氏　　名	Tanit Boonsiri（たにっと　ぶーんしり）
学位の種類	博士 (医学)
学位記番号	甲第612号
学位授与年月日	令和2年3月16日
学位授与の要件	自治医科大学学位規定第4条第2項該当
学位論文名	**オキサシリン感性 *mecA* 陽性黄色ブドウ球菌におけるβラクタム薬高度耐性化機構の解明**
論文審査委員	(委員長)　教授　國田　智 (委員)　教授　輿水崇鏡　教授　畠山修司

論文内容の要旨

1　研究目的

Emergence of *Staphylococcus aureus* bacterial strains carrying β-lactam resistance gene (*mecA*) but are phenotypically susceptible to β-lactam antibiotic oxacillin (OS-MRSA) is an ongoing hidden problem in clinical settings worldwide. Owing to its susceptibility to oxacillin, OS-MRSA might be misidentified as methicillin-susceptible *S. aureus* (MSSA) in routine clinical laboratory where *mecA* detection is unavailable. In addition, despite being fundamentally susceptible to β-lactam treatment, OS-MRSA prone to develop β-lactam resistance following antibiotic therapy due to *mecA* carriage. These will ultimately lead to β-lactam treatment failure. Currently, the mechanisms underlying generation of β-lactam resistance remain unknown. Therefore, this study aims to unravel the genetic determinants associated with the acquisition of β-lactam resistance in OS-MRSA.

2　研究方法

1. Determination of the characteristics of OS-MRSA

Oxacillin MIC and *mecA* carriage in all 43 clinical OS-MRSA isolates collected from Japan and Taiwan between 1998 and 2015 were re-determined by E-test and PCR, respectively.

2. Isolation of oxacillin-resistant mutants

All OS-MRSA strains were exposed to oxacillin by E-test. Individual colony of resistant mutant induced from each OS-MRSA strain were collected and purified before determination of oxacillin MIC by E-test.

3. Genome analysis of OS-MRSA and its mutants

Genomic DNA of OS-MRSA strains and their mutants were prepared using phenol-chloroform method and QIAamp DNA mini kit. Whole genome sequences (WGS) were determined by Illumina Miseq. The sequence data were compared and analyzed using CLC Genomics Workbench ver 9.

4. Mutants construction

mecA-overexpressing strain was constructed by transforming plasmid-encoded wide-type *mecA* into the parental OS-MRSA strain. Deletion of *mecA* or *blaI* in the parental strain and its mutants were achieved with allelic replacement method.

5. *mecA* transcription

OS-MRSA strain and its mutants were cultured to an OD_{600} of 0.3 before treated with oxacillin or distill water for 1 hour. Total RNA prepared from the treated cultures were converted to cDNA and *mecA* expression was determined by qRT-PCR.

6. Transcriptome analysis

OS-MRSA strain and its mutants cultured to an OD_{600} of 0.3 were grown in the presence/absence of oxacillin to mid-log phase before the cells were harvested for RNA extraction. After depletion of rRNA, double-stranded cDNA was synthesized. RNA-sequencing was performed by Illumina Miseq.

7. Determination of cellular ATP level

The ATP levels of OS-MRSA strain and its mutants grown to mid-exponential phase were measured using a BacTiter Glo kit.

3 研究成果

1. Characteristics of OS-MRSA

All 43 studied strains maintained the characteristics of OS-MRSA, which is *mecA* positive, but were susceptible to oxacillin with MICs ranging from 0.125-2 μg/mL. WGS revealed a high genomic diversity among these isolates, whereby they were classified into 7 phylogenetic clades, 11 MLST types (ST1, ST5, ST8, ST59, ST89, ST91, ST121, ST338, ST772, ST1516 and ST-nontypeable), and 4 SCC*mec* types (II, IVa, IVc and V). Among the 43 isolates, 34 (79%) carried *bla* operon that can be classified into 2 types (*bla* operon-1 and -2).

2. Oxacillin-resistant mutants isolated from OS-MRSA

Although resistant colonies grown within inhibition zone can be generated from all parental OS-MRSA strains, not all selected colonies displayed increased oxacillin MIC. Finally, a total of 100 *in vitro*-selected mutants with increased oxacillin MICs (ranged between 1-256 μg/mL) isolated from 26 parental OS-MRSA strains were used for downstream study.

