論文の内容の要旨

論文題目　A Novel Two-component System Contributing to Staphylococcal Virulence and Cell Wall Integrity

(黄色ブドウ球菌の病原性と細胞壁の完全性に寄与する新規二成分制御系)

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Introduction:

Upon bacterial infection, the bacteria should sense and respond appropriately to environmental conditions in hosts; nutrient concentration, pH and to the defense system deployed by the host such as the antimicrobial peptides, complement system, opsonins or other components, which interact with the cell wall and/or bacterial membrane. To respond to these conditions, the membrane associated-proteins including nutrient receptors and two-component sensor-kinase and regulator systems play important roles. Virulence of pathogenic bacteria is also largely determined by membrane-associated proteins and secreted toxins. Therefore, the integrity of the membrane and/or cell wall of bacteria should be consequently required for the efficient function of these response and virulence determinants.

In this study, based on the standpoint that the virulence of *S. aureus* could be impaired by the dysfunction of some membrane-associated proteins, I searched for the virulence genes.

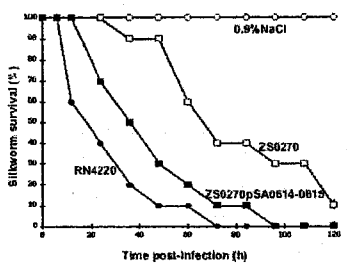
1) Identification of an *S. aureus* gene responsible for chlorpromazine-sensitivity and decreased virulence against silkworm larvae

I speculated that mutants for membrane-associated proteins might be obtained if I selected chlorpromazine-sensitive (CPZ^s) strains, as CPZ is a drug perturbing membrane fluidity. I isolated one hundred mutants by means of ethylmethane sulfonate treatment. These mutant cells grow under normal conditions of culture but not in presence of CPZ. Out of one hundred, sixteen strains displayed a decreased killing ability compared to the parent strain, in a fifth instar silkworm larvae-infection assay. Then the wild type genes that complement CPZ^s phenotype were screened from whole genomic DNA library of *S. aureus*. I present here, one strain named ZS0270 out of three strains that were complemented for CPZ^s phenotypes. A plasmid pSA0614-SA0615 harboring the genes SA0614 and SA0615 but not a vector pKE515 plasmid complemented CPZ^s phenotype (Table.1). In silkworm infection assay, parent RN4220 killed the infected silkworm within 20h whereas ZS0270 took 60h, suggesting the decreased virulence of ZS0270 (Fig.1). The plasmid also complemented the decreased virulence of ZS0270. DNA sequence analysis revealed that ZS0270 harbored a single amino acid substitution mutation in the gene locus SA0614,Gly59Glu. To confirm the responsibility of the mutation for the CPZ^s and the decreased virulence, phage transduction experiments were carried out. The results demonstrated that both phenotypes were co-transduced with a certain frequency with a drug-resistant marker inserted near to the SA0614 gene. Therefore, I concluded that the SA0614 gene confers tolerance to CPZ on *S. aureus* and contributes to *S. aureus* virulence against silkworm larvae.

Table.1. Transformation of ZS0270 with a multi-copy plasmid pSA0614-0615 rescued the mutant strain from CPZ-sensitivity

Strains	Plasmids	Colony number ratio: CPZ 0.15mM/0mM
RN4220	-	1
ZS0270	-	<0.0001
ZS0270	pKE515	<0.0001
ZS0270	pSA0614-0615	0.8 ± 0.05

Fig.1. Partial complementation of ZS0270 with plasmid pSA0614-0615 slightly increased the killing ability of *S. aureus* against silkworm



2) A two-component system SA0614 – SA0615 involved in *S. aureus* tolerance to CPZ and contributing to virulence

The genome database of *S. aureus* suggested that the SA0614 gene encodes a histidine kinase-activated transcriptional regulator of a two-component system and SA0615 encodes a cognate membrane-integrated histidine-kinase, whose function has not been reported. The insertional disruptants of each gene were constructed, tested for sensitivity to CPZ and decreased virulence. The mutants were unable to grow at a CPZ concentration over 0.08 mM while parent strain grew stably until CPZ concentration 0.15 mM

(Fig2A). The virulence assay on Fig.2B shows that killing of silkworms infected by the deletion mutants was 3 times delayed compared to parent RN4220. These results suggest that the two-component system is responsible for tolerance to CPZ and virulence.

