氏　　名	Thitiananpakorn Kanate（てぃてぃあなんぱこーん　かねーと）
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学位論文名	**MRSA におけるダプトマイシンとバンコマイシン交差耐性メカニズムの解明**
論文審査委員	(委員長)　教授　加藤大智
	(委　員)　教授　萩原弘一　　教授　國田　智

論文内容の要旨

1　研究目的

Staphylococcus aureus, being one of the ten pathogens commonly associated with multidrug resistance (especially methicillin-resistant *S. aureus*, MRSA), is renowned for its ability to cause various difficult-to-treat infections in humans. The emergence of MRSA resistant to vancomycin (VCM), the last-line antibiotic against MRSA infections, further limits the scarcely available treatment options.

Daptomycin (DAP) is an antibiotic newly approved for treating complicated MRSA infections due to its high bactericidal activity and low propensity for resistance. Nevertheless, DAP nonsusceptibility developed during the course of treatment for MRSA infections has been reported. In spite of different mechanisms accounting for DAP and VCM resistance, MRSA with reduced susceptibility to both DAP and VCM, known as DAP/VCM cross-resistance, have been isolated. VCM inhibits bacterial cell-wall (CW) synthesis by binding to D-ala-D-ala residues of CW peptidoglycan, while DAP inserts its lipophilic tail into the cell membrane (CM) to cause potassium leakage, thereby leading to cell membrane depolarization. However, the mechanism(s) conferring *S. aureus* resistance to the two different classes of antibacterial agents remains unknown.

Unless the mechanism(s) of DAP/VCM cross-resistance are clarified, development of new therapeutic drugs will be hindered and in turn increased morbidity and mortality due to MRSA infections is inevitable. Therefore, we will focus on unraveling the mechanisms of cross-resistance in *S. aureus* to DAP and VCM, as this will facilitate the identification of novel target sites and development of new therapeutic agents, contributing to the management of difficult-to-treat bacterial infections.

2　研究方法

<1: Bacterial strains and culture conditions >

A total of 33 clinical MRSA strains, comprising 12 sets of DAP susceptible (DS) and nonsusceptible (DNS) isolates (each set collected from the same patient in Japan before and after DAP treatment), were included in this study. In addition, we also studied on 32 VISA (vancomycin-intermediate *Staphylococcus aureus*) strains collected worldwide from 9 countries, all of which are clinical isolates from patients receiving vancomycin therapy.

<2: Antimicrobial susceptibility profiling >

Firstly, we determined the minimum inhibitory concentration (MIC) of DAP and VCM to all isolates by using both Etest methods. According to MIC tests, antimicrobial resistance pattern of each studied strain will be classified as follows;

1) DAP/VCM cross-resistance

2) DAP nonsusceptibility

3) VCM resistance

<3: Whole genome sequencing for single nucleotide polymorphisms (SNPs) analysis>

Whole genome sequences of paired DS and DNS MRSA strains selected from each of the 12 sets of patients' isolates were determined using MiSeq system (illumina) for comparative genomic analysis. DS parental strains were sequenced with mate pair sequencing. Genome-wide comparison between DS and DNS isolates were then performed using CLC genomics workbench program. Following genome comparison, point mutation(s) in genetic sequences of DNS strains as compared to their corresponding susceptible parental strains, SNPs, will be identified. The functions of mutated gene(s) found in each patterns of antimicrobial resistance will be annotated by database searches and data mining in order to delineate the bacterial physiology causing drug resistance.

< 4: Construction of mutants by allelic replacement >

pKOR1 plasmid carrying the mutated gene(s) presumably involved in DAP/VCM cross-resistance will be constructed and transformed into sensitive strains. Furthermore, resistant strains harboring gene mutation(s) will be reverted to susceptible strains through introduction of wild-type gene(s). All mutated constructs will be verified by Sanger sequencing. Phenotypic alteration(s) in both transformed strains, such as the MICs as well as CW thickness and CM surface charge, will then be observed and compared with parental strains.

< 5: Genotypic and phenotypic characterization of DAP/VCM cross-resistance strains>

Differential gene expression between paired DS and DNS strains selected from the 12 sets of patients' isolates will be determined with transcriptome analysis and gene expression quantification. Since DAP and VCM are known to be targeting bacterial CM and CW respectively, it would be likely for genes associated with CM/CW biosynthesis to be differentially expressed in DNS strains compared to DS strains. To consolidate our findings, genetic determinant(s) that contribute to bacterial resistance (differentially expressed genes) will be verified phenotypically. In case of phenotypic experiment, the thickness of CW or the property of CM is investigated. Increased thickness of CW was previously reported to reduce bacterial susceptibility towards VCM. Hence, we will measure the size of CW by using transmission electron microscope (TEM). On the other hand, alteration of membrane surface charge is involved in DAP nonsusceptibility and that can be measured by using cytochrome *c* assay.

3 研究成果

We found that all 12 DNS strains exhibiting cross-resistance carried mutations in *mprF*, while one DNS strain with resistance to only DAP carried a *lacF* mutation. On the other hand, among the 32 VISA strains isolated from patients treated with VCM, 5 out of the 18 strains showing cross-resistance to VCM

and DAP carried a *mprF* mutation, while 14 strains resistant to only VCM had no *mprF* mutation. Moreover, substitution of *mprF* in a DS strain with mutated *mprF* resulted in cross-resistance and vice versa. The *mprF* mutation elevated lysyl-phosphatidylglycerol (L-PG) production, positive membrane surface charges, and CW synthetic pathways. These results demonstrated that the *mprF* mutation contributed to cross-resistance to VCM and DAP in MRSA.

