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研究課題名（和文）Temperature-driven regulation system of hypermucoviscosity in carbapenem-resistant Klebsiella pneumoniae

研究課題名（英文）Temperature-driven regulation system of hypermucoviscosity in carbapenem-resistant Klebsiella pneumoniae

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研究成果の概要（和文）：高粘着性(HMV) は、通常rmpAまたはrmpA2遺伝子によって制御され、Klebsiella pneumoniae(Kp)の毒性によく関連する。本研究では、HMVレベルが低い場合はrmpA/rmpA2に関連し、37°CでHMVを発現するが、HMVレベルが高い場合は関連しなく、室温でHMVを発現することがわかりました。
また、Kpは数種類のバクテリオシンも保有する。分離株の32.8%が1 種類以上のバクテリオシンを保有した。Microcin E492が最も多く(14.4%)、幅広い活性スペクトルを示した。Cloacinは7.2%で検出され、主にクレブシエラ属に対して抗菌効果を示した。

研究成果の学術的意義や社会的意義
Our findings facilitate the discovery of new factors related to HMV and explain different virulence tactics of Kp infection.
Findings about bacteriocins will facilitate studies on competition within microflora and the potential applications of bacteriocins in treating multidrug-resistant bacteria.

研究成果の概要（英文）：Hypermucoviscosity (HMV) is a phenotype commonly associated with hypervirulence of Klebsiella pneumoniae (Kp), which is usually regulated by rmpA or rmpA2 genes. Our analysis showed that the low HMV level is usually related to rmpA/rmpA2, while the high HMV level is not. Strains carrying rmpA/rmpA2 are likely to express HMV at 37° C, whereas those negative for these genes are likely to express HMV at room temperature.
Kp also produces several kinds of bacteriocins that have antimicrobial effects against other species. We found that 32.8% of isolates carried at least one bacteriocin type. Microcin E492 was the most prevalent type (14.4%) and had a wide spectrum of activity. Cloacin-like bacteriocin was detected in 7.2% of strains and exhibited inhibitory effect against mainly Klebsiella spp. Other bacteriocins, such as microcin S-like, microcin B17, and klebicin C-like, were detected at lower rates and had limited inhibitory activity.

研究分野：microbiology

キーワード：Klebsiella pneumoniae　virulence factor　hypermucoviscosity　antimicrobial resistance　antimicrobial peptide　bacteriocin

１．研究開始当初の背景

Klebsiella pneumoniae (Kp) is currently regarded as a significant threat to global health because of the emergence of hypervirulent (hv) clones causing severe community-acquired infections and multidrug-resistant (MDR) clones related to hospital outbreaks. Kp infections can be community- or hospital-acquired, leading to serious diseases such as pneumonia, primary pyogenic liver abscess or distinctive invasive syndrome. The virulence and resistance determinants are distributed in distinct subpopulations of Kp; however, the emergence of Kp with both carbapenem-resistant and hv phenotypes was recently reported in China, South and South-East Asian countries, and various other regions globally. The geographical focus of this convergence is likely to occur in Asia because of the common existence of both hv and MDR clones. As Japan is a part of Asia, hv/MDR Kp might have been disseminated into the main continent and evolved. In West Japan, carbapenemase-producing Kp has emerged in the past decade; these strains carry extended-spectrum β-lactamases (ESBL) ($bla_{CTX-M-2}$) and carbapenemase (bla_{IMP-6}) and are resistant to all β-lactam antibiotics, except imipenem. Nevertheless, no studies have provided a complete molecular epidemiological investigation of the Kp population in Japan so far, leaving a gap in knowledge about the specific regional population and diversity.

A variety of Kp virulence factors have been identified, including capsular polysaccharide, colibactin, ferric ion uptake, salmochelin (*iro*) and aerobactin (*iuc*). hvKp strains display an increased ability to acquire iron than classical Kp (cKp) strains due to the synthesis of iron-acquisition factors, such as aerobactin or salmochelin, that 'steal' the iron from the host. Another factor reported to contribute to the virulence of Kp is the hypermucoviscosity (HMV) phenotype. HMV has traditionally been attributed to overexpression of the capsule, which assists these bacteria in colonizing the mucosa and protects them from phagocytosis and human defensin-mediated bactericidal activity. The HMV phenotype is sometimes associated with the hypervirulence of Kp, although not all Kp with the HMV phenotype are hvKp. The *magA* gene (mucoviscosity-associated gene A) was first identified to code for a factor responsible for HMV,but was later found to be specific to the capsular serotype K1. Nevertheless, strains other than the K1 serotype (*magA*-negative) have also been reported to have HMV and hv phenotypes, suggesting that another factor may be associated with the HMV phenotype besides *magA*. Recent studies have suggested that unknown factors other than capsule production also play a role in the HMV phenotype. The KpnO porin, an outer membrane protein, was found to contribute to capsular polysaccharide production in the hvKp NTUH-K2044. However, it remains unclear whether there is any difference in the distribution of this factor between hvKp and cKp. The HMV phenotype is reportedly enhanced by expression of the plasmid-borne loci *rmpADC* (where *rmpA* is the regulator of the mucoid phenotype A) or *rmpA2*, which are considered some of the major factors involved in the HMV phenotype.

