Rapid evolution bacteria reveal pathogenic mechanisms and clinical risk

Toyotaka Sato (sato.t@vetmed.hokudai.ac.jp)
Hokkaido University
Kojiro Uemura
Sapporo Medical University School of Medicine
Kotaro Aoki
Toho University School of Medicine
Soh Yamamoto
Sapporo Medical University School of Medicine
Noriko Ogasawara
Sapporo Medical University School of Medicine
Atsushi Saito
Sapporo Medical University School of Medicine
Takayuki Wada
Osaka Metropolitan University
Koji Kuronuma
Sapporo Medical University School of Medicine
Chie Nakajima
Hokkaido University International Institute for Zoonosis Control
Yasuhiko Suzuki
Hokkaido University https://orcid.org/0000-0002-1313-1494
Motohiro Horiuchi
Hokkaido University
Hirofumi Chiba
Sapporo Medical University School of Medicine
Satoshi Takahashi
Sapporo Medical University School of Medicine
Shin-ichi Yokota
Sapporo Medical University School of Medicine https://orcid.org/0000-0002-0831-3429

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Abstract

Bacteria continually evolve, as postulated in Darwin's theory of evolution[1-3]. Previous studies have evaluated bacterial evolution in retrospect, but this approach is based on only speculation[4-6]. Cohort studies are reliable but require a long duration[7-11]. Here, using hypermutable strains, we established a rapid and integrated bacterial evolution analysis, RIBEA, based on serial passaging experiments, whole-genome and transposon-directed sequencing, and in vivo evaluation to monitor bacterial evolution for one month in a cohort. RIBEA enabled the elucidation of the mechanism of clinical progression and the potential clinical risk associated with the development of invasive ability and antimicrobial resistance in the major respiratory pathogen *Klebsiella pneumoniae*[12]. RIBEA also revealed novel bacterial factors (via the identification of gene mutations that occurred during evolution) contributing to serum and antimicrobial resistance. Our results demonstrate that RIBEA enables the prediction of bacterial evolution and identification of clinically high-risk bacterial strains, clarifying the associated mechanisms of pathogenicity and antimicrobial resistance development.

Text

Bacteria emerged approximately 3.5 billion years ago and have continued to evolve according to the theory of evolution described in Charles Darwin's "Origin of Species", similar to human evolution[1-3]. This means that the history of human-bacterial coexistence and bacterial infections is an evolutionary battle between bacteria and humans[1]. Many clinicians are trying to overcome bacterial infections, but there is no sign of convergence on a universal approach.

For pathogenic and opportunistic bacteria, the evolution of pathogenicity, such as the acquisition of virulence factors and toxins and enhancing gene mutations, influences human health. In addition, bacteria have also evolved antimicrobial resistance (AMR), which has become a major concern worldwide due to the acquisition of antimicrobial resistance genes and resistance-conferring gene mutations[13,14]. Scientists have tried to retrospectively uncover the evolutionary mechanism of bacterial pathogenesis and the development of AMR by collecting clinical isolates[4-6]. Additionally, some researchers have tried to monitor pathogen evolution by cohort studies *in vitro* and/or *in vivo*[7-11]. Although these studies have succeeded in uncovering the parts of the mechanism, they have not achieved a comprehensive understanding because retrospective analysis yields only speculative results, and cohort studies are reliable but time-consuming. Therefore, innovations must be developed to overcome these problems and uncover the bacterial evolution mechanism to benefit human health. One of the best solutions is constructing a rapid analytical system to observe the details of bacterial evolution.

Klebsiella pneumoniae (Kp) is the main bacterium that causes lower respiratory tract infections, urinary tract infections, and bloodstream infections[12]. In 2019, more than 0.6 million deaths were caused by AMR-associated Kp infections, the third most prevalent bacterial species among the cases of AMR-associated deaths[14]. Based on clinical progression, Kp can be divided into two variants, classical and hypervirulent[15]. Hypervirulent Kp generally exhibits a hypermucoviscous (HMV) phenotype[15] that is well known as an clinical important phenotype for Kp causing invasive syndromes such as liver abscess, meningitis, pleural empyema, or endophthalmitis[16,17]. In contrast, previous studies reported that the majority (67.9%) of bacteraemias are caused by non-HMV-Kp infections, which are prevalent in hospital-acquired bacteraemias[17]. In addition, in the latest meta-analysis, no significant difference was observed in mortality between HMV- and non-HMV-Kp cases[18]. These observations suggest the clinical impact of non-HMV-Kp. Although the characteristics (K1 and K2 serotypes) and pathogenicity (possession of virulence factors such as *rmpA*, *rmpA2*, *iutA*, *iroN*, and the virulence IncHIB plasmid)[16,19,20] of HMV-Kp are well understood, evaluations of the actual impact and potential risk of clinical progression have never been established for non-HMV-Kp infections. Therefore, non-HMV-Kp is a logical target for assessing the associated potential risk.

Accordingly, we previously reported a non-HMV-Kp bloodstream infection that rapidly developed multidrug resistance during the infection[21]. By bacteriological analysis, we revealed that the null mutation in *mutS* accompanied this development. MutS is a DNA mismatch repair enzyme that immediately corrects erroneous nucleotide sequences and facilitates faithful DNA replication with MutL and MutH[22,23]. This observation implies that we it is possible to predict bacterial evolution according to accelerated gene mutation frequency by the MutS functional disruption.

This study aims to establish a rapid and integrated bacterial evolution analysis (RIBEA) that enables us to monitor the long-term evolution of bacterial pathogenicity and antimicrobial resistance within one month by constructing and utilizing hypermutable bacteria. RIBEA comprises serial passaging experiments, whole-genome sequencing (WGS), transposon-directed sequencing (TraDIS),

and *in vivo* evaluation. This approach revealed the potential risk of non-HMV-Kp infections by revealing the clinical progression and antimicrobial resistance mechanisms. RIBEA also enabled the identification of novel serum and antimicrobial resistance factors (via detection of gene mutations that actually occurred during evolution) and revealed that some factors are more critical than those factors previously well known and believed to play a significant role[4,24]. Thus, we propose that RIBEA is a beneficial tool for the observation of bacterial evolution in front of our eyes.

Results

High risk of bloodstream infection by non-HMV-Kp

To evaluate clinical impact of non-HMV-Kp, we first aimed to determine the clinical risk of total Kp infections. Our 5-year retrospective study of Kp infectious cases in a university hospital revealed that these infections were associated with more severe clinical signs in terms of hospitalization, antimicrobial use, and 60-day mortality in immunosuppressed patients and those with bacteraemia due to Kp infections (Fig. 1a and Supplementary Table 1). Therefore, immunosuppressant use and bloodstream infections are key risk factors for Kp infections.

Next, we evaluated the proportion of Kp clinical isolates derived from bloodstream infections. To conduct this analysis, we first performed the string test to distinguish HMV- and non-HMV-Kp isolates from among 277 clinical isolates. Among the 277 isolates, 29 (10.5%) were string test (HMV) positive. The prevalence of HMV isolates for each isolation site ranged from 0 to 17% (Fig. 1b). Notably, none of the HMV isolates were collected from blood samples. Serum susceptibility was determined according to the minimum inhibitory concentration (MIC) of human serum to estimate Kp survival ability in blood. Kp clinical isolates exhibited varied serum MICs, ranging from ≤16 to >64%, and more than 40% of the total isolates were serum resistant (Fig. 1c). In the comparison of serum resistance between HMV and non-HMV populations, there was no significant difference (Fig. 1d). These observations indicate that the potential risk of causal bloodstream infections and serum resistance is associated with non-HMV-Kp rather than HMV Kp.

Next, we evaluated the gene mutation frequency of Kp clinical isolates (Fig. 1e). We found that the gene mutation frequencies of Kp clinical isolates were diverse, ranging from 5.5×10^{-10} to 4.4×10^{-6} across the sites of infection. The mutation frequency was significantly higher in the non-HMV group than in the HMV group (Fig. 1f). These observations indicate that Kp clinical isolates consisted of a genetically heterogeneous population, with a higher propensity for the non-HMV phenotype. We focused on Kp clinical isolates from respiratory specimens for further analysis due to the majority of isolates being derived from these samples and the fact that respiratory Kp infections are the source of bloodstream Kp infections (Extended Data Fig. 1). We found no specific associations between gene mutation frequency and HMV phenotype, sequence type (ST), antimicrobial resistance, or serum susceptibility, suggesting that gene mutation frequency is an independent and nonfocused factor in respiratory Kp infection risk.

