Studies on the role of the putative phosphorylation consensus sequence YXNX in the tandem repeat domains of the *Staphylococcus aureus* Mu50 Extracellular Adherence Protein (Eap)

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1 List of Abbreviations

ABBREVIATIONS	
σ^B	RNA polymerase subunit Sigma B
2L4 WT	three domain Mu50 Eap fragment, domains 2 to 4
2L4D3	2L4, having a mutation in domain 3
2L4D4	2L4, having a mutation in domain 4
3L4 WT	two domain Mu50 Eap fragment, domains 3 and 4
3L4D3	3L4, having a mutation in domain 3
3L4D4	3L4, having a mutation in domain 4
aa	amino acid(s)
agr	accessory gene regulator
AhpC	alkylhydroxide reductase
Arp 2/3	actin-related proteins 2/3
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CatA	Catalase A
CFT-1 cells	bronchial epithelial cells of a patient with cystic fibrosis
cfu	colony forming unit(s)
CHIPS	chemotaxis inhibitory protein of *Staphylococcus aureus*
ClfA	clumping factor A
ClfB	clumping factor B
Cna	collagen adhesion protein
Coa	coagulase
CR3	complement receptor 3
CV	column volume
D3M	domain 3 of the Mu50 Eap harboring a mutation
D4M	domain 4 of the Mu50 Eap harboring a mutation
dsDNA	double strand deoxyribonucleic acid
DTT	dithiothreitol
DMSO	dimethylsulfoxide
Eap	extracellular adherence protein

ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EdU	ethynyl-2- deoxyuridine
Efb	extracellular fibrinogen-binding protein
EGF	epidermal growth factor
EMBL	European Molecular Biology Laboratory
Emp	extracellular matrix binding protein
Eno	enolase
EPEC	enteropathogenic *Escherichia coli*
Erk	extracellular signal-regulated kinases
EU	endotoxin units
FAME	fatty acid-modifying enzyme
FBS	fetal bovine serum
Fg	fibrinogen
FL	full length
FLD3	full length Mu50 Eap harboring a mutation in domain 3
FLD4	full length Mu50 Eap harboring a mutation in domain 4
FLIPr	FPR-like 1 inhibitory protein
Fn	fibronectin
FnBPA	fibronectin-binding protein A
FnBPB	fibronectin-binding protein B
FPLC	fast performance liquid chromatography
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GRB2	growth factor receptor-bound protein 2
GTP	guanosine-5'-triphosphate
H_2O_2	hydrogen peroxide
HaCat	keratinocyte cell line
HEK 293	Human Embryonic Kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	intercellular adhesion molecule-1

IgG	immunoglobulin G
IgM	immunoglobulin M
IL-4	interleukin-4
IPTG	isopropyl-β-D-thiogalactopyranoside
IU	international unit
K_D	dissociation constant
KGF	keratinocyte growth factor
LAL	limulus amebocyte lysate
LB	Luria Bertani medium
LPS	lipopolysaccharides
LPXTG	Consensus motif found in bacterial proteins that are covalently attached to the bacterial cell-wall, sortase A recognition sequence
MAP	MHC analogues protein
MAPK	mitogen-activated protein kinases
mAU	milli-absorbance unit
MEK	MAPK/Erk kinase
MHA	Müller Hinton agar
MHC	major histocompatibility complex
MOI	multiplicity of infection
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin sensitive *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
Ni^{2+}-NTA	nickel nitrilotriacetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
Pkn	protein-kinase
PMSF	phenylmethylsulphonylfluoride

psi	pound-force per square inch
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acids
rpm	revolutions per minute
RTKs	receptor tyrosine kinases
sae	*S. aureus* exoprotein expression
Sak	staphylokinase
sar	staphylococcal accessory regulator
Sas proteins	*S. aureus* surface proteins
SCIN	staphylococcal complement inhibitor
SCVs	small colony variants
Sdr protein	serin-aspartic acid rich proteins
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
SERAM	secretable expanded repertoire adhesion molecule
SH2	Src Homology 2
SOS	(son of sevenless) guanine nucleotide exchange factor
Spa	*Staphylococcus aureus* protein A
src	rous sarcoma oncogene cellular homologue
TAE	tris-acetate-EDTA
TB	Terrific broth
TBS T	tris buffer saline containing 0.1% Tween-20
TCR	T-cell receptor
TF	tissue factor
Th1/2	T helper cell 1/2
TNF	tissue necrosis factor
TSST	toxic shock syndrome
UV	ultraviolet visible
VEGF	vascular endothelial growth factor
Vn	vitronectin
WT	wild type

Standard abbreviations for amino acids

A	Ala	Alanine
L	Leu	Leucine
R	Arg	Arginine
K	Lys	Lysine
N	Asn	Asparagine
M	Met	Methionine
D	Asp	Aspartic acid
F	Phe	Phenylalanine
C	Cys	Cysteine
P	Pro	Proline
Q	Gln	Glutamine
S	Ser	Serine
E	Glu	Glutamic acid
T	Thr	Threonine
G	Gly	Glycine
V	Val	Valine
H	His	Histidine
W	Trp	Tryptophan
I	Ile	Isoleucine
Y	Tyr	Tyrosine

Units

Current strength	Ampere	A
Concentration	Molar	M
	Milimolar	mM
	Micromolar	µM
Length	Centimeter	cm
Mass	Gram	G
	Microgram	µg
Molecular weight	Dalton	Da

	Kilodalton	kDa
Tension	Volt	V
Temperature	degree Celsius	°C
Volume	Litre	L
	Millilitre	ml
	Microlitre	µl
Wave length	Nanometer	nm
Time	Second(s)	s
	Minute(s)	min
	Hour(s)	hr

2 Summary

Staphylococcus aureus is a major human pathogen and a leading cause for both community and hospital acquired infections. The bacterium produces several virulence factors including the extracellular adherence protein (Eap), a member of the secreted expanded-repertoire adhesive molecules (SERAMs). With the majority of the secreted protein being bound back to the bacterial cell surface, Eap binds to a number of host cell matrix molecules, and posses some immunmodulatory and antiangiogenic properties. Depending on the genetic background of *S. aureus*, Eap consists of 3 to 6 repeats of the so-called Eap domain, a 110 amino acids spanning polypeptide sharing some homology to bacterial super-antigens. Although Eap is widely studied, only little is known about its structural and functional characteristics. The computational analysis of the Eap amino acid sequences performed in our laboratory identified a conserved YXNX motif in some of the Eap repeats, which might serve as putative tyrosine phosphorylation sites. To test whether and how the mutation of the tyrosine residue of this motif might affect the activities of this protein, we focused on the Eap of strain Mu50, which consists of four tandem repeats having YXNX motif in domains 1, 3 and 4. Eight different mutants were created by converting tyrosine to phenylalanine in the YXNX motifs of domains 3 and 4. This resulted in the construction of full-length Eap derivatives being mutated in domain 3 (FLD3) and domain 4 (FLD4), two Eap domain fragments consisting of domains 2 to 4 and having a mutation in domain 3 (2L4D3) and 4 (2L4D4), respectively, two Eap domain fragments consists of domains 3 and 4, having mutations in domain 3 (3L4D3), and 4 (3L4D4), respectively, and the single domain 3 (D3M) and 4 (D4M) mutants. All Eap derivatives were expressed in *Escherichia coli* strain BL21(DE3), and the proteins were purified by affinity- and size-exclusion chromatography. As adherence and internalization of bacteria into host cells are considered to be important steps during infection, we examined the role of the Eap derivatives on these two processes by the attachment and internalization of *S. aureus* strain SA113 into HaCaT cells that were preincubated with the Eap derivatives. We observed that the preincubation of HaCaT cells with the mutated full-length and 2 domain Eap fragments (FLD3, FLD4, 3L4D3, and 3L4D4) clearly reduced the adherence and internalization of *S. aureus* into HaCaT cells if compared to the wild-type proteins (FL and 3L4), whereas all single domain Eap (both wild-type and mutant derivatives) showed no effect. In contrast, none of the mutations in the YXNX motifs of Eap domains 3 or 4 of the full-length Eap and the two domain Eap fragments (3L4D3 and 3L4D4) significantly altered the Eap effect on

either the keratinocyte growth factor stimulated ERK phosphorylation, or the proliferation of HaCaT cells, suggesting that the putative YXNX motifs of domains 3 and 4 exert an effect on some but not all activities of this protein. The molecular mechanism(s) by which the YXNX motifs affect the Eap activities still need to be identified.

3 Zusammenfassung

Staphylococcus aureus ist ein weitverbreitetes humanpathogenes Bakterium und ein wichtiger Verursacher von ambulant erworbenen und nosokomialen Infektionen. Der Erreger bildet eine Vielzahl von Virulenzfaktoren, zu denen auch das extrazelluläre Adhäsionsprotein Eap gehört. Dieses zu der Familie der SERAM (**s**ecreted **e**xpanded-**r**epertoire **a**dhesive **m**olecules) gehörende Protein spielt eine wichtige Rolle bei der Infektion und Kolonialisierung der eukaryotischen Wirtszelle und verfügt neben seinen adhäsiven auch über immunmodulatorische Eigenschaften. Eap wurde bislang nur in *S. aureus* gefunden und ist in Abhängigkeit vom genetischen Hintergrund des jeweiligen Stammes aus 3 bis 6 Wiederholungen der sogenannten Eap-Domäne aufgebaut, eines 110 Aminosäuren umfassenden Polypeptids mit Ähnlichkeiten zu bakteriellen Superantigenen. Trotz zahlreicher Studien ist bislang wenig über den Zusammenhang zwischen Struktur und Funktion dieses Proteins bekannt. Bioinformatische Untersuchungen der verfügbaren Eap-Sequenzen in unserem Labor ergaben, dass einige Eap-Domänen ein konserviertes YXNX Motiv enthalten, bei dem es sich um eine mögliche Tyrosin-Phosphorylierungsstelle handeln könnte. Die vorliegende Arbeit beschäftigt sich mit der Frage, ob Mutationen in diesem Motiv die Aktivität von Eap beeinflussen. Alle Untersuchungen wurden mit dem Eap des Stammes Mu50 durchgeführt, das aus vier Eap-Domänen besteht, von denen außer der zweiten alle Domänen ein YXNX Motiv enthalten. Um die Frage nach der Bedeutung der Tyrosine (Y), der mutmaßlichen Phosphorylierungsstellen in den YXNX Motiven des Mu50 Eap zu beantworten, wurden acht Mutanten generiert, bei denen die Tyrosine in den YXNX Motiven der Domänen 3 und 4 durch Phenylalanine ersetzt wurden. Bei den Mutanten handelte es sich um zwei Volllängen- Eap Varianten, die je eine Mutation in Domäne 3 (FLD3) bzw. Domäne 4 (FLD4) tragen, sowie sechs verkürzte Eap Mutanten. Von den verkürzten Varianten bestehen zwei aus den Domänen 2 bis 4 und tragen je eine Mutation in Domäne 3 (2L4D3) bzw. Domäne 4 (2L4D4); zwei weitere umfassen nur die Domänen 3 und 4 und tragen ebenfalls je eine Mutation in Domäne 3 (3L4D3) bzw. Domäne 4 (3L4D4). Die letzten beiden Mutanten

bestehen nur aus einer einzigen, die Mutation tragenden Domäne (D3M und D4M). Alle Eap Varianten wurden heterolog in dem *Escherichia coli* Stamm BL21(DE3) exprimiert und über Affinitäts- und Größenausschluss-Chromatographie aufgereinigt.

Da die Adhäsion eines Bakteriums an die Wirtszelle und seine anschließende Internalisierung wichtige Schritte bei der Entstehung einer Infektion darstellen, sollten die Eap-Mutanten auf ihre Funktionalität hinsichtlich dieser beiden Prozesse untersucht werden. Dazu wurde die Anhaftung bzw. Internalisierung des *S. aureus* Stamms SA113 an bzw. in HaCaT Zellen verfolgt, die mit den gereinigten Eap Mutanten präinkubiert wurden. Dabei zeigte sich, dass eine Präinkubation der HaCaT Zellen mit den beiden Volllängen-Eap Mutanten FLD3 und FLD4 sowie den verkürzten Eap Mutanten 3L4D3 und 3L4D4 jeweils zu einer deutlich reduzierten Adhäsion und Internalisierung der Bakterien im Vergleich zum jeweiligen Wildtyp (FL und 3L4) führte. Bei einer Präinkubation der Zellen mit den Eap Einzeldomänen D3 bzw D4 hingegen konnten weder bei den jeweiligen Wildtyp-Varianten noch bei den Mutanten (D3M bzw. D4M) Auswirkung auf Adhäsion und Internalisierung beobachtet werden. Darüber hinaus konnte gezeigt werden, dass sich Mutationen des YXNX Motivs der Eap Domänen 3 oder 4 weder auf die Aktiviät des Eap hinsichtlich des durch den Keratinozyten-Wachstumsfaktor stimulierten ERK Signalweges noch hinsichtlich der Proliferation der Wirtszellen auswirkten. Diese Befunde lassen darauf schliessen, dass Eap an den Tyrosinen der YXNX Motive phosphoryliert werden könnte und dies die Aktivität des Proteins beeinflusst, weisen zugleich aber auch darauf hin, dass diese mutmaßliche Phosphorylierung nicht für alle Aktivitäten des Eap gleichermaßen benötigt wird. Weiterführende Studien sind erforderlich, um den molekulare Mechanismus aufzuklären, durch den die YXNX Motive die Aktivität des Eap beeinflussen.

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6 Introduction

6.1 Staphylococcus aureus

Staphylococcus aureus is a facultative anaerobic, gram-positive coccus. First described in 1880, the name *Staphylococcus* comes from the Greek word *staphyle*, meaning a bunch of grapes and reflecting its microscopic morphotype (Figure 1). On blood agar plates, it forms large, round, golden-yellow colonies, that are often surrounded by hemolytic areas. The carotenoid pigment staphyloxanthin is responsible for the characteristic golden colour in *S. aureus*, and is the etymological root of the bacteria's name; *aureus*, which means "golden" in Latin.

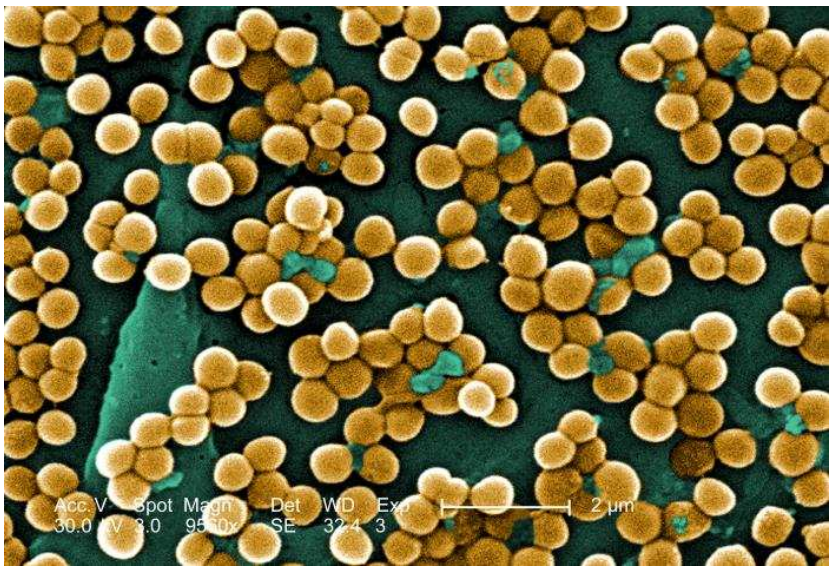


Figure 1: *Staphylococcus aureus* under scanning electron microscope. (Adapted from www.healthhype.com/staphylococcus-aureus.html).

S. aureus is a catalase-positive as it produces the enzyme "catalase", which converts hydrogen peroxide (H_2O_2) to water and oxygen. This particular enzymatic reaction is used to distinguish staphylococci from enterococci and streptococci. Within the staphylococci, *S. aureus* is usually differentiated from other staphylococcal species by the coagulase test. *S. aureus* produces the enzyme "coagulase" that causes clot formation, whereas most other *Staphylococcus* species, like *S. epidermdis, S. saprohyticus, S. lugdunensis, S. schleiferi, and S. caprae*, are coagulase-negative.

S. aureus is a frequent commensal of humans. It is estimated that two thirds of the healthy population are transiently or permanently colonized by this organism, although pronounced variations depending on the geographic, ethnic, social and medical factors may exist. The bacterium is usually found in the nose and on the skin, but can be recovered from other moist regions of the body (e.g. the inguinal and perineal area, axilla, forehead, vagina and colon) as well. In the majority of cases, the bacterium does not cause disease, however, damages of the skin or other injuries may allow the bacterium to overcome natural protective mechanisms of the body, and may lead to infections. Although more than 30 staphylococcal species may lead to infections in humans, most infections are caused by *S. aureus* (Deurenberg et al., 2008).

6.2 Pathogenesis and virulence factors

S. aureus is a versatile and harmful pathogen of both hospital- and community acquired infections. It causes a variety of suppurative (pus-forming) infections and toxinosis in human. Pathogenicity of *S. aureus* is caused by the coordinate expression of a large arsenal of virulence factors, which can lead to superficial skin lesions such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles and abscesses up to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis (Fred, 2000). *S. aureus* may also cause food poisoning by releasing heat-stable enterotoxins into food products (Ortega et al., 2010).

S. aureus produces a wide range of virulence factors, which are either surface associated or secreted. An important step in many types of infections is the bacterial adhesion to host tissues, which helps the bacteria to colonize and invade into host cells. The virulence factors of *S. aureus* have been comprehensively reviewed by T. Chavakis and colleagues (Chavakis et al., 2007), who categorized them into seven groups as illustrated in Figure 2.

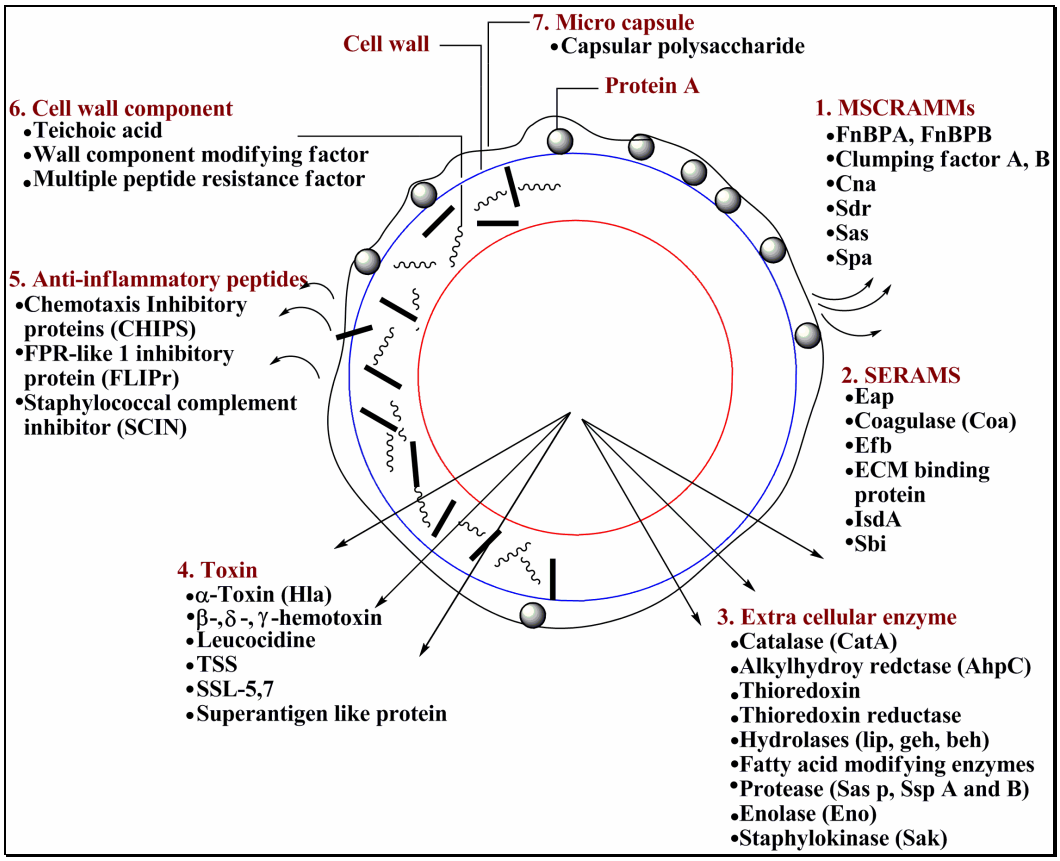


Figure 2: Virulence determinants of *Staphylococcus aureus*. *S. aureus* expresses many potential virulence factors (Chavakis et al., 2007). (1) **Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)** including Fibronectin-binding protein A and B (FnBPs), clumping factors A and B, collagen-binding adhesin, serin-aspartic acidrich proteins (Sdr protein), *S. aureus* surface proteins (Sas proteins) and *S. aureus* protein A (Spa). (2) **Secreted expanded repertoire adhesive molecules (SERAMs)** including Eap (extracellular adherence protein), coagulase (Coa), extracellular matrix binding protein (Emp), extracellular fibrinogen-binding proteins (Efb) (3) **Extracellular enzymes** including catalases, alkylhydroxide reductase, thioredoxins and thioredoxin reductases, glycerol ester hydrolases, fatty acid-modifying enzymes, various proteases (e.g. V8 protease, aureolysin, staphopain), O-acetyltransferase, phosphatidylinositol -phospholipase C, staphylokinase and enolase. (4) **Toxins** including α-toxin, β-hemolysin (also known as sphingomyelinase C), δ-hemolysin, bicomponent toxins: γ-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M, toxins with superantigen activity (staphylococcal enterotoxins, TSS), staphylococcal superantigen-like protein-5, staphylococcal superantigen-like protein-7, formylpeptides. (5) **Anti inflammatory peptides** including chemotaxis inhibitory proteins (CHIPS), FPR- like I inhibitory protein (FLIPr), staphylococcal complement inhibitor (SCIN). (6) **Cell wall components** including teichoic acid, wall component modifying factors, multiple peptide resistant factors. (7) **Micro capsule** including capsular polysaccharide.