Nevertheless, the relationship between the *rmpA* genes and the HMV phenotype remains unclear, as some HMV-positive isolates do not harbour these genes. In this context, the regulatory mechanism of HMV in Kp is not fully understood.

２．研究の目的

From the viewpoint of clinical practice, an effective diagnostic tool to predict hvKp and its related characteristics could provide valuable early warnings about the hypervirulence and potential metastatic infections; therefore, comprehensive knowledge of the genomic population, virulence determinants and resistance determinants may significantly contribute to the infection control strategy. Furthermore, elaborating our understanding of the regulatory mechanisms of HMV and virulence of Kp may advance the development and implementation of new chemotherapies targeting these factors, which would improve the prognosis of serious infections. From our preliminary data, we found that temperature was a key factor affecting the HMV of Kp. Here, we present a genomic epidemiological study of hypermucoviscous Kp that examines the genomic characteristics (sequence types, capsular types, *rmpA*/*rmpA2*, virulence genes and resistance genes) of hypermucoviscous Kp in correlation with the temperature-dependent HMV phenotype.

３．研究の方法

(1) Bacterial isolates

A total of 236 Kp isolates, obtained from patient specimens, such as blood, respiratory tract (RT), urine, bile, pus or puncture fluid from different hospitals in the Kansai (southern-centre area of Japan) and Chugoku (middle-west area of Japan) regions between 2006 and 2017, were used for HMV evaluation by string tests. Strains were collected after completion of routine microbiological diagnostics.

(2) Modified string test

The isolates were cultured on agar containing 5 % sheep blood (Becton, Dickinson) and incubated at 37 ° C and room temperature (20-25 ° C). Each blood agar plate was divided into four parts, and a full loop of each bacterial strain was streaked evenly in each quarter with a 2 mm inoculating loop. After 24, 48 and 72 h of incubation, a string test was performed with a cotton swab. A 5-mm-diameter cotton swab was used to collect all the colonies of a strain within the area and was stretched upward. The string test was deemed positive when a viscous string of ≥5 mm was generated. The string test at each time point was repeated five times, and the mean string length was recorded. The maximum string length at three time-points of each strain was used for data analysis. The strains showing negative string tests at all growth conditions were excluded, and 170 Kp isolates with a positive string test in at least one condition were included in subsequent investigations.

(3) DNA extraction, whole-genome sequencing and genomic analysis

All Kp isolates were cultured overnight in Luria-Bertani (LB) broth at 37 ° C, followed by total DNA extraction using the phenol-chloroform method. Libraries were constructed using Nextera DNA kits (Illumina), and whole-genome sequencing (WGS) was performed using Illumina MiSeq, generating 150 bp paired-end reads. *De novo* assemblies were generated using Shovill v1.0.9 and subsequently annotated using the PATRIC RAST-tk-enabled Genome Annotation Service. The whole-genome sequences of our isolates were analysed using Kleborate v2.1.0 with the Kaptive option for their sequence types, K (capsule) serotype prediction, virulence loci and antimicrobial resistance (AMR) genes. The multi-locus sequence type (MLST) alleles (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) and sequence type (ST) profiles that had not been previously described were submitted to the curator of the official Kp BIGSdb-Pasteur database (http://bigsdb.pasteur.fr/klebsiella/) to assign new designations. The virulence and resistance scores were automatically calculated using Kleborate based on the types of virulence and AMR genes carried by each isolate. From the results of Kleborate, 14 isolates were identified as *K. quasipneumoniae* or *K. variicola* and hence were excluded from this study. The remaining 156 isolates were confirmed as *K. pneumoniae* and were used for further analysis.