3. Mutations associated with oxacillin resistance

The comparative genome analysis between the 100 selected mutants and their parents revealed a total of 141 mutations with 118 variants in 46 coding regions and 8 intergenic regions. Genes involved in RNA polymerase (*rpoBC*), purine biosynthesis (*guaA*, *prs*, *hprT*, *rsh*), glycolysis (*pykA*, *fbaA*, *fruB*), protein quality control (*clpXP*, *ftsH*) and tRNA synthase (*lysS*, *gltX*) were frequently mutated among these mutants. No mutations were identified in *mecA* and its regulatory elements (*mec* and *bla* operons).

4. *mecA* contributed to reduced oxacillin susceptibility in OS-MRSA

JMUB217 strain and its mutants were selected as the representing strains for profound

study because they carried a variety of mutated genes and is the only strain harboring two types of *bla* operons. JMUB217 transformed with plasmid carrying *mecA* shown increased oxacillin MIC from 0.75 to 48 μg/mL, whereas the deletion of *mecA* in JMUB217 and its 3 mutants carrying *rpoB* or *rpoC* mutation exhibited a drop in oxacillin MIC to 0.38 μg/mL.

5. *mecA* transcription is not directly correlated with oxacillin-reduced susceptibility

Under oxacillin-induced condition, the *mecA* expression showed strong correlation with oxacillin MIC ($r_s = 0.78$, $p < 0.0001$). However, its expression levels were not dramatically increased in all mutants compared with the parent.

6. *blaI* had slight effect on oxacillin-reduced susceptibility

Single-gene knockout of *blaI*-1 or *blaI*-2 in strain JMUB217 did not affect its oxacillin MIC, but double-gene knockout of *blaI*-1 and *blaI*-2 caused increased oxacillin MIC from 0.75 to 2 μg/mL, though it is still susceptible to oxacillin.

7. Integration of transcriptome and WGS analysis highlights that stringent-like response (SLR) is responsible for reduced oxacillin susceptibility

Gene expression profiles of 5 representative mutants of JMUB217 carrying *rpoB*, *rpoC*, and *rpiA* mutation were compared with their parent. Overall, most of the genes associated with glycolysis, pentose phosphate, purine, folate, and tRNA synthesis were downregulated among the mutants. Part of the genes involved in protein quality control, autolysin, and peptidoglycan synthesis were also downregulated. Meanwhile, several genes related to antibiotic resistance were upregulated, particularly *mecA*, *blaZ*, and *blaR1*. The integrated analysis of WGS and transcriptome implicated that mutations identified by WGS in each mutant resulted in partial loss of gene function which imitated the downregulation of these genes in gene expression profiles of transcriptome analysis in the representative mutants.

8. Mutations occurred in the mutants have no fitness cost

Except for JMUB217-21 ($GuaA^{I249fs}$), doubling times of the other mutants were approximately equal to the wild type and showed a positive correlation with oxacillin MIC ($r_s = 0.47$, $p = 0.0205$).

9. Normal growth rate shown in the mutants might be restored by increased ATP production

The ATP levels of mutants showed a positive correlation with the oxacillin MIC ($r_s = 0.6047$, $p = 0.0017$), and that might render the mutants to restore normal growth.

4 考察

Although oxacillin resistance in OS-MRSA was caused by increased *mecA* expression, the level of *mecA* was not uniformly increased in all representative studied strains. We postulated that OS-MRSA had a more complex regulatory pathway than just direct *mecA* signaling. Herein, oxacillin exposure induced mutations in genes associated with a wide range of central metabolic pathways such as *rpoBC*, *guaA*, *rsh*, and etc. These gene mutation profiles were similar to those observed in typical MRSA which were reported to be associated with classic stringent response (SR) and resulted in oxacillin resistance. The combination of oxacillin

exposure-derived mutation analysis and transcriptomic analysis illustrated that oxacillin resistance of OS-MRSA can also be induced by another pathway, namely “the stringent-like response”. This pathway mimics the induction of classic SR via amino acid starvation. However, unlike classic SR, the new pathway showed downregulation of genes responsible for branched-chain amino acid and (p)ppGpp synthesis. These findings provided evidence that the SLR does not require the accumulation of a regulator (p)ppGpp to regulate the expression of gene profiles. Instead, the lower intracellular GTP/GDP level may play a key role in oxacillin resistance via subsequent upregulation of *mecA* expression by *blaR1*, as well as influencing the expression of genes involved in autolysis and peptidoglycan synthesis. Moreover, the resistance-associated mutations exhibited a relatively low fitness cost, explaining for the easy selection of β-lactam–resistant OS-MRSA mutants during antibiotic treatment.