Construction of rapid-evolution bacteria

To uncover the pathogenesis of non-HMV-Kp, we constructed hypermutable bacteria by *mutS* deletion to establish RIBEA. We selected a non-HMV strain, namely, SMKP838, derived from a patient with pneumonia, which belongs to a major clone, ST 45, causing respiratory non-HMV-Kp infections[25]. As anticipated, the *mutS*-deletion SMKP838 mutant accerelated a mutation frequency up to 824-fold compared with that of the parent SMKP838, and this frequency (7.7×10^{-6}) was defined as hypermutable (Extended Data Fig. 2a). We used this mutant for serial passaging experiments in the presence of human serum or antimicrobial agents to observe the adaptive evolution in the blood or during antimicrobial treatment.

The hypermutable *mutS*-deletion mutant rapidly acquired serum resistance (on day 6) and continued developing higher serum resistance, which reached a plateau at a serum MIC of 70% after 13 days (Extended Data Fig. 2c). In contrast, the parent (wild-type) strain did not exceed the breakpoint of serum resistance for 20 days. A time-killing assay demonstrated that the *mutS* deletion retained greater survival ability in the presence of human serum than the wild type by accumulating gene mutations (Extended Data Fig. 2d and e). This rapid bacterial evolution was also seen in the serial passaging experiments for ciprofloxacin, amikacin, and meropenem, clinically important antimicrobial agents against Kp infections (Extended Data Fig. 3a). The *mutS*-deletion mutant rapidly acquired antimicrobial resistance within 5 days, whereas wild-type SMKP838 did not exceed the breakpoints during passage for 20 days. A drastic increase in gene mutations occurred in the *mutS*-deletion mutant during serial passaging (Extended Data Fig. 3b). Therefore, these observations suggest that the ability to acquire serum and antimicrobial resistance in non-HMV-Kp relies on impaired DNA repair

ability, and this rapid bacterial evolution approach is useful to determine the influence of non-HMV-Kp evolution on the ability to cause infection in different sites and the outcomes of antimicrobial treatment.

Interestingly, the number of gene mutations and genes that had mutations varied depending on the selective pressures (Extended Data Fig. 3b and c). Although the development of serum resistance did not influence antimicrobial susceptibility, the development of antimicrobial resistance decreased serum resistance (Extended Data Fig. 3d and e). Thus, this approach enabled us to identify distinct bacterial evolution depending on the environment.

Integrated analysis of serum resistance

We hypothesized that the bacterial factors contributing to serum resistance in non-HMV-Kp could be extrapolated from among the gene mutations occurring during serial passaging in the presence of human serum. However, we could not readily identify serum resistance genes due to the numerous accumulated gene mutations. Thus, we performed transposon-directed sequencing (TraDIS) because TraDIS can comprehensively detect the bacterial factors contributing to survival in different environments (Extended Data Fig. 4a). We successfully identified the difference in the abundances of detected transposon-inserted genes depending on the medium conditions by TraDIS (Extended Data Fig. 4b and c). The numbers of significantly enriched or depleted transposon-inserted genes in 4% and 8% serum (620 and 794 genes, respectively, vs. plain medium) were much higher than that in the presence of surfactant protein A (SPA) (only 3 genes), which is a large multimeric antimicrobial protein found in the airways and alveoli of the lung[26] (Extended Data Fig. 4d and e). This result suggests that human serum exerts a stronger selective pressure than lung antimicrobial substances. Among the genes detected by TraDIS, the decreased abundance of genes in serum suggests putative serum resistance genes in non-HMV-Kp.

Next, we merged the data for genes that accumulated nonsynonymous mutations in serum-resistant-*mutS*-deletion SMKP838 mutants after serial passaging in the presence of human serum and the data for genes detected by TraDIS (Fig. 2a). Thus, we identified a total of 22 genes that were shared between the serial passaging and TraDIS data (Supplementary Table 2). Next, we constructed specific-gene deletion SMKP838 mutants and measured their serum MIC to determine the change in serum susceptibility. Among the genes, we observed gene-deletion mutants that enhanced serum resistance (from a serum MIC of 14% to 20 or 22%) compared with that of the parent SMKP838 strain (Fig. 2b). Therefore, we finally identified four genes, *ramA* (encodes a DNA-binding transcriptional regulator), *LOCUS_10060* (encodes a putative sugar transferase), *LOCUS_14270* (encodes a pyruvate kinase), and *LOCUS_16740* (encodes a gamma-glutamylcyclotransferase), that are bacterial factors that contribute to serum resistance in non-HMV-Kp. These observations indicated that the integration of serial passaging experiments using rapidly evolving bacteria and TraDIS could be used to identify the contributing gene mutations that actually occurred during bacterial evolution.

RIBEA in non-HMV-Kp clinical isolates

To evaluate whether RIBEA reveals the actual bacterial evolution that occurs in clinical isolates, we next performed a serial passaging experiment in the presence of human serum for 20 days for randomly selected serum-sensitive HMV-Kp clinical isolates with extremely high, high and low mutation frequencies (Fig. 3a). Similar to the laboratory-derived *mutS*-deletion mutant, a hypermutable clinical isolate, SMKP590, acquired serum resistance the earliest, after 3 days of passaging. The acquisition of serum resistance was also seen in five Kp (including one *K. quasipneumoniae*) highly mutable isolates. In contrast, poorly mutable isolates did not develop serum resistance during 20-day passaging (p <0.05).

By WGS, we found that SMKP590 gradually accumulated gene mutations along with the increase in serum resistance, and we finally detected 74 gene mutations after 20 days of passaging (Fig. 3b). Interestingly, the number of novel and accumulated mutations in genes increased or decreased, and the number of nonsynonymous mutations was also uniform throughout the passaging (Fig. 3c and d). When we integrated and compared these data with the TraDIS data for SMKP838, we identified that 24 of 103 nonsynonymous mutations that occurred during passaging were associated with serum resistance (Extended Data Fig. 5 and Supplementary Table 3). Taken together, these observations suggest that bacterial adaptative evolution of clinical isolates is also associated with mutation frequency, and the current integrated approach is useful for prediction and/or identification of currently high-risk clones.

Evaluation of rapidly evolved non-HMV-Kp in vivo

We established a mouse pneumonia model to evaluate the pathogenicity of rapidly evolved non-HMV-Kp by serial passaging experiments. First, we used SMEK838 and the *mutS*-deletion mutant to establish intrabronchial infection. We found that the infection was not established without immunosuppression, as the mice eradicated these strains from their lungs without the development of

any symptoms (Fig. 4a), suggesting that immunocompetent mice are protected; this was not unexpected, as non-HMV-Kp is an opportunistic pathogen[27]. Thus, we established immunosuppressed mice. This immunosuppression drastically enhanced the bacterial load in the lungs (Fig. 4a) and blood 32 h after infection (Fig. 4b). Thus, non-HMV-Kp can cause pneumonia and invade the bloodstream in immunosuppressed hosts. We used this immunosuppression pneumonia model and compared the efficacy of ciprofloxacin treatments between mice infected with the wild-type and hypermutable mutant strains (Fig. 4c).

In contrast with the loads prior to ciprofloxacin treatment, bacterial loads of the wild-type strain and the *mutS*-deletion mutant (day 0) were drastically reduced after ciprofloxacin treatment in the lung (Fig. 4d), and no viable colonies were observed from the blood of infected mice after treatment (Fig. 4e). In contrast, the ciprofloxacin-resistant SMKP838 mutant derived from serial passaging in the presence of ciprofloxacin on day 19 [Δ*mutS*_CIP^R (day 19)] (Extended Data Fig. 6) maintained its bacterial load in both the lung and blood after ciprofloxacin treatment. Notably, we observed spontaneous development of serum-resistant clones only from *mutS*-deletion SMKP838 mutants (Fig. 4f and g). These observations indicate that enhancement of the mutation frequency in non-HMV-Kp results in the production of antimicrobial- and serum-resistant mutants *in vivo* and affects clinical outcomes.