Whole-genome SNP analysis was performed with CSIPhylogeny 1.4 from the Center for Genomic Epidemiology with default settings. A phylogenetic tree was created and was annotated with the Interactive Tree of Life (iTOL). Related nodes within the phylogenetic tree were clustered using RAMI. With the threshold of 0.1, RAMI produced 48 clusters, among which eight predominant clusters were found, and their key features were compared.

(4) Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v28.0.1.0. Comparisons between either Class I and Class III isolates, group A and group B isolates, or RT and blood isolates were performed using the chi-square test and Fisher's exact test (for key features) and Mann-Whitney test for mean string length. Mean string lengths at 37 ° C and room temperature between the *rmpA/rmpA2* positive and negative groups were analysed using the Mann-Whitney test and Wilcoxon signed-rank test.

４．研究成果

(1) Distribution of multi-locus STs and predicted capsular serotypes (K) within the HMV Kp population

Genotypic analysis using WGS on 156 HMV Kp strains showed that HMV Kp was highly diverse, comprising 58 STs and belonging to 35 known K-loci and four unknown K-loci. Twelve (20.69%) of the 58 STs had not been previously identified (ST4994, ST5049, ST5064, ST5069, ST5072, ST5134, ST5192, ST5193, ST5194, ST5195, ST5196 and ST5198). The most prevalent STs were ST23 (*n*=27, 17.3%), ST65 (*n*=19, 12.2%), ST86 (*n*=15, 9.6%), ST268 (*n*=9, 5.8%), ST37 (*n*=6, 3.8%) and ST375 (*n*=5, 3.2%). The most prevalent K-loci, which accounted for >50 % of the isolates, were KL2 (*n*=42, 26.9%), KL1 (*n*=29, 18.6%), KL20 (*n*=11, 7.1%) and KL57 (*n*=9, 5.8%). While ST23, ST65 and ST268 were each associated

with a single K-locus (KL1, KL2 and KL20, respectively), some other STs were associated with multiple K-loci; for example, ST37 included four KL136 and two KL38. Furthermore, 67.2 % (39/58) and 42.9 % (15/35) of the STs and known K-loci, respectively, were represented by a single isolate.

(2) AMR determinants and phenotypes

In this collection, we detected 21 isolates (13.5%) that carried ESBL and/or carbapenemase genes. Among them, 13 isolates carried both ESBL and carbapenemase genes, seven isolates carried only ESBL, and two isolates carried only carbapenemases. The ESBL genes included $bla_{CTX-M-2}$ (n=11, 52.4%), bla_{SHV-27} (n=4, 19.0%), $bla_{CTX-M-15}$ (n=3, 14.3%) and $bla_{CTX-M-65}$ (n=2, 9.5%), and the carbapenemase genes included bla_{IMP-6} (n=12, 57.1%) and bla_{KPC-2} (n=2, 9.5%).

Half of the ESBL- and/or carbapenemase gene-carrying isolates (n=11/21) carried both $bla_{CTX-M-2}$ and bla_{IMP-6}, along with *aacA4*, *aadA2*, *sul1* and *tetA*, all of which showed resistance to cephalosporins, meropenem and doripenem, as well as tobramycin and minocycline. Furthermore, four of these isolates (MS5293, MS5294, MS5265 and MS5291) also carried mutations associated with fluoroquinolone resistance (GyrA-83F, GyrA-87N and ParC-80R), resulting in resistance to levofloxacin and ciprofloxacin.

(3) Genotypic convergence of AMR and HMV

Phylogenetic analysis revealed dominant clusters corresponding to the dominant STs described above and represented well-known hvKp and cKp clones: hv-KL2-ST65 and -KL2-ST375 (cluster 1), -KL1-ST23 (cluster 2) and -KL2-ST86 (cluster 3) possessed *rmpA*/*rmpA2* and other virulence determinants (*ybt, clb, iuc* and *iro*), but only a few carried ESBL/carbapenemases (Fig. 1). Additionally, all KL2-ST65 and KL1-ST23 isolates carried an intact *rmpA* and a truncated *rmpA2*, while the other clusters did not show a consensus pattern. In contrast, the other clusters, including KL136-ST37 and KL38-ST3, KL47-ST11, KL24-ST45, and KL64-ST147 (clusters 5, 6, 7 and 8, respectively), mostly lacked *rmpA*/*rmpA2* and all other acquired virulence loci except *ybt,* but had a high prevalence of ESBL and/or carbapenemases (10/13 strains, 76.9%). Another cluster comprising KL20-ST268 and KL62-ST36 (cluster 4) displayed characteristics similar to those of cluster 2 (KL1-ST23), with a high prevalence of *rmpA*/*rmpA2* and other virulence determinants. These findings were generally consistent with the traditional view of Kp, for which acquired virulence and acquired resistance genes are usually found in distinct subsets of the population. However, we also identified several so-called ‘convergent’ isolates harbouring both acquired virulence and acquired AMR genes.

