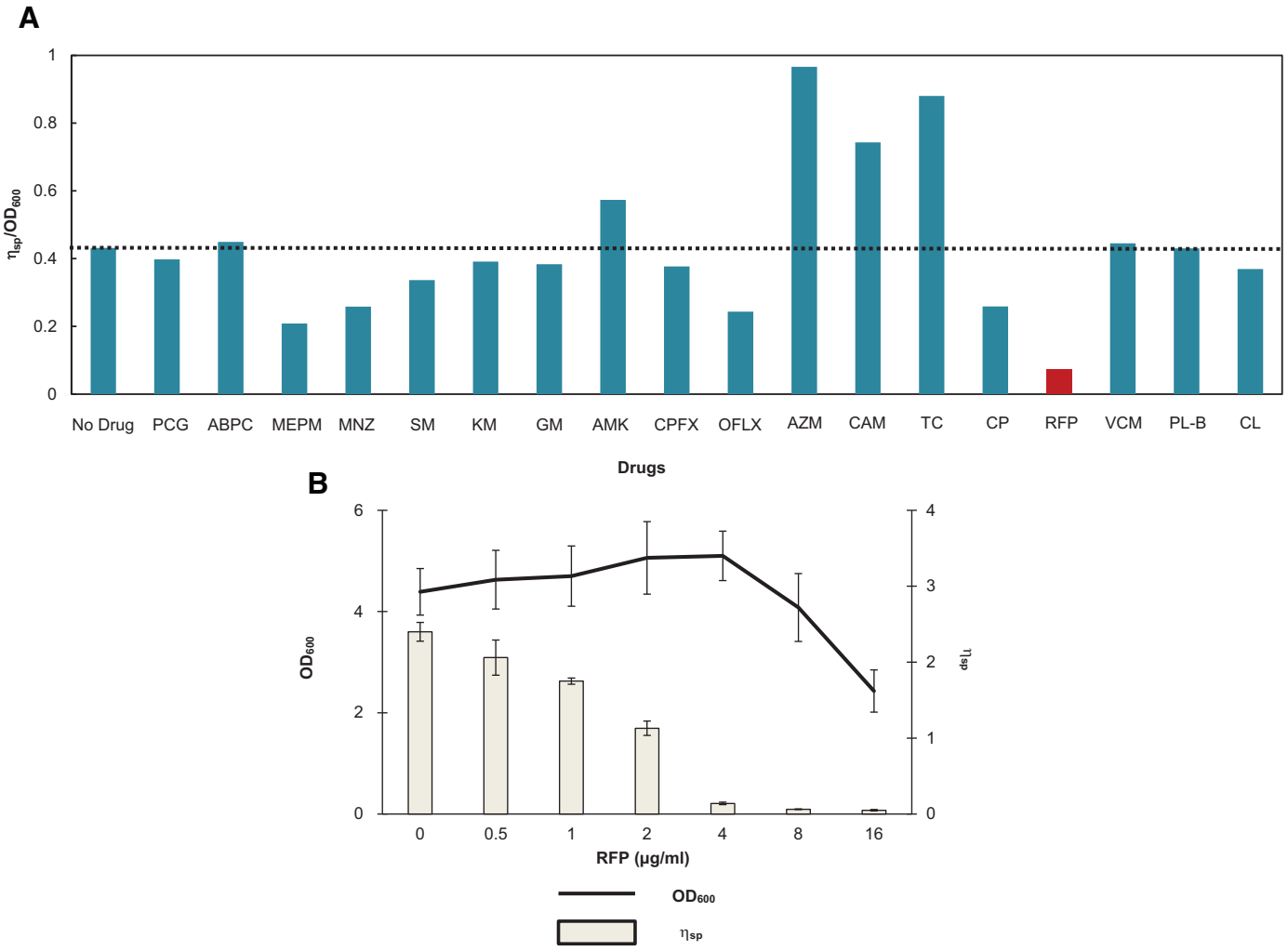


Fig. 1. (A) Effect of 18 antimicrobial agents on the mucoviscosity of hypervirulent *Klebsiella pneumoniae* (OCU_hvKP1). The dashed line indicates the mucoviscosity level of hvKP grown without an antimicrobial agent. PCG, benzylpenicillin; ABPC, ampicillin; MEPM, meropenem; MNZ, metronidazole; SM, streptomycin; KM, kanamycin; GM, gentamicin; AMK, amikacin; CPFX, ciprofloxacin; OFLX, ofloxacin; RFP, azithromycin; CAM, clarithromycin; TC, tetracycline; CP, chloramphenicol; RFP, rifampicin; VCM, vancomycin; PL-B, polymyxin B; CL, colistin. (B) Concentration dependence of RFP activity on the growth inhibition and anti-mucoviscosity against hvKP. Values presented are the mean and standard deviation from three independent experiments. OD_{60}, optical density at 600 nm; η_{sp}, specific viscosity.