6.2.1 Secreted expanded repertoire adhesive molecules (SERAMs).

S. aureus produces a number of secreted bacterial proteins with extracellular matrix binding properties, the so-called SERAM. Although these secreted bacterial proteins are structurally unrelated, they share common functional features. SERAMs have the ability to bind to host factors and/or mediate bacterial adhesion to host molecules, cells, or tissues, and they do usually interfere with host defense mechanisms (Chavakis et al., 2005). The SERAM family also include the extracellular adherence protein (Eap), coagulase (Coa), the extracellular matrix binding protein (Emp) and the extracellular fibrinogen binding protein (Efb).

6.2.1.1 *S. aureus* coagulase (Coa)

The coagulase is a secretable 60-70 kDa protein containing a variable amino acid sequence comprising a prothrombin binding site, and a conserved region with multiple domain repeats. Prothrombin binding results in the formation of an active 'staphthrombin' complex, which converts fibrinogen to fibrin, and since decades, this reaction provides a hallmark for the microbiological diagnosis of *S. aureus* infections. Additionally, coagulase has been identified as a platelet-binding protein of *S. aureus*. In terms of pathogenesis, coagulase is thought to cause the production of a fibrin layer which contributes to the formation of a focal staphylococcal abscess, thus localizing the infection and protecting the organisms from phagocytosis.

6.2.1.2 Extracellular matrix binding protein (Emp)

The extracellular matrix binding protein (~40 kDa) has been shown to interact with fibronectin, fibrinogen, collagen, and vitronectin (Lee, 2001). Bacterial mutants deficient in Emp production were shown to attach with immobilized fibrinogen and fibronectin to a lower extent than the wild-type, and this difference could be complemented with the intact protein (Chavakis et al., 2005).

6.2.1.3 Extracellular fibrinogen binding proteins (Efb)

This small protein (15.6 kDa) was shown to bind to the α-chain of fibrinogen under *in vitro* conditions, however, it seems not to contribute to bacterial adherence to fibrinogen or fibrin *in vivo* . Instead, it binds and inhibits complement factor C3b and C3d, blocks opsono-phagocytosis, binds to platelets, and blocks the fibrinogen-induced platelet aggregation (Shannon et al., 2006). Efb is constitutively produced by all *S. aureus* strains investigated so far. Furthermore, in rat model Efb was shown to play a role in the pathogenesis of wound infections (Palma et al., 1996, 1999).

6.2.1.4 Extracellular adherence protein (Eap)

The extracellular adherence protein (Eap) will be discussed in more detail in 6.4.

6.3 Host pathogen interactions

Since *S. aureus* is a primarily colonizing commensal of human, this bacterium must gain an entrance into its host to exert its pathogenic activity. Then it starts to establish an infection usually only under immune suppressive conditions and begins to express toxins and tissue degrading enzymes, which allow the pathogen to passage through tissue barriers. However, the high rates of relapse occurring after *S. aureus* infection, suggested that the bacterium may possess the ability to internalize and persist inside host cells. Likewise, *S. aureus* has been shown to internalize into a variety of non-professional phagocytic cell types, such as osteoblasts, fibroblasts, epithelail cells, and endothelial cells (Hagger et al., 2003). The binding of the bacterium to host cells and extracellular matrix (ECM) components is governed by cell wall-anchored proteins, such as the Fn-binding proteins FnBPA and FnBPB, the clumping factors (ClfA, ClfB), and cell wall-associated factors such as Eap. Internalization of *S. aureus* into the eukaryotic cells, however, is thought to rely mainly on the fibronectin-mediated bridging between Fn-binding proteins FnBPA or FnBPB on the surface of staphylococci, and α5β1 integrin on host cell surface (Dziewanowska et al., 1999; Sinha et al., 1999). The invasion is thought to occur through a ′zipper type′ mechanism in which the bacteria bind directly or indirectly to the host cell receptors, which trigger the internalization of the receptors together with the bacteria. The bacteria are completely internalized in a time-, dose-, and temperature-dependent manner driven

by F-actin rearrangement in the host cell (Sinha et al., 1999), involving intracellular signaling such as tyrosine phosphorylation, MAPK activation (ERK-1 and ERK-2 phosphorylation), and Src kinase activation (Sinha et al., 2005).

After invasion, endosome-localized *S. aureus* may escape into the cytoplasm (Bayles et al., 1998; Menzies and Kourteva, 1998; Sinha et al., 2005) or replicate inside the endosomes (Kahl et al., 2000). The ability of *S. aureus* to persist within a host cell depends heavily on the control of exoprotein production by the bacterium. For example, *S. aureus* strain Cowan I, which weakly expresses exoproteins, survives within HEK 293 cells for days without damaging the host cells (Sinha et al., 1999), whereas *S. aureus* strain Newman, a high exotoxin producer, is highly cytotoxic and rapidly (within hours) destroys its host-cells. Consequently, the intracellular passage through endothelial cells seems to increase the formation of the so called "small colony variants" (SCVs), which are characterized by a reduced metabolism and virulence determinant production. These regulatory and metabolic adaptations may results into intracellular persistence of the bacteria and recurrent of disease (Vann et al., 1987).

6.4 Eap (extracellular adherence protein)

Eap is one of the most extensively studied SERAM of *S. aureus*, which was also designated as Map (MHC class II analogous protein) or P70. Youssif et al. (1991), described a highly cationic 70 kDa (p70) protein from the cell surface of *S. aureus* strain Wood 46 that possessed a high affinity for the glomerular basement membrane of rats. Boden and Flock (1992) and McGavin et al., (1993) also reported about a 60-70 kDa staphylococcal surface protein in strains FDA574 and Newman, respectively which showed broad extracellular matrix binding properties by binding to fibrinogen, fibronectin and bone sialoprotein. Palma et al., (1999) finally characterized and named this protein as Eap, and confirmed its broad spectrum of binding activities to host cell components. Although the size of Eap varies considerably between strain to strain, the protein was found in more than 97% of all the clinical isolates of *S. aureus* (Hussian et al., 2001). Lacking the LPXTG motif found in MSCRAMMs, the secreted protein is linked to the bacterial cell surface by binding to the cell wall-associated neutral phosphatase (Nptase) (Palma *et al.*, 1999; Flock and Flock, 2001; Hussain *et al.*, 2002). This property enables EAP to promote

adherence of *S. aureus* to host components as well as cells, including fibroblasts and epithelial cells

Depending on the growth conditions, the expression of Eap *in vitro* appears to be maximal during the late exponential phase (Harraghy et al., 2005) or stationary phase (Joost et al., 2009), and the expression is tightly regulated by the *sae* two-component system (Harraghy et al., 2005). In addition, large differences have been observed for the production of Eap, with strain Newman producing considerably more Eap than all other strains tested to date (Hussain et al., 2001), due to a mutation in *saeS*, encoding the sensor-kinase of this two-component system (Adhikari et al., 2008).

6.4.1 Structural organization of Eap

Eap was first characterized from strain FDA574, and was shown to consists of 689 amino acids including a signal peptide sequence (Jonsson et al., 1995), and a 110-amino acid domain that is repeated six times (Figure 3). Each domain includes a 31 amino acid sub-domain with great similarity to the N-terminus of β-chains of many major histocompatibility complex (MHC) class II molecules, which led to the name Map for MHC analogous protein. Sequencing of the *eap* gene of strain Newman identified an open reading frame which was in its N-terminal region homologous to the Map sequence characterized by Palma et al. (1999), but lacked the C-terminal domain found in the Map sequence of FDA574. Hussain et al., (2001) later on suggested that Eap and Map represent strain to strain variations of the same protein.

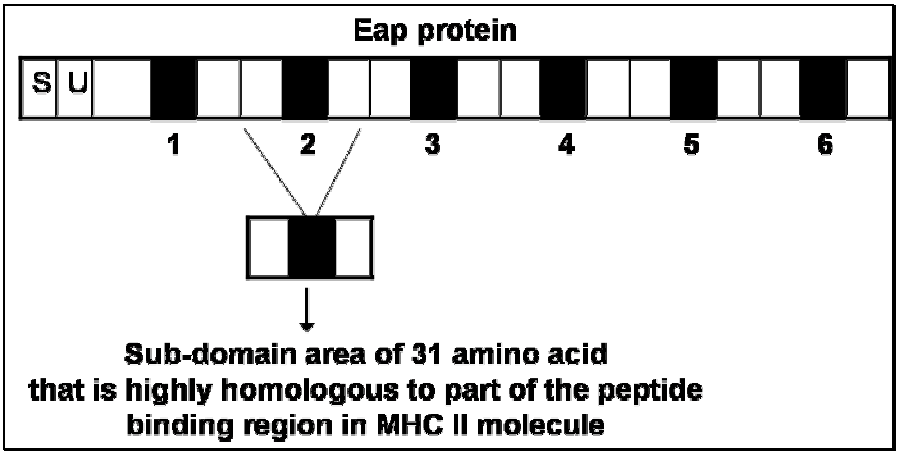


Figure 3: Structure of 72 kDa Map protein of *S. aureus* strain FDA 574. The protein consists of a signal sequence (S) of 30 amino acids followed by a unique sequence (U) of 19 amino acids of yet unknown function, and six repeated domains (1-6) of approximately 110 amino acids. Within each domain, there is a subdomain of 31 amino acids that is highly homologous to part of the peptide binding region in MHC class II molecule (modified after Jonsson et al., 1995).

The crystal structure of the superantigen SEC bound to TCR β-chain was studied and suggests a presence of a potential ligand binding site within Eap (Fields et al., 1996). Moreover, the crystal structures of EAP domains were also solved and revealed a core fold that is comprised of an alpha-helix lying diagonally across a five stranded, mixed beta-sheet (Geisbrechts et al., 2005) which is depicted in Figure 4.

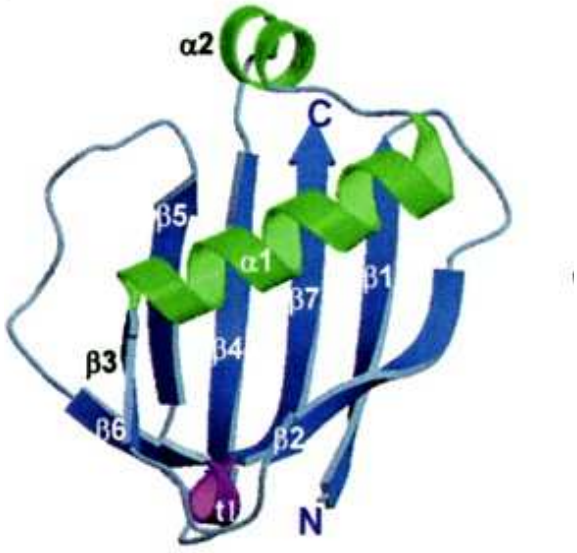


Figure 4: The three-dimensional crystal structure of Eap2 (Mu50). The EAP domain structures consist primarily of a fourturn α-helix lying diagonally across one face of a fivestranded, mixed β-sheet and resemble an open hand holding a roll of coins (Geisbrecht et al, 2005).

6.4.2 Properties of Eap

6.4.2.1 Binding to plasma proteins

Eap has a broad binding spectrum, and can bind to plasma proteins, eukaryotic cell receptors, and to itself. It is known to interact with at least seven plasma proteins such as vitronectin, fibronectin, fibrinogen, collagen, thrombospondin, prothrombin or osteopontin (McGavin et al., 1993; Hussian et al., 2002, Hansen et al., 2006, Palma et al., 1999).

6.4.2.2 Rebinding to the bacterial surface

Since Eap is a secreted protein, it can rebind to the bacterial surface (Palma et al., 1999), however, the respective binding sites on the bacterial surface are not unequivocally identified. Externally added Eap was shown to rebind to *S. aureus* by the endogenous surface located Eap to create Eap-Eap interactions. However, this rebinding process was found to be independent from the endogenous Eap production (Hussain et al., 2002), as Eap-negative mutants were found to bind exogenous Eap like the wild-type strain, suggesting that there might be several Eap-binding structures on the cell surface that are capable of binding Eap. One of these targets is neutral phosphatase (Flock and Flock, 2001). Similarly, Kreikemeyer and colleagues identified two *S. aureus* cell surface proteins of 82 kDa and 50 kDa, respectively, which were capable of binding to Eap (Kreikemeyer et al., 2002). Moreover, Vuong et al., (2002) showed that binding of Eap to the *S. aureus* cell wall was dependent on the D-alanylation of teichoic acids, as Eap was not bound to the surface of a *S. aureus dlt* (defective in the D-alanylation of teichoic acids) mutant, presumably due to altered electrostatic interactions.

6.4.2.3 Agglutination of bacteria

The capacity of Eap to multimerize also supports the bacterial aggregation, which is believed to contribute to the pathogenesis of this organism (Palma et al., 1999; Hagger et al., 2003). It was observed that externally added Eap enhanced the agglutination of *S. aureus* (Hussian et al., 2002), and that the self-binding property of Eap contributed to this process (Hussian et al., 2008).

6.4.2.4 Role of Eap in adherence and internalization

Eap contributes to the adhesion of *S. aureus* to eukaryotic cells. Exogenously added Eap significantly enhanced the adherence of *S. aureus* to fibroblasts and epithelial cells, probably due to its dual affinity for plasma proteins on the cell surface and for the bacterium itself (Hussian et al., 2002). In addition, Eap also contributes to the internalization of *S. aureus* into eukaryotic cells (Hagger et al., 2003).

6.4.2.5 Interaction with ICAM-1

The endothelial cell adhesion molecule ICAM-1, which is also present on fibroblasts and epithelial cells, appears to be a major host "receptor" for Eap on eukaryotic cells. Eap was shown to inhibit the ICAM-1–integrin interactions and to block the ICAM-1-dependent leukocyte adhesion to the endothelium, resulting in the disruption of integrin-dependent leukocyte recruitment during *in vitro* and *in vivo* conditions, suggesting that Eap might serve as a potent anti-inflammatory factor (Athanasopoulos et al., 2006). In addition, Eap seems to affect the transendothalial migration, and the ICAM-1-mediated upregulation of NFκB activity as well as the expression of tissue factor (Athanasopoulos et al., 2006)

Eap is also thought to have an important role in the pathogenesis of *S. aureus* by impairing wound healing, which is frequently seen in chronically *S. aureus*-infected wounds or ulcera (Madsen et al., 1996; Grimble et al., 2001). Eap was found to interfere with the recruitment of leukocytes into the infected tissue, and Eap-treated wounds were reported to show an impaired neovascularization. The protein was also shown to affect the formation of new blood vessels and capillary tube formation *in vitro*, indicating that Eap might inhibit cell proliferation (Sobke et al., 2006).

6.4.2.6 Role of Eap in immune response

Beside its anti-inflammatory and antiangiogenetic properties, Eap seems to exert some immunomodulatory functions as well by interfering with T-cell functions by different means, thereby promoting immune tolerance to *S. aureus* and staphylococcal disease. Eap is capable of inducing a time and dose-dependent increase in IgM and IgG synthesis in PBMC (peripheral

blood mononuclear cell) cultures (Jahreis et al., 1995), and to induce an increased interleukin (IL)-4 synthesis in this cell type. In addition, Eap was reported to inhibit a delayed type hypersensitivity response and to induce T-cell death (Lee et al., 2002).

6.5 Cell signaling

The process of signaling within and between cells is critical for the living organism. The binding of most signaling molecule to its receptor initiates a series of intracellular reactions that regulate virtually all aspects of cell behavior including metabolism, movement, proliferation and differentiation. Investigations on the molecular mechanisms of these pathways has thus become a major area for research, especially with respect to host-pathogen interactions, as many pathogens utilize and exert their effects via these regulatory systems.

6.5.1 The mitogen activated protein kinase pathway

A wide variety of cellular stimuli, including growth factors, cytokines and extracellular matrix components, transmit signals to the nucleus via pathways involving tyrosine phosphorylation (Neel and Tonks, 1997; Pawson, 1995). The mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) cascade is a major signaling system by which the cell transduces extracellular signals into intracellular responses. The MAPK pathway (Figure 5) leads to a wide range of cellular responses, including cellular growth, differentiation, survival, inflammation and apoptosis. It is activated in response to many mitogenic stimuli, such as epidermal growth factors (EGF) and platelet derived growth factors (PDGF). The activation of these cascades classically involves ligand binding to receptor tyrosine kinases (RTKs) (1). Binding of growth factors to RTKs stimulates the autophosphorylation of specific tyrosines on the receptor. The phosphorylated receptor then interacts with an adaptor protein such as GRB2, which in turn, recuits SOS (son of sevenless) to the plasma membrane (2). SOS is a guanine nucleotide exchange factor which displaces GDP from Ras (GTPase), subsequently allowing the binding of GTP (3). GTP-bound Ras associates with the serine/threonine kinase Raf-1 (4), which is then activated by phosphorylation. Raf phosphorylates and activates the downstream kinase by phophorylating MEK (5). Phosphorylated MEK in turn phosphorylates ERK (6). Phosphorylated ERK moves from the cytoplasm into the nucleus where it subsequently phosphorylates a number

of transcription factors, including Elk-1 (7). Phosphorylated transcription factors turn on transcription (gene expression) of specific sets of target genes leading to the cell proliferation. The activity of Ras is limited by the hydrolysis of GTP back to GDP by GTPase activating proteins (GAP) (Figure 6).

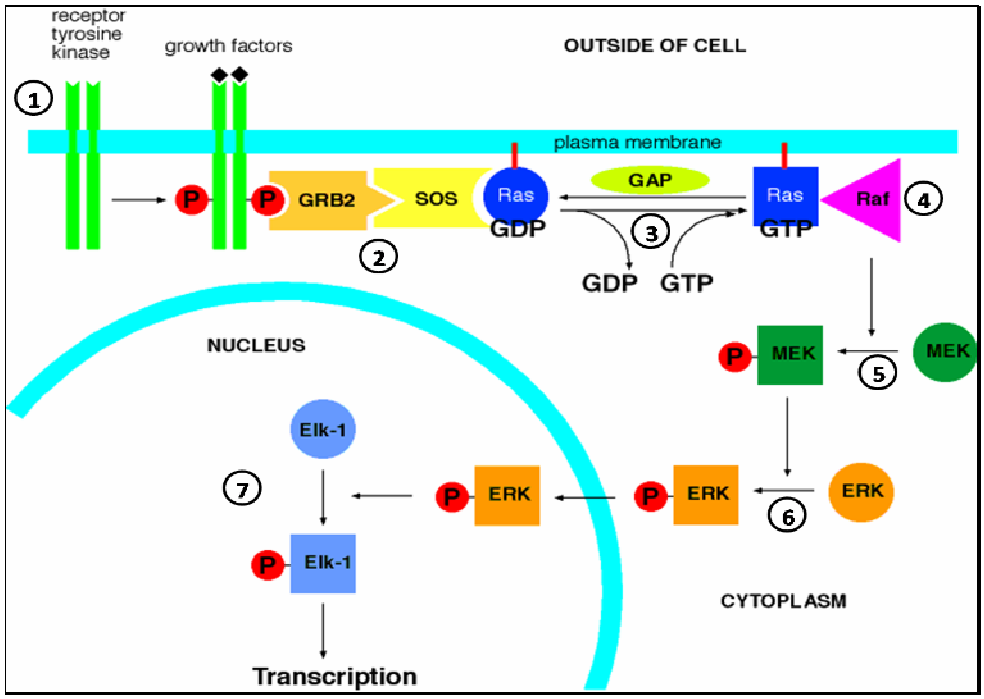


Figure 5: The mitogen-activated protein kinase pathway. (1) Cell membrane has receptor tyrosine kinases (RTKs). (2) Binding of growth factor to receptor activates the tyrosine kinase activity of the cytoplasmic domain of the receptor and phosphorylated on tyrosine residues. GRB2 binds to the guanine nucleotide exchange factor SOS (3) GRB2 SOS complex promotes the removal of GDP from a member of the Ras which then bind GTP and become active. (4) Activated Ras activates RAF (5) RAF kinase phosphorylates and activates MEK (6) MEK phosphorylates and activates an "extracellular signal-regulated kinases" (ERK) (7) ERKs are known to activate many transcription factors, such as ELK1. (Adopted from Vandebroek and Schrijvers (2007), with slight modifications).

6.5.2 Ras signaling pathway

Ras is a G protein with a molecular weight of 20-40 kDa. Like other G proteins, Ras can switch between GTP-bound and GDP-bound states (Figure 6) (Malumbres and Barbacid, 2002). Transition from the GDP-bound to the GTP-bound state is catalyzed by the guanine nucleotide exchange factor (GEF), which induces the exchange between the bound GDP and the cellular

GTP. The reverse transition is catalyzed by a GTPase-activating protein (GAP), which induces hydrolysis of the bound GTP. Ras or more specially, Ras/raf, is a key inducer which exerts a plethora of effects like apoptosis, translation, transcription, cell cycle progression, golgi trafficking vesicle formation, cell-cell junctions- and cytoskeletons modifications. Eap inhibits the activation of Ras by interfering with the Ras/Raf/MEK/ERK pathway (Sobke et al., 2006).