The 21 AMR-HMV isolates belonged to 14 different STs and 13 corresponding K-loci. These STs included AMR-background STs such as ST37 (n=4, 19.0%), ST11 (n=2, 9.5%), ST45 (n=2, 9.5%) and ST147 (n=2, 9.5%) (coloured in green), the hypervirulent-background STs such as ST65 (n=2, 9.5%) (coloured in blue), and some other miscellaneous STs such as ST268, ST36, ST134 and ST611.

Among 21 AMR-HMV isolates, five were positive for the *rmpA* (N2531, N2576, N454 and N579) or *rmpA2* (MS5288) genes and some other virulence genes (MS5288, N2531 and N2576: yersiniabactin, colibactin, aerobactin and salmochelin; N454 and N579: aerobactin and salmochelin).

(4) HMV phenotype and rmpA/rmpA2 genes

We detected 101 isolates carrying the *rmpA* gene, among which three isolates (3.0%) possessed truncated *rmpA* genes. Conversely, 88 isolates carried *rmpA2* genes, 73 (83.0 %) of which were truncated *rmpA2* genes. Thirteen strains (8.3%) carried both intact *rmpA* and intact *rmpA2*. Altogether, 100 isolates harboured an intact *rmpA* and/or *rmpA2* [henceforth referred to as the *rmp* (+) group], and 56 isolates carried a truncated or were negative or for both *rmpA* and *rmpA2* [henceforth referred to as the *rmp* (-) group]. A comparison of the mean string length between the *rmpA*-positive and *rmpA*-negative groups revealed no significant difference in string length at 37 ° C, but the string length at room temperature in the *rmp* (-) group was significantly higher than that of the *rmp* (+) group (Mann-Whitney test, P<0.001). Within the *rmp* (+) group, the mean string length at 37 ° C was significantly higher than that at room temperature (Wilcoxon signed-rank test, P<0.001), whereas, within the *rmp* (-) group, the mean string length at room temperature was significantly higher than that at 37 ° C (Wilcoxon signed-rank test, P<0.001).