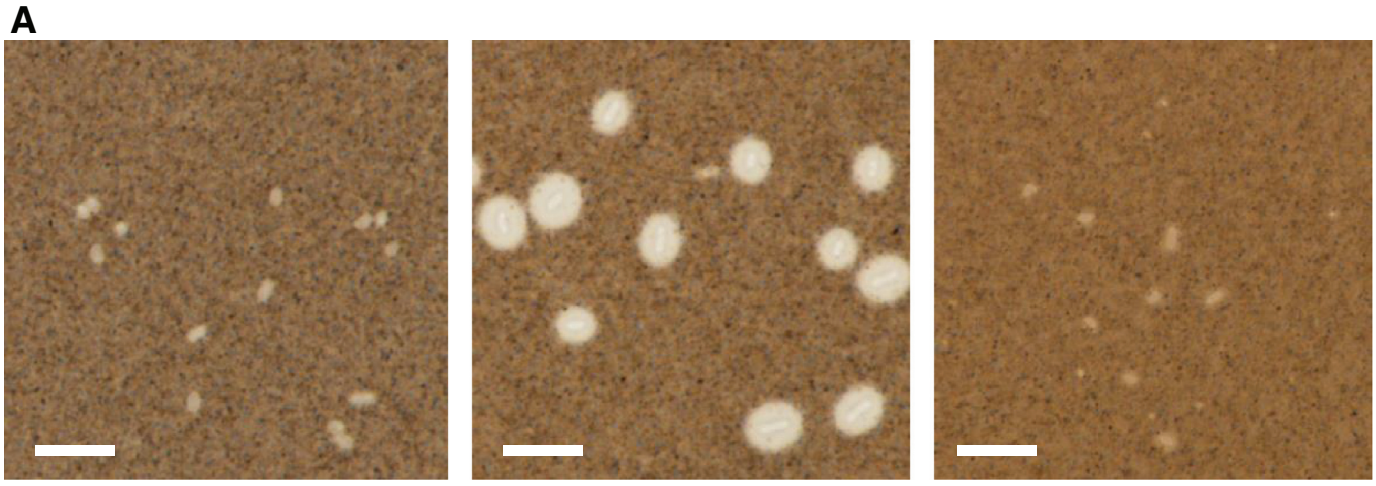
2.7. Statistical analyses

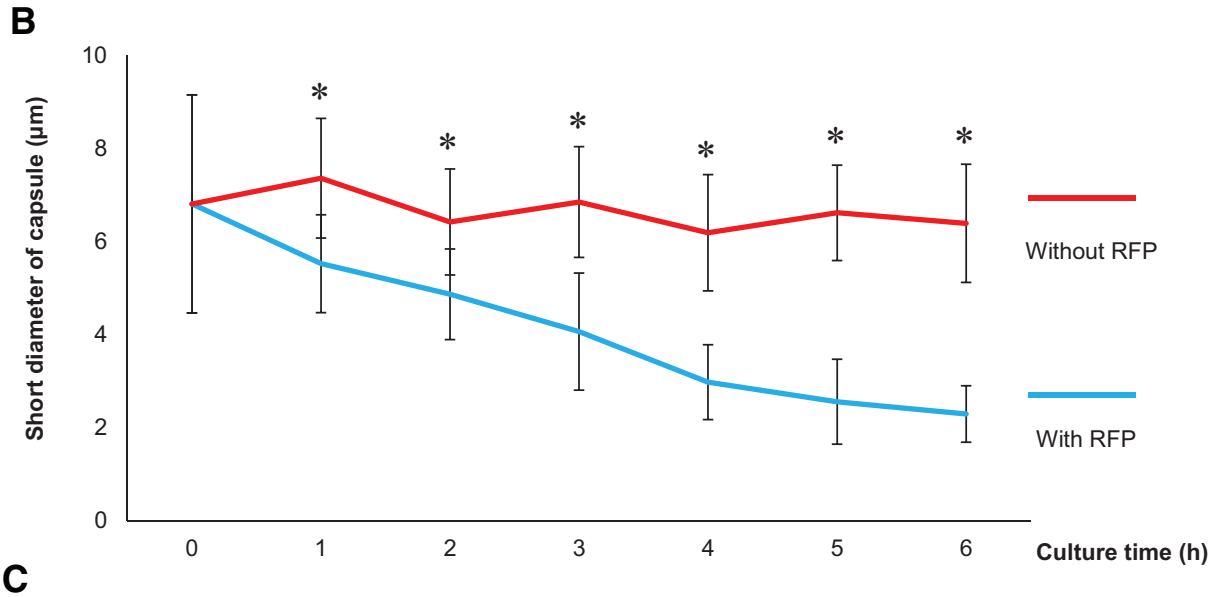
Mann–Whitney *U*-tests were performed using EZR v.1.37 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical interface for R v.3.4.1 (The R Foundation for Statistical Computing, Vienna, Austria). The tests were used for analysing the data presented in the figures. A *P*-value of <0.05 indicated that the difference in values for two given groups was statistically significant.