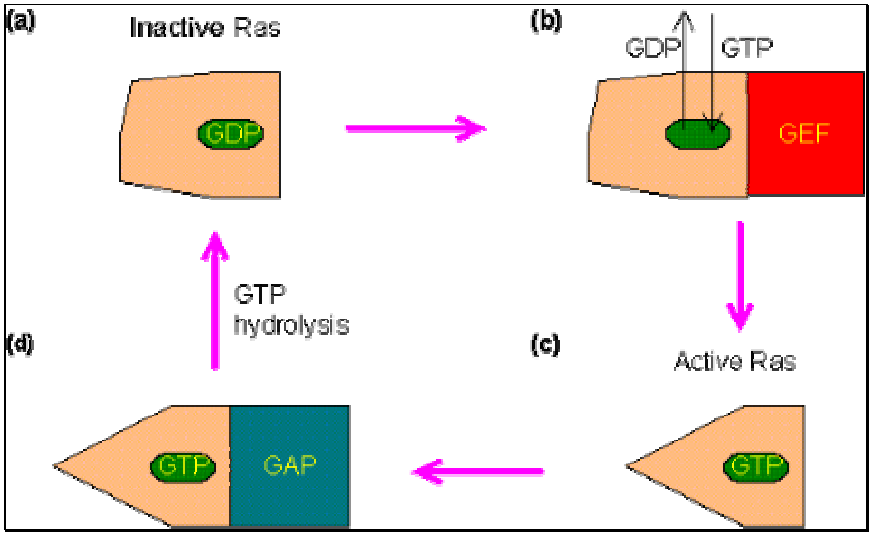


Figure 6: Cycling of the Ras protein between active and inactive states. Ras signaling pathway. (a) Inactive form of Ras. (b) Removal of GDP by guanine nucleotide exchange factor (GEF) and adition of GTP. (c) Active form of Ras (d) GTPase-activating protein (GAP) which induces hydrolysis of the bound GTP into inactive form (Yarwood et al., 2006).

6.6 Protein phosphorylation in bacteria

Like in eukaryotes, phosphorylation of proteins is an important regulatory mechanism in bacteria. However, it was believed that eukaryotes use the Ser/Thr/Tyr phosphorylation for signal transduction, while bacteria use the Asp/His phosphorylation systems. The first serious crack in this picture came out with the characterization of the first serine/threonine eukaryotic-like protein-kinase (Pkn1) in the cyanobacterium *Anabaena* PCC 7120 (Zhang, 1993) and *Myxococcus xanthus* (Hanlon et al., 1997). Today, the widespread presence of eukayotic-like Ser/Thre kinase- and phosphatase-genes in bacteria is indisputable. Concerning the existence of bacterial proteins being phosphorylated at tyrosine residues, the first indication was reported in 1982 for *Escherichia coli* (Wang et al., 1982).

A diverse strategies have been developed by pathogenic bacteria to interact with host cells to manipulate their behaviors, and thus to survive and propagate within its host. During the process of pathogenesis, phosphorylation of proteins at serine, threonine and tyrosine residues occurs at different stages including cell-cell interactions and adherence, translocation of bacterial effectors into host cells, and changes in the host cellular structure and function. The role of protein phosphorylation on serine/threonine/tyrosine residues and its effect on regulation of a variety of cellular functions has been the subject of intensive investigations (Hanlon et al., 1997; Motley et al., 1999; Peirs et al., 1997; Verma and Maurelli, 2003; Grangeasse et al., 2007; Ravichandran et al., 2009; Teqtmeyer et al, 2011), and is thought to play a great role in the process of pathogenesis (Cozzone, 2005). During the initiation of an infection, the bacterial pathogen has first to attach to the host cell. This is followed by pathogen invasion, survival and replication inside the host cell, which may end in cell intoxication and death of the eukaryotic cell. Although the mechanism to protect itself from host cell defense varies from species to species, certain common tactics are apparent that affect changes in gene expression, signal transduction pathways, cytoskeleton organization and vacuolar trafficking. During the invasion process, protein phosphorylation occurs at different stages, either within the bacterial pathogen or at/in the host cell. Several bacterial pathogens, such as enteropathogenic *Escherichia coli* (EPEC), *Helicobacter pylori*, *Chlamydia trachomatis*, *Anaplasma phagocytophilum* and *Bartonella henselae*, use secretion systems to inject tyrosine kinase substrates into host cells (Kenny et al., 1997; Schulein et al., 2005; Segal et al., 1999) (Figure 7). After translocation, these proteins are usually phosphorylated on tyrosine residues by host cell kinases. Once phosphorylated, they react with other cytosolic proteins to modulate host cell functions for the benefit of the pathogen (Backert and Selbach, 2005).

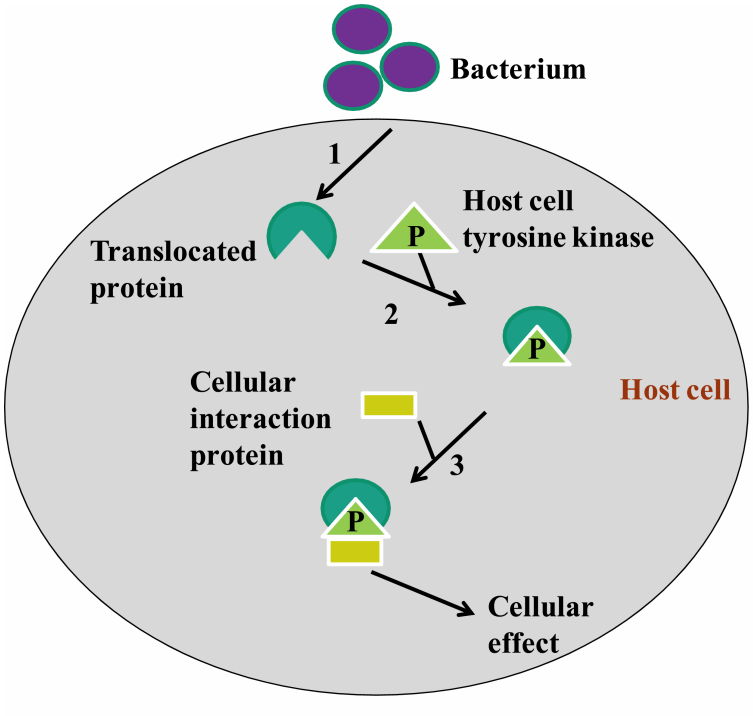


Figure 7: Interaction partners of tyrosine phosphorylated bacterial proteins. (1) Bacterial pathogens inject proteins into mammalian cells. (2) Proteins are phosphorylated on tyrosine residues by a host cell kinase. (3) Phosphorylated proteins interact with other cytosolic proteins and interefere with host cell function (modified after Selbach et al., 2009).

6.7 Selection of YXNX motif of Eap

In bacteria, protein phosphorylation on tyrosine residues has been reported to involve in the control of heatshock response (Klein et al., 2003), adaptation to cold (Ray et al., 1994), adaptation to light (Warner and Bullerjahn, 1994), flagellin export (South et al., 1994), cell aggregation and sporulation (Frasch and Dworkin, 1996), and cell division and differentiation (Wu et al., 1999). However, there are only a few reports about bacterial effector proteins being tyrosine phosphorylated by eukaryotic kinases, and these bacterial proteins vary widely in their functions. Well studied tyrosine phosphorylated bacterial proteins include: The translocated intimin receptor Tir of the enteropathogenic *E. coli* (EPEC), which is required for attaching/effacing (A/E) lesion formation, induced change in the cytosol of infected host cell and induced nuclear signaling events (Kenny et al., 1997; DeVinney et al., 2001; Savkovic et al., 1997); the cytotoxin associated antigen A CagA of *Helicobacter pylori*, which modulates cellular signaling cascades of host cells (Puls et al., 2002; Segal et al., 1999); the translocated actin recruiting phosphoprotein Tarp of *Chlamydia trachomatis*, which helps in the actin-driven

uptake of bacteria by host epithelial cells (Clifton et al., 2004); the Bartonella exported protein D (BepD) of *Bartonella henselae*, which promotes cellular invasions and interfere signaling processes (Schulein et al., 2005); and the AnkA homolog of *Anaplasma phagocytophilum*, a 190 kDa immunoreactive protein with ankyrin-like repeats that interact with host chromatin and nuclear components to downregulate key host defence genes (Ijdo et al., 2007).

Because of the limited studies performed on the conserved region of the putative docking site for tyrosine phosphorylation, clear conclusions for the selection of such sites at the molecular-level are difficult to establish. However, in the nematode *Caenorhabditis elegans* it was found that SOC-2 and SHOC-2 proteins, which are rich in leucine repeats, contains two YXNX motifs, which are shown as a potential tyrosine-phosphorylated docking sites for the Src homology 2 domain of SEM-5/GRB2 (Selfors et al., 1998). However, tyrosine phophorylation is not required for SOC-2 functions in *vivo* and tyrosine phosphorylation was not observed in SHOC-2 in response to FGF stimulations (Selfors et al., 1998). Moreover, it was also shown that the phosphorylation of Tyr-1009 of the platelet derived growth factor receptor (PDGFR) is involved in binding to GRB2, the adaptor protein composed out of two Src homology 3 (SH3) domains and one Src homology 2 (SH2) domain, which plays a critical role in the regulation of Ras (Lowenstein et al., 1992; Buday et al., 1994). The amount of the PDGFR bound to the GRB2 beads was found to be significantly reduced by the tyrosine mutant Y1009F. Furthermore, during cell signaling it was also shown that GRB2 binds to Tyr-1068 (YINQ) in the EGF receptor and to the insulin receptor substrate 1 (IRS-1), where the conserved-domain of YXNX at Y-727, Y-895, and Y-939 (Barbacid et al., 1987; Skolnik et al., 1993; Smith et al., 1986) was available. In addition, it was also shown that GRB2 also binds to Shc (Rozakis-Adcock et al., 1992; Skolnik et al., 1993), apparently through a phosphorylated YVNV motif.

A recent study by Sylvain Kerdudou at the Institute of Medical Microbiology and Hygiene, University of Saarland Hospitals, identified putative YXNX motifs in domains 1, 3 and 4 of the Mu50 Eap; with the YXNX motifs of domain 3 and 4 being highly conserved among all Eap amino acid sequences being available to date (unpublished PhD thesis). Interestingly, in this yet unpublished PhD work, S. Kerdudou showed in BiaCore studies that the domains 3 and 4 of the Mu50 Eap exhibited a higher interaction with β-arrestin (one of the alternative molecule of the heterotrimeric G proteins used by the seven transmembrane receptor family involved in the

transmission of extracellular signals across the plasma membrane to the cytosol) and NFAT-5 (a nuclear factor of activated T-cells, which is currently the only known mammalian transcription factor that regulates gene expression in response to extracellular hypertonicity, allowing the optimal cell proliferation under isotonic conditions). Furthermore, Eap fragments having domain 4 possessed anti-angiogenic activities, while this was not the case with Eap fragments lacking this domain (Preissner and Herrmann, unpublished results). Therefore, we speculated that the conserved YXNX motifs of these domains might serve as potential phosporylation sites, which may modulate the activity of this protein. This hypothetical background was the starting point for this work.

7 Objectives

7.1 General objective

To construct mutants, lacking the tyrosine residues at the conserved YXNX motifs of the Mu50 Eap domains 3 and 4, and study the impact of the mutations on cellular processes known to be affected by Eap.

7.2 Specific objectives

1. To perform site directed mutagenesis at the conserved YXNX motifs of the full-length Eap protein (FLWT), three-domain (2L4WT), two-domain (3L4WT), and the single-domain domain (D3WT) and (D4WT) of Mu50 Eap.
2. To express and purify the wild-type and mutated Mu50 Eap fragments.
3. To study the effect of the wild-type and mutated Mu50 Eap variants on adherence and internalization of bacteria into HaCaT cells.
4. To study the effect of the Mu50 Eap wild-type and mutant proteins on ERK signaling and proliferation of HaCaT cells

8 Materials and Methods

8.1 Materials

The instruments, chemicals, enzymes, kits, reagents and other accessories used in this work were illustrated in Appendix II.

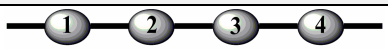
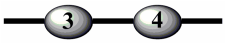
8.1.1 Bacterial strains

The *Escherichia coli* laboratory strain DH5α (Stratagene) was used for the transformation and plasmid amplification. The heterologous expression of the recombinant Mu50 Eap and the different variants of the mutated Eap were performed in *E. coli* BL21 (DE3) (Novagen). *S. aureus* strain SA113 (provided by A. Peschel, Tübingen) was used for the adherence and internalization assays.

8.1.2 Vectors

In this work, a set of pT7HMT expression vectors (Geisbrecht et al., 2006) having the insert of Mu50 *eap* open reading frame (full-length FL) and different *eap* fragment(s) were provided by Dr. B. V. Geisbrecht, (Division of cell biology and biophysics, school of biological science, University of Missouri-Kansas City, USA). The Mu50 Eap FL and different Eap fragments used in this study are shown in Table 1. The details of the vectors are illustrated in Appendix I. 1.

Table 1: *Staphylococcus aureus* Mu50 Eap domains. The schematic representation and the length of the domains used in this work are shown.

Mu50 Eap Wild type	**Abbrevation**	**Diagrammatic representation**	**Remark Length of amino acid**
Full-length	FLWT		476
Three domain having domain 2, 3 and 4	2L4WT		367
Two domain having domain 3 and 4	3L4WT		263
Single domain 1	D1WT		MAP1*, 109
Single domain 2	D2WT		MAP2*, 110
Single domain 3	D3WT		MAP3*, 109
Single domain 4	D4WT		MAP4*, 104

Note: MAP* - MHC Analog Protein. The length of Eap repeats and the length of the amino acids are retrieved from www.uniprot.org/uniprot/Q99QS1.html. The Eap repeats of position 45-153, 154-263, 264-372 and 373-476 amino acids are described as MAP1, MAP2, MAP3 and MAP4, respectively (Kuroda et al., 2001).

8.1.3 Oligonucleotides

The oligonucleotides used to amplify the Mu50 *eap* and to construct the different *eap* mutants were purchased from MWG Biotechnology, Germany. The list of the primers that were used for the study is described in Table 2.

Table 2: List of mutagenic and sequencing primers for the point mutation of YXNX motifs of *Staphylococcus aureus* Mu50 Eap. Nucleotides leading to a point mutation in the Mu50 Eap are shown in red.

Description	**Forward**	**Reverse**	**Sequence (5´→3´)**
Primers for amplification of Eap domain 1			
RRY-5	X		AAAGTTAAATCAGTTTTATTCTTTAATAGAGGTATTAG
RRY-6		X	CTAATACCTCTATTAAAGAATAAAACTGATTTAACTTT
Primers for amplification of Eap domain 3			
RRY-3	X		CAAATAAACCTTGGACAAATTTCAAAAATTTAACTAGTC
RRY-4		X	GACTAGTTAAATTTTTGAAATTTGTCCAAGGTTTATTTG
Primers for amplification of Eap domain 4			
RRY-1	X		CAATTAATTAGTTACAAATTTTTAAACGACAAAGTG
RRY-2		X	CACTTTGTCGTTTAAAAATTTGTAACTAATTAATTG
Primers for sequencing			
Description	**For**	**Rev**	**Sequence (5´→3´)**
T7 prom	X		T TAATACGACTCACTATAGGG
T7 term		X	GCTAGTTATTGCTCAGCGG

8.2 Methods

8.2.1 Sequence analysis

The Eap amino acid sequences of 14 different strains of *S. aureus* [Mu50 (SAV1938), RF122 (SAB1873c), N315 (SA1751), JH9 (A5IUA7), CI-7 (AJ243790), USA300 (ABX29943), MSSA476 (SAS1861), MW2 (EFK80959.1) , 8325 (ABD31208), COL (AAW36971), FDA547 (Q53599), Newman mapN (AJ132841), MRSA252 (SAR2030) and Wood46 map-W (AJ245439)] were retrieved from the EMBL homepage and aligned using the CLUSTAL W program (www.ebi.ac.uk/clustalw).

8.2.2 Preparation of competent cells

Competency of bacterial cells for uptake of foreign DNA can be achieved by chemical or electro-physical manipulations of cells. In this work, both types of cells were used.

8.2.2.1 Preparation of chemically competent cells

Chemically competent cells were used for protein expression and plasmid preparation purposes. The chemically competent cells were made from *E. coli* DH5α and BL21(DE3), respectively. An overnight culture of the respective *E. coli* strain was used to inoculate a 250 ml culture composed of Medium A (Appendix III. 1) to an optical density at 600 nm (A_{600}) of 0.1. The culture was incubated at 20-25°C in a water bath by shaking at 150 rpm, and cell densities were monitored every hour until A_{600} reached 0.6. The culture was collected in centrifuge tubes and kept on ice for 10 min. It was harvested by centrifugation at 2500 *g* for 10 min at 4°C. The pellet was suspended in 80 ml of ice cold Medium B (Appendix III. 1), and incubated on ice for 10 min. After a further centrifugation at 2500 *g* for 10 min, the cells were resuspended in 20 ml of ice cold Medium B. Afterward, to this cell suspension 1.4 ml of DMSO was added drop by drop, and the mixture was kept on ice for an additional 10 min. Aliquots of 200 µl volume of the competent cells were transferred into a chilled sterile 1.5 ml microfuge tube. The cells were frozen with liquid nitrogen and were stored at -70°C.

8.2.2.2 Preparation of electro-competent cells

E. coli DH5α electro-competent cell were used for the site directed mutagenesis. An overnight seed culture (4 ml) was used to inoculate a 400 ml LB (Difco) main culture, which was grown at 37°C by shaking at 180 rpm, until the absorbance at A_{600} reached 0.6. The culture flask was incubated on ice for 30 min and subsequently centrifuged at 2000 *g* for 15 min at 4°C. The bacterial pellet was dissolved in 400 ml of ice cold sterile distilled water and centrifuged as mentioned before. The pellet was then resuspended in 200 ml of ice cold sterile distilled water and centrifuged as before. The resulting bacterial cell pellet was resuspended in 20 ml of ice cold sterile 10% glycerol and centrifuged as before. The pellet was finally dissolved in 2 ml of ice cold sterile 10% glycerol and was divided into aliquots of 50 µl in microfuge tubes. The tubes were immediately frozen in liquid nitrogen and stored at -80°C. The transformation frequency of the competent cell was 3 x 10^{10} cells/ml.

8.2.3 Transformation of *E. coli*

8.2.3.1 Heat shock transformation

Heat shock transformation was applied to introduce the plasmid DNA into the chemically competent cells (Sambrook and Russell, 2001). The competent cells were defrosted on ice for 10 min and mixed with 1 µl (20-100 ng) of plasmid DNA. The mixture was incubated on ice for 30 min and transferred into a 42°C water bath for 45 sec, followed by incubation on ice for 5 min. 250 µl of SOC medium (Appendix III. 1) was added to the cells and the mixture was incubated at 37°C for an additional hour at 180 rpm. 100 µl of the culture were finally plated on LB agar plates containing kanamycin (50µg/ml). The plates were incubated at 37°C overnight.

8.2.3.2 Electroporation

Higher transformation efficiency is generally required for the site directed mutagenesis purpose, which can be achieved by using electro-competent cells. In this method, plasmid DNA is introduced into bacterial cells by introducing a dielectric breakdown of cell membranes.

Electro-competent cells were thawed on ice and mixed with 2 µl (50 ng) of plasmid DNA. The suspension (~100 µl) was transferred to a chilled 0.1 cm electroporation cuvette, which was

flicked to remove air bubbles. The cuvette was placed into an electroporator and the electric pulse was applied (200Ω and 1.8kV). Cells were immediately resuspended in 500 µl of SOC medium, incubated at 37°C for 1 hr and plated on nutrient agar plates containing kanamycin (50 µg/ml).

8.2.4 Site directed mutagenesis

In order to convert Tyrosine (Y) to Phenylalanine (F) in YXNX motifs of domains 3 and 4 of the Mu50 Eap, the Quick-Change Site-Directed Mutagenesis Kit (Stratagene Ltd, Cambridge, UK) was used according to manufacturer's instruction. The assay relies on *Pfu* DNA polymerase replicating both plasmid strands with high fidelity. The procedure was started with a PCR reaction using a recombinant vector with an inserted gene of interest as template and two oligonucleotide primers containing the desired point mutation. The primers were extended during temperature cycling by *Pfu* DNA polymerase. This incorporation of primers generated a mutant plasmid containing staggered nicks. The treatment of the amplification product with D*pn*I resulted in the digestion of the parental DNA template. Oligonucleotide primers were designed as illustrated in Table 2, introducing the point mutations at amino acid 296 and 403 of the Mu50 Eap open reading frame, respectively, leading Y to F exchanges in the respective gene products.

Table 3: The reaction components and the PCR conditions employed during the site directed mutagenesis.

Components	Volume	Components	Volume
plasmid DNA (50 ng)	X µl	Primer 2 (0.2 µM)	1 µl
10 x *Pfu* reaction buffer	5 µl	*Pfu* DNA polymerase (2.5 U/µl)	0.5 µl
dNTPs (100 µM each)	2 µl	Water (dd H_2O)	Up to 50 µl
Primer 1 (0.2 µM)	1 µl		

The mutated plasmids were amplified using the following conditions: 95ºC for 30 sec, followed by 20 cycles of 95ºC for 30 sec, 55ºC for 1 min and 68 ºC for 6 min. The final cycle was 25°C for 2 min. In the case of Mu50 Eap of single domain, the elongation time was reduced to 2 min.

After PCR, the samples were placed on the ice for an additional 2 min to cool the reaction to ≤ 37ºC.

8.2.4.1 Transformation of mutagenesis product

To remove the unmutated parental DNA, the PCR samples were treated with the restriction enzyme *Dpn*I (1 µl) for 1 hr at 37ºC, which cuts only methylated DNA strands. An aliquot of electro-competent cells *(E. coli* DH5α) was thawn on ice, and 2 µl of the *Dpn*I treated plasmid sample was mixed with the competent cells. The tube was flicked carefully to mix the sample and was incubated on ice for an additional 30 min. After this, the sample was transformed by the electroporation method as described above (section 3.2.3.2). After selection of well-isolated colonies on an agar plate containing kanamycin, plasmids were extracted and purified as mentioned below. The mutations were confirmed by sequencing (4 base-lab, Germany).