In support of this hypothesis, both serum-sensitive and serum-resistant *mutS*-deletion mutants caused enhanced mortality compared with wild-type SMKP838 ($p = 0.0246$) (Fig. 4h). Moreover, the mortality caused by the serum-resistant *mutS*-deletion mutant was higher than that caused by the serum-sensitive *mutS*-deletion SMKP838 ($p = 0.0012$), and a higher bacterial load of the serum-resistant *mutS*-deletion mutant was observed in the blood (Fig. 4i). Severe bacterial masses were present in the livers and kidneys of mice infected with the *mutS*-deletion mutant (Fig. 4j). Collectively, these results suggest that this *in vivo* model is suitable for the evaluation of the clinical risk of rapidly evolving non-HMV-Kp.

Evaluation of RIBEA for internationally spreading high-risk non-HMV-Kp

Finally, we tried to evaluate the utility of RIBEA for currently important bacterial clones in clinical settings. In recent decades, high-risk non-HMV-Kp clones such as ST11 and ST258, which exhibit multidrug resistance, have spread worldwide and become a major clinical problem[28]. We previously reported the presence of *mutS* mutations in ST11 and ST258[21]. This finding suggests that the worst-case scenario is that these international high-risk non-HMV-Kp clones develop pathogenicity by accumulating gene mutations[4]. We constructed a multidrug-resistant ST258 mutant that contained a stop codon in *mutS* in the BIDMC1 strain (Fig. 5a). The BIMDC1 *mutS* mutant exhibited a drastically enhanced mutation frequency (Fig. 5b) and rapidly acquired serum resistance after 3 days of passaging (Fig. 5c). In contrast, its bacterial growth kinetics were decreased (Fig. 5d). During serial passaging, the BIDMC1 *mutS* mutant accumulated more gene mutations than the wild type (Fig. 5e). Finally, we observed that the *mutS* mutant killed mice significantly more rapidly than the wild type (Fig. 5f). Taken together, these observations suggest that RIBEA enables the prediction of the clinical risk of internationally distributed high-risk multidrug-resistant bacteria.

Discussion

Due to the difficulty of observing bacterial evolution closely and for a long enough time, elucidating the mechanisms of pathogenesis and the potential risk in the field of infectious diseases remains an important focus[7-11]. Heterogeneity is associated with bacterial colonization[11], pathogenesis[29], and antimicrobial resistance[30,31]. In particular, it is well known that gene mutation frequency is associated with the progression of cystic fibrosis in *P. aeruginosa* and *H. influenzae*, and highly mutable strains and hypermutators have fitness and pathogenesis advantages[32-36]. However, controversial observations have also been reported indicating that hypermutator strains are less virulent than wild-type *P. aeruginosa*[37,38]. Therefore, it is necessary to establish a method that allows us to observe bacterial evolution in cohorts over a specific study period to gain a better understanding of the role of mutation frequency in bacterial infection[39,40].

Bacterial evolution assays using hypermutable strains have already been established[21,39]. However, the identification of novel bacterial factors among evolved bacteria is very challenging due to the numerous accumulated gene mutations. Therefore, no previous studies covering all the genetic variations has been performed during the evolution period for the identification of bacterial factors, and previous studies have always had a limited focus on inferable genes, resulting in the focus on genes that have not received any association or contribution in the past among the numerous detected genes being out of scope or lower priority[4,21,31,36,39]. These limitations are bottlenecks in the comprehensive elucidation of bacterial ecology. RIBEA can solve this problem for a one-month period (Fig. 6a). By this approach, we successfully covered all gene mutations occurring during bacterial evolution and identified novel

bacterial factors that contribute to pathogenesis. Importantly, this rapid and integrated approach can be used to select and identify the genes that are actually required for bacterial evolution.

Using the rapid-bacterial evolution method, we succeeded in dramatically accelerating the speed of the adaptive evolution of bacteria (more than 800-fold higher frequency than the wild-type strain in one day) and caused selection pressure-dependent evolution with an increase in gene mutations. Thus, we were able to observe the details of bacterial evolution, which sometimes takes decades or centuries, within only two weeks (the time to reach a plateau in phenotype during serial passaging experiments).

A previous study using *Escherichia coli* reported that the selection pressure provided by an environment is more essential for the evolution of novel traits than the mutational supply experienced by wild-type and mutator strains[41]. Consistent with this finding, evaluating non-HMV-Kp as the bacterial evolution model showed that the presence of human serum was more impactful than surfactant protein A. This is explained by antibacterial components within serum, including complement (forms the membrane attack complex) and antimicrobial peptides[42]. Thus, the environment (infection site) greatly affects evolution speed. Antimicrobial pressure is also a harsh environment for bacterial survival, but we revealed that non-HMV-Kp can overcome growth restriction by clinically important antimicrobial agents via the accumulation of gene mutations. Therefore, a rapid serial passaging experiment is also helpful in identifying the environments that promote bacterial evolution.

By the construction of the rapid bacterial evolution method, we also determined that although serum-resistant mutants exhibited decreased bacterial growth, they did not exhibit antimicrobial susceptibility, contrary to the development of antimicrobial resistance enhancing serum sensitivity. Thus, RIBEA supports the notion that a given combination of gene mutations during bacterial evolution does not affect bacterial fitness but significantly increases virulence, as shown in mouse models. Overall, RIBEA can reveal the changes in bacterial characteristics during bacterial evolution within a cohort.

Previous studies have reported a high mortality rate of lower respiratory tract infection and subsequent bacteraemia in non-HMV-Kp infections[14,43,44], suggesting the importance of understanding the mechanisms of clinical progression in non-HMV-Kp infections. RIBEA showed that non-HMV-Kp can adapt to the pressure of human serum and antimicrobial agents during dissemination from the lung to the blood; specifically, gene mutations contributed to serum and antimicrobial resistance. This finding indicates that non-HMV-Kp causes more severe conditions during infection and the loss of antimicrobial therapy efficacy. Importantly, this phenomenon was robustly observed for hypermutators and in immunosuppressed hosts, which was demonstrated in a previous clinical case report[21]. Therefore, RIBEA is also useful for clarifying the mechanisms of clinical progression of bacterial infection, as it was revealed that non-HMV-Kp has more risk to immunosuppressant-using and/or immunodeficient hosts and that gene mutations of non-HMV-Kp affect the infection outcome.

In addition, we revealed that these identified gene mutations, such as those in *wecC*, *wzc gnd,* and *wbaP*, are already harboured in non-HMV-Kp clinical isolates to a certain degree, as well-known components of serum resistomes[4,8,24,45,46]. Another important observation is that the non-HMV-Kp clinical isolates consist of a more heterogeneous population in terms of gene mutation frequency than HMV-Kp isolates. This finding indicates that some isolates with high hypermutation frequencies can evolve serum and antimicrobial resistance, consistent with RIBEA results derived using *mutS*-engineered mutants. Collectively, we conclude that RIBEA can mirror the present and/or future of clinical bacterial isolates and estimate the potential risk, suggesting that non-HMV-Kp cannot be underestimated in clinical settings (Fig. 6b).

The limitations of this study are that this integrated approach does not consider the influence of the acquisition of exogenous factors such as virulence plasmids and horizontally transferred antimicrobial resistance genes[47]. In addition, the accumulation of gene mutations is not only a survival strategy for bacteria, as shown by enhanced persistence[48]. Evaluation of these persistent cohorts in the short term is also needed. A comprehensive evaluation of these systems will bring us closer to understanding bacterial evolution and survival strategies.