3. Results

First, the four hvKP strains used in this study were characterised. MLST analysis revealed that all of the hvKP isolates were ST23, which is the major sequence type of CG23. PCR detection of a colibactin cluster further revealed that all of the isolates belonged to the CG23-I sublineage. The MICs of 18 antimicrobial agents against each of the strains determined by the broth microdilution method are shown in Table 2. These data demonstrate that the antimicrobial susceptibility patterns of the strains were similar to that of typical non-hvKP strains, thus showing good susceptibility to β-lactams 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}) for the four hvKP strains (OCU_hvKP1, OCU_hvKP2, OCU_hvKP3 and OCU_hvKP4) was approximately ten times higher compared with that for a non-hvKP control strain (0.44, 0.23, 0.54 and 0.38 vs. 0.04, respectively). The mucoviscosity of OCU_hvKP1 in the presence and absence of each antimicrobial agent at $0.25 \times$ MIC was then compared (Fig. 1A). This concentration was chosen 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 with that in the absence of the antimicrobial agent. It was noted that the RFP concentration used in this experiment (8 μg/mL, i.e. $0.25 \times$ MIC) had only a small effect on the growth of the bacteria. The effect of RFP on the other three hvKP strains was also tested and similar results were obtained. 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 with RFP treatment.





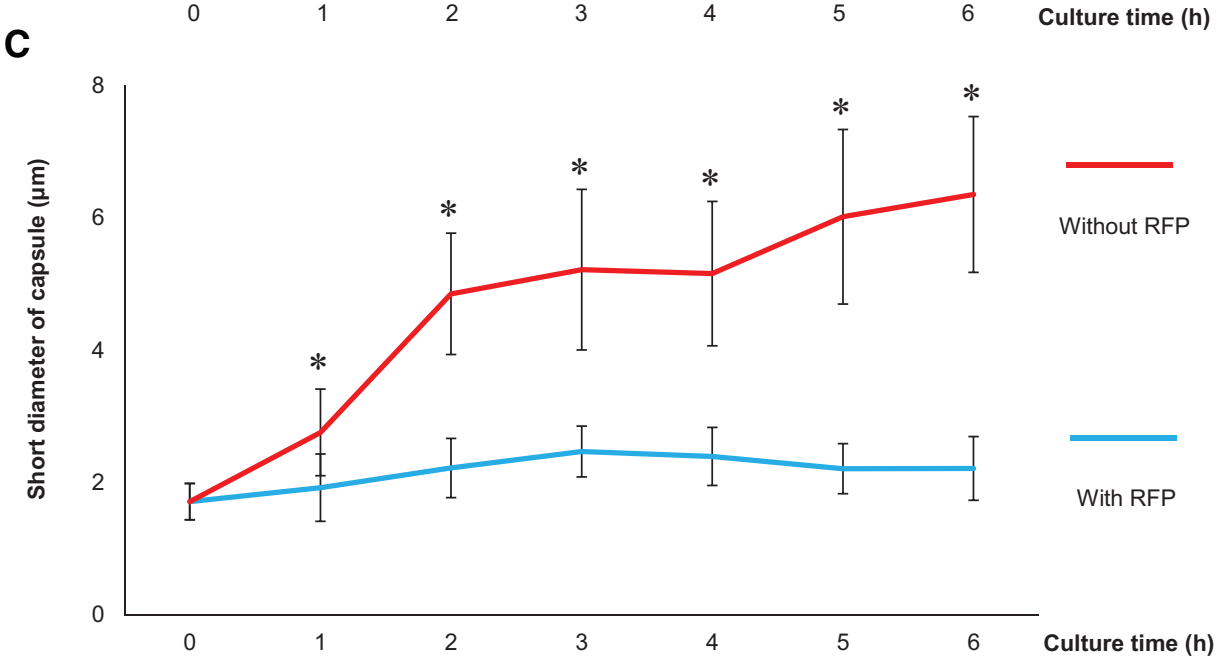


Fig. 2. Effect of rifampicin (RFP) on the thickness of the hypervirulent *Klebsiella pneumoniae* (hvKP) capsule. (A) Microscopic evaluation of the capsule visualised using India ink staining. Representative images are shown for a non-hvKP control strain grown in the absence of RFP (left), OCU_hvKP1 grown without RFP (centre) and OCU_hvKP1 grown in the presence of 8 μg/mL RFP (right). Scale bar, 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 the standard deviation. (C) Temporal changes in the capsule thickness after removal of RFP. At least 50 cells were measured at each time point. Bars indicate the standard deviation. * $P < 0.001$ (Mann–Whitney *U*-test).