4 考察

The results suggest that *mprF* mutation plays a main role in generation of MRSA with cross-resistance to DAP and VCM during DAP therapy, but partially in the MRSA strains that are generated to be cross-resistance during VCM chemotherapy. *mprF* mutation have already reported on bacterial protection from cationic antimicrobial peptides but resistance to VCM from *mprF* mutation is still. VISA strains display thickened CW to allow increased binding of VCM to false targets in peptidoglycan (affinity trapping), thereby contributing to their reduced VCM susceptibility. Therefore, one possible pathway leading to DAP-nonsusceptibility in VISA strains maybe increased CW thickness. However, as shown by our TEM analysis, only 1 DNS strain in DAP/VCM cross-resistance has thickened CW. Neither the remaining 11 sets of DNS strains carrying *mprF* mutation nor DNS strain with only DAP-nonsusceptibility carrying *lacF* mutation showed increased thickness of CW. This phenomenon might due in part to the small range of VCM MIC changes observed between DS and DNS strains. On the other hand, DAP/VCM cross-resistance has been reported in both laboratory-derived and clinical isolates with no phenotypic characteristic of CW thickening. It is therefore indicated that DAP/VCM cross-resistance is not resulting from a single contributing factor; while increased CW thickness is associated with DAP/VCM cross-resistant VISA strains, alteration in membrane surface changes is more likely the causative factor of DAP/VCM cross-resistance in DNS strains.

There are two domains of MprF protein, lysinylation domain and flippase domain. Normally, negatively charge membrane can alter to positively charge membrane by lysinylation domain that add lysine into phospholipid of membrane. The function of flippase domain translocates the positively charge membrane to outer membrane against antimicrobial peptides such as cathelicidins or defensins. Phenotypic alteration of membrane surface charges via *mprF* mutation is a commonly reported bacterial evolution to resist positively-charged drugs, such as CAMPs and DAP. Interestingly, VCM molecules contain ionizable group, amine and carboxylic group, that also display positive charge when administered. Moreover, disruption of negatively-charged wall teichoic acids by deletion of *dltABCD* operon involving in alanylation of teichoic acids were reported to increase the drug susceptibility of *S. aureus* Sa113 to both CAMPs, such as α-defensins or nisin, and glycopeptides, such as VCM or teicoplanin. Besides the mechanism of increased CW thickness, cross-resistance mechanism seems to be involved in alteration of membrane surface charges. Our results found that every DNS isolates in DAP/VCM cross-resistance carried *mprF* mutation showed reduction of negative surface charges in CM. This alteration of bacterial membrane surface charges is resulted from increased positively-charged membrane from L-PG production. Furthermore, RNA-sequence analysis showed changes in gene expression that enhance fatty acid biosynthesis in DNS strain, suggesting that changes in membrane surface charge via enhanced levels of

L-PG production can contribute to DAP/VCM cross-resistance.

5　結論

This study suggested that DAP/VCM cross-resistance in MRSA is associated with *mprF* mutation. The reduction of DAP and VCM susceptibility is mainly mediated by alteration of CM surface charge through increased L-PG production, while increased CW thickness is marginally involved. Besides, we also revealed a novel pathway leading to DAP-nonsusceptibility which is not related to the common genetic determinant *mprF*. In our study, single DAP-nonsusceptibility is believed to be caused by alterations in cellular metabolisms ensued from *lacF* mutation.

論文審査の結果の要旨

学位論文表題：MRSA におけるダプトマイシンとバンコマイシン交差耐性メカニズムの解明

本論文は、メチシリン耐性黄色ブドウ球菌(MRSA)の治療において見られるダプトマイシンとバンコマイシンの交差耐性のメカニズムの解明を試みたものである。

この研究で申請者は、12 人のダプトマイシン単独治療を受けた患者の治療前後の MRSA を分離し、1）11 人の患者から分離した 12 株でバンコマイシンに対する交差耐性を獲得していること、2）全ゲノムシークエンスの比較解析を行い、交差耐性株では全て *mprF* 遺伝子の、主に Lysinylation domain に変異が認められたこと、3）*mprF* 遺伝子の変異体をダプトマイシン感受性株に導入することで交差耐性が誘導されること、4）*mprF* 遺伝子の変異は細菌の細胞膜表面電荷に変化をきたし、それが交差耐性の獲得に関与すること、5）バンコマイシン単独投与によって交差耐性を獲得した MRSA においても *mprF* 遺伝子の変異が認められること、を明らかにした。

本研究は多岐にわたる研究手法を駆使して行われており、得られた結果は明快であった。本研究を通して得られた結果は細菌学分野において重要な研究成果であると言え、博士の学位を授与するに相応しいものと高く評価できる。

以上のことから、本申請論文は学位論文として合格であると判定された。

なお、審査員からいくつかの修正点が指摘されたが、それらは全て適切に修正されたことが確認された。

最終試験の結果の要旨

申請者は学位論文に沿った研究内容の発表を行った。発表は大変分かりやすいもので、プレゼンテーションの構成も理解しやすいように作られていた。発表時間は約 30 分間と予定通りであった。

申請者の発表の後に、薬剤耐性の定義、血清中の薬剤濃度と薬剤耐性との関連、遺伝子変異部位と薬剤耐性メカニズムの関連、薬剤耐性を獲得することによって細菌にもたらされる不利益等について約 30 分間にわたって活発な質疑応答が行われた。申請者は各質問に対して適切に答えており、研究結果の的確な解釈に加えて、同分野の研究背景や研究の進展について多くの知識を得てきていることが確認された。時間を通して有意義な質疑応答が行われたと考える。

論文審査および最終試験から、申請者が研究者として充分な知識と研究遂行能力を有すると評価し、審査員全員一致で最終試験に合格と判断した。