8.2.4.2 Plasmid extraction

The plasmid DNA was purified using the Qiagen Miniprep kit according to manufacturer's instruction. A well isolated colony was picked from a LB-kanamycin plate with a sterile loop and placed into 5 ml of LB medium containing kanamycin (50 µg/ml). The culture was incubated overnight at 37ºC with an agitation at 230 rpm. The overnight culture (1.5 ml) was transferred to a 1.5 ml Eppendorf tube and centrifuged for 1 min at 13,000 rpm. The supernatant was removed and the pellet was resuspended in 250 µl of buffer P1, followed by an addition 250 µl of buffer P2 (both buffers were provided by the kit). The sample was mixed by inverting the tube for 4 to 6 times, and lysis was allowed to occur for 2 min. The lysis step was stopped by adding 350 µl of the N3 solution (provided by the kit), inverting the tube 4 to 6 times, and incubating the mixture for 5 min on ice. The solution was then centrifuged for 10 min at 13,000 rpm at room temperature. The supernatant was transferred to a Qiagen column and the tube was centrifuged for 1 min at 13,000 rpm. The column was washed with 750 µl of PE buffer (provided in the kit) and centrifuged twice to remove the buffer solution. The elution of the DNA was performed by adding 50 µl of sterile water to the column and incubating the column at room temperature for 1 min, followed by a centrifugation at 13,000 rpm for 1 min. The concentration of the plasmid was determined by measuring the absorption at 260 nm in a spectrophotometer (Sambrook and

Russell, 2001) . The principle of this method is based on the absorption ability of UV light by the ring structure of purines and pyrimidines in the DNA or RNA. An aliquot of DNA was send for sequencing; the rest was stored at -20°C.

8.2.4.3 Agarose gel electrophoresis

Agarose gel electrophoresis is an effective method to separate DNA fragments for analytical and preparative purposes. The negative charges of DNA molecules help to migrate towards the anode in an electrical field. The migration depends on the size (base pair), the conformation of the DNA (either relaxed or super-coiled) and also on the pore size of the gel. To detect the DNA, ethidium bromide is often used, which intercalates between the stacked base pairs and can be visualized by ultraviolet illumination.

For the separation of DNA fragments of 1000 to 8000 bp of length, a 0.8% melted agarose solution in 1 x TAE (Appendix III. 3) containing 0.5 µg/ml of ethidium bromide was poured into the mold. The solidified gel was transferred into the electrophoresis tank and was covered with 1 x TAE buffer. DNA samples (5-50 µl) mixed with DNA loading buffer (5:1) (Appendix III. 3) were loaded on the gel. To determine the sizes of the DNA fragments, a 1 kb DNA ladder (Invitrogen) was loaded on each gel. Electrophoresis was performed at 100 V for 45 min. The gel was examined and photographed under by ultraviolet light.

8.2.5 Recombinant Mu50 Eap expression and purification

8.2.5.1 Heterologous gene expression of His_6-Eap

E. coli strain BL21(DE3) was used as heterologous hosts for the expression of Eap and its variants. 100 ng of the respective Mu50 Eap plasmid was mixed with the *E. coli* competent cells. Transformation was carried out by heat shock as described above. The transformation product (100 µl) was spread onto a LB plate containing kanamycin and incubated at 37ºC for 24 hr. Following this, a well isolated single colony was picked from the plate, inoculated into 10 ml LB containing kanamycin (50 µg/ml), and grown overnight at 37ºC with an agitation at 230 rpm. At the following day, the main culture was started by diluting the overnight culture 1:100 into 250 ml of fresh Terrific Broth (TB) (Appendix III. 1) and incubating the mixture at 37°C and 230

rpm. When the optical density (A_{600}) of the culture reached 1.0, the protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was grown at 37ºC and 230 rpm for an additional 18 hr to achieve a maximal cell density, before the cells were harvested by centrifugation at 5000 rpm.

8.2.5.2 Bacterial cell lysis

The harvested cell pellets were resuspended in denaturing lysis buffer (5% of the original culture volume) and lysed by stirring (Appendix III. 3) at 300–500 rpm for 30 min at room temperature. The solubilized-denatured proteins were separated from the cell debris by centrifugation at 25,000 *g* for 30 min.

8.2.6 Protein purification

8.2.6.1 Affinity chromatography

The affinity chromatography is based on the formation of specific reversible complexes between the chromatographic matrix and the molecule of interest. The matrix is represented by a suitable polymer, carrying functional group-ligands, which bind the target molecules, whereas all other non-specific molecules will pass through the column. In order to purify the proteins of interest, the column is first equilibrated with binding buffer. This step is followed by sample loading and washing steps, which remove all proteins that are not specifically bound to the matrix. The bound molecules are finally eluted from the column either by inducing physico-chemical changes, such as the change of ionic strength, pH, temperature, or by adding chaotropic agents like urea, ethylene glycol, detergents or ethanol, or by adding a competing substance, such as imidazole.

In this study, protein purification was carried out at room temperature using a fast performance liquid chromatography (FPLC) unit (BioRad, Biologic Duo Flow) equipped with a flow cell UV-vis monitor and a fraction collector. The affinity chromatography was carried out with a Ni^{2+}–NTA column (GE Healthcare), which exhibits a high binding capacity for histidine-tagged proteins. A schematic view on interactions between 6xHis-Tag, Ni^{2+}-NTA, imidazole and EDTA with corresponding dissociation constants (K_d) (Knecht et al., 2009) is shown in Figure 8.

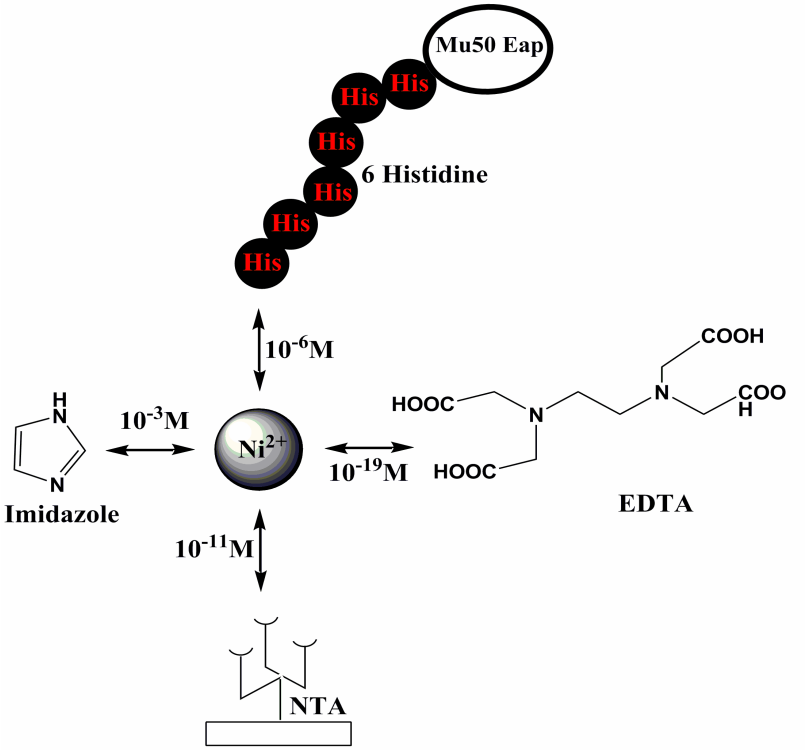


Figure 8: An illustration of the interactions that are involved in the purification with a Ni^{2+}-NTA affinity column. The high-binding affinity between Ni^{2+} and NTA (K_d value of 10^{-11} M) allows a mild elution with moderate imidazole concentrations (K_d of 10^{-3} M). The K_d value of imidazole is low enough to displace bound 6xHis tags, but is not too high to strip the Ni^{2+} off the column. This can be performed by an injection of EDTA (K_d of 10^{-11} M) leading to a complete recovery of the column material (adapted and modified from Knecht et al., 2009).

8.2.6.1.1 Purification of Eap by denature method

Affinity chromatography was performed by using a Ni^{2+}–NTA column for FPLC. The clear supernatant of the solubilized cell extract was loaded to a 5 ml Ni^{2+}–NTA column joined to FPLC, which had previously been equilibrated to room temperature in denaturing wash buffer (Appendix III. 3). After loading the sample, non-specifically bound proteins were removed from the column by applying 5 column volume (CV) of denaturing wash buffer. The tagged proteins were eluted from the resin with 2.5 CV of denaturing elution buffer (Appendix III. 3). During the purification, the first 0.5 CV of eluate were discarded since they contained negligible amounts of protein.

8.2.6.1.2 Protein refolding

Following the purification under denaturing conditions, the denatured protein samples were drawn into an appropriate syringe with a needle, and refolded by quickly injecting the entire amount into a 10-fold excess volume of native buffer (Appendix III. 3) at room temperature with rapid stirring. The diluted samples were allowed to stir for 5-10 min at room temperature.

8.2.6.1.3 Native purification

The refolded protein samples were reapplied to the Ni^{2+}–NTA column that had been regenerated according to manufacturer's suggestions and pre-equilibrated with a native wash buffer (Appendix III. 3). The excess urea was removed by washing with 5 CV of native wash buffer, and the bound, refolded proteins were eluted with 2.5 CV of native elution buffer (Appendix III. 3). The proteins were concentrated using Amicon Millipore Centricon according to manufacturer's instructions.

8.2.6.2 Gel filtration chromatography

In gel filtration, samples are loaded on top of a column packed with gels of cross-linked polymers with a known pore size, such as dextran. An elution buffer with pH and ionic strength suitable for the sample preservation is passed from the top of the column with large molecules being eluted faster, as smaller molecules are retarded by the matrix. The speed of elution directly affects the effectiveness of the separation with slower speeds giving better resolutions.

In this work, gel filtration was performed using a pre-packed Superdex 75 10/300 column (GE healthcare) and the BioRad FPLC system, which should allow separating proteins of molecular masses between 3 and 70 kDa. The column was set at room temperature, and prior to the loading of the sample, it was washed with 5 CV of phosphate buffer (Appendix III. 3) that had been degassed and filtered. During the filtering process, the eluent was analyzed for conductivity, pH and UV absorbance, and results were plotted on chromatograms. 200 µl of the concentrated Ni^{2+}–NTA purified native protein was injected into the FPLC and passed through the Superdex column at the rate of 500 µl per min. The sample was passed through a UV detecter at 254 nm and each

fraction (500 µl) was collected in a fraction collecter (Biorad). The chromatogram was obtained by plotting the changes in UV absorbance (mAU) vs. eluent per sample volume (ml).

8.2.7 Quantitation of endotoxin from purified recombinant Mu50 Eap

8.2.7.1 Removal of endotoxin from purified Eap

Endotoxin is an unwanted and often encountered by product of recombinant proteins purified from *Escherichia coli*. The inherent toxicity of endotoxins makes their removal an important step for downstream applications of the protein in several biological assays. In this work, putative residual *E. coli* endotoxin contaminating the purified Eap protein fraction was removed using an EndoTrap Blue column (Cambrex, Walkersville, MD). The column was charged with 6 CV of regeneration buffer (provided by the manufacturer) and equilibrated with 6 CV of equilibration buffer (provided by the manufacturer). Following equilibration, the purified Eap protein sample was loaded onto the column and allowed to pass through by gravity flow. Fractions were collected immediately after the sample was loaded. The collected fractions were concentrated using Millipore Centricon as manufacturer's instruction.

8.2.7.2 Measurement of endotoxin in the purified Eap samples

The limulus amebocyte lysate (LAL) assay was used to quantify the amount of endotoxin present in protein samples following the purification of Eap. The QCL-1000 kit (Lonza) was used according to the manufacturer's instructions.

A standard curve of endotoxin was created ranging from 0.2-1.0 endotoxin units/ml. The purified stock of endotoxin was vortexed vigorously for 15 min prior to dilution, and each standard dilution was vortexed for an additional two min before transferring it to the next tube. Samples were diluted directly in the tube in duplicate, and 10-fold dilutions ranging from pure sample to 1:1000 were created. Each sample and the standard were prewarmed to 37°C before an equivalent amount of LAL reagent was added and incubated for 10 min at 37°C. Chromogenic substrate solution was then added to the tubes and incubated for an additional 6 min at 37°C. The assay was stopped by the addition of 50 µl of 10% SDS. The absorbance of each sample was determined at 405 nm on the spectrophotometer (BioTek Instruments) and the values were

compared to the standard curve of known endotoxin concentrations. The linear regression equation of the best fit line through the standard curve points was calculated by Microsoft Excel, and then used to determine the endotoxin concentration of the purified protein samples.

8.2.8 Protein quantification

In this work, protein determination was done either by the Bradford method or by a BCA assay as described below.

8.2.8.1.1 Bradford protein assay

The protein concentrations in the cell culture samples were determined with a Bradford protein assay, using bovine serum albumin (BSA) as a protein standard (Bradford 1976). The standard curve was made of the readings of eight different dilutions of the stock solution of BSA (1 mg/ml) with final concentrations of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.08 mg/ml, respectively. For the protein determination, Bradford solution (Bio-Rad Protein Assay) was 5-fold diluted in water, and 250 µl were added to the cuvettes containing 10 µl of the standard or samples. The solutions were mixed thoroughly and incubated at room temperature for 5 min. The respective samples were tested in duplicate, and the absorbance of standard and samples were measured using a spectrophotometer (Gene Quantpro) at 595 nm.

8.2.8.2 BCA protein assay

Protein concentrations were also determined using the BCA (Bicinchoninic Acid) assay protein quantitation kit. The principle of the BCA assay is a colorimetric assay which involves the reduction of Cu^{2+} to Cu^{+} by peptidic bonds of proteins. Bicinchoninic acid chelates Cu^{+} ions with very high specificity to form a water soluble purple colored complex (Figure 9). The reaction was measured by the optical absorbance at 562 nm. Protein concentrations were calculated in reference to a standard curve obtained with BSA.

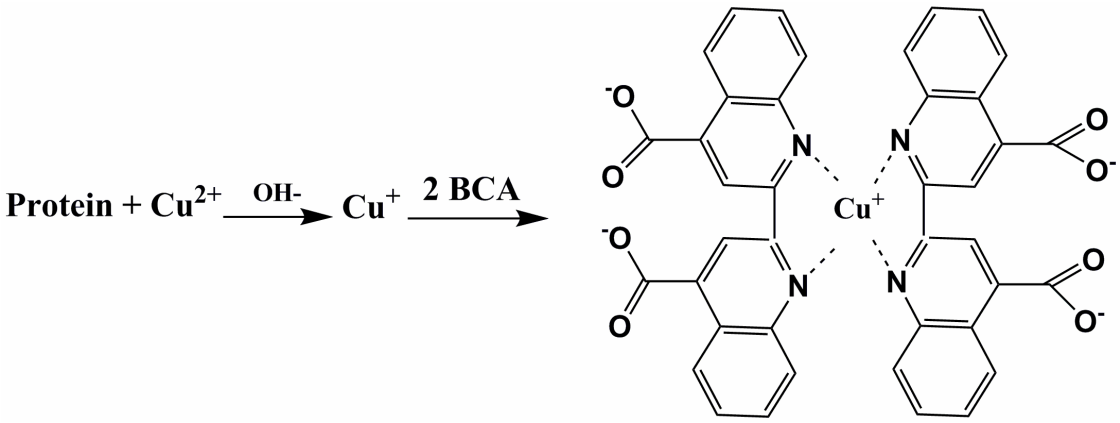


Figure 9: BC Assay reaction

The working reagent was prepared by mixing 50 volumes of BCA reagent A with 1 volume of BCA reagent B (50:1). 100 µl of the standard protein fractions and samples were mixed with 2 ml of working reagent, respectively, incubated at 37°C for 30 min, and measured at 562 nm on the spectrophotometer (Unicam Helios Instruments). The average absorbance values for the standard curve were plotted, and the linear regression equation for the best fit line through the points was calculated by Microsoft Excel. Average sample absorbance values in the range of the standard curve were interpolated to determine the protein concentration.

8.2.9 Sodium Dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE)

The discontinuous gel electrophoresis was used to monitor the status of Eap expression and the purity of the Eap obtained after several steps of the purification procedure. Proteins were separated according to their charge and molecular mass, using Laemmlis' discontinuous gel electrophoresis method (Laemmli, 1970), with a 10 % separating gel (Appendix IV) and a 5% stacking gel (Appendix IV).

Briefly, a portion of the Eap protein sample was mixed with 2xSDS loading buffer (Appendix IV) (1:1 v/v) and the sample was heated to 100°C for 5 min. After cooling down for 2 min on ice, samples were applied to the slots (wells) of the stacking gel and were run for 1 hr and 30 min at 120 V until the tracking dye (bromophenol blue) front reached to the bottom of the gel. The pre-stained broad range protein marker (Fermentas life science) served as a reference molecular

weight marker. To visualize the protein bands, the gel was stained with Coomassie staining solution (Appendix IV) for 30 min at room temperature on a shaker and then incubated in destaining solution (Appendix IV), which was changed every 30 min until the protein bands became clearly visible.

8.2.10 Cell culture

Human keratinocytes were grown and maintained in the keratinocyte growth medium MCBD 153 basal medium (BIOCROM AG), supplemented with 10% fetal bovine serum (FBS) obtained from PAA lab (GmbH), and 1% penicillin /streptomycin (10000U/10000 µg/ml) solution in a humid atmosphere containing 5% CO_2 and 95% air at 37ºC. All cells were split twice a week. Prior to splitting of the cells, they were washed twice with 5 ml PBS (Appendix III. 3). Cells were detached from the petri-dishes (10 cm) by adding pre-warmed 1x trypsin-EDTA (2 ml; PAA lab GmbH) and incubated at 37°C with 5% CO_2 for 10-15 min and collecting the cells by centrifugation. The cells were resuspended into fresh medium and transferred into new petri-dishes containing fresh medium (10 ml) (cells were diluted by a factor <10). Cell cultures were tested routinely for mycoplasma contaminations using the PCR mycoplasma Test Kit (AppliChem) according to the manufacturer's recommendations.

8.2.10.1 Freezing cell lines for long term storage

After trypsination with pre-warmed 1x trypsin-EDTA and addition of prewarmed MCBD 153 basal medium, cells were transferred to a falcon tube and centrifuged at 1000 g. In order to prevent the formation of ice crystals within the cells, pellets were resuspended in media containing 20% DMSO, and were slowly cooled by freezing the cells at –20°C for 1 hr, followed by an overnight incubation at –70°C. On the following day, the cells were stored either in a liquid nitrogen cell incubator or at –70°C.

8.2.10.2 Determination of cell numbers and cell viability

Cells were counted by a trypan blue method using the Countless Automated Cell Counter (Invitrogen). Cell samples were mixed with trypan blue, loaded into countless cell counting

chamber slides (Stuart Scientific Colony Counter), and applied to the cell counter, thereby determining the live/dead ratio of cells and the total cell concentration per milliliter.

8.2.11 Cell culture assays

8.2.11.1 Adherence assay

The adherence and internalization assay was performed as described by Haggar et al., (2003) with slight modifications. Briefly, HaCaT cells were seeded ($1x10^5$cells/well) into 24-well plates and incubated at 37°C in presence of 5% CO_2 until the cell layer reached confluence. Cells were washed with PBS (Appendix III. 3), and 300 µl of standard medium supplemented with the purified Eap samples (WT and mutant samples) at a concentration of 30 µg/ml (final concentration) were added. The cultures were incubated for 1 hr and washed with PBS as described above. 300 µl of fresh standard medium containing 10^7 bacteria (multiplicity of infection of 100) were added per well. After additional 2 hr and 30 min of incubation, media were removed and wells were washed three times with PBS to remove non-adherent bacteria. In the next step, 200 µl of trypsin/EDTA were added per wells to detach the cells, which were subsequently lysed by the addition of 800 µl of sterile water, and sonicated at 75 watt for 15 sec. The bacteria were serially diluted and plated on MHA plates. After 24 hours of incubation, colonies were counted, and CFUs were determined.

8.2.11.2 Internalization assay

For the internalization assay, the adhesion assay protocol was followed with the one exception that after incubation for 2 hr and 30 min, the old media were replaced by adding 200 µl of fresh media containing lysostaphin (20 µg/ml final concentrations) and the cell cultures were incubated for an additional hr.

8.2.11.3 ERK phosphorylation assay

8.2.11.3.1 Cell assay for ERK signaling

The ERK phosporylation assays were performed essentially as described (Sobke et al., 2006). Briefly, HaCaT cells were seeded into 6 well plates with a cell-density of $2x10^6$cells/well, and cultured for 24 hr in MCBD 153 containing 10% FBS and 1% penicillin/streptomycin. After this incubation, old media were replaced by fresh medium containing 0.5% FBS and further incubated overnight. On the following day, old media were replaced by basal medium containing 0.05% BSA (Sigma), and the cells were incubated for additional 4 hr before Eap was added. After an additional hr of incubation, ERK phosphorylation was induced by adding keratinocyte growth factor (KGF) at a concentration of 50 ng/ml and incubating the culture for 8 min. After that, cells were washed twice with ice-cold PBS, lysed on ice in RIPA buffer (Appendix V) and then centrifuged at 10,000 *g* for 10 min at 4ºC to remove all insoluble material. The supernatants were collected and total cellular protein contents were determined using Bradford and BCA assays. Equal amounts of protein (30 µg) were subjected to Western blot analyses using anti-phospho-ERK (Thr202/Tyr204) antibodies.

8.2.11.3.2 Western blot of ERK signaling

Proteins were transferred from the SDS-gel onto a nitrocellulose transfer-membrane (Protran®, Whatman) by a semi-dry blotting method in a BioRad Transfer-blot SD system using the manufacturers protocols. The blots were subsequently blocked for 1 hr with 5% skim milk in Tris-buffer saline (Appendix III. 3) supplemented with 0.1% Tween 20, followed by an overnight incubation at 4°C with a 1:1000 dilution of anti-phospho-ERK (Thr202/Tyr204) antibody purchased from Cell Signalling Technology (Beverly MA). On the following day, membranes were washed 3-times with TBS, followed by incubation at 25°C for 1 hr with a 1:4000 dilution of the secondary antibody, a horseradish peroxidase-conjugated anti-mouse IgG antibody (Biorad). After additional three rounds of washing, blots were equilibrated in detection buffer, overlayed with an ECL solution (Amersham Bioscience) and incubated for 2 min. at RT, before the chemiluminescence signals were captured using a ChemiDoc XRS-system -system (BioRad).