In conclusion, in this study, the adaptive evolution of bacteria according to Darwin's theory of evolution was demonstrated in a short time, and prediction of bacterial adaptation while identifying causal factors was made possible. This prediction is also helpful for assessing the bacterial clones we should be aware of today, as shown here regarding the health risk of the internationally distributed high-risk multidrug-resistant non-HMV-Kp clone ST258. Therefore, our established rapid and integrated bacteriological approach represents a beneficial and suitable analysis to elucidate the mechanisms of bacterial survival, adaptation, and infection and for predictions outcomes of infection by various pathogenic bacteria and multidrug-resistant bacteria. Furthermore, this technology is

useful for elucidating the ecosystem of nonpathogenic bacteria, such as those in nature and the environment. Thus, RIBEA and its derivatives have the potential to accelerate our understanding of bacterial evolution along with human evolution and to become valuable tools for predicting the future of the Earth's ecosystem, which is largely responsible for determining human life.

Declarations

Data availability

NGS data are available on the NCBI database as following accession numbers; DRR503736-DRR503819 for genomic sequences of Kp clinical isolates, DRR503832-DRR503857 for genomic sequences of serial passaging experiments in SMKP838 and the *mutS* deletion mutants, DRR504916-DRR505017 for genomic sequences of the serial passage experiment in Kp clinical isolates, and DRR503820-DRR503831 for TraDIS data.

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Author contributions

T.S. and K.U. developed the concept, and T.S. conducted all experiments. S.T. collected and identified the bacterial strains. K. U, S.Y., N.O., and A. S. conducted experiments using mice. K.U., T.S., K.A., T. W., C.N., and Y. S. conducted genomic experiments and analysis. K.U., T.S., and S.Y. wrote the manuscript. K. K., H. C., M. H., and S.Y. supervised this study.

Competing interest

All authors declare no competing interests.

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Methods

Clinical epidemiology

Clinical epidemiology analysis was performed using 695 Kp infections reported from 2017 to 2022 at Sapporo Medical University Hospital, including 393 “Colonization” and 302 “Infection” cases. “Infection” cases were analysed by classifying them into the presence or absence of immunosuppression, site of infection, and presence or absence of bacteraemia. Contingency tables were analysed using Fisher’s exact test. A p value < 0.05 was considered to indicate significance.

Bacterial isolation, antimicrobial susceptibility testing, and string test

A total of 277 Kp strains were isolated from clinical specimens from hospitalized patients at Sapporo Medical University between 2017 and 2021. These clinical specimens comprised 100 urine samples, 113 respiratory samples, 12 blood samples, and 52 other samples (drainage, tongue coating, skin, vaginal lubricant, pus, and bile). Identification of Kp (*K. pneumoniae* subsp.) was performed by MALDI Biotyper (Bruker Corporation, Billerica, USA). BIDMC_1, a carbapenem-resistant Kp strain isolated at the Beth Israel Deaconess Medical Center (BIDMC), was provided by BEI Resources (NIAID, NIH, USA).

The antimicrobial susceptibility of Kp strains was tested by the broth microdilution method, and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) recommendations. In this study, the following antimicrobial agents were used: ciprofloxacin (Wako Pure Chemical Industry, Tokyo, Japan), ciprofloxacin hydrochloride monohydrate (Tokyo Chemical Industry, Tokyo,

Japan), amikacin (Wako Pure Chemical Industry, Tokyo, Japan), kanamycin (Wako Pure Chemical Industry, Tokyo, Japan), and meropenem (Wako Pure Chemical Industry, Tokyo, Japan).

HMV strains were defined by a positive string test as previously described[1]. A single colony grown overnight on Mueller-Hinton II agar was fished, and the formation of a string > 5 mm in length was defined as a positive result. For detection of hypervirulence factors (serotypes K1 and K2, *rmpA*, *rmpA2*, *iutA*, *iroN*, and an IncHIB plasmid), multiplex PCR was performed as previously described[2].

Serum susceptibility

In this study, we used human serum from individual healthy donors (Cedarlane Laboratories Ltd, Burlington, Canada). The serum MIC was defined as the minimum % serum concentration that prevented the visible growth of microorganisms. We set the resistance breakpoint at 32%, and isolates that exhibited more than 48% of serum MIC were defined as serum-resistant isolates because this concentration is the composition of the total blood in humans. Kp strains were grown in 0.5 ml of tryptic soy broth from an overnight culture. The strains were diluted 10^{-4}-fold (10^5 CFU/mL) and incubated in plates with different serum concentrations for each well. After 20 h, colonies were visually confirmed because wells with high serum concentrations had high optical density (OD600 nm).

Measurement of mutation frequency

Mutation frequency was measured by rifampicin assay[3]. The Kp isolates were cultured overnight in tryptic soy broth. The solution was concentrated 10-fold and plated onto plain or 100 mg/L rifampicin-containing Mueller Hinton II agar plates, and the plates were cultured at 37°C for 24 h. After cultivation, the number of colony-forming units (CFU) that grew on the agar plates was counted. The gene mutation frequency was calculated as [CFU on the rifampicin-containing MH agar plate]/[CFU on the plain MH agar plate]. We defined mutator types as hyper (> 10^{-7}), high (from 10^{-8} to 10^{-7}), moderate (from 10^{-9} to 10^{-8}), and low (<10^{-9}). Student's t test was used for the statistical analysis. A p value < 0.05 was considered to indicate significance.

Serial passaging experiments

Serial passaging experiments were performed by incubating Kp isolates (SMKP838, SMKP590, and BIDMC) in 96-well plates with MHBII containing certain concentrations (serial dilutions from original concentrations) of human serum or antimicrobial agents (ciprofloxacin, amikacin, and meropenem), as previously described[4]. For the experiments using other Kp clinical isolates, we selected 19 serum-susceptible Kp isolates (serum MICs were from 8 to 16%) from among the hyper- (n = 1), high- (n = 9; contains one *K. quasipneumoniae*), and low-mutators (n= 9) in the serial passaging experiment in the presence of human serum. In the ciprofloxacin assay, we selected 22 ciprofloxacin-susceptible Kp clinical isolates (ciprofloxacin MICs were from 0.03 to 0.25 mg/L) from among the hyper- (n = 1), high- (n = 11; contains one *K. quasipneumoniae*), and low-mutators (n = 10). We picked the well with the highest concentration (sub-MIC) of human serum or antimicrobial agent in which the bacteria grew and diluted the bacterial culture 100-fold with 0.85% NaCl. Then, 1 µL of the dilution was inoculated in 96-well plates containing 100 µL of MHBII with various concentrations of human serum or antimicrobial agents and cultivated at 37°C for 24 h. This serial passaging was repeated for 20 days in triplicate.

Time-killing assay

Single colonies of SMKP838 and the *mutS* mutant strains were grown overnight in TSB medium. The culture solution was adjusted to a final concentration of 1 × 10^5 CFU/mL and incubated for 0-24 h with each serum-containing solution (1/4 × MIC, 1 × MIC, or 2 × MIC) or in solution without serum (Ctl) at 37°C without shaking. The assay result was determined at 0 min, 30 min, 1 h, 3 h, 6 h, and 24 h.

WGS

Genomic DNA was isolated by a DNeasy Blood & Tissue Kit (Qiagen, Hulsterweg, The Netherlands). The DNA library was prepared by a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) for sequencing 300 bp paired-end reads according to the manufacturer's protocol. An Illumina MiSeq was used for WGS. CTX-M genes were identified using assembled genome data by Resfinder (https://www.genomicepidemiology.org). MLST was performed using the Institute Pasteur MLST database and software (https://bigsdb.pasteur.fr/klebsiella/). Fast average nucleotide identification (FastANI) against the type strain genome was utilized for species identification. Core-genome single-nucleotide polymorphism (SNP)-based phylogenetic analysis was conducted: the Kp ATCC 35657 genome (accession number: CP015134.1) was used as a mapping reference. Mapping and core-genome extraction were performed using BWA version 0.7.17 with the "bwasw" option, SAMtools version 1.6 with the "mpileup" option, and VerScan version

2.3.9 with the “mpileupcns” option. The exclusion of estimated homologous recombination regions was performed using ClonalFrameML version v1.11-2. Snp-dists was used to count the pairwise SNP distance. A phylogenetic tree was generated using FastTree version 2.1.11 and FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). The number of accumulated gene mutations during serial passaging experiments was analysed by mapping the genome reads to the reference genome (wild-type strain on day 0) obtained from WGS, followed by basic variant detection using CLC Genomics Workbench 21 (QIAGEN).

