EFFECTS OF DOMAIN MANIPULATION IN THE STAPHYLOCOCCAL PHAGE 88 ENDOLYSIN ON LYTIC EFFICIENCY AND HOST RANGE

By

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Chairman : Adelene Song Ai-Lian, PhD
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Endolysins are bacteriophage (phage) lytic enzymes responsible for phage-mediated lysis of bacterial peptidoglycan (PGN). Phage therapy for Multidrug-Resistant *Staphylococcus aureus* (MDRSA) infections is currently emerging due to antibiotic resistance. However, using whole phages for therapy has presented several drawbacks including resistance by the bacterial host hence the use of phage endolysins instead. Staphylococcal phage endolysins typically consist of two types of domains: Enzymatically-Active Domains (EAD) and Cell Wall-Binding Domain(s) (CBD). The EADs cleave PGN whereas the CBD localizes endolysins to PGN. The Staphylococcal Phage 88 endolysin (NXEndo88) consists of two EADS: an N-terminal Cysteine, Histidine-dependent Amidohydrolase/Peptidase (CHAP) domain and a central Amidase-2 domain, as well as one CBD: a C-terminal Src Homology 3b (SH3b) domain.

The host strain of the phage is *Staphylococcus aureus* PS88, an MDRSA. Domain manipulations encompassing truncations and swapping among different endolysins have been shown to alter endolysin lytic efficiency and host range. Therefore, the purpose of this study was to determine the effects of domain manipulations on the lytic efficiency and host range of NXEndo88. Five mutants were generated – three truncation mutants: 1) CHAP, 2) CHAPAmidase and 3) CHAPSH3; and two chimera mutants containing the tandem Cpl7 CBDs from a streptococcal endolysin, LambdaSa2-ECC: 4) CHAPAmidase-Cpl7Cpl7 and 5) Endo88-Cpl7Cpl7.

The protein constructs were cloned into the pET28a plasmid vector and expressed in *E. coli* BL21(DE3). Proteins were then purified using Nickel-Nitrilotriacetic Acid (NTA) affinity chromatography for His-tagged proteins. Host range and lytic efficiency were subsequently assessed via plate lysis assays and turbidity reduction assays. All truncated mutants exhibited reduced lytic activity compared to NXEndo88 towards the host strain, *Staphylococcus aureus* PS88. The same pattern was observed in three other staphylococci (*Staphylococcus aureus* Mu50, *Staphylococcus epidermidis* and *Staphylococcus hominis*) as well as *Enterococcus faecalis*.

Outside these two bacterial genera, no lytic activity was detected. The CHAPAmidase and CHAPSH3 constructs exhibited varying specific activities between *S. aureus PS88* and *S. epidermidis*, which may be attributed to strain-specific PGN features. Next, the chimera mutants (CHAPAmidase-Cpl7Cpl7 and Endo88-Cpl7Cpl7) were tested against two strains, *S. aureus PS88* and *Streptococcus agalactiae*, as the addition of the Cpl7Cpl7 domains (specific to streptococci) were predicted to extend their host range to lyse streptococci. However, lytic activity was only detected against *S. aureus PS88*. Both chimeras had reduced lytic activity compared to NXEndo88, which may be attributed to structural instability.

In conclusion, all domains of the NXEndo88 endolysin are interdependent for maximum lytic efficiency, where removal of Amidase-2 and/or SH3b reduced lytic efficiency. As for the chimera constructs, it appears that swapping or adding the Cpl7 CBDs from a streptococcal endolysin was insufficient to expand the host range. Multi-faceted studies on the factors influencing endolysin functionality are necessary for the development and optimization of targeted antimicrobials against MDRSA.