8.2.11.4 Proliferation assay

HaCaT cell ($2x10^3$ cells per 100 µl) were seeded into 96 well plates in a MCBD 153 medium supplemented with 0.5% fetal bovine serum and incubated at 37ºC for 24 hr in a humid atmosphere containing 5% CO_2 and 95% air. Afterwards, the old media were replaced by fresh media containing 0.2% BSA and 30 µg/ml of Eap and further incubated for a night. On day three, cells were incubated with EdU (ethynyl-2- deoxyuridine) at a concentration of 10 mM and incubated for 24 hr. On day four, the EdU incorporation was measured with the Click-iT-EdU Microplate Assay kit (Invitrogen). All samples were assayed in triplicate. Fluorescence signals were measured with the Wallac Victor-2 microtiter well plate reader (Perkin Elmer Life Science) at excitation/emission wavelengths of 490/510 nm.

8.3 Statistical methods

An unpaired two sided Mann Whitney U test was used, with a threshold of statistical significance assumed at *P* values of <0.05 (*) and <0.01 (**).

9 Results

9.1 Bioinformatics study

All *S. aureus* strains tested to date produce Eap, which varies considerably in its size between different isolates. The protein is usually composed of 4 to 6 tandem repeats, with only few exceptions. In this study, the amino acid sequences of Eap from 14 different strains of *S. aureus* were retrieved and a multiple sequence alignment was performed as described in the 'Methods' section. The alignment result is shown in Appendix I. 3.

Based on the sequence alignment of the Eaps, five different Eap domain-types were identified (type I, II, III, IV and V). The corresponding sequence alignments of the domains are shown in Figure 10-14 and their comprehensive diagrammatic representation is shown in Figure 15. *S. aureus* stains having small variants of this protein were composed of Eap domain types that were not redundant, while the larger Eap variants usually contained repeats of one or two domain types, with the domain types 3 and 4 being duplicated in most of the cases. For example, the Eap of *S. aureus* strain Mu50 is composed of four domains: domain 1 of type I, domain 2 of type II, domain 3 of type III, and domain 4 of type IV. The Eap of strain RF122 is composed of three domains; domain 1, domain 2 and domain 3. The alignment of each domain with respect to the strain Mu50 showed the presence of type I, type II and type IV domains, respectively, in which the domain 3, though being third domain, showed the highest identity/similarity with type IV (Table 4). Similarly, the Eap of strain Wood 46 is composed of 6 domains; domain 1 (type I), domain 2 (type II), domain 3 and domain 4 (both type III), and domain 5 and 6 (both type IV), suggesting a duplication of these two domains during the evolutionary process of the strains.

Table 4: The comparative study of the individual domain of *S. aureus* strain RF122 with respect to the strain Mu50. The pairwise sequence alignment was performed to find the identity/similarity of the individual domain. The domain 1 (D1), domain 2 (D2) and domain 3 (D3) of RF122 showed the highest identity/similarity with type I, type II and type IV domains with respect to the strain Mu50, and the values are shown in bold.

	Identity/Similarity		
***S. aureus* strain**	**RF122**		
Mu50	D1	D2	D3
D1 (Type I)	**85.2/95.5**	84.1/95.5	56.8/77.3
D2 (Type II)	83.0/95.5	**97.7/98.9**	60.2/79.5
D3 (Type III)	53.4/77.3	51.1/76.1	60.2/75.0
D4 (Type IV)	62.5/78.4	62.5/76.1	**92.0/97.7**

The sequence alignment also revealed that the putative tyrosine phosporylation sites of Eap identified by S. Kerdudou (unpublished PhD thesis), the YXNX motifs, were more or less conserved in the Eap domain types I, III and IV (Figure 10-15), whereas they were absent in domain types II and V. The highest conservation of the YXNX motif was found in the domain of type III, which was present in all strains harboring this type followed by the domain type IV, where the YXNX motif was found in 12 out of 14 isolates (Figure 15). However, the YXNX motif was less conserved in the domain type I, being present in 10 of the 14 analyzed isolates only (Figure 15). Interestingly, Eap variants that are composed of more than four repeats did not show the conserved YXNX motifs in all the duplicated type III and IV domains (Figure 15).

```
Mu50_D1          IPYTITVNGTSQNILSSLTFNKNQNISYKDIENKVKSVLYFNRGISDIDLRLSKQAEYTV 60
RF122_D1         VPYAITVNGTSQNILSSLTFNKNQNISYKDLEDRVKSVLKSDRGISDIDLRLSKQAKYTV 60
N315             IPYTITVNGTSQNILSSLTFNKNQNISYKDIENKVKSVLYFNRGISDIDLRLSKQAEYTV 60
JH9Map_D1        IPYTITVNGTSQNILSSLTFNKNQNISYKDIENKVKSVLYFNRGISDIDLRLSKQAEYTV 60
CI7_D1           IPYTITVNGTSQNILSSLTFNKNQQISYKDIENKVKSVLYFNRGISDIDLRLSKQAKYTV 60
USA300_D1        VPYAITVNGTSQNILSSLTFNKNQNISYKDLEDRVKSVLKSDRGISDIDLRLSKQAKYTV 60
MSSA476_D1       IPYTITVNGTSQNILSSLTFNKNQNISYKDIENKVKSVLYFNRGISDIDLRLSKQAEYTV 60
MW2_D1           IPYTITVNGTSQNILSSLTFNKNQNISYKDIENKVKSVLYFNRGISDIDLRLSKQAEYTV 60
8325_D1          VPYAITVNGTSQNILSSLTFNKNQNISYKDLEDRVKSVLKSDRGISDIDLRLSKQAKYTV 60
COL_D1           VPYAITVNGTSQNILSSLTFNKNQNISYKDLEDRVKSVLKSDRGISDIDLRLSKQAKYTV 60
FDA574_D1        IPYTITVNGTSQNILSSLTFNKNQQISYKDIENKVKSVLYFNRGISDIDLRLSKQAKYTV 60
Newman_D1        IPYTITVNGTSQNILSSLTFNKNQQISYKDIENKVKSVLYFNRGISDIDLRLSKQAKYTV 60
MRSA252_D1       IPYTITVNGTSQNILSSLTFNKNQQISYKDIENKVKSVLYFNRGISDIDLRLSKQAKYTV 60
Wood46_D1        IPYTITVNGTSQNILSSLTFNKNQNISYKDIENKVKSVLYFNRGISDIDLRLSKQAEYTV 60

Mu50_D1          HFKNGTKRVIDLKSGIYTADLINTSDIK 88
RF122_D1         YFKNGAKRVIDLKAGIYTADLINTSEIK 88
N315             HFKNGTKRVIDLKSGIYTADLINTSDIK 88
JH9Map_D1        HFKNGTKRVIDLKSGIYTADLINTSDIK 88
CI7_D1           HFKNGTKRVVDLKAGIHTADLINTSDIK 88
USA300_D1        YFKNGTKKVIDLKAGIYTADLINTSEIK 88
MSSA476_D1       HFKNGTKRVIDLKSGIYTADLINTSDIK 88
MW2_D1           HFKNGTKKVIDLKSGIYTADLINTSDIK 88
8325_D1          YFKNGTKKVIDLKAGIYTADLINTSEIK 88
COL_D1           YFKNGTKKVIDLKAGIYTADLINTSEIK 88
FDA574_D1        HFKNGTKRVVDLKAGIHTADLINTSDIK 88
Newman_D1        HFKNGTKRVVDLKAGIHTADLINTSDIK 88
MRSA252_D1       HFKNGTKRVVDLKAGIHTADLINTSDIK 88
Wood46_D1        HFKNGTKRVIDLKSGIYTADLINTSDIK 88
```

Figure 10: The Clustal W2 alignment of the amino acid sequences of Eap domain type I from fourteen different strains of *Staphylococcus aureus* species. The 14 different strains of *S. aureus* Eap include Mu50 (SAV1938), RF122 (SAB1873c), N315 (SA1751), JH9 (A5IUA7), CI-7 (AJ243790), USA300 (ABX29943), MSSA476 (SAS1861), MW2 (EFK80959.1) , 8325 (ABD31208), COL (AAW36971), FDA547 (Q53599), Newman mapN (AJ132841), MRSA252 (SAR2030) and Wood46 map-W (AJ245439). The conserved -YXNX- motif of domain type I is represented in red. The diagrammatic representation of the conserved domain with its domain type is illustrated in second part of this figure (Figure 15).

```
Mu50_D2          VPYTITVNGTSQNILSNLTFNKNQNISYKDLEGKVKSVLESNRGITDVDLRLSKQAKYTV 60
RF122_D2         VPYTITVNGTSQNILSNLTFNKNQNISYKDLEGKVKSVLESNRGIIDVDLRLSKQAKYTV 60
N315_D2          VPYTITVNGTSQNILSNLTFNKNQNISYKDLEGKVKSVLESNRGITDVDLRLSKQAKYTV 60
JH9_D2           VPYTITVNGTSQNILSNLTFNKNQNISYKDLEGKVKSVLESNRGITDVDLRLSKQAKYTV 60
CI7_D2           VPYTITVNGTSQNILSNLTFKKISKLVIKILENNVKSVLKSNRGITDVDLRLSKQAKFTV 60
USA300_D2        VPYTITVNGTSQNILSNLTFNKNQNISYKDLEDKVKSVLESNRGITDVDLRLSKQAKYTV 60
MSSA476_D2       VPYTITVNGTSQNILSNLTFNKNQNISYKDLEGKVKSVLESNRGITDVDLRLSKQAKYTV 60
MW2_D2           VPYTITVNGTSQNILSNLTFNKNQNISYKDLEGKVKSVLESNRGITDVDLRLSKQAKYTV 60
8325_D2          VPYTITVNGTSQNILSNLTFNKNQNISYKDLEDKVKSVLESNRGITDVDLRLSKQAKYTV 60
COL_D2           VPYTITVNGTSQNILSNLTFNKNQNISYKDLEDKVKSVLESNRGITDVDLRLSKQAKYTV 60
FDA574_D2        VPYTITVNGTSQNILSNLTFKKNQQISYKDLENNVKSVLKSNRGITDVDLRLSKQAKFTV 60
Newman_D2        VPYTITVNGTSQNILSNLTFKKNQQISYKDLENNVKSVLKSNRGITDVDLRLSKQAKFTV 60
MRSA252_D2       VPYTITVNGTSQNILSNLTFKKNQQISYKDLENNVKSVLKSNRGITDVDLRLSKQAKFTV 60
Wood46_D2        VPYTITVNGTSQNILSNLTFNKNQNISYKDLEDKVKSVLESNRGITDVDLRLSKQAKYTV 60

Mu50_D2          NFKNGTKKVIDLKSGIYTANLINSSDIK 88
RF122_D2         NFKNGTKKVIDLKAGIYTANLINSSDIK 88
N315_D2          NFKNGTKKVIDLKSGIYTANLINSSDIK 88
JH9_D2           NFKNGTKKVIDLKSGIYTANLINSSDIK 88
CI7_D2           NFKNGTKKVIDLKAGIYTANLINTGDIK 88
USA300_D2        NFKNGTKKVIDLKSGIYTANLINSSDIK 88
MSSA476_D2       NFKNGTKKVIDLKSGIYTANLINSSDIK 88
MW2_D2           NFKNGTKKVIDLKSGIYTANLINSSDIK 88
8325_D2          NFKNGTKKVIDLKSGIYTANLINSSDIK 88
COL_D2           NFKNGTKKVIDLKSGIYTANLINSSDIK 88
FDA574_D2        NFKNGTKKVIDLKAGIYTANLINTGGIK 88
Newman_D2        NFKNGTKKVIDLKAGIYTANLINTGDIK 88
MRSA252_D2       NFKNGTKKVIDLKAGIYTANLINTGDIK 88
Wood46_D2        NFKNGTKKVIDLKAGIYTANLINSSDIK 88
```

Figure 11: The Clustal W2 alignment of the amino acid sequences of Eap domain type II from fourteen different strains of *Staphylococcus aureus* species as mentioned in Figure 12. There is no conserved -YXNX- motif in domain type II. The diagrammatic representation of the conserved domain with its domain type is illustrated in second part of this figure (Figure 15).

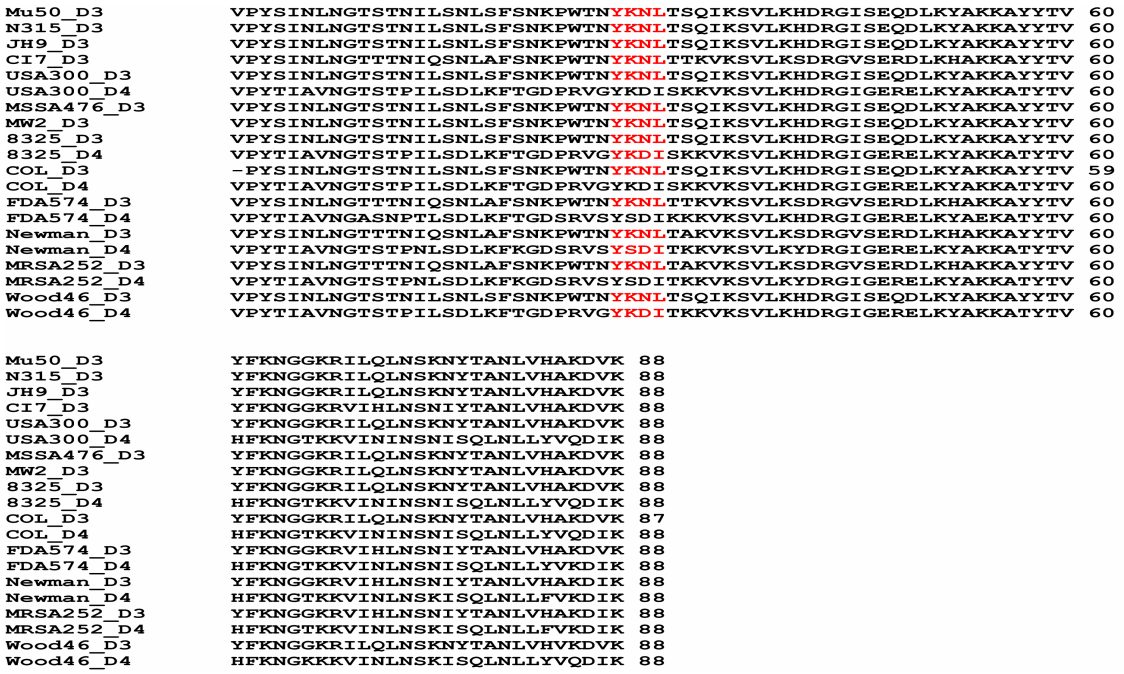


Figure 12: The Clustal W2 alignment of the amino acid sequences of Eap domain type III from fourteen different strains of *Staphylococcus aureus* species as mentioned. The conserved -YXNX- motif of domain type III is represented in red. The diagrammatic representation of the conserved domain with its domain type is illustrated in second part of this figure (Figure 15).

Figure 13: The Clustal W2 alignment of the amino acid sequences of Eap domain type IV from fourteen different strains of *Staphylococcus aureus* species as mentioned. The conserved -YXNX- motif of domain type IV is represented in red. The diagrammatic representation of the conserved domain with its domain type is illustrated in second part of this figure (Figure 15).

```
CI7_D5          VKFPVTINGFSNLVSNEFAFLHPHKITTNDLNAKLRLALASDQGITKHDIGLSERTVYKV 60
COL_D6          VKFPVTINKFENIVSNEFVFYNASKITINDLSIKLKSAMANDQGITKHDIGLAERAVYKV 60
FDA574_D6       VKFPVTINGFSNLVSNEFAFLHPHKITTNDLNAKLRLALRSDQGITKHDIGLSERTVYKV 60
Newman_D6       VKFPVTINGFSNVVSNEFAFLHPHKITTNDLNAKLRLALASDQGITKHDIGLSERTVYKV 60
MRSA252_D6      VKFPVTINGFSNVVSNEFAFLHPHKITTNDLNAKLRLALASDQGITKHDIGLSERTVYKV 60

CI7_D5          YFKDGSSKFVDLKAAKQDSKVFKATDIK 88
COL_D6          YFKNGSSKYVDLKTEYKDERVFKATDIK 88
FDA574_D6       YFKDGSSKLEDLKAAKQDSKVFKATDIK 88
Newman_D6       YFKDGSSKFVDLKAAKQDSKVFKATDIK 88
MRSA252_D6      YFKDGSSKFVDLKAAKQDSKVFKATDIK 88
```

Figure 14: The Clustal W2 alignment of the amino acid sequences of Eap domain type V from fourteen different strains of *Staphylococcus aureus* species as mentioned. There is no conserved -YXNX- motif in domain type V. The diagrammatic representation of the conserved domain with its domain type is illustrated in second part of this figure (Figure 15).

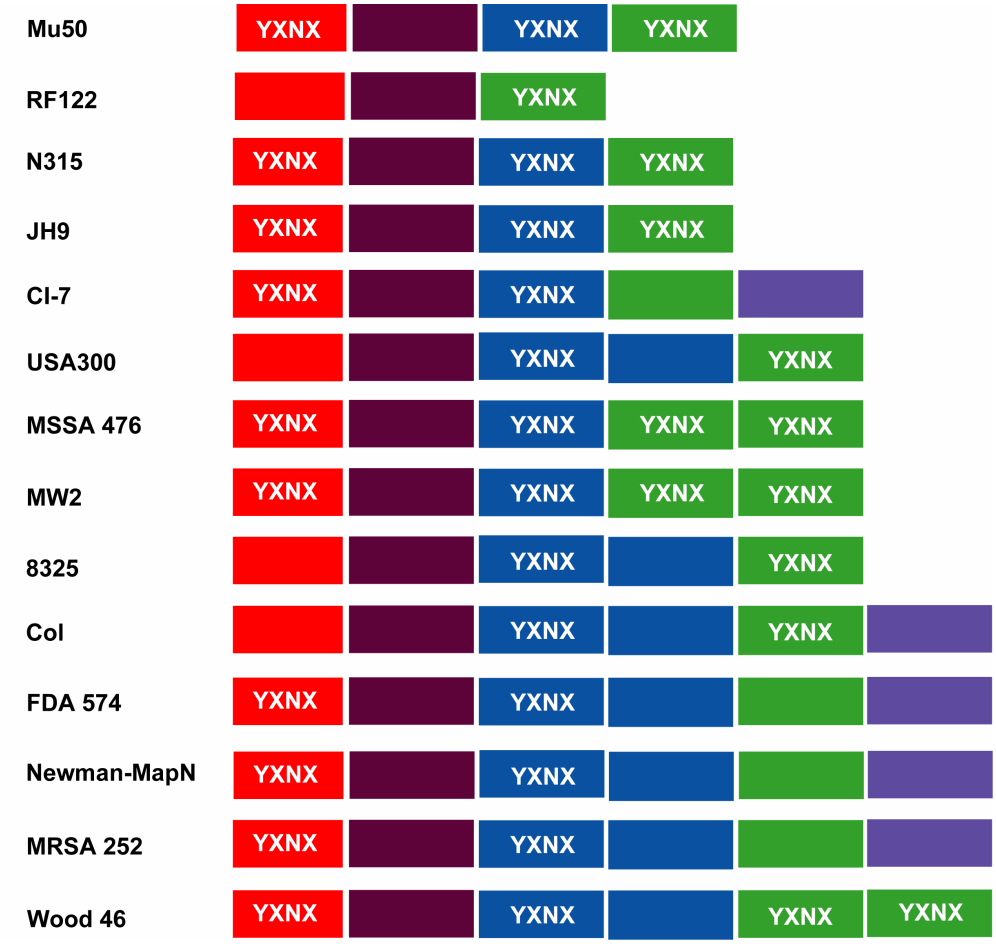


Figure 15: Distribution of the Eap domain types in 14 different *Staphylococcus aureus* strains. The domain type I (red), type II (magenta), type III (blue), type IV (green) and type V (purple) are shown. The type I, type III and type IV domain have conserved -YXNX- motifs, in which (Y) is tyrosine, (N) is asparagine and X is variable.

9.2 Selection of Mu50 Eap and generation of its derivatives

In this work, we focused mainly on the Eap of *S. aureus* strain Mu50 (Mu50 Eap) for the following reasons: The Eap of strain Mu50 is well characterized and shown to be functional (Geisbrecht et al., 2005; Hammel et al., 2007). Moreover, it harbors three YXNX motifs in the domain types I, III, and IV. Interestingly, it also lacks the duplications of the domain types found in other derivatives, thereby reducing the workload to mutate all YXNX motifs present in the Eap of interest. Additionally, the expression-plasmids harboring the wild type of Mu50 Eap and all single domains were already available (Geisbrecht et al., 2005), which accessed us to create desired mutations in the constructs.

In addition, the amino acid tyrosine is an important site for the phosphorylation which might play an important role during cell signaling. The phosphorylation of tyrosine can modulate the functional activity and/or promote interaction with other molecules (Schlessinger, 2000). As a result, in this study we speculated that the phosphorylation of tyrosine residues in the conserved YXNX motifs might play an essential role for Eap functions.

In reference to the above mentioned experimental and theoretical assumptions, the tyrosine residues of the conserved YXNX motif in domains 3 and 4 of Mu50 Eap were mutated to phenylalanine at Y296F and Y403F, respectively, with the mutagenic primers mentioned in Table 2. The point mutations were done for the full-length (FL) Mu50 Eap, and for single, double, and triple domain constructs as depicted in Figure 16 and Appendix I.1). As a result of our mutagenesis attempts, eight mutants were created having point mutations leading to tyrosine to phenylalanine exchanges in the FL Eap of domain 3 (FLD3) and domain 4 (FLD4), the domains 2 to 4 containing fragments 2L4 mutated in domain 3 (2L4D3) and domain 4 (2LD4), the domains 3 and 4 containing fragments 3L4 mutated in domain 3 (3L4D3), and domain 4 (3L4D4), and the single domain 3 mutant (D3M) and domain 4 mutant (D4M). All mutations were confirmed by sequencing (see Appendix I. 2).

