
UNIVERSITI PUTRA MALAYSIA

# TOXIN ANTITOXIN SYSTEM AS AN ANTIMICROBIAL TARGET FOR ANTIBIOTIC RESISTANT STAPHYLOCOCCUS AUREUS

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# TOXIN ANTITOXIN SYSTEM AS AN ANTIMICROBIAL TARGET FOR ANTIBIOTIC RESISTANT STAPHYLOCOCCUS AUREUS

**By**

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**TOXIN ANTITOXIN SYSTEM AS AN ANTIMICROBIAL TARGET FOR ANTIBIOTIC RESISTANT STAPHYLOCOCCUS AUREUS**

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**February 2014**

**Chairman: Professor Zamberi bin Sekawi**
**Faculty: Medicine and Health Sciences**

Antibiotic-resistant bacteria have become a global concern and new strategies to control pathogenic bacteria are urgently needed. Toxin antitoxin (TA) system is defined as a regulator system consist of toxin that neutralized by cognate antitoxin. In theory, activation of the toxin or inhibition of the antitoxin within a bacterial TA system could provide a potent new antibiotic therapy. TA systems can increase pathogen stress tolerance and certain TA loci have be characterized in a small number of Methicillin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococcus (VRE), *E. coli,* and *Pesudomanas aeruginosa.* Here it is determined the prevalence of TA system in a large number of independently isolated clinical isolates of antibiotic resistant *S. aureus* from diverse locations, then functionality of dominant TA system is evaluated and the antitoxin is subjected for silencing by anti*MazE* Peptide Nucleotide Acid (PNA) subsequently the suicide of bacteria by toxin is determined. To evaluate potential TA loci as therapeutic targets, it was screened the plasmid and chromosome sequences of 1000 clinical isolates of *S. aureus* from Milad hospital in Iran and 60 MRSA clinical isolates from Hospital Kuala Lumpur (HKL) in Malaysia for the presence of TA loci. Plasmid-borne *MazEF* TA loci were present in all tested, antibiotic resistant *S. aureus* strains in Iran and MRSA in Malaysia while when DNA were subjected as template, 21.2% of Milad hospital and 22.6% of HKL isolates were positive for *MazEF* TA system. In addition, plasmid transformation confirmed *MazEF* TA loci harboured on plasmid. Additionally, RT-PCR analysis revealed that the transcripts were produced from *MazEF* TA loci, suggesting that these loci are functional in the clinical isolates. Toxin transcript expression levels were increase when bacteria were grown under stressful conditions. Furthermore, cellular ATP levels are decreased consistent with *MazF* toxin expression and activity. The ATP

results were confirmed by turbidity analysis. To activate toxin expression, it was targeted the *MazE* antitoxin mRNA using peptide nucleic acid (PNA) oligomers. The anti-*MazE* oligomers were bactericidal against drug-resistant *S. aureus* containing *MazEF* and did not inhibit strains lacking *MazEF*. Therefore, *MazEF* TA loci are widespread in drug-resistant strains of *S. aureus* and are plasmid-borne, and activation of toxin activity by silencing of the antitoxin gene provides a means to selectively kill drug resistant strains.

**Keywords:** Toxin antitoxin system, *MazEF*, *Staphylococcus aureus,* Peptide Nucleotide Acid.

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**SISTEM ANTITOKSIN TOKSIN BAGAI SASARAN ANTIMIKROBIAL UNTUK STAPHYLOCOCCUS AUREUS PERINTANG ANTIBIOTIK**

Oleh

**SOBHAN GHAFOURIAN**

**February 2014**

**Pengerusi:** **Professor Zamberi bin Sekawi**
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Bakteria yang mepunyai ketahanan antibiotik menjadi keperihatinan global masa kini dan strategi baru amat diperlukan dalam mengawal bakteria yang patogenik. Toksin-antitoksin sistem (TA) ditakrifkan sebagai satu system pengawal an yang terdiri daripada toksin yang dineutralkan oleh antitoksin berpadanan. Secara teorinya, pengaktifan toksin or perencatan antitoxin di kalangan toksin-antitoksin bakteria membolehkan penghasilan antibiotik baru yang berkesan. Sistem TA boleh meningkatkan tekanan toleransi pada pathogen dan lokus TA tertentu telah dikategorikan dalam sebilangan kecil Methicillin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococcus (VRE), *Escherichia coli*, and *Pseudomonas aeruginosa*. Kelaziman system TA dalam jumlah yang besar dari sampel klinikal MRSA dari pelbagai lokasi telah ditentukan, kemudian fungsi dominan sistem TA dinilai dan antitoksin adalah tertakluk untuk penyenyapan oleh *antiMazE* Asid Nukleotida Peptida, seterusnya bakteria yang matidisebabk antoksin ditentukan.