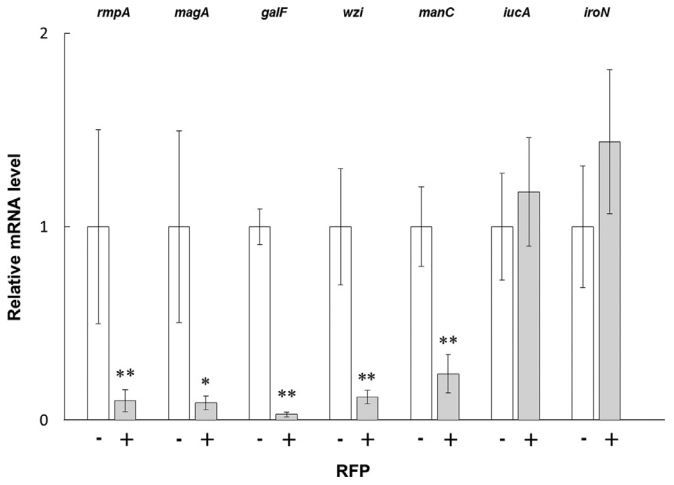


Fig. 3. Effect of rifampicin (RFP) on the expression levels of capsule- and siderophore-related genes. Expression levels in hypervirulent *Klebsiella pneumoniae* (hvKP) grown without RFP were set to 1.0 for each gene. The values presented are mean relative mRNA levels from four (*magA*), six (*rmpA* and *iucA*) or five (*galF, wzi, manC* and *iroN*) independent experiments. Bars indicate the standard deviation. * $P < 0.05$; ** $P < 0.01$ (Mann–Whitney *U*-test).

The lowest concentration of RFP that could exert a mucoviscosity-suppressing activity was then determined. 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 (Fig. 1B). These findings clearly demonstrated that RFP treatment had a strong mucoviscosity-suppressing activity against hvKP strains that was observed at even subinhibitory concentrations.

Negative staining with Indian ink and light microscopy (Supplementary Fig. S1) were applied to the hvKP strains as well as 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 (Fig. 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 (Fig. 2A). This indicated that RFP suppressed hvKP mucoviscosity by inhibiting CPS synthesis. The change in capsule thickness over time following the addition or removal of RFP was then monitored. The thickness of the hvKP capsule gradually decreased following the addition of RFP until a minimum thickness was reached after 6 h (Fig. 2B). Conversely, when RFP was removed from an overnight culture of hvKP, the capsule slowly regained its thickness over several hours (Fig. 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 synthesise CPS.