Keywords: Endolysin, domain manipulation, DNA cloning, host range, lytic efficiency

SDG: GOAL 3: Good Health and Well-being

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN PENGUBAHSUAIAN DOMAIN ENDOLISIN FAJ STAFILOKOKUS 88 TERHADAP AKTIVITI LISIS DAN JULAT PERUMAH

Oleh

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Endolisin ialah enzim litik bakteriofaj (faj) yang bertanggungjawab bagi melisis peptidoglikan (PGN) bakteria oleh faj-perantara. Terapi faj untuk rawatan jangkitan *Staphylococcus aureus* Rintang Pelbagai Ubat (MDRSA) kini berkembang akibat rintangan antibiotik. Walau bagaimanapun, menggunakan faj utuh untuk terapi telah menunjukkan beberapa kelemahan termasuk rintangan oleh perumah bakteria. Oleh itu, penggunaan endolisin faj sebagai agen antimikrob lebih berpotensi. Endolisin faj *Staphylococcus* biasanya terdiri daripada dua jenis domain: domain pencernaan (EAD) dan domain pengikat dinding sel (CBD). EAD berfungsi dalam memutuskan ikatan PGN manakala CBD mempertingkat lokalisasi endolisin kepada PGN. Endolisin Faj Stafilokokus 88 (NXEndo88) terdiri daripada dua EAD: domain Sisteina, Amidohidrolase/Peptidase yang bergantung kepada Histidina (CHAP) dan domain Amidase-2 serta satu CBD: domain Src Homologi 3b (SH3b). Strain perumah faj adalah bakteria rintang antibiotik, *S. aureus* PS88.

Pengubahsuaian domain yang merangkumi pemangkasan domain serta penukaran domain antara endolisin berbeza terbukti dapat mengubah aktiviti lisis dan julat perumah endolisin. Hasil penemuan ini dapat menjelaskan fungsi domain serta membolehkan penghasilan agen antimikrob khusus dan efektif. Oleh itu, objektif kajian ini adalah untuk mengenalpasti kesan pengubahsuaian domain terhadap aktiviti lisis dan julat perumah endolisin NXEndo88. Lima mutan telah dihasilkan – tiga mutan pangkasan: 1) CHAP, 2) CHAPAmidase, 3) CHAPSH3 serta dua mutan kimera yang mengandungi CBD Cpl7Cpl7 endolisin streptokokus, LambdaSa2-ECC: 4) CHAPAmidase-Cpl7Cpl7 dan 5) Endo88-Cpl7Cpl7. Konstruk protein telah diklonkan ke dalam vektor plasmid pET28a dan diekspreskan dalam *Escherichia coli* BL21(DE3). Protein seterusnya ditulenkan melalui kromatografi keafinan nikel-asid nitriloasetik (Ni-NTA) bagi protein yang mengandungi His-tag. Seterusnya, julat perumah dan kecekapan litik telah dinilai melalui ujian lisis plat dan ujian penurunan kekeruhan.

Semua mutan yang dipangkas menunjukkan aktiviti litik yang berkurangan berbanding dengan NXEndo88. Corak yang sama diperhatikan dalam tiga stafilokoki lain (*Staphylococcus aureus* Mu50, *Staphylococcus epidermidis*, *Staphylococcus hominis)* serta *Enterococcus faecalis*. Aktiviti lisis terhadap strain selain dua genera tersebut tidak dikesan. Konstruk CHAPAmidase dan CHAPSH3 menunjukkan tahap aktiviti lisis yang berbeza terhadap *S. aureus* PS88 dan *S. epidermidis*. Hal ini mungkin disebabkan oleh ciri-ciri spesifik PGN antara kedua-dua strain tersebut.

Seterusnya mutan kimera (CHAPAmidase-Cpl7Cpl7 dan Endo88-Cpl7Cp7) telah diuji terhadap dua strain, *S. aureus* PS88 dan *Streptococcus agalactiae*, kerana penambahan domain Cpl7Cpl7 dijangka akan meluaskan julat perumahnya untuk melisiskan streptokokus. Walau bagaimanpun, aktiviti lisis hanya dikesan terhadap *S. aureus* PS88. Kedua-dua kimera menunjukkan aktiviti lisis yang lebih kurang berbanding dengan NXEndo88, yang mungkin disebabkan oleh reka bentuk konstruk yang kurang stabil. Kesimpulannya, kesemua domain endolisin NXEndo88 saling bergantung untuk mencapai aktiviti lisis maksimum, di mana pemadaman Amidase-2 dan SH3b mengurangkan kecekapan aktiviti lisis.