3)The two-component system SA0614- SA0615 functions to cell wall integrity

The sensitivity of the insertional disruptants to CPZ can be explained by that (i) the two-component system function in membrane homeostasis upon CPZ-induced damage, or (ii) bacterial membrane became more exposed to CPZ because of an altered cell wall structure. To verify these hypotheses, the insertional disruptants were tested for sensitivity to membrane damaging agents such as ionic and non-ionic detergents and cell wall damaging agents. The mutants were strikingly sensitive to the non-ionic detergent including Triton-X 100 (fig.3A) and NP-40 but not to ionic detergents SDS or deoxycholate. The mutants were also more sensitive to lysozyme, a cell wall degrading agent, compared to parent strain RN4220 (fig.3B). In these cases, non-ionic detergents and CPZ killed the bacteria near micelle-forming concentration whereas ionic detergents killed the bacteria at less than micelle-forming concentration. In all, these results suggest that the cell wall may be altered, resulting to easier exposure of the membrane to the drugs. To confirm the altered cell wall integrity in the by disruption of the SA0614-SA0615 genes, I determined the amount of cell wall peptidoglycan in the mutants and observed a decrease in peptidoglycan amount in the mutant strains compared to parent (Fig.4). To ask further whether the peptidoglycan alteration resulted from an increased autolysis activity, I performed a zymography analysis of the autolysins of the cell wall of the mutants to determine any increase in activity. The bacteriolytic enzyme profiles that the activity of autolysins was not increased, suggesting that the decreased amount of peptidoglycan may not be caused by an increased synthesis of autolysins.

Fig.2.Deletion of SA0614 or 0615 resulted to CPZ-sensitivity (A) and decreased the killing ability of *S. aureus* (B) mutnats against silkworm

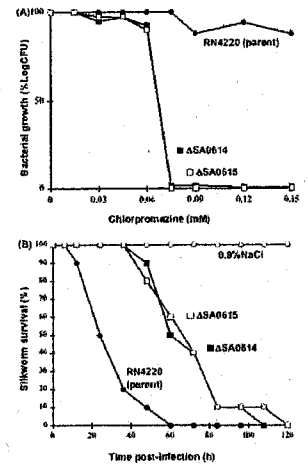


Fig.3.Deletion of SA0614 or 0615 resulted to a sensitivity to (A) Triton-X 100 and (B) Lysozyme

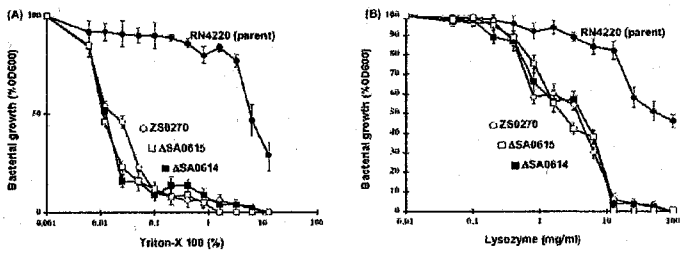
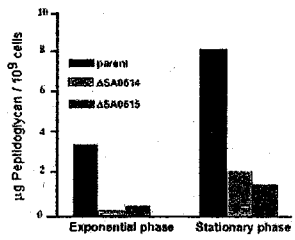


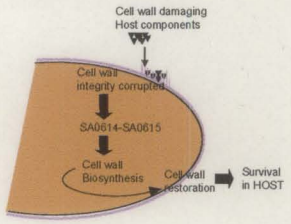
Fig.4.Decreased of cell wall Peptidoglycan amount from *S. aureus* mutants ΔSA0614, ΔSA0615 but not parent



Discussion:

In this study, I showed that the novel two-component system formed by SA0614-SA0615 contributes to the production of sufficient amount of bacterial cell wall peptidoglycan but also for virulence. The sufficient amount of cell wall peptidoglycan might allow *S. aureus* to cover the membrane appropriately and to resist cell wall damaging components. This finding suggests that the thickness of cell wall peptidoglycan of Gram-positive bacteria contributes to their pathogenesis, probably by preventing the cell wall and membrane from host attacks.

Fig.5. Perspective of this work



Publication:

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