Bagi menilai potensi lokus TA sebagai target terapi, plasmid and jujukan kromosom telah diuji terhadap 1000 isolat klinikal, di mana isolate ini adalah *S. aureus* yang telah diambil dari Hospital Milad di Iran. Selain itu juga, sebanyak 60 isolat klinikal terdiri daripada MRSA telah diambil dari Hospital Kuala Lumpur (HKL) di Malaysia bagi mengesan lokus TA.

Plasmid-borne lokus TA MazEF telah dikesan dalam semua ujikaji sama ada dalam ketahanan terhadap antibiotik bagi sub-species *S. aureus* dari Iran mahupun MRSA di Malaysia. Sementara itu, sebanyak 21.2% isolat dari hospital Milad dan 22.6% isolat dari HKL telah dikesan positif untuk system TA *MazEF*. Tambahan lagi, transformasi plasmid mengesahkan lokus *MazEF* telah ditumpukan ke dalam plasmid.

Tambahan pula, analisis daripada RT-PCR menjelaskan transkripsi telah dihasilkan daripada lokus TA *MazEF*, ini menerangkan bahawa lokus ini telah berfungsi dalam isolat klinikal. Paras ekspresi bagi transkrip toksin telah meningkat apabila bakteria membiak di bawah beberapa keadaan yang stres. Di sampling itu, paras ATP sel telah menurun secara konsisten terhadap ekspresi toksin dan aktiviti *MazF*.

**Kata kunci:** sistem Toksin antitoksin, *MazEF, Staphylococcus aureus*, Asid Nukleotida Peptida.

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## DECLARATION

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| | |
|---|---|
| TA system | Toxin antitoxin system |
| *DNA* | Deoxyribonucleic acid |
| MRSA | Methicillin Resistant *Staphylococcus aureus* |
| VRE | Vancomycin Resistant Enterococcus |
| MSSA | Methicillin Sensitive *Staphylococcus aureus* |
| *RNA* | Ribonucleic acid |
| PNA | Peptide Nucleotide Acid |
| PMO | Phosphorodiamidate Morpholino Oligomer |
| LNA | Locked Nucleic Acid |
| VRSA | Vancomycin Resistant *Staphylococcus aureus* |
| PSK | Post Segregation Killing |
| PCD | program cell's death |
| HKL | Hospital Kuala Lumpur |
| MIC | Minimal Inhibitory Concentration |
| PCR | Polymerase Chain Reaction |
| RT-PCR | Reverse Transcriptase PCR |
| RT-qPCR | Real-Time quantitative PCR |
| ATP | Adenosine Tri Phosphate |
| CTT | Catalase Tube Test |
| MSA | Monnitol Salt Agar |