Bagi konstruk kimera pula, penambahan domain Cpl7Cpl7 kepada domain NXEndo88 mungkin tidak mencukupi untuk memperluas julat perumah. Kajian selanjutnya diperlukan untuk mengoptimumkan aktiviti kimera. Selain itu, kajian pengenalpastian faktor yang mempengaruhi aktiviti endolisin diperlukan untuk membolehkan pembangunan agen antimikrob yang berfungsi terhadap MDRSA.

Kata Kunci: Endolisin, pengubahsuaian domain, pengklonan DNA, julat perumah, kecekapan litik

SDG: *GOAL 3: Good Health and Well-being*

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LIST OF ABBREVIATIONS

~	Approximately
ºC	degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
AP	Alkaline Phosphatase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
CBD	Cell Wall-Binding Domain
CHAP	Cysteine, Histidine-dependent Amidohydrolase/Peptidase
CoNS	Coagulase-Negative staphylococci
Da	Dalton
D-ala-gly	D-alanine-glycine
ddH_2O	Double-deionized water
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EAD	Enzymatically-Active Domain
EDTA	Ethylenediaminetetraacetic Acid
× g	Gravity force
GFP	Green Fluorescent Protein
GlcNAc	N-acetylglucosamine
GroP	Glycerol phosphate
h	Hour
IMAC	Immobilized Metal Ion Affinity Chromatography
IPTG	Isopropyl-β-D-Galactopyranoside

kb	Kilobase pair
kDa	Kilodalton
L	Litre
LB	Luria-Bertani
MDR	Multidrug-Resistant
MDRSA	Multidrug-Resistant *Staphylococcus aureus*
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MurNAc	N-acetylmuramic acid
MWCO	Molecular Weight Cut Off
NBT	Nitro blue tetrazolium chloride
NCBI	National Centre for Biotechnology Information
NTA	Nitrilotriacetic acid (NTA)
ng	Nanogram
nm	Nanometre
OD	Optical density
PCR	Polymerase Chain Reaction
PES	Polyether-sulfonate
PGN	Peptidoglycan
PVDF	Polyvinylidene difluoride
RboP	Ribitol phosphate
RE	Restriction enzyme
RNase	Ribonuclease
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
PGN	Peptidoglycan
PVDF	Polyvinylidene difluoride
RboP	Ribitol phosphate
RE	Restriction enzyme

RNase	Ribonuclease
s	Second
SH3b	Src Homology 3b
SOE-PCR	Splicing by Overlap Extension-Polymerase Chain Reaction
TAE	Tris-Acetate EDTA
Tm	Melting temperature
V	Volt
VISA	Vancomycin-Intermediate *Staphylococcus aureus*
w/v	Weight per volume
WTA	Wall Teichoic Acid
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background

The ability of lytic bacteriophages (phages) to specifically kill Multidrug-Resistant (MDR) bacteria without harming the natural microflora has long been exploited in antimicrobial treatment as phage therapy (Hatfull et al., 2022). Due to reports of phage resistance and other pitfalls, phage proteins that do not elicit bacterial resistance have garnered much interest as prospective antimicrobials. Chief of these is the endolysin, expressed by phages for internal degradation of bacterial peptidoglycan (PGN) and subsequent lysis of cells, enabling release of viral progeny at the end of the phage life cycle (Abdelrahman et al., 2021; Murray et al., 2021).

Externally, endolysins are able to replicate this same effect of bacterial lysis in Gram-positive bacteria by way of the exposed PGN, unhindered by outer-membranes as opposed to Gram-negative bacteria (Oechslin et al., 2022). One of the Gram-positive pathogens at the fore of the antibiotic crisis requiring new therapies is MDR *Staphylococcus aureus* (MDRSA). Recognized as a serious threat by the Centers for Disease Control and Prevention, resistance to last-resort antibiotics like vancomycin has been reported (Centers for Disease Control and Prevention (U.S.), 2019; Cong et al., 2020). Furthermore, the COVID-19 pandemic has seen a sharp increase in hospital admissions for MDRSA infections (Weiner-Lastinger et al., 2022).