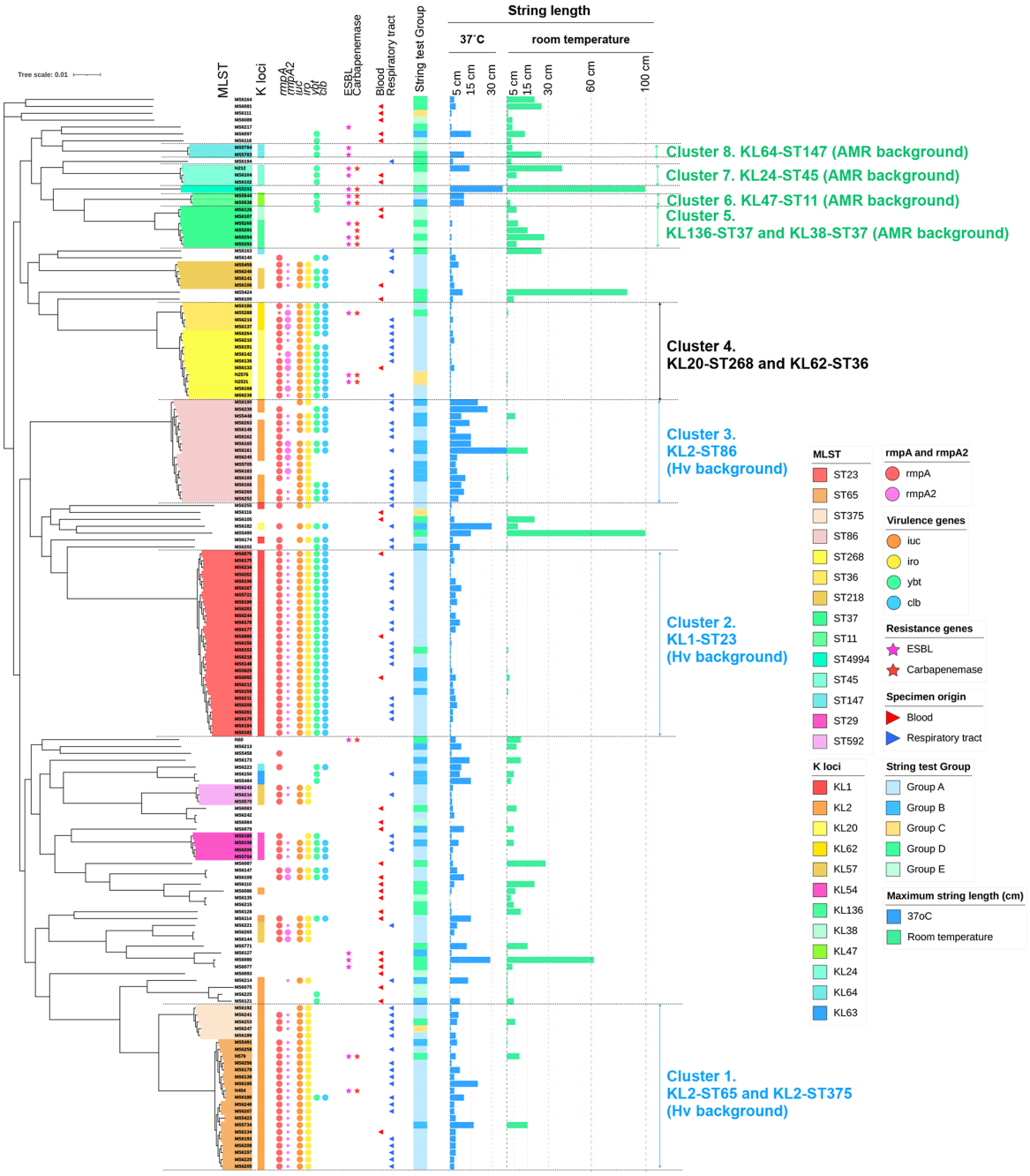

Figure 1. Phylogenetic analysis of 156 HMV K. pneumoniae isolates. Isolates are annotated with the datasets of sequence types (ST), capsular locus (KL), virulence genes, resistance genes, specimen origins, string test group, and maximal string length at 37 ° C and room temperature, from left to right. Different STs are highlighted in different colours, and the most prominent clusters are marked as follows: cluster 1, KL2-ST65 and KL2-ST375; cluster 2, KL1-ST23; cluster 3, KL2-ST86; cluster 4, KL20-ST268 and KL62-ST36; cluster 5, KL136-ST37 and KL38-ST37; cluster 6, KL47-ST11; cluster 7, KL24-ST45; and cluster 8, KL64-ST147. For the *rmpA* and *rmpA2* genes, the large circle indicates an intact gene, while the small circle indicates a truncated gene

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５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Le Mi Nguyen-Tra、Kayama Shizuo、Wyres Kelly L.、Yu Liansheng、Hisatsune Junzo、Suzuki Masato、Yahara Koji、Terachi Tsuneko、Sawa Kana、Takahashi Shin、Okuhara Toshihiko、Kohama Kunihiko、Holt Kathryn E.、Mizutani Tetsu、Ohge Hiroki、Sugai Motoyuki	４．巻 8
２．論文標題 Genomic epidemiology and temperature dependency of hypermucoviscous Klebsiella pneumoniae in Japan	５．発行年 2022年
３．雑誌名 Microbial Genomics	６．最初と最後の頁 1
掲載論文のDOI（デジタルオブジェクト識別子） 10.1099/mgen.0.000827	査読の有無 有
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１．著者名 Le Mi Nguyen-Tra、Nguyen Thao Huu-Huong、Trinh Van Minh、Nguyen Tam Phuc-Bao、Kawada-Matsuo Miki、Kayama Shizuo、Sugai Motoyuki、Komatsuzawa Hitoshi	４．巻 8:e0086323
２．論文標題 Comprehensive Analysis of Bacteriocins Produced by the Hypermucoviscous Klebsiella pneumoniae Species Complex	５．発行年 2023年
３．雑誌名 Microbiology Spectrum	６．最初と最後の頁 1
掲載論文のDOI（デジタルオブジェクト識別子） 10.1128/spectrum.00863-23	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

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１．発表者名 Mi Nguyen-Tra Le
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１．発表者名
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Temperature-dependent hypermucoviscosity and genomic population structure of Klebsiella pneumoniae

３．学会等名
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〔図書〕　計0件

〔産業財産権〕

〔その他〕
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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関