5　結論

This study discloses the genetic pathway responsible for acquisition of oxacillin resistance in OS-MRSA with diverse genetic backgrounds. Our results suggested that OS-MRSA was rendered oxacillin resistance by the SLR and the ensuing expression of antibiotic resistance genes (*mecA*, *bla* operon, etc.). The relatively low fitness cost conferred by mutations may fuel the easy selection of resistant mutants of OS-MRSA during the course of antimicrobial treatment. Therefore, *mecA* detection is important for identification of OS-MRSA in clinical laboratory to prevent misdiagnosis and could be a strategy for better treatment of MRSA infection.

論文審査の結果の要旨

本論文は、oxacillin 感性の *mecA* 陽性黄色ブドウ球菌（OS-MRSA）がβ-ラクタム薬耐性を容易に獲得する現象に着目し、その耐性獲得機構を解明したものである。耐性遺伝子 *mecA* を保有しながら抗菌薬に感性を示す OS-MRSA は、抗菌薬曝露により速やかに耐性化し、抗菌薬治療が失敗する危険性を有することから、近年臨床的にも問題とされており、その耐性機構解明は診療上も意義深い。

この研究において申請者は、１）OS-MRSA 臨床分離株の薬剤耐性および関連遺伝子の解析、２）抗菌薬曝露により OS-MRSA から分離した oxacillin 耐性変異株のゲノム解析による変異遺伝子の同定、３）逆遺伝学解析による OS-MRSA の oxacillin 耐性獲得における *mecA* および *blaI* 遺伝子の関与の検証、４）oxacillin 耐性変異株での *mecA* 発現量と oxacillin MIC の正の相関の検証、５）RNA-seq 解析による OS-MRSA と oxacillin 耐性変異株の抗菌薬曝露に伴う発現変動遺伝子の同定、６）oxacillin 耐性変異株の増殖速度および細胞内 ATP 量の解析等を行った。そして、これらの実験結果より、OS-MRSA の oxacillin 耐性獲得には、PBP2a 産生遺伝子である *mecA* の発現増加が必須であり、この *mecA* 発現の誘導に stringent-like response（SLR）経路が重要な役割を果たしているとの結論に至った。また、本研究では、抗菌薬曝露によって耐性化した OS-MRSA

の多面的な特性が明らかになり、OS-MRSA を MSSA と鑑別する上での *mecA* 検出の重要性が示唆された。以上の研究成果は、抗菌薬耐性の機構を解明する上で重要な知見を包含するとともに、MRSA の診断や治療の開発に資するところも大きいと考えられることから、博士の学位を授与するに相応しいものと評価できる。

なお、本学位論文中の用語や表現ならびに一部のデータ表記に関して、委員から修正の指摘があった。再提出された論文を審査委員全員で検討し、適切に修正されていることを確認の上、合格と判定した。

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。発表内容としては、研究背景と意義、研究目的とその解明に向けた実験方法と結果、および申請者の導いた仮説を含む考察が明確かつ論理的に示された。発表後に、実験材料と実験手技、実験結果の解釈、特に供試臨床分離株の由来、同定された変異遺伝子や変異部位と oxacillin 耐性獲得の分子機構、速やかな耐性獲得をもたらす機序、β-ラクタム薬に対する交叉耐性、鑑別診断法の現状と開発状況などについて、審査委員との間で質疑応答を行った。一部の質問に対して申請者の回答根拠に不明確な部分も見受けられたが、全般的に適切な応答が行われた。

以上のような論文審査および最終試験の結果から、申請者が博士の学位に相応しい科学的知識と研究遂行能力、ならびに論理的思考と発表能力を有すると評価し、審査委員全員一致で最終試験に合格と判定した。