However, most naturally-occurring endolysins against MDRSA may not possess optimal properties availing it for antimicrobial usage. Therefore, endolysins are sometimes engineered to enhance its properties. Towards this goal, domain manipulations of these endolysins are commonly undertaken to shed light on domain function, enabling the development of specialized, recombinant endolysins. Domain engineering is feasible as endolysin domains are modular in nature: retaining function upon domain rearrangement and truncations as well as domain swapping among endolysins of different species (chimera-genesis) (Park et al., 2023; Schmelcher et al., 2012b).

Staphylococcal endolysins typically possess two types of domains with distinct functions: Enzymatically-Active Domains (EAD) and a Cell Wall-Binding Domain (CBD). EADs are responsible for bond cleavage in bacterial PGN, whereas the CBD is thought to localize the EADs to PGN, increasing access to the cut-sites (Nelson et al., 2012). The endolysin in this study, the Staphylococcal Phage 88 (NXEndo88) endolysin has a tri-domain architecture composed of two EADs: A Cysteine, Histidine-dependent Amidohyrolase/Peptidase (CHAP) domain followed by a central Amidase-2 domain. The third domain is a CBD, the Src Homology 3b (SH3b) domain. Most staphylococcal endolysins in the literature are similarly structured, where each domain is known to play a carefully curated role in PGN lysis, as has been demonstrated in previous studies (Benešík et al., 2018; Horgan et al., 2009; Son et al., 2018). The CHAP domain is the most enzymatically potent domain in terms of PGN cleavage, without which the endolysin is almost always defunct (Becker et al., 2015; Son et al., 2018).

The role of Amidase-2 is more elusive with reports of both enzymatic cleavage and binding. However, its importance in functionality varies according to endolysin species. The SH3b domain is purported to be involved in localization, as well as curtailing endolysin host range to only PGN substrates possessing the cognate binding sites (Benešík et al., 2018; Mitkowski et al., 2019).

In theory, CBD removal could potentially increase endolysin host range to encompass other bacterial species, however this result is not uniform across the gamut of staphylococcal endolysins. In the context of domain swapping, CBD exchange between endolysins targeting different bacterial genera produces recombinant endolysins known as 'chimeras'. These chimeras mostly retain the host ranges of the parental endolysins while exhibiting increased lytic efficiency. One endolysin-based product (Gladskin©) is currently in the market to treat chronic MDRSA eczema, demonstrating its therapeutic potential (Haddad Kashani et al., 2018; Totté et al., 2017).

1.2 Problem statement

Multidrug-Resistant (MDR) *Staphylococcus aureus* (MDRSA) infections represent one of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens recalcitrant to antibiotics, which account for most nosocomial infections. As a mounting health concern, the discovery of safe products that successfully eliminate pathogenic bacteria without eliciting resistance is imperative.

Novel antimicrobials including bacteriophage endolysins have demonstrated tremendous therapeutic potential without inducing bacterial resistance. However, naturally-occurring endolysins may not function optimally in terms of lytic efficiency and host range. Domain engineering has been previously undertaken to improve these properties and subsequently determine the factors contributing to host specificity. The domains in a modular staphylococcal endolysin are multi-functional but the contribution of each domain to host range and lytic activity is still unclear due to poor consensus. Therefore, this study was conducted to determine the effect of domain manipulations on the existing host range and lytic efficiency of the Staphylococcal Phage 88 endolysin (NXEndo88).

1.3 Objectives

The purpose of this study was to ascertain the effect of domain truncations of the Staphylococcal Phage 88 (NXEndo88) endolysin on lytic efficiency and host range. It was also investigated if domain swapping between NXEndo88 and the CBDs of a streptococcal endolysin, LambdaSa2-ECC, could expand the host range to include streptococci. The specific objectives of this study are:

i. To construct domain manipulations in the Staphylococcal Phage 88 endolysin.
ii. To clone and express recombinant endolysin mutants in BL21(DE3) *Escherichia coli* cells.
iii. To determine the effect of domain manipulation on the lytic efficiency and host range of each mutant.

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