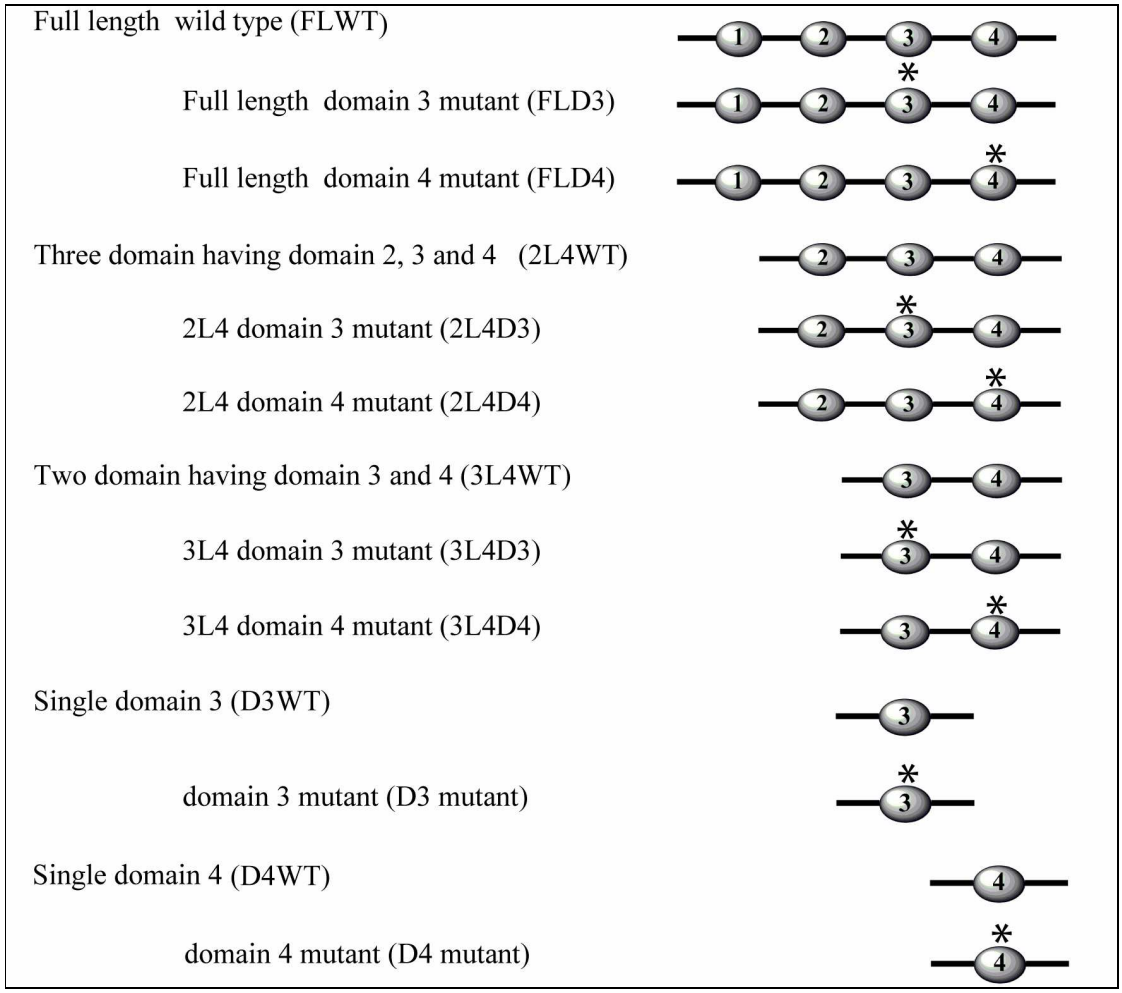


Figure 16: Schematic representation of the Mu50 Eap wild-type and its mutants used in this study. Eight Eap mutants were constructed by exchanging Tyrosine (T) to phenylalanine (F) in the putative YXNX motifs of domains 3 and 4, respectively. The asterisk '*'represents the point mutation that has been introduced.

9.3 Heterologous expression of the recombinant wild-type and mutant Mu50 Eaps

E. coli strain BL21(DE3) was used as a heterologous hosts for the expression of the Mu50 Eap and its different mutant derivatives. The Mu50 FL *eap* sequence and the different Mu50 Eap fragments were sub-cloned into the plasmid pT7HMT (Geisbrecht et al, 2005) into the *Sal*I/*Not*I restriction sites (Appendix I.1).

In order to determine the optimal conditions for the production of large amounts of the recombinant proteins, expression trials were performed using 5 ml cultures of the transformed *E. coli* host cells. The yield of the protein expression was first tested in the *E. coli* strain

BL21(DE3)pLys but the expression was very low in this expression host. Therefore, we used strain BL21(DE3) to optimize the expression conditions. The addition of the inducer IPTG to the bacterial culture at different growth stages identified an A_{600} of 1 as the best induction time-point. These studies also revealed that a high shaking speed (230 rpm) and a culture/flask volume of 1:10 were beneficial for a high Eap expression yield. In addition, an incubation temperature of 37°C was found to be the best temperature for the expression of this protein.

The recombinant proteins of all Eap constructs were expressed accordingly, and whole cell extract fractions of the uninduced and induced cultures were tested on SDS-PAGEs to confirm the over-expression of Eap and its derivatives. The representative picture for the Mu50 FL Eap is shown in Figure 17.

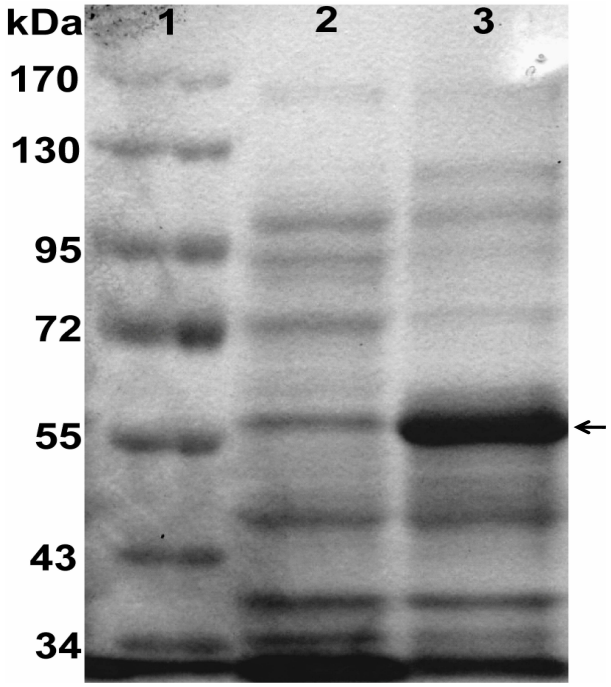


Figure 17: Heterologous expression of the wild-type Mu50 Eap. Coomassie blue-stained SDS-PAGE of Mu50 Eap wild-type protein. Lane 1 - prestained protein ladder (Fermentas), lane 2 - before induction, lane 3 – after induction (18 hr). The numbers in the column represents the molecular weight (kDa) and the arrow denotes the expression of the target protein (~60 kDa)

After optimizing the expression conditions, large scale batch cultures were carried out in 2 L culture flasks following the growth conditions as mentioned above. The cells were harvested and were stored at -20°C until purification.

9.4 Purification of the wild-type and mutant recombinant Mu50 Eap

The purification of the recombinant Eap derivatives of *S. aureus* Mu50 was performed by affinity chromatography (Ni^{2+}-NTA column) followed by size exclusion chromatography (superdex-75) using a FPLC (fast performance liquid chromatography) unit (BioRad) equipped with a fraction collector. The system simultaneously analyzes the eluent for conductivity, pH and UV absorbance. The working pressure of the column was below 40 psi.

In the first step of the purification, the supernatants of cell lysates were applied to a Ni^{2+}-NTA column under denatured condition, and the hexahistidine-tagged proteins were eluted following a single, stringent washing step (Figure 18; data for FLD4 shown). Purifying proteins under denatured conditions has several advantages, as it helps to stabilize labile proteins by inactivating proteolytic enzymes which might be present in the crude sample extracts, and it also reduces the binding of contaminating proteins to the resin.

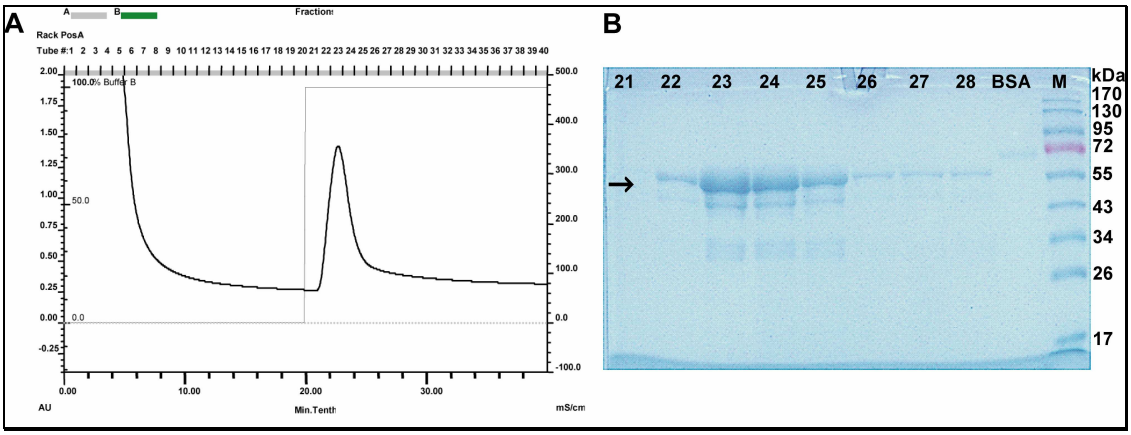


Figure 18: Denature purification of the Mu50 Eap protein. A. FPLC chromatogram of the Mu50 full-length domain 4 (FLD4) mutant by Ni^{2+}-NTA affinity chromatography. The tube numbers on top represent the collected fraction during purification. The x-axis and y-axis represent the absorbance at 280 nm and time (min), respectively. The peak at tubes 22 and 23 is the major purified fractions of Mu50 Eap (FLD4). **B. SDS-PAGE of fractions 21 to 28 of the Mu50 Eap FLD4 collected from Ni^{2+}-NTA affinity chromatography**. The arrow represents the target protein. The 'M' represents the prestained protein ladder (Fermentas) and BSA (bovine serum albumin) is used as a control.

Following the initial denatured purification step, the Eap proteins were refolded in a native buffer and reapplied to a Ni^{2+}-NTA column that was pre-equilibrated with native wash buffer. After washing, the proteins were eluted by a native elution buffer, and fractions were collected using

the fraction collector. Aliquots of each fraction were applied to SDS-PAGE to identify the fraction containing the highest concentration of the protein of interest (Figure 19; data for FLD4 shown).

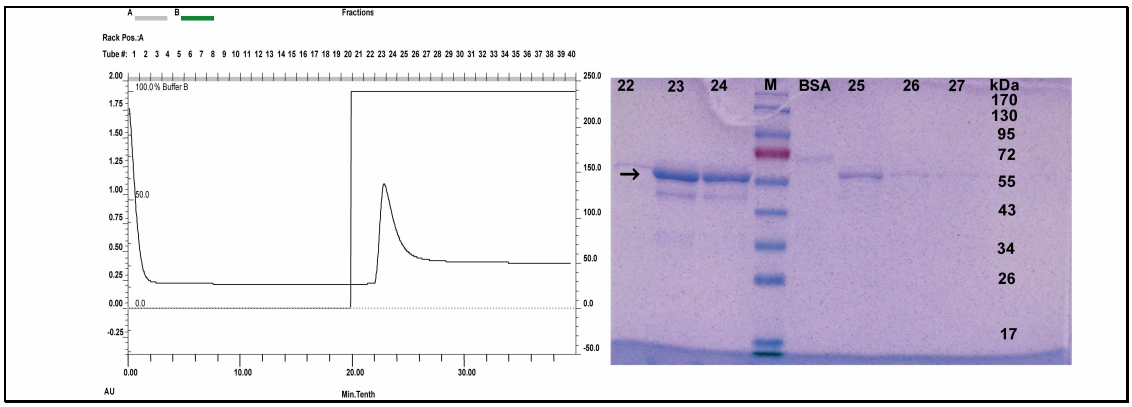


Figure 19: **Native purification of Mu50 Eap protein. A. FPLC chromatogram of the Mu50 full-length domain 4 (FLD4) mutant by Ni-NTA affinity chromatography.** The tube numbers on top represent the collected fraction during purification. The x-axis and y-axis represent the absorbance at 280 nm and time (min), respectively. **B. SDS-PAGE of the Mu50 FL D4 mutant fractions 22 to 27 obtained by the Ni-NTA affinity chromatography.** The arrow represents the target protein. The 'M' represents the prestained protein ladder (Fermentas).

In the next step, fractions of interest were pooled and concentrated to the final volume of approximately 2-3 ml using 10 kDa and 30 kDa cutoff centricons (Amicon Millipore), respectively, according to the molecular weight of the recombinant Eap proteins (full-length and fragments). In the last step, the concentrated proteins were applied to a Superdex-75 column (Figure 20; data for FLD4 shown).

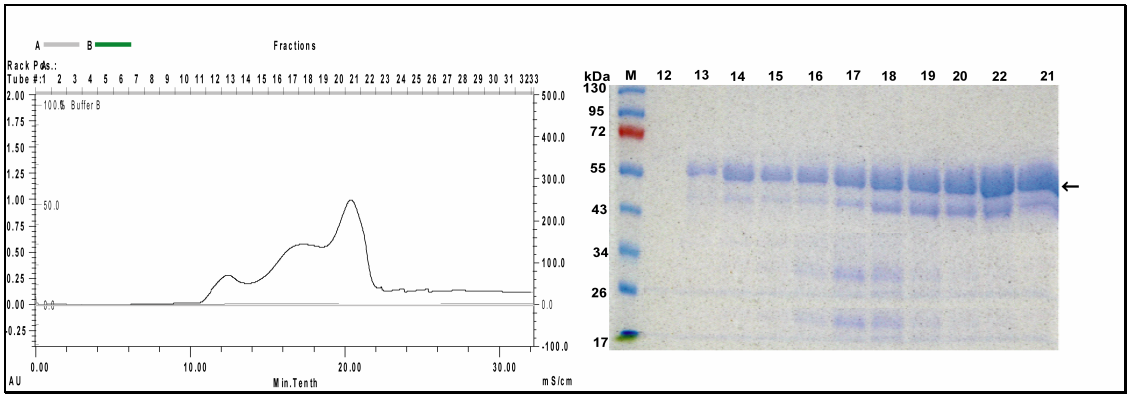


Figure 20: A. Purification of Mu50 full-length Domain 4 (FLD4) mutant by size exclusion chromatography. The tube numbers on top represent the collected fraction during purification. The x-axis and y-axis represent the absorbance at 280 nm and time (min), respectively. **B. SDS-PAGE of Mu50 FLD4 mutant.** Lane 2 to 12 represents the respective fractions (12 to 21) collected during the size exclusion chromatography. The low molecular bands present in the fractions were either impurities or degraded Eap. The arrow represents the target protein. The 'M' represents the prestained protein ladder (Fermentas).

The eluted fractions were again collected into different tubes, pooled and concentrated as mentioned before, and the protein contents of the concentrated samples were determined by the BCA method. The purified Mu50 Eap derivatives are shown in Figure 21 and Table 5, respectively.

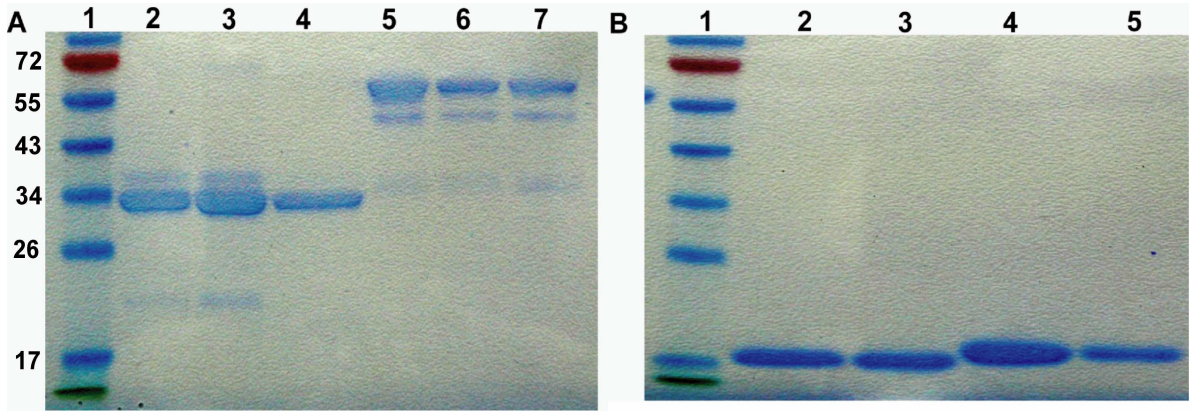


Figure 21: SDS-PAGE of purified Mu50 Eap derivatives. A. lane 1: marker; lane 2: two domains having domain 3 and 4 fragment wild-type (3L4 WT); lane 3: two domains having domain 3 and 4 fragment with the mutation in domain 3 (3L4D3), lane 4: two domains having domain 3 and 4 fragment with the mutation in domain 4 (3L4D4), lane 5: full-length wild-type (FLWT), lane 6: full-length domain 3 mutant (FLD3), lane 7: full-length domain 4 mutant (FLD4). **B**. lane 1: marker, lane 2: single domain 3 wild-type (D3WT), lane 3: single domain 3 mutant (D3M), lane 4: single domain 4 wild-type (D4WT), lane 5: single domain 4 mutant (D4M).

9.5 Endotoxin purification

The endotoxin LPS (lipopolysaccharide), generated by Gram-negative bacteria such as *E. coli*, is toxic to eukaryotic cells and may have severe inhibitory effects in eukaryotic cell assays. However, endotoxin concentrations below 0.1 endotoxin units (EU) per µg protein are considered to be non effective in cell culture assays. Since LPS contaminations are frequently encountered in protein samples obtained from *E. coli* cultures, all Eap proteins were purified by using EndoTrap Blue columns (Cambrex, Walkersville, MD) as described by the manufacturer, and the residual endotoxin contents of the samples were subsequently tested using the limulux test system (Lonza). After the EndoTrap purification step, all samples showed endotoxin concentrations that were below 0.0032 EU per 30 µg of protein (Table 5).

Table 5: Protein and endotoxin concentrations of Mu50 Eap samples. The concentrations of the different Mu50 Eap wild-type and mutant protein samples were identified by the BCA method after size exclusion chromatography and endotoxin purification. The amount of endotoxin was calculated for 30 µg of the corresponding Eap sample.

Mu50 Eap	Conc. of Eap (µg/µl)	Conc. of endotoxin (EU/ml)	Conc. of endotoxin per 30 µg of protein (EU/30 µg)
FL WT	0.958	0.104	0.0032
FLD3 mutant	1.858	0.088	0.0014
FLD4 mutant	1.684	0.072	0.0012
3L4 WT	1.733	0.050	0.0008
3L4 D3 mutant	2.219	0.090	0.0012
3L4 D4 mutant	1.519	0.009	0.0001
D3 WT	2.06	0.059	0.0008
D3 mutant	2.26	0.067	0.0008
D4 WT	1.838	0.077	0.0012
D4 mutant	1.764	0.102	0.0017

9.6 Cell culture experiments

9.6.1 Adherence assay

Previous studies showed that externally added Eap of strain Newman could enhance the binding of *S. aureus* to fibroblasts and epithelial cells (Hussian et al., 2008). However, the effect of the wild-type and mutated Eap of Mu50 in the adherance of *S. aureus* has not been studied so far. Therefore, in this study, we examined the role of externally added Eap and its derivatives on the adherence of *S. aureus* SA113 to HaCaT keratinocyte cells. HaCaT cells grown to confluency were inoculated with 30 µg/ml of the Eap samples and incubated for 1 hr. After the Eap treatment, cells were washed with PBS to remove unbound Eap, and subsequently inoculated with *S. aureus* SA113 with an MOI of 100, and incubated for 2 hr 30 min. After the second incubation step, cells were trypsinized, sonicated, and the lysates were plated on agar plates to determine the colony forming units representing the adherent (Figure 22) and internalized bacteria.