Bacterial growth determination

Bacterial growth was monitored by measuring the turbidity (that is, the optical density at 600 nm [OD600]) using an Infinite M200 PRO multimode microplate reader (Tecan, Kawasaki, Japan). Strains were grown in 0.5 ml of TSB (Becton Dickinson) overnight at 37°C, and 1×10^5 CFU/ml bacteria were cultured in 0.1 ml of MHBII broth (Becton Dickinson) in a 96-well plate at 37°C with shaking at 140 rpm for 16 h. Bacterial growth curves were created based on measurements every 10 min for 16 h.

Transposon-directed insertion site sequencing (TraDIS)

The SMKP838 transposon library was constructed using the EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit (Epicentre, Wisconsin, USA). The bacteria with transposase introduced by electroporation (2.5 kV/cm, 200 Ω, and 25 μF) were selected by the formation of colonies on MHII agar containing 50 mg/l kanamycin. Over 100,000 colonies were collected, pooled, and frozen at -80°C in TSB with 10% glycerol as stock solutions until use. The transposon mutant library (10^6 cfu/mL) was inoculated into 1 mL of plain MHBII, MHBII containing 4% or 8% serum, or MHBII containing 40 mg/L surfactant protein A (SPA) and cultured at 37°C for 20 h. Total DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Total DNA (500 ng) was used to prepare the DNA library for TraDIS using an NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). After fragmentation, end repair, 5' phosphorylation, dA-tailing, and adaptor ligation, and size selection (275-475 bp) according to the manufacturer’s protocol, the transposon-inserted genes were amplified by PCR using NEBNext Ultra II Q5 Master Mix (New England Biolabs), 20 nM NEBTnF2fas (5'-TCGACCTGCAGGCATGCAAGCTTCAGGGTTGAGATGTG-3') and NEBTn5-700 (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3') primers, and 20 ng of fragmented DNA as the template with following condition: initial denature at 98°C for 30 sec, 22 cycles of 98°C for 10 sec and 72°C for 1 min 15 sec, and final extension at 72°C for 2 min. After the purification of the PCR product using AMPure XP beads (Beckman Coulter, Brea, CA, USA), enrichment PCR was performed by using a KAPA HiFi HotStart Library Amplification Kit (Roche, Basel, Switzerland), 20 nM NEBNext i700 primers including NEBNext Multiplex Oligos for Illumina (New England Biolabs) and NEBTn5-501-3 (5'-AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGACCTGCAGGCATGCAAGCTTC-3'), and 20 ng of the purified DNA as the template with following condition: initial denature at 98°C for 45 sec, 10 cycles of 98°C for 15 sec, 60°C for 30 sec, and 72°C for 10 sec, and final extension at 72°C for 30 sec. The PCR products were purified and size selected (average: 650 bp) using AMPure XP beads. These products were pooled, and a NovaSeq600 was used for TraDIS. TraDIS analysis was performed according to a previous study[5], and a false discovery rate-adjusted p value (FDRp) < 0.05 (vs. plain medium) was defined as significant.

Genes with significantly lower detected levels in serum or SPA samples (> 2-fold vs. plain medium) were considered putative serum or SPA resistance genes.

Construction of gene deletion mutants

The*mutS*-deletion SMKP838 mutant and each serum resistance gene were generated by the λ-Red recombinase system, as previously described, using pKD46-hyg[6,7]. Each gene was replaced with Mini genes containing kanamycin resistance cassettes (Gene Bridges, Heidelberg, Germany) and 50 bases corresponding to the upstream and downstream regions of the target genes. The gene deletions were confirmed by PCR using specific primers.

Mouse models of lung and bloodstream infection

Ten- to 12-week-old female BALB/c mice were anaesthetized and infected transbronchially with a microsprayer (TORAY PRECISION, Tokyo, Japan) with 50 μl of a 1×10^8 CFU/ml solution. The mice were immunosuppressed by intraperitoneal injection of 250 mg/kg five days prior to infection and 125 mg/kg one day prior to infection with cyclophosphamide monohydrate (lot No. SKE6784; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). In the treatment group, mice were injected subcutaneously with 100 mg/kg ciprofloxacin monohydrochloride (TOKYO CHEMICAL INDUSTRY, Tokyo, Japan) 1 and 24 hours after infection. After 32 hours in the

nontreated group and 48 hours in the treated group or when a 'prelethal critical endpoint' had been reached, mice were euthanized by cervical dislocation. Lungs were washed with sterile PBS and homogenized with a gentleMACS Dissociator (Miltenyi Biotec). Homogenates were plated for determination of the number of CFU per lung. Blood was collected by puncturing the jugular vein. A drop of blood was added to 1 ml of PBS, vortexed, and then cultured on an appropriate plate. In the experiments, wild type-derived strains were selected by seeding on MHII agar containing 100 mg/l ampicillin sodium and Δ*mutS*-derived strains on MHII agar containing 50 mg/l kanamycin to eliminate the effects of other indigenous and environmental bacteria. Fifty colonies from each specimen were randomly selected, and their serum MIC was measured.

Construction of mutS-mutated BIDMC_1

Since the λ-Red recombination system was unable to generate *mutS*-deficient strains of BIDMC1, we used pORTMAGE and constructed *mutS*-mutated BIDMC1 (BIDMC1 MutS_Tyr37STOP)[8]. Hygromycin-integrated pORTMAGE was generated as previously described[9]. Transformants were produced by electroporation. Oligonucleotides (90 bp) for *mutS* containing the C111T mutation were designed using the MEGA Oligo Design Tool: MegamutS, CGCAACATCCTGACATTCTGCTGTTTTACCGGATGGGGGATTTTTA**a**GAGCTATTTTATGACGATGCGAAACGCGCCTCGCAGCTGCTCG; bold "a" indicates an introduced base. Gene replacement was confirmed by direct DNA sequencing.

Ethics statement

This study was approved by the Sapporo Medical University Hospital Institutional Review Board (IRB No. 272-70) and Sapporo Medical University Animal Care and Use Committee (Nos. 17-137, 18-083, and 20-006).

Statistical analysis

We used Prism 9 to calculate the significance of differences. Unpaired, two-tailed Student's *t* test or a two-tailed Mann–Whitney U test was used to compare two groups, and Dunn's comparison test followed by the Kruskal–Wallis test was used to compare three or more groups. In addition, the log-rank test was used for survival data analysis. Statistical methods and *P* values are described in each figure. A *P* value < 0.05 was considered to indicate significance. In addition, Fisher's exact test and Student's *t* test were used in the clinical analysis to compare two groups.