We hypothesised that RFP represses cps genes by repressing rmpA. Thus, expression levels of rmpA, magA, galF, wzi and manC in hvKP grown with or without RFP were examined. Expression levels of *iucA* and *iroN*, genes involved in aerobactin and salmochelin siderophore systems, respectively, were also measured 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 with that in the absence of RFP (Fig. 3). In contrast, RFP showed no inhibitory effect on either *iucA* or *iroN*. These observations are consistent with the 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, temporal changes in the level of transcript for *rmpA* following inoculation into fresh medium were monitored (Fig. 4). Changes in the transcription level of *magA* as a representative *cps* gene were also monitored. When hvKP was grown without RFP, the level of *rmpA* transcripts was low at 2 h post-inoculation but dramatically increased over a period between 2–6 h. Strikingly, no increase in the *rmpA* transcript level 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 transcript level was detected between 2–4 h, after which it remained constant for ≥6 h. However, in the presence of RFP a decrease in the amounts of transcripts was detected between 2–4 h. The reason that the transcript level was higher at 2 h in the presence of RFP compared with 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 due to 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 this 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 [36,37]. Of these methods, the string test is merely a quantitative method and is not applicable for the current study. Similarly, the limitations regarding quantitation and reproducibility of OD_{600} measurements would not allow us to distinguish subtle differences in hvKP that may be caused by the test antimicrobials. As for the measurement of glucuronic acid, we avoided this method since we were unsure whether this sugar accurately reflected the mucoviscosity of CPS. Therefore, instead of using 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 measures bacterial EPS viscosity, including the capsular material, independent of the polysaccharide composition included. In the first screening of candidate antimicrobials, η_{sp} values divided by OD_{600} were used for comparison of their anti-mucoviscosity effect. However, one should be cautious in using OD_{600} for normalisation 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, this 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 meropenem, ofloxacin and chloramphenicol also demonstrated some activity to suppress mucoviscosity, whilst others showed mucoviscosity-promoting activity. These observations may be reflective of the actual activity of these agents in affecting 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. azithromycin, clarithromycin, tetracycline and chloramphenicol) tended to cause