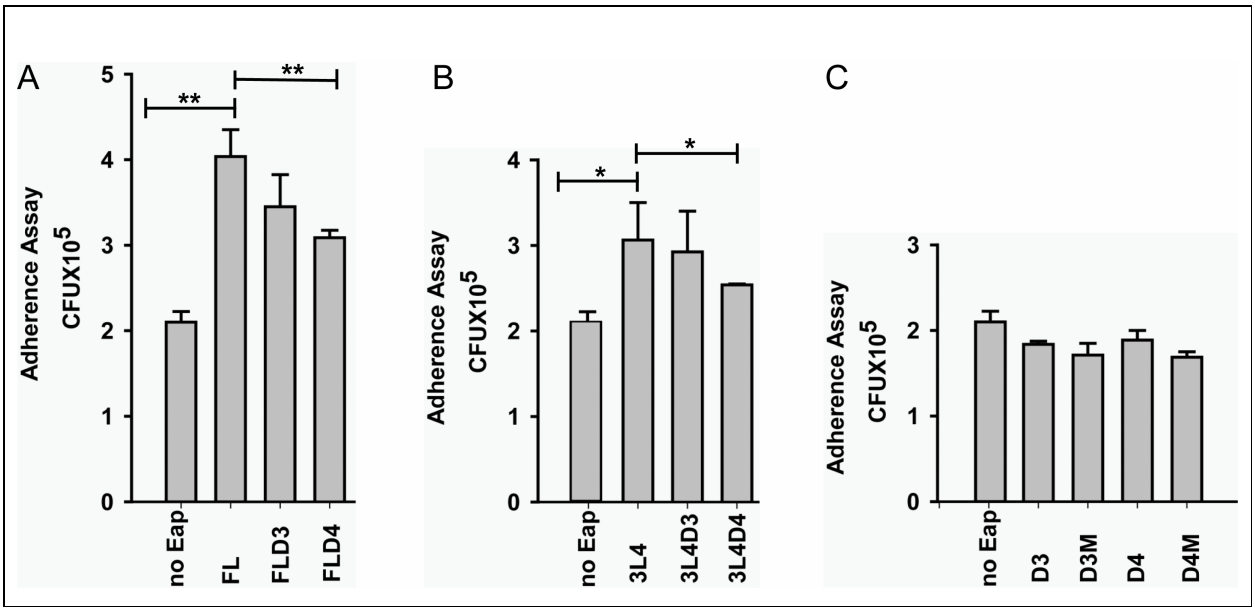


Figure 22: Adherence of bacteria to keratinocytes after the preincubation with Eap. Confluent layers of keratinocytes were incubated for 1 h with 30 µg/ml of the (A) FLWT, FLD3, FLD4; (B) 3L4WT, 3L4D3, 3L4D4; (C) D3WT, D3M, D4WT and D4M, of the Mu50 Eap, washed with PBS, inoculated with strain SA113 (MOI of 100) and incubated at 37°C for 2 hr and 30 min. Bacteria were detached and viable counts were determined. Data represent the means and the error bars show standard deviation of 3 independent experiments performed in duplicate. Statistical significances between the wild-types and their derivatives were determined using the

Mann-Whitney U-Test. *P* values of individual wild-types versus their mutants were <0.05 (∗) and <0.01 (∗∗).

This assay revealed a clear difference between wild-type and mutant proteins in terms of adherence of bacteria to keratinocytes. In correlation with previous findings (Hussian et al., 2008), we detected an increased adherence of *S. aureus* to keratinocytes after preincubation of the eukaryotic cells with the full-length wild-type Eap (FL) (*P*<0.01) (Figure 22. A). Incubating the HaCaT cells with the two-domain 3 and 4 fragment of Eap (3L4WT) also increased the binding of *S. aureus* to the keratinocytes (*P*<0.05) (Figure 22. B), although this effect was not as pronounced as that observed with the full-length protein. However, treating the keratinocytes with the single domains 3 and 4, respectively (Figure 22. C) did not alter the binding capacity of *S. aureus* to the HaCaT cells, indicating that at least a tandem repeat of the Eap domains is required for this effect.

Interestingly, treating HaCaT cells with the FLD3 and FLD4 mutant proteins showed a decreased adherence of *S. aureus* to the eukaryotic cells, if compared to the wild-type FL protein, and this decrease in adherence was found to be more pronounced with the full-length protein being mutated in domain 4 (*P*<0.01) (Figure 22. A). Similarly, treating the HaCaT cells with the two domain Eap fragment with the mutation in domain 4 (3L4D4) yielded a less pronounced increase in adhesion of SA113 to the keratinocytes than incubating the eukaryotic cells with the wild-type fragment (3L4WT) (Figure 22. B). However, no such effect was observed with the two-domain fragment being mutated in the YXNX motif of domain 3, which induced an increase in adhesion that was comparable to the wild-type fragment. Like the single domains D3WT and D4WT neither the mutated variant D3M nor D4M showed the change in the binding capacity of *S. aureus* to the HaCaT cells (Figure 22. C).

9.6.2 Internalization assay

The Eap-stimulated attachment of *S. aureus* to fibroblast and epithelial cells is also reported to increase the internalization of the bacterium into the eukaryotic cells for strain Newman (Hussain et al., 2008). Thus, we were curious whether and how the mutations of Eap could sustain this effect. Consequently, we carried out an internalization assay that essentially mimicked the

adhesion assay described above, with the expectation that all attached but not internalized bacteria were lysed by lysostaphin treatment after the second incubation period. The level of internalization was again determined by assaying the colony forming units on agar plates (Figure 23).

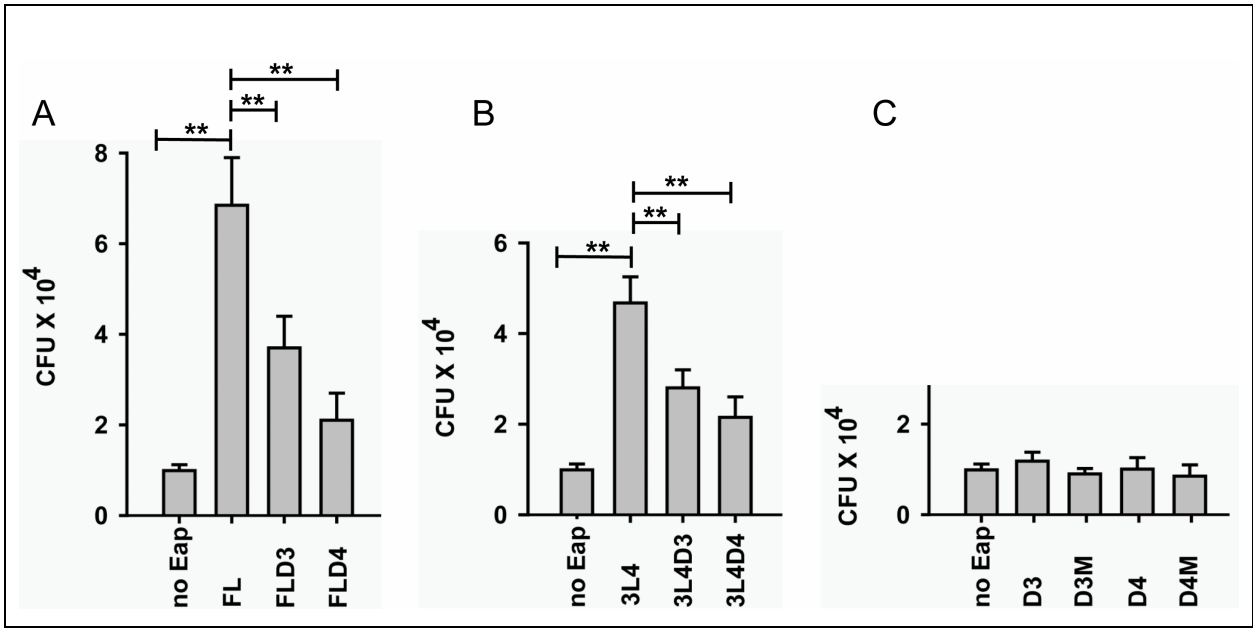


Figure 23: Internalization of bacteria to keratinocytes after preincubation with Eap. Confluent layers of keratinocytes were incubated for 1 hr with 30 μg/ml of the (A) FLWT, FLD3, and FLD4; (B) 3L4WT, 3L4D3 and 3L4D4; (C) D3WT, D3M, D4WT and D4M, of the Mu50 Eap, washed with PBS, inoculated with strain SA113 (MOI of 100) and incubated at 37°C for 2 hr and 30 min. To eliminate adherent bacteria, wells were further incubated with lysostaphin to kill the extracellular bacteria. Cells were detached and lysed, and bacterial viable counts were determined. Data represent the means and the error bars show standard deviation of 3 independent experiments performed in duplicate. Statistical significances between the wild-types and their derivatives were determined using the Mann-Whitney U-Test. *P* values verses the control were <0.01 (∗∗) and <0.05 (∗).

Similar to the adhesion assays, we also observed clear differences in the internalization assays after the incubation of keratinocytes with the WT- and mutated-Eaps. The preincubation of the HaCaT cells with the full-length Eap protein yielded an almost 7-fold increase in internalization of SA113 into this cell type ($P<0.01$) (Figure 23. A). However, the treatment of HaCaT cells with the mutated full-length proteins FLD3 and FLD4, significantly lower the internalization of SA113 into the keratinocytes if compared to the wild-type protein ($P< 0.01$) (Figure 23. A). Once again, we observed that the mutation in domain 4 seemed to inhibit the stimulative effect of the

Eap more than the mutation of domain 3. Similarly, by incubating the HaCaT cells with the two-domain fragments, the strongest increase in invasiveness was observed with the unmutated fragment, 3L4WT (P<0.01) (Figure 23. B), followed by the domain 3 mutant, while the domain 4 mutant 3L4D4 showed the smallest effect. In comparison to the full-length protein (FLWT), the 3L4WT fragment was less effective (P<0.05) in inducing the internalization of SA113 into keratinocytes. No differences in invasiveness were observed after the treatment with the single mutant derivatives (Figure 23. C), which did not seem to stimulate the uptake of bacteria by the eukaryotic cells.

9.6.3 ERK phosphorylation assay

Eap was shown to interfere with the ERK phosphorylation pathway in endothelial cells (Sobke et al., 2006). To discern whether the activation of the ERK pathway is inhibited or reduced by Eap in HaCaT cells, we explored the phosphorylation of ERK 1/2 in response to Eap. For this purpose, lysates of HaCaT cells, which were stimulated with 50 ng/ml of keratinocyte growth factor (KGF) were immunoblotted with a phosphospecific antibody directed against the activated forms of ERK 1/2 (phosphos Thr202/Tyr204). Similar to the observed results in endothelial cells (Sobke et al., 2006), we detected an increase in ERK phosphorylation in keratinocytes upon stimulation with the cognate growth factor, in which the treatment of HaCaT cells with KGF (50 µg/ml) showed an increased phosphorylation of ERK 1/2 with a maximum at 8 min after the addition of the growth factor (Figure 24).

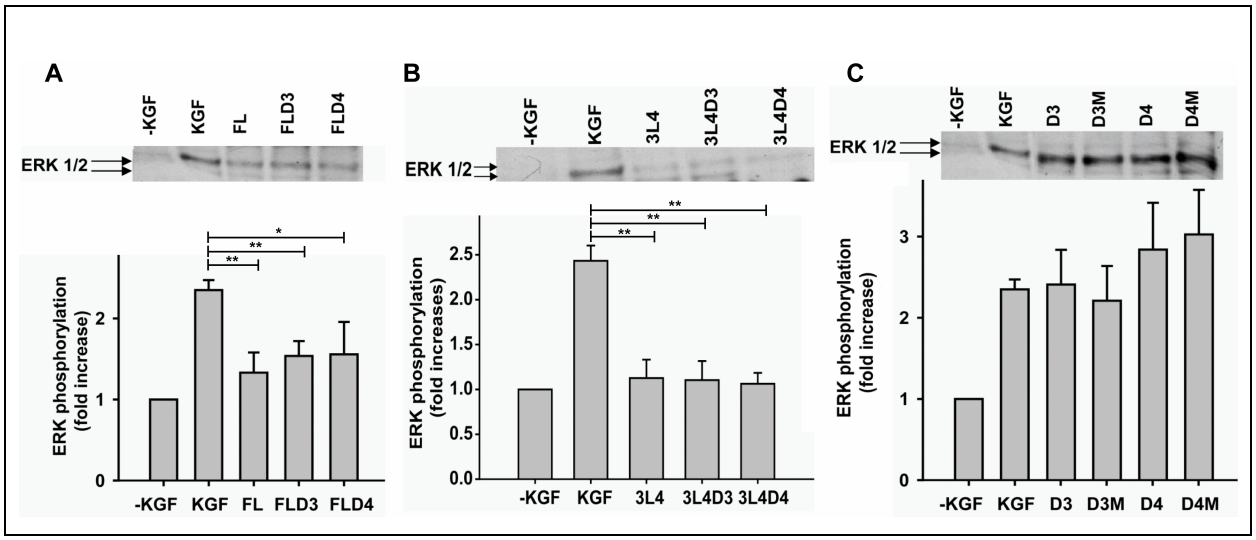


Figure 24: Effect of Eap on KGF induced ERK phosphorylation. Serum deprived HaCaT cells were left untreated or incubated with different Mu50 Eaps; (A) FLWT, FLD3 and FLD4, (B) 3L4WT, 3L4D3 and 3L4D4 (C) D3WT, D3M, D4WT and D4M, of Mu50 Eap (30 µg/ml of each) for 1 hr before the ERK phosphorylation was induced with 50 ng/ml KGF for 8 min. After cell lysis, 30 µg of total cell protein per lane were analysed by SDS-PAGE on a 10% gel and used for Western blot analysis with antibodies specific to the phosphorylated form of ERK 1/2. Densitometric evaluation of the ERK 1/2 phosphorylation signals normalized against the phosphorylation signal in the uninduced control (-KGF)**.** Data represent the means and the error bars show the standard deviation of 3 independent experiments performed in duplicate. *P* values verses the control (KGF) were <0.05 (∗) and <0.01 (∗∗).

Similar to the situation observed for endothelial cells (Sobke et al., 2006), we found that the preincubation of keratinocytes with the wild-type Eap (FLWT) largely allowed to suppress the KGF-induced ERK phosphorylation ($P<0.01$). Interestingly, preincubating the HaCaT cells with the mutated full-length Eap derivatives FLD3 ($P<0.01$) and FLD4 ($P<0.05$) reduced the KGF-stimulated phosphorylation of ERK to a similar extent as the wild-type Eap (Figure 24. A), and the same effect was observed for the two domains Eap fragment protein 3L4WT and its mutant variants 3L4D3 and 3L4D4, which suppressed the KGF-stimulated phosphorylation of ERK (Figure 24. B). The treatment of the cells with single domain variants D3 and D3M of Eap, on the other hand, did not show any interference with the KGF triggered ERK1/2 phosphorylation, while the domain 4 variants (D4 and D4M) showed a slightly increase in ERK1/2 phosphorylation of the keratinocytes, although this was not statistically significant (Figure 24. C).

9.6.4 Proliferation assay

A previous study indicated that Eap affects the proliferation of peripherial blood mononuclear cells (PBMCs) in a concentration-dependent manner (Hussain et al., 2008). Since we were also interested to see the effect on the growth factor stimulated proliferation of keratinocytes, we assayed the KGF response of HaCaT cells in presence and absence of Eap by using an EdU incorporation test.

We first determined the impact of the Eap concentration on HaCaT cell proliferation (Figure 25). For this purpose, HaCaT cells were incubated for 24 hr with the increasing concentration (2.5 to 100 µg/ml) of the full-length wild-type Eap, before the cells were treated with 10 mM EdU. HaCaT cells incubated with 2.5 µg/ml of Eap seemed to display a slight increase in proliferation, however, this was not statistically significant. Incubating the keratinocytes with higher concentration of the Eap (5-100 µg/ml), on the other hand, reduced the proliferation in a dose dependent manner, and this effect became statistically significant with concentrations above 10 µg/ml. Keratinocytes incubated with a proteinase K treated Eap sample showed a proliferation rate similar to the KGF-stimulated control, confirming that this effect is indeed Eap-dependent.

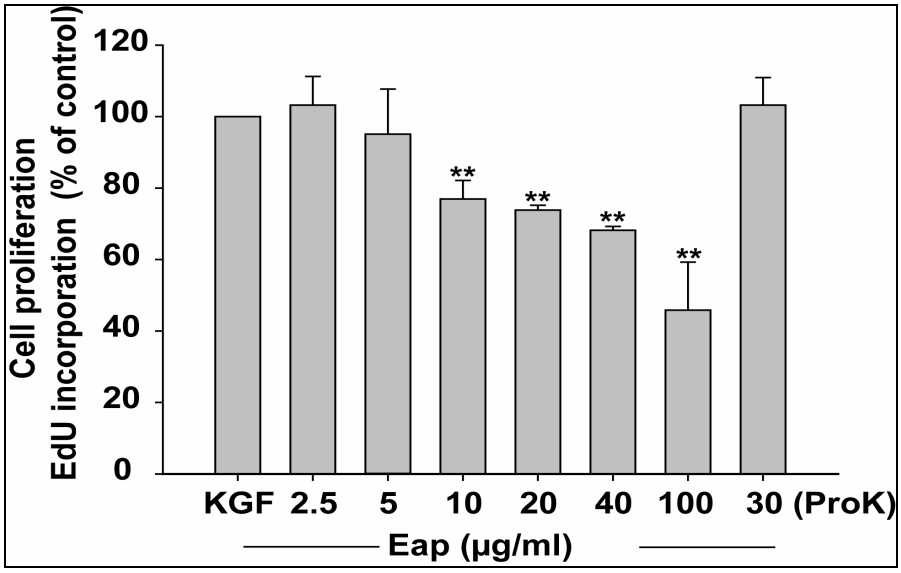


Figure 25: Proliferation of HaCaT cell in the absence/presence of increasing concentrations of Mu50 FL WT Eap. The HaCaT cells were treated without and with increasing concentration of Mu50 Eap FL. The value indicated below each bar represents the concentration of Eap used. The cells were treated with 10 mM EdU and the proliferation of the cells was measured by a plate reader. Data represent the means and the error bars show standard deviation of 2 independent experiments performed in duplicate. The *P* value verses the control (KGF) was <0.01 (∗∗).

In the second step, the effect of the Eap derivatives was tested (Figure 26). HaCaT cells that were treated with keratinocytes growth factor showed a 100% proliferation after 24 hr of growth, whereas the cells that were treated with the full-length Eap derivatives (FLWT, FLD3, and FLD4) and the domains 3 and 4 fragment derivatives (3L4WT, 3L4D3, and (3L4D4) showed a reduced proliferation, which was below 55% ($P<0.01$). In addition, HaCaT cells incubated with the single domain Eap variants (D3WT, D4WT, D3M and D4M) were less efficient in reducing the proliferation rates in comparison with the full-length and two domain derivatives, although the proliferation rates were still below the control cells that were treated with only KGF ($P<0.05$). However, no apparent differences in proliferation were observed between the keratinocytes that were treated with the wild-type Eaps and their mutant variants.

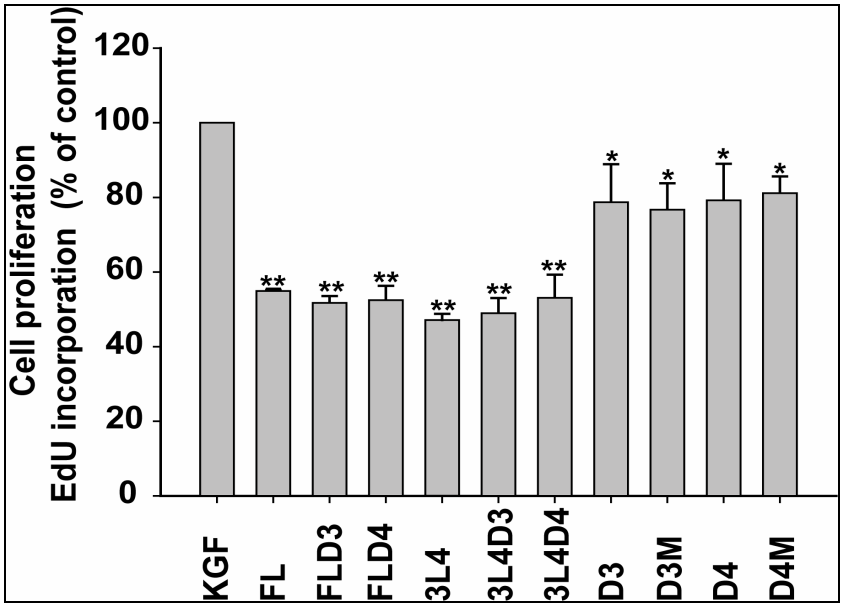


Figure 26: Proliferation of HaCaT cell in the presence of the Eap derivatives. The HaCaT cells were incubated for 24 hr with KGF and with Eap samples; FLWT, FLD3, FLD4, 3L4WT, 3L4 D3, 3L4D4, D3WT, D3M, D4WT and D4M of Mu50 Eap (30 μg/ml of each). After that, cells were treated with 10 mM EdU and the proliferation of the cells was measured by a plate reader at 490/510 nm. Proliferation of HaCaT was expressed as percentage of the untreated control. Data represent the means and the error bars show standard deviation of 4 independent experiments performed in duplicate. *P* values verses the control (KGF) were <0.01 (∗∗) and <0.05 (∗).

10 Discussion

Staphylococcus aureus is a one of the leading etiological agent for nosocomial infections and a number of community borne diseases. The extracellular adherence protein (Eap), a member of the family of SERAM, is one of the potent virulence factors, which is exclusively present in all *S. aureus* strains analyzed to date. This protein is known to exhibit a broad spectrum of binding capacities, and was shown to interfere with host cell signaling processes. Especially the latter properties of Eap, together with the observation that this protein harbors conserved YXNX motifs, which are discussed as potential tyrosine phosphorylation sites in a couple of eukaryotic proteins, led us to the assumption that Eap might be subject to tyrosine phosphorylation, which in turn may alter the activity of this protein. Here, we report about the construction and characterization of Eap mutants derived from strain Mu50, in which tyrosine residues of the conserved YXNX motifs of domains 3 and 4 were changed to phenylalanine.

10.1 Selection and study of Mu50 Eap domain containing conserved motif

The comparison of the amino acid sequences of the Eap domains derived from fourteen different strains of *S. aureus* identified five types of domains (designated as type I to V). In this study, we focused on the Eap of strain Mu50, which is composed of four Eap domains of type I, II, III, and IV. It is believed that the variation in the tandem repeats of Eap domain could be due to the duplication from the original single monomer during evolutionary processes.