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Figures

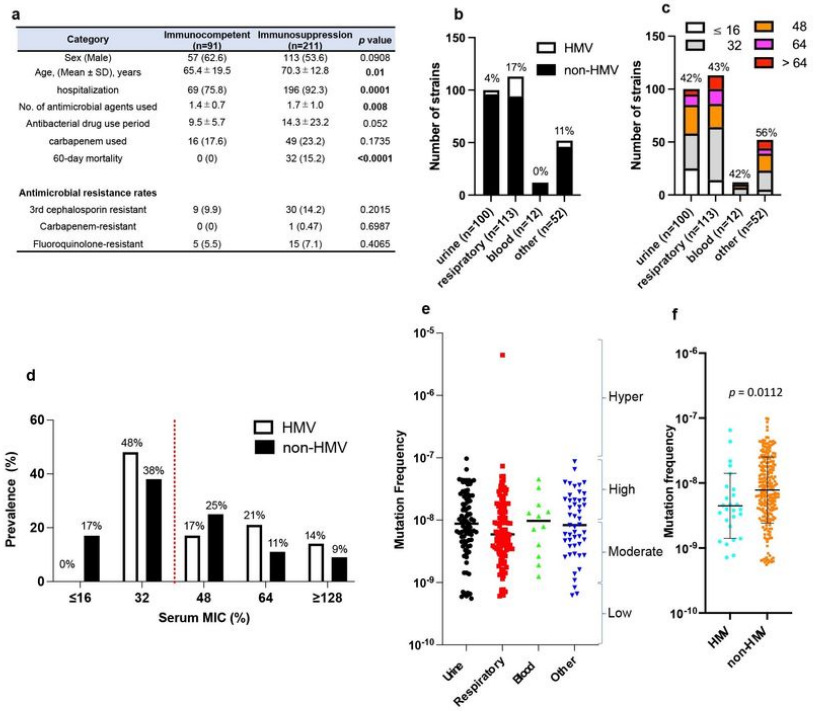

Fig. 1. Association of HMV, serum susceptibility, and gene mutation frequency in Kp clinical isolates. **a**, Influence of Kp infection among immunocompetent and immunosuppressed patients. The clinical histories of the Kp-infected patients were retrospectively analysed by Fisher's exact test and Student's t test. The parentheses indicate the prevalence (%). A *p* value <0.05 was considered significant. **b**, Number of HMV-Kp and non-HMV-Kp isolates derived from clinical specimens. The percentage indicates the prevalence of HMV-Kp isolates. **c**, Susceptibility to human serum of Kp clinical isolates. Values indicate the minimum inhibitory concentration (MIC) of human serum (%, vol/vol in MHBII). Isolates with MICs of more than 48% serum were defined as serum-resistant isolates. The percentage indicates the prevalence of serum-resistant isolates. **d**, Serum MIC distributions in HMV-Kp and non-HMV-Kp clinical isolates. The y-axis shows the prevalence of isolates among the HMV and non-HMV groups. The broken red line shows the breakpoint of serum resistance. The significance was analysed by Fisher's exact test, and no significant difference in serum resistance between the HMV and non-HMV groups was observed ($p = 0.5548$). **e**, Measurement of the gene mutation frequency of Kp clinical isolates by rifampicin assay. Gene mutation frequencies were measured by counting the number of rifampicin-resistant mutants as the number of colony-forming units (CFUs) that grew on 100 mg/L rifampicin-containing MHII agar after cultivation at 37° C for 24 h: [CFUs on rifampicin-containing MH agar plate]/[CFUs on plain MH agar plate]. We defined mutators as low ($<10^{-9}$), moderate (from 10-9 to 10-8), high (from 10^{-8} to 10^{-7}), and hyper ($> 10^{-7}$) by their mutation frequencies. A hypermutator was detected from respiratory clinical specimens (a non-HMV isolate, namely, SMKP590; mutation frequency: 4.43×10^{-6}). The significance was analysed by the Kruskal-Wallis test, and no significant difference in gene mutation frequencies among each isolation site was observed ($p = 0.2519$). **f**, Comparison of the mutation frequency between HMV-Kp and non-HMV-Kp clinical isolates. We divided Kp clinical isolates into HMV (n= 26) and non-HMV (n= 241) groups and compared the gene mutation frequency observed in **e**. We removed the value of the mutator strain to evaluate the majority. Geometric means with geometric standard deviations are indicated, and Student's t test was used for the statistical analysis.

Figure 1

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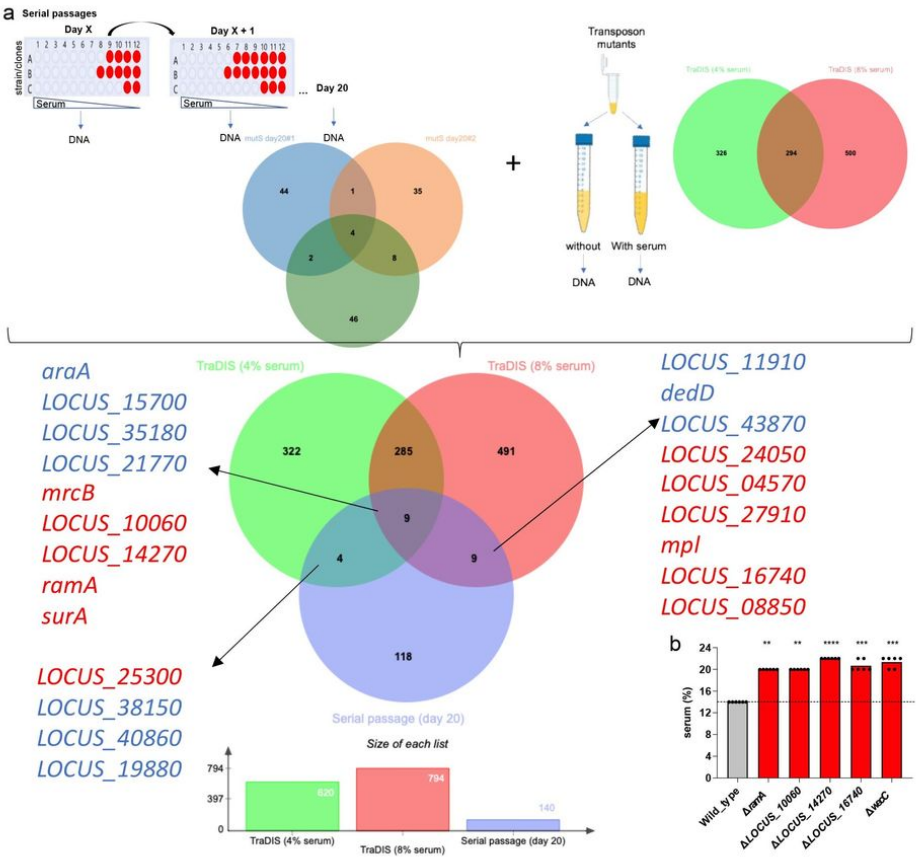

Fig. 2. Integrated analysis of serial passaging experiments and TraDIS for identification of serum resistance genes in which mutations accumulated during bacterial evolution.
We used the non-HMV-Kp strain SMKP838 as a parent strain. **a**, Venn diagram representing the integration of a total of 140 mutant genes identified in the serial passaging experiment in the presence of human serum (Supplementary Fig. 2e) and genes associated with serum resistance derived from TraDIS analysis performed in the presence of 4% (620 genes) and 8% serum (794 genes) (Supplementary Fig. 4d). Genes that are putative serum resistance genes by functional disruption (less than -2-fold difference in detection in the presence of human serum vs. without human serum in TraDIS, FDRp<0.05) are shown in red. Genes that are putative serum susceptibility genes (more than 2-fold difference in detection in the presence of human serum vs. without human serum in TraDIS, FDRp<0.05) are shown in blue. **b**, Serum susceptibility of SMKP838 gene deletion mutants. With this approach, we detected four novel genes (*ramA*, *LOCUS_10060*, *LOCUS_14270*, and *LOCUS_16740*) that contribute to serum resistance. We included *wecC*, which is known to contribute to serum resistance[45], as a control. The significance was calculated using Dunn's multiple comparisons test following the Kruskal–Wallis test, ANOVA; ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 2

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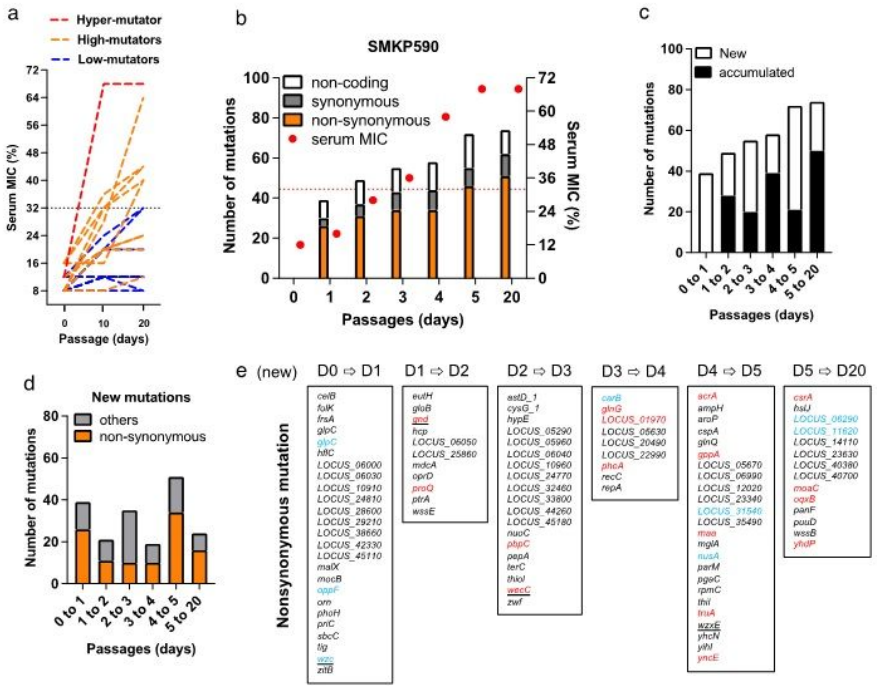