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

The decade of the 1940s witnessed the introduction of penicillin, the first antimicrobial agent that was effective against *Staphylococcus aureus* (Kirby, 1944). Subsequently, resistance to penicillin was observed (Demerec, 1945) and during the two decades following the 1940s, penicillin resistant *S. aureus* became a worldwide concern (Rountree *et al.*, 1954). In 1959, methicillin was used for treatment of infections arising from penicillin resistant *S. aureus*. However, in 1961, the first Methicillin Resistant *Staphylococcus aureus* (MRSA) was reported (Jevons, 1961). Toxin-antitoxin (TA) systems, which were first described in the mid 1980s, are regulatory loci that encode a toxin and its corresponding antitoxin. The toxin and antitoxin may be an RNA or protein, but in all TA systems reported to date, the antitoxin is unstable, and the toxin is stable. TA loci appear to be more common in pathogenic bacteria, but many plasmids contain TA loci. One role of TA loci is to stabilize plasmids within cells, but they also play a role in stress resistance (Aizenman et al., 1996). Plasmid stability is conferred by TA elements; at the time of cell division, all daughter cells will inherit stable toxin molecules or toxin-encoding mRNA, but only those cells that inherit plasmid DNA will be able to replenish sufficient antitoxin to survive. This process is termed 'post-segregational killing' (PSK) (Ogura & Hiraga, 1983; Faridani *et al.*, 2006). By this way, the bacteria that contain the TA loci on plasmid (commonly these plasmids also harbored the antibiotic resistant genes) will survive and the bacteria without plasmid containing TA loci and antibiotic resistant genes will be killed. Antisense therapy, which is sequence dependent, silences a specific gene. The antisense components are analogs of mRNA; therefore, this technology is involved in the inhibition of gene expression. Many techniques are available for antisense therapy that use different RNA analogs, such as phosphorodiamidate morpholino oligomers (PMOs), locked nucleic acids (LNAs) and peptide nucleic acids (PNA). Among these, the properties of PNAs make it particularly appropriate for antisense therapy in bacteria. This technique is applied for molecular bioengineering, therapeutic methods and antibiotics (Lee & Roth, 2003; Janson & Duringm 2006; Rasmussen *et al.*, 2007). The structure of PNAs is similar to that of DNA or RNA, except that the nucleobases are changed to a pseudopeptide (Nielsen *et al.*, 1991) following the Watson and Crick base-pairing rule; however, PNAs can bind DNA and RNA (Jensen *et al.*, 1997).

## 1.2 Statement of problem

Resistance to antibiotics develops through mutations of target sites or the acquisition of antibiotic resistant genes from other pathogens. To date, multiple drug resistance is the biggest challenge in the management of infectious diseases. While researches on development of new antibiotics are rapidly progressing, identification of new drug targets in microbes are also very important for effective infection control. The dissemination of antibiotic-resistance genes among nosocomial pathogens such as streptococci, staphylococci and *Enterobacteriaceae* has led to many cases of treatment failure. MRSA and vancomycin resistant *Staphylococcus aureus* (VRSA) are now global medical challenges. A global increase in MRSA worldwide and an increase in VRSA in Iran, the United States and India (Aligholi*et al.,* 2008; CDC, 2002; CDC, 2004; Perichon & Courvalin, 2009) have been reported. The identification of new bacterial drug targets is an important component in the effort to develop new drugs (Drews, 1996). Toxin antitoxin system could be a potent target for antibiotic therapy. In theory, the activation of a toxin or inhibition of an antitoxin is an attractive strategy for antimicrobial therapy (DeNap & Hergenrother, 2005; Engelberg-Kulka *et al.,* 2004). Amita and colleagues demonstrated that 5% of bacterial cells were viable and 95% were killed after toxin activation because the increased toxin could not be neutralized by the antitoxin. However, when co-expressing *mazE (*antitoxin) and neutralizing *mazF* (toxin), 85% of the cells were viable because the toxin was neutralized and inhibited by the antitoxin (Amitai *et al.,* 2004). Hence, artificial disruption of antitoxin can lead to bacterial cell killing. However, the most important step for potency of TA system, as an antibacterial target, is to identify a TA system that is prevalent in all resistant clinical strains and determine its functionality. While the analysis of TA system can be instructive, until now, there is no information on the prevalence and identity of TA systems in a large panel of *S. aureus* clinical isolates. Therefore, it is necessary to study a TA system that is prevalent and transcribed in all clinical antibiotic resistant *S. aureus* and evaluate this target for antimicrobial therapy.

## 1.3 Statement of objectives

### 1.3.1 General objective

To evaluate the antitoxin *(MazE*) as an attractive antimicrobial target for the eradication of antibiotic resistant *S. aureus.*

### 1. 3. 2 Specific objectives

1. To determine the prevalence of different TA systems, their location on plasmid or chromosome and the dominant TA system in clinical isolates of antibiotic resistant *S. aureus***.**

2. To evaluate functionality of TA system in all clinical isolates of antibiotic resistant *S. aureus.*

3. To evaluate effect of *MazF* toxin against antibiotic resistant *S. aureus.*

### 1.4 Statement of hypothesis

Based upon these data, it hypothesized that the identification of a dominant TA system in antibiotic resistant *S. aureus* will provide a novel antibacterial target against *S. aureus* infections.

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