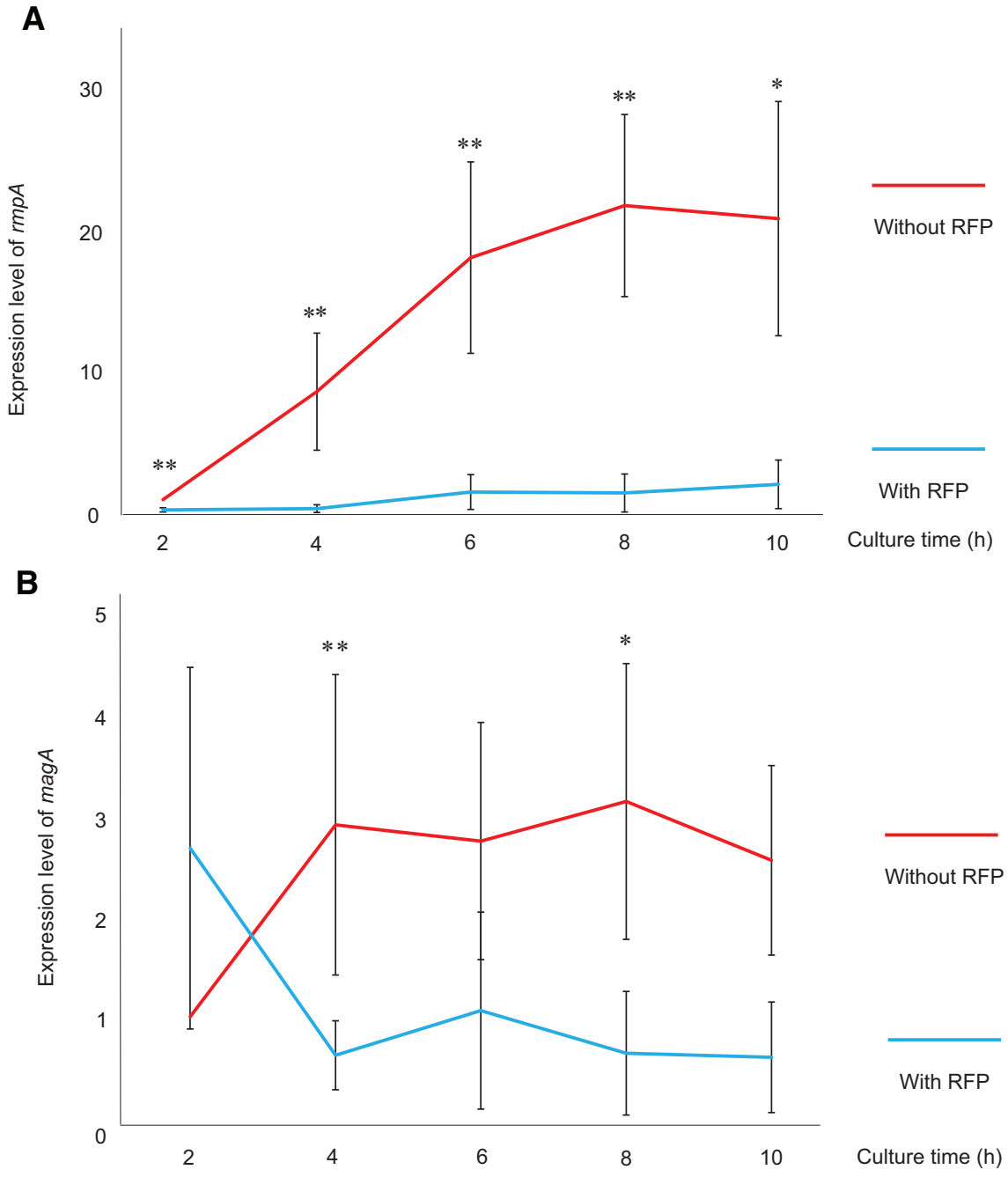


Fig. 4. Time-dependent change of the transcript levels of (A) *rmpA* and (B) *magA* under growth with or without rifampicin (RFP). Expression levels at 2 h of growth without RFP were set to 1.0 for each gene. Presented values are mean relative mRNA levels from four or five independent experiments. Bars indicate the standard deviation. * P <0.05; ** P < 0.01 (Mann–Whitney U-test).

relatively large deviations of η_{sp}/OD_{600} values. In the presence of these agents, severe growth inhibition was observed even at 0.25 × MIC. In the presence of azithromycin, clarithromycin, tetracycline and chloramphenicol, the cell densities only reached OD_{600} values of 2.4, 2,9, 3.0 and 1.4, respectively, following overnight cultivation, when it should have reached approximately 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, meropenem, metronidazole and ofloxacin caused a decrease of the η_{sp}/OD_{600} value without inhibiting bacterial growth. We assume that these agents might indeed have some anti-mucoviscosity activity, although their specificity appears to be much lower than that of RFP.

In addition to viscosity measurements, India ink staining was also used 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 a capsule [38]. 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, a search of the literature revealed no study using a negative staining method to compare the capsule thickness of hvKP and non-hvKP strains. The results of the current study 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 [39]. 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 the *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 of these promoters [15]. We hypothesised that the effect of RFP on hvKP may be exerted via repression of *rmpA*. If this is the case, all of the *cps* genes are expected to be repressed by RFP treatment. Thus, the influence of RFP on the expression levels of *rmpA, magA, galF, wzi* and *manC* was examined. As expected, qPCR revealed a reduced transcription level of *rmpA* in hvKP grown in the presence of RFP. Moreover, all of 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 means by which RFP causes a 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 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 hypothesise that hvKP treatment would be more efficient if the mucoviscosity of hvKP could be reduced by administration of RFP. Klebsiella pneumoniae is generally not susceptible to RFP, as exemplified by the antimicrobial susceptibility test on the hvKP isolates in the current study (Table 2). In addition, this study demonstrated that the anti-mucoviscosity effect of RFP on hvKP cells is reversible. We therefore would not expect that administration of RFP alone to be sufficient as a therapy for hvKP infection. Instead, we assume the use of RFP as a concomitant drug in 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* (anti-MRSA) agents in the treatment of MRSA infection, especially for refractory infections such as osteomyelitis and prosthetic joint infections [40]. 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 a strong anti-mucoviscosity activity against CG23-I hvKP. We suggest that RFP may serve as a potential antivirulence agent for this problematic pathogen. Development of a new treatment strategy using RFP as an antivirulence agent will require molecular-based experiments to reveal the mechanisms of RFP activity as well as in vivo experiments using animal models to verify its efficacy.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.05.018.

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