Fig. 3. Impact of the gene mutation frequency on the development of serum resistance in non-HMV-Kp clinical isolates.

a, Serial passaging experiments in the presence of human serum. Fifteen serum-susceptible non-HMV-Kp clinical isolates (serum MICs ranged from 8 to 16%) from among hyper- (n = 1), high- (n = 9; contains one K. quasipneumoniae), and low-mutators (n= 9) were inoculated in 96-well plates that contained serial dilutions of human serum (from 4 to 68%) in MHBII and cultured at 37° C for 24 h. After cultivation, we picked the well with the highest serum concentration in which the isolates grew (sub-MIC) and diluted the culture with 0.85% NaCl at 100-fold. One microlitre of the dilution was inoculated into 100 μL of MHBII that contained higher serum concentrations (from MIC to 68%) in 96-well plates and cultured at 37° C for 24 h. This step was repeated for 20 days. The red circles indicate the wells in which the strains/mutants grew. Every day, we measured the serum MIC of each selected mutant. Compared with the number of low-mutators, the number of high-mutator isolates that acquired serum resistance was significantly higher as determined by Fisher's exact test (p = 0.015). **b**, Serum susceptibility and accumulated gene mutations in the hypermutable isolate, SMKP590, during a serial passaging experiment. The serial passaging experiment in the presence of human serum was performed as shown in **a**. We measured the serum MICs (red circles) and number of gene mutations (orange, grey, and white show nonsynonymous mutations, synonymous mutations, and gene mutations in noncoding regions, respectively) of the SMKP590 mutants obtained during serial passaging in the presence of human serum. **c**, Number of novel and accumulated gene mutations in SMKP590 during serial passaging experiments in a. Novel and accumulated gene mutations were counted by comparison with those of the day before. **d**, Number of nonsynonymous and other gene mutations during serial passaging experiments of SMKP590 in a. Other genes contained synonymous mutations and gene mutations in noncoding regions. **e,** Gene mutations accumulated during the serial passaging experiments of SMKP590 in **a**. Red and blue show significantly enriched and depleted genes in human serum-containing medium according to detection by TraDIS analysis (Supplementary Fig. 5). The underlines show the known genes associated with serum resistance[44-46].

Figure 3

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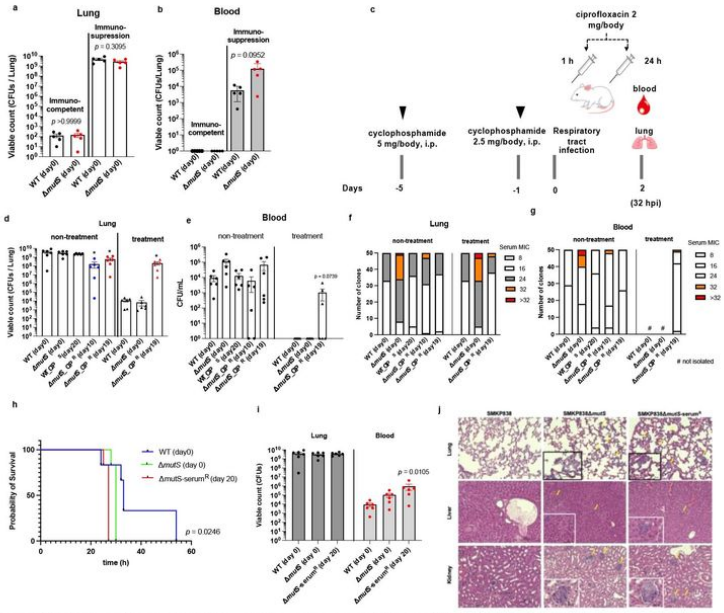
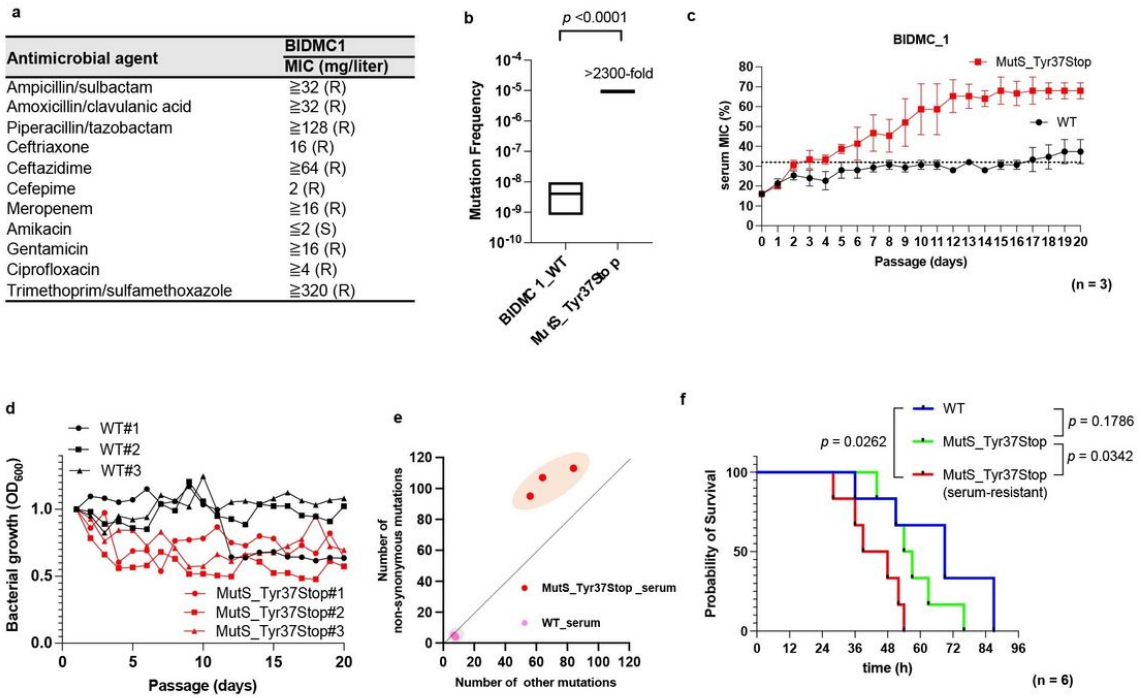


Fig. 4. Influence of gene mutations on the pathogenesis in non-HMV-Kp-infected mice.
a and **b**, Construction of an intrabronchial infection mouse model. Non-HMV-Kp SMKP838 and the *mutS* deletion mutant were used for infection. Ten- to 12-week-old female BALB/c mice with or without immunosuppression were intraperitoneally injected with 250 and 125 mg/kg cyclophosphamide monohydrate five and one day prior to infection, respectively. SMKP838 and the *mutS* deletion mutant (in 50 µl containing 1×10^8 CFU/mL) were inoculated intrabronchially with a microsprayer. After 32 h of infection or when a sublethal critical endpoint had been reached, the numbers of viable bacteria in the lungs (**a**) and blood (b) were counted. The geometric means with geometric standard deviations of n = 5 biologically independent experiments are indicated. The significance was calculated with a nonparallel two-tailed Student's t test. **c**, Intrabronchial infection mouse model in established immunosuppressed mice in this study. Ciprofloxacin monohydrochloride (100 mg/kg) was subcutaneously administered 1 and 24 h after infection. i.p., intraperitoneal administration. hpi, hours post-infection. The results shown in **d**-**g** were observed in this model. **d** and **e**, Numbers of viable bacteria in the lungs (**d**) and blood (**e**) with or without ciprofloxacin treatment. Non-HMV-Kp SMKP838 and the *mutS* deletion mutant are indicated as wild type (WT) and Δ*mutS*, respectively. We used the parent strains (day 0) and ciprofloxacin-susceptible (CIP^S) or ciprofloxacin-resistant (CIP^R) mutants observed after 10, 19, or 20 days of serial passaging experiments in the presence of ciprofloxacin (Supplementary Figs. 3 and 6). Geometric means with geometric standard deviations are indicated, and one-way ANOVA followed by Dunnett's multiple comparison test was used for the statistical analysis (**d** and **e**, n = 6 biologically independent experiments). **f** and **g**, Serum susceptibility of SMKP838 and the *mutS* deletion mutants after infection of mice. We picked 50 colonies per mutant from the lungs (**f**) and blood (**g**) 32 h after infection of the mice with or without ciprofloxacin treatment and measured their serum MICs. **h**, Lethality of non-HMV-Kp SMKP838 and the evolved mutants in mice. Non-HMV-Kp SMKP838 [WT (day 0)], the *mutS* deletion mutant [Δ*mutS* (day 0)], and the serum-resistant *mutS* deletion mutant [Δ*mutS*-$serum^R$ (day 20)] were used for the infection of mice. Δ*mutS*-$serum^R$ (day 20) was observed during the serial passaging experiment with human serum on day 20 (serum MIC: 72%) (Supplementary Fig. 2c). The log-rank test was used for the statistical analysis vs. WT (day 0) (n = 6 biologically independent experiments). **i**, The number of viable bacteria in the lungs and blood 32 h after infection or when a sublethal critical endpoint had been reached (n = 6 biologically independent experiments). The geometric means with geometric standard deviations are indicated, and one-way ANOVA followed by Dunnett's multiple comparison test vs. WT (day 0) was used for the statistical analysis. **j**, Histological examination of tissues from immunosuppressed mice infected with the parent SMKP838 and the *mutS* deletion mutants. The lungs, livers and kidneys were evaluated 24 h after infection. Representative histological images of haematoxylin and eosin (H&E)-stained organs (lung, liver and kidney) are shown (n = 3 biologically independent experiments). The yellow arrows indicate examples of clumps of bacteria.

Figure 4

See image above for figure legend.



Antimicrobial agent	BIDMC1 MIC (mg/liter)
Ampicillin/sulbactam	≧32 (R)
Amoxicillin/clavulanic acid	≧32 (R)
Piperacillin/tazobactam	≧128 (R)
Ceftriaxone	16 (R)
Ceftazidime	≧64 (R)
Cefepime	2 (R)
Meropenem	≧16 (R)
Amikacin	≦2 (S)
Gentamicin	≧16 (R)
Ciprofloxacin	≧4 (R)
Trimethoprim/sulfamethoxazole	≧320 (R)

Fig. 5. Influence of bacterial evolution on the outcomes of internationally spreading high-risk multidrug-resistant non-HMV-Kp infection.

We used the non-HMV-Kp strain BIDMC1 as a representative for the internationally spreading high-risk clone ST258. **a**, Antimicrobial susceptibility of BIDMC1. S and R indicate susceptible and resistant, respectively. **b**, Gene mutation frequency of BIDMC1 and the *mutS* nonsense mutable mutant (BIDMC1 MutS_Tyr37Stop). A rifampicin assay was performed to measure the gene mutation frequency. Floating bars represent maximum and minimum values, and lines represent geometric means (n = 3 biologically independent experiments). Student's t test was used for the statistical analysis. **c**, Serial passaging experiments of BIDMC1 and the *mutS* mutant in the presence of human serum. BIDMC1 (wild-type, WT) and BIDMC1 MutS_Tyr37Stop were used. Geometric means with geometric standard deviations for biological independent triplicate experiments are indicated **d**, Bacterial growth of the BIDMC1-derived mutants during the serial passaging experiment in **c**. We subjected three clones of both WT and MutS_Tyr37Stop. The bacterial growth was measured as turbidity (OD600) after 16 h of cultivation in MHBII. **e**, Number of accumulated gene mutations of BIDMC_1 and BIDMC_1 MutS_Tyr37Stop in a. f, The survival rate in immunosuppressed mice that were intrabronchially infected with BIDMC_1, MutS_Tyr37Stop, or serum-resistant MutS_Tyr37Stop. The mouse infection model was the same as that described in Fig. 4c. The log-rank test was used for the statistical analysis.

Figure 5

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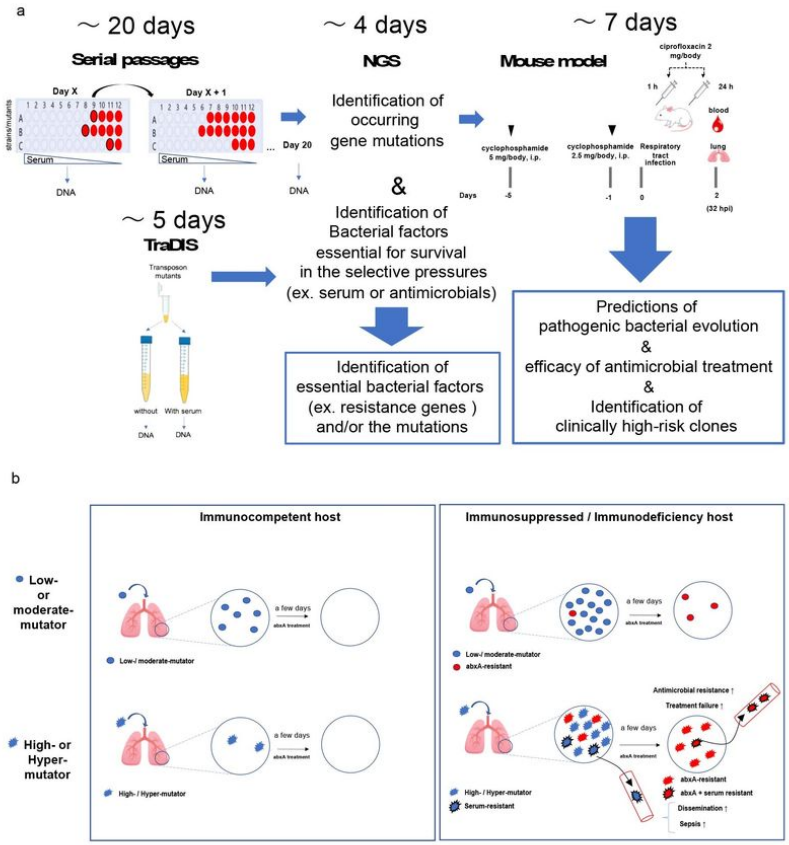

Fig. 6. Scheme of RIBEA and identification of the clinical risk of evolved bacteria.
a, Scheme of RIBEA developed in this study. We integrated serial passaging experiments to monitor the rapid bacterial evolution by using hypermutable strains in selective environments, such as different sites of infections and in the presence of antimicrobial agents, whole genome sequencing (WGS) for identification of accumulated gene mutations during bacterial evolution, transposon-directed sequencing (TraDIS) analysis for identification of bacterial factors that potentially contributed to survival in the selective environments (identification of resistance genes), and an *in vivo* model to evaluate the pathogenesis of the evolved bacteria and determination of the potential clinical risk. RIBEA can be completed within approximately one month. **b**, The mechanism of pathogenicity development in evolved bacteria. In this study, we revealed the pathogenesis mechanism and the potential clinical risk of evolved non-HMV-Kp, which is a principle bacterial pathogen. Non-HMV-Kp does not exhibit typical clinical symptoms upon infection and is eradicated by innate immune defence in immunocompetent hosts. In immunosuppressed (and/or immunodeficient) hosts, non-HMV-Kp can cause lower respiratory tract infections. Non-HMV-Kp with the potential for bacterial evolution (hyper and high gene mutation frequency) and evolved non-HMV-Kp enhance the clinical impact by increasing dissemination from the primary site (lung) to the blood with or without the development of antimicrobial (abxA) resistance, which leads to a decrease in the treatment efficacy of antimicrobial agents. Therefore, by RIBEA, we revealed the potential and the current clinical risk presented by bacteria that have a high gene mutation frequency during infection.

Figure 6

See image above for figure legend.

Supplementary Files

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