During the Eap sequences analysis, we found that only the domain types I, III and IV contained YXNX motifs, with the YXNX motif of domain type III being the most conserved motif, followed by the YXNX motif of domain type IV (Figure 10-15). Moreover, the pronounced anti-angiogenic activity observed with domain 3 and 4 (type III and IV) fragments of Eap from strain Mu50, respectively, highlighted the importance of these domains (K. T. Preissner, unpublished data). In addition, the solved crystal structure of Mu50 Eap (Geisbrecht et al., 2005) also provides detailed structural information for this protein. Therefore, our study focused on the Eap of strain Mu50. Here, different mutated derivatives of the Mu50 Eap, FLD3 (mutation in domain 3 of full-length), FLD4 (mutation in domain 4 of full-length), 3L4D3 (mutation in domain 3 of two domain Eap 3L4), 3L4D4 (mutation in domain 4 of two domain Eap 3L4), D3 (single domain 3

mutant) and D4 (single domain 4 mutant) were created from their respective wild-type (WT) variants in the expression vector pT7HMT by site directed mutagenesis, and the corresponding proteins were expressed in *E. coli* BL21(DE3).

10.2 Study on the effect of Mu50 Eap and its derivatives on human keratinocytes

The entry of the pathogen into the host cell is initiated by bacterial binding to the host cell surfaces, which is thought to trigger or activate signals in the host cell that mediate the bacterial entry (Finlay and Falkow, 1997). A variety of molecules and macromolecular structures, collectively known as adhesins, mediate adherence of bacteria to cell surfaces. A wide range of mammalian cell surface compounds, including proteins, glycolipids, and carbohydrates, can serve as receptors for bacterial adhesions (Kenny et al., 1997). Like other pathogenic bacteria, *S. aureus* has developed various mechanisms allowing it to adhere to host components. It possesses a number of specific surface proteins which help the bacterium to attach to host factors such as plasma proteins (fibrinogens, fibronectins, collagen, vitronectin and thrombospondin) as well as to other host cell components.

10.2.1 Effect of Eap on adherence of bacteria

It has been shown that Eap act as an adhesion factor for *S. aureus* allowing it to attach to various cell types such as fibroblasts and epithelial cells (Flock et al., 2001; Hussian et al., 2002; Kreikemeyer et al., 2002). In this study, the effect of different Mu50 wild-type and mutant proteins on adherence of *S. aureus* to eukaryotic cells was studied. In particular, we were very interested to determine whether the change of the putative phosphorylation sites in domains 3 and 4 - the tyrosine residues in the conserved YXNX motifs of Eap - might affect bacterial adherence to keratinocytes. We found that preincubation of keratinocytes with the full-length wild-type Mu50 Eap (FLWT) and the unmutated domain 3 and 4 fragment, 3L4WT, increased the adherence of *S. aureus* to keratinocytes, and this adhesion phenotype was reduced with the mutated versions of these derivatives (FLD3, FLD4, 3L4D3 and 3L4D4) (Figure 22). However, the preincubation of HaCaT cells with single domains Eap (D3WT and D4WT) and their mutated counterparts (D3M and D4M) did not increase the adhesion of *S. aureus* to the eukaryotic cells, suggesting the need of at least two Eap repeats for this type of host cell interaction. Likewise, the

addition of full-length Eap of *S.aureus* Newman was able to enhance the adherence of *S. aureus* to fibroblast but the single domain fragments (D1 to D5) were not able to show this effect (Hussian et al., 2008), which correlates well with our findings.

10.2.2 Effect of Eap on internalization of bacteria

The evasion of the host immune system by internalization into host cells is a strategy used by a number of Gram-positive bacteria including *S. aureus*, *Listeria monocytogenes*, *Streptococcus pyogenes* and *Enterococus faecalis* (Ireton et al., 1996; Isberg et al., 1990; Wells et al., 2000). In addition, *S. aureus* can invade a variety of nonprofessional phagocyte cell types including cultured endothelial cells (Vercellotti et al., 1984; Ogawa et al., 1985), epithelial cells (Dziewanowska et al., 1999), fibroblasts and kidney cells (Sinha et al., 1999). Moreover, it was shown that the *S. aureus* FnBPs play a crucial role in the internalization process by promoting an actin-mediated phagocytosis of the adherent bacteria (Dziewanowska et al., 1999; Peacock et al., 1999; Sinha et al., 1999). However, bacteria lacking FNBPs can still internalize, although at a lower rate, and it was suggested that Eap might contribute to this process (Hagger et al., 2003).

Following our adhesion findings, we observed that the preincubation of keratinocytes with the Mu50 full-length Eap significantly enhanced the internalization of SA113 into keratinocytes. Interestingly, this effect appeared to be reduced when the mutated full-length variants FLD3 and FLD4 were used, with FLD4 showing a significantly decreased internalization if compared to the wild-type (Figure. 23 A). Similarly, preincubation of HaCaT with the mutated two-domain Eap fragments 3L4D3 and 3L4D4 yielded a decreased internalization of bacteria than the preincubated with the 3L4 wild-type fragment, with the differences between 3L4FL and 3L4D4 being again significant Figure. 23 B. Neither of the single domain Eap fragments (D3WT, D3M, D4WT and D4M) (Figure. 23 C) affected the internalization rate of *S. aureus*, suggesting that at least two domains of this protein need to be present to exert such an effect. The latter findings are in line with a recent study on Eap from strain Newman (Hussian et al., 2008), in which the authors observed an enhanced invasion of *S. aureus* into fibroblast in presence of the full-length protein and an Eap fragment consisting of the domains 1 to 3, while the co-cultivation of *S. aureus* and fibroblasts with all single domains (D1 to D5) did not affect the invasiveness. Our findings presented here now suggest that the tyrosine residues of the YXNX motifs might be

involved in this internalization process. Interestingly, in our study, the decrease in internalization was observed with both full-length mutants $P< 0.01$, (the FLD3 mutant having the mutation in the YXNX motif of domain 3, and the FLD4 mutant having the mutation in the YXNX motif of domain 4) compared to the wild-type suggesting both motifs to be involved in this phenomenon. Thus, it is tempting to speculate that the inactivation of both tyrosine residues (domain 3 and domain 4) in the Mu50 Eap would lead to an even larger reduction in the internalization, a hypothesis that was tried to be addressed by the construction of the respective double mutant, FLD3-D4, which, however, was not obtained in the time frame of this thesis.

10.2.3 Effect of Eap on proliferation of HaCaT cells

The effect of Eap on the proliferation of eukaryotic cells has been addressed by several studies (Sobke et al., 2006; Hussain et al., 2008; Wang et al., 2010). Eap was shown to inhibit the T-cell proliferation, the DTH (Delayed-Type Hypersensitivity) response, and to induce T-cell death by apoptosis (Lee et al., 2002). In this work, we studied the effect of Mu50 Eap and its variants on the proliferation of keratinocytes. Here, we observed that the proliferation of keratinocytes was affected by the Mu50 Eap in a dose-dependent manner (Figure 25). Interestingly, our findings correlated well with the results obtained on proliferation of PBMC in the presence of Newman Eap where the higher concentrations inhibited this process (Hussain et al., 2008).

By analyzing the effects of the Mu50 Eap derivatives on the proliferation of HaCaT keratinocytes (Figure 26), we found that the mutated variants had a similar effect on the proliferation of keratinocytes as their respective wild-type versions. However, the single domains were less efficient in suppressing the KGF-induced proliferation than the two-domain and full-length variants. These findings suggest that the tyrosines of the YXNX motifs are not likely to be involved in the Eap effect on cell proliferation. However, as we have only mutants in one of the YXNX motifs, we cannot exclude that the remaining tyrosines present in the other two domains might be sufficient to induce the inhibitory effect of Eap on cell proliferation. Nevertheless, it appears that at least two domain repeats of the protein are needed to exert this effect on HaCaT cells; this finding contrasts with the results reported by Hussain et al. (2008) who demonstrated that the treatment of PBMCs with Eap monomers results in a decreased proliferation rate of this cell type.

10.2.4 Effect of Eap on KGF induced ERK phosphorylation

Eap is known to inhibit the Ras-dependent signaling pathway by interfering with the Ras-GTP formation (Sobke et al., 2006). Thus, in this work, we were interested see the effect of Mu50 Eap and its derivatives on the KGF-induced ERK 1/2 phosphorylation in HaCaT cells. Similar to the proliferation assay, we found that the full-length Eap protein and its mutated derivatives (FLWT, FLD3, FLD4), as well as all the two domain Eap fragments (3L4WT, 3L4D3 and 3L4D4), were able to repress the KGF-induced phosphorylation of ERK 1/2. The single domain Eap fragments (D3WT, D4WT, D3M, and D4M) did not interfere with the KGF-induced ERK 1/2 phosphorylation, suggesting once again that at least a repeat of two Eap domains is needed for activity (Figures 24). Additionally, it became clear that the modifications of the tyrosine (Y) in the YXNX motifs of domains 3 and 4 did not alter the Eap effect on ERK phosphorylation, indicating that the putative phosphorylation of these motifs might not be required for this type of Eap activity. However, we cannot exclude the fact that the exchange of tyrosine to phenylalanine in one of the YXNX motif present in Eap was not sufficient to suppress the effect of Eap on ERK signaling indicating once again the need of YXNX double or triple mutants to answer this question.

10.3 Potential role of the YXNX motif in Eap

Protein phosphorylation at tyrosine residues is nowadays considered a fundamental process in the pathogenesis of a number of microorganisms (Cozzane et al., 2004; Manai et al., 1983). Several bacterial pathogens have been shown to use secretion systems to inject tyrosine kinase substrates into host cells to manipulate the host cell functions (Selbach et al., 2009). The *Helicobacter pylori* protein CagA, for instance, is translocated into gastric epithelial cells, where it is phosphorylated by Src- and Abl-tyrosine kinases (Poppe et al., 2007; Selbach et al., 2002; Stein et al., 2002; Tammer et al., 2007). Once phosphorylated, CagA binds to Src homology SH2-containing proteins, thereby activating multiple pathways which stimulate among others actin polymerization and pedestal formation (Covacci and Rappuoli, 2000).

Based on our findings presented in this study, we hypothesize that the *S. aureus* Eap can be phosphorylated, and that this affects the activity of the secreted protein, especially during the process of adhesion and internalization. Support for this assumption is given by preliminary

findings made by S. Bur (unpublished data), showing that the treatment of the full-length Eap (FLWT) with acetylphosphate reduces the internalization rate of *S. aureus* into keratinocytes. Other preliminary data indicate on the other hand that this exoprotein is secreted by the bacterium in an unphosphylated state, as Western blot analyses with an antibody specific to phosphor-tyrosines did not react with Eap (V. Molle, unpublished data). Taking into account that the secreted Eap can rebind to the bacterial cell surface via the cell wall-anchored neutral phosphatase, one can assume that it might be of interest for the bacterium to excrete Eap in an unphosphorylated state in order to exerts its full activity. Once bound to the eukaryotic cell, Eap might become phosphorylated at its YXNX motifs by kinases provided by the host cell, which in turn enhances the adhesion/internalization process. Whether this phosphorylation is mediated by eukaryotic cell kinases present on the cell surface or takes place within the cytosol of the eukaryotic cell, still needs to be identified. Here, both possibilities are feasible, since the extracellular Eap is effectively bound the cell membrane of keratinocytes, taken up by the eukaryotic cell and released into the cytosol (I. Joost and S. Bur, unpublished data).

In analogy to CagA of *H. pylori* and considering the above mentioned unpublished data of our laboratory, it is feasible that the phosphorylation of Mu50 Eap (Eap-P) at tyrosine residues in YXNX motifs might promote binding of Eap-P to Src homology (SH2) containing proteins such as GRB2, thereby increasing actin polymerization, which in turn enhances the bacterial uptake (the hypothetical functions of Eap on internalization and cell signaling are illustrated in Figure 27). Other functions of Eap, like the interference with the KGF-stimulated ERK phosporylation or the regulatory circuits that are effective during proliferation either do not require the phosphorylation of the tyrosines of the YXNX motifs of Eap, or the phosphorylation of some but not all the YXNX motifs present in this protein are sufficient to be effective in the underlying eukaryotic cell signaling pathways. Experiments are ongoing in order to address these questions.

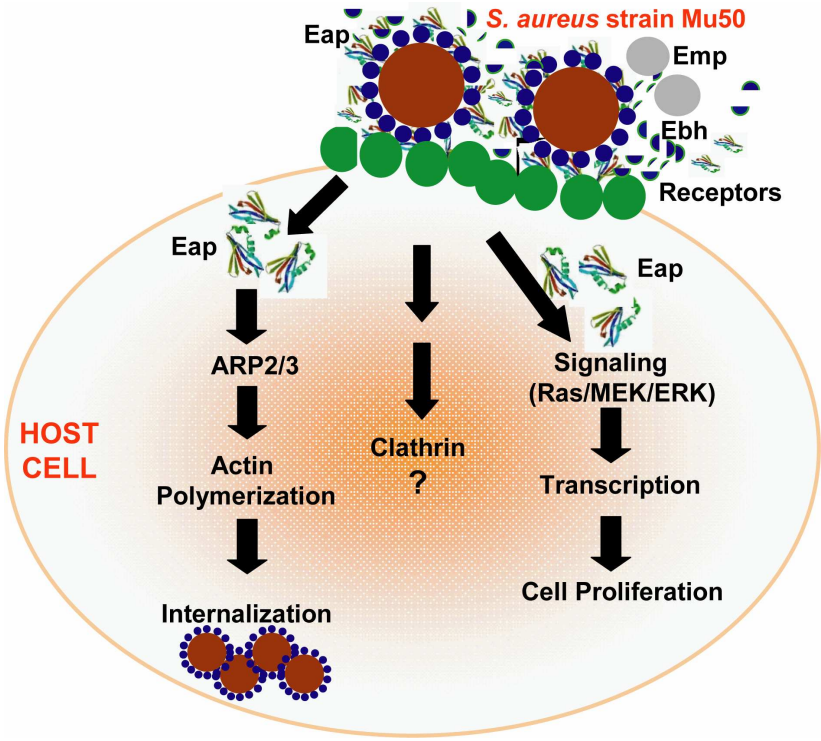


Figure 27: Cellular functions of Eap. Eap might interact with Clathrin to modulate the intracellular trafficking of vesicles, or it is bound by and translocated into the eukaryotic cell by yet unidentified ligands/mechanisms, where it is (in part) tyrosine phosphorylated by a eukaryotic cell kinase. Phosphorylated Eap (Eap-P) might bind to Src homology (SH)2-containing proteins present in the cytoplasm to activate the Arp2/3 (a seven subunit protein play a role in actin cytoskeleton) pathway which enhances actin polymerization and subsequently increases the internalization of bacteria. Additionally, Eap (and Eap-P) interferes with the MAP signaling cascade to alter the transcription of nuclear genes. In the figure, Emp - Extracellular matrix binding protein; Ebh – Extracellular matrix binding protein homologue.

Consequently, the scientific interest in bacterial pathogenesis has been growing in recent years because of the increasing bacterial resistance to antibiotics and the emergence of new microbial diseases. Tyrosine phosphorylation in bacteria represents an important regulatory device of bacterial physiology. As a result, it is essential to improve our understanding of the molecular and cellular mechanisms of bacterial pathogenesis in order to allow a better understanding of this process. In this context, the study of the conserved YXNX motifs in the Mu50 Eap and the phosphorylation on conserved tyrosine residues in this domain might involve a new concept for cell signaling and pathogenesis and sets the foundation for a novel approach of the problem.

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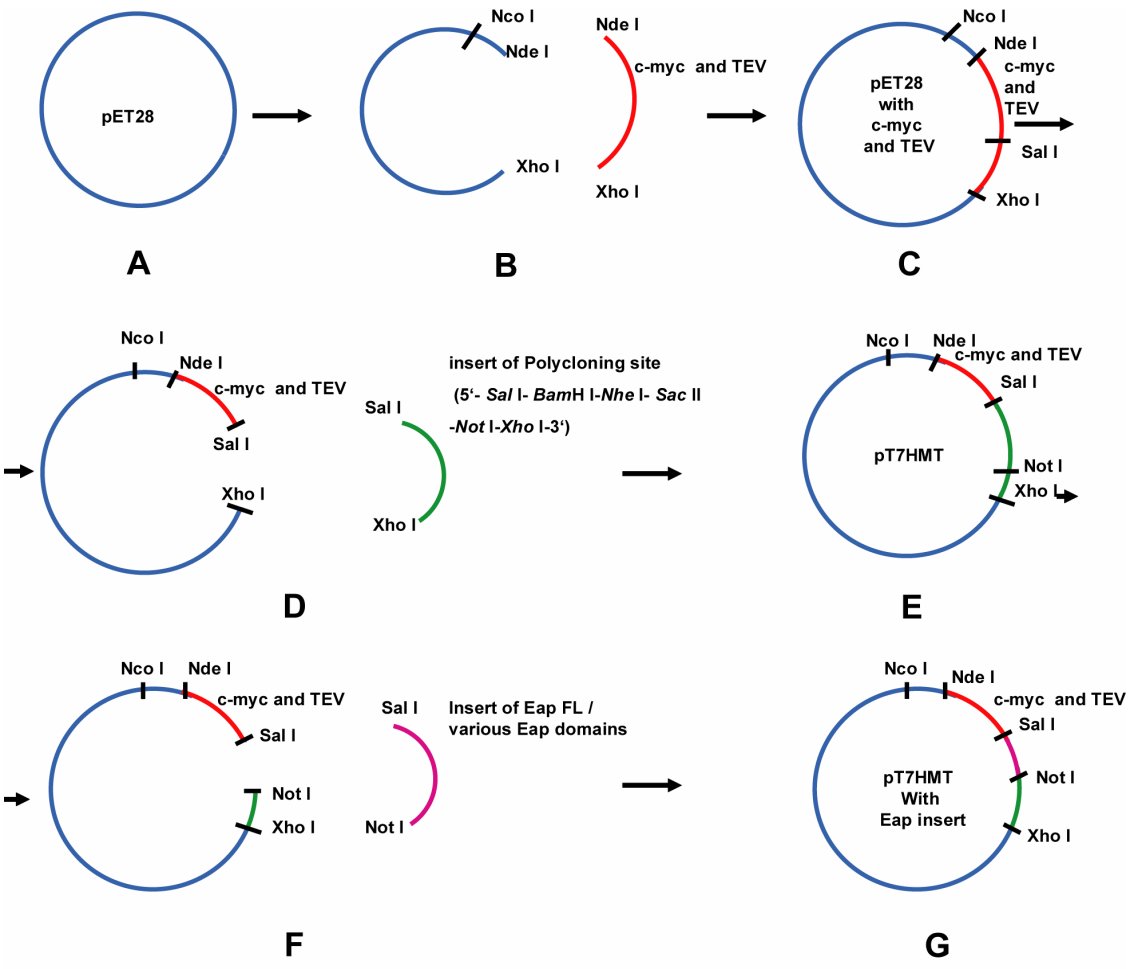
12 Appendix

APPENDIX I . 1



Schematic diagram of molecular cloning of Mu50 Eap domain-insert in pT7HMT vector starting from expression vector pET28 (picture derived after Geisbrecht et al, 2006). The expression plasmid pET28 (A) was digested with *Nde*I and *Xho*I, and an insert (c-myc and TEV) digested with same restriction enzyme was ligated (B) to form a construct having pET28 with c-myc and TEV (C). The vector was designed with *Sal*I and *Xho*I, and an insert of polycloning site digested with same restriction enzymes was ligated (D) to form a expression construct pT7HMT (E). The expression construct of Mu50 Eap full length (FL) and its various domains were prepared in pT7HMT. For this, the vector was digested with *Sal*I and *Not*I, and an insert of Eap FL (and tandem repeats) digested with same restriction enzymes were cloned (G).

APPENDIX I . 2

Sequencing results of mutated plasmid of Mu50 Eap and its repeats

1. FLD3

```
Mu50            AACATTTTATCGAATCTTTCATTTTCAAATAAACCTTGGACAAATTACAAAAATTTAACT 900
FLd3_rry8       AACATTTTATCGAATCTTTCATTTTCAAATAAACCTTGGACAAATTTCAAAAATTTAACT 73
                ********************************************* ************

Mu50            AGTCAAATAAAATCAGTACTGAAGCATGATAGAGGTATTAGTGAACAAGATTTAAAATAT 960
FLd3_rry8       AGTCAAATAAAATCAGTACTGAAGCATGATAGAGGTATTAGTGAACAAGATTTAAAATAT 133
                ************************************************************

Mu50            GCTAAGAAAGCTTATTATACTGTTTATTTTAAAAATGGTGGTAAAAGAATCTTACAGTTA 1020
FLd3_rry8       GCTAAGAAAGCTTATTATACTGTTTATTTTAAAAATGGTGGTAAAAGAATCTTACAGTTA 193
                ************************************************************
```

2. FLD4

```
Mu50            AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 1200
FLd4_rry8       AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 376
                ************************************************************

Mu50            TACAAATATTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 1260
FLd4_rry8       TACAAATTTTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 436
                ******* ****************************************************

Mu50            CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 1320
FLd4_rry8       CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 496
                ************************************************************
```

3. 2L4D3

```
Mu50            AATCAATTCAAGTGATATTAAAAGTATCAATATTAACGTAGATACAAAAAAACATATCGA 779
2L4d3prom       AATCAATTCAAGTGATATTAAAAGTATCAATATTAACGTAGATACAAAAAAACATATCGA 490
                ************************************************************

Mu50            AAATAAAGCTAAAAGAAACTATCAAGTTCCATATTCAATTAATCTAAATGGTACATCTAC 839
2L4d3prom       AAATAAAGCTAAAAGAAACTATCAGGTTCCATATTCAATTAATCTAAATGGTACATCTAC 550
                ********************** *********************************

Mu50            AAACATTTTATCGAATCTTTCATTTTCAAATAAACCTTGGACAAATTACAAAAATTTAAC 899
2L4d3prom       AAACATTTTATCGAATCTTTCATTTTCAAATAAACCTTGGACAAATTTCAAAAATTTAAC 610
                ********************************************* ************
```

4. 2L4D4

```
Mu50            AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 1200
2L4d4term       AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 596
                ************************************************************

Mu50            TACAAATATTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 1260
2L4d4term       TACAAATTTTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 656
                ******* ****************************************************

Mu50            CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 1320
2L4d4term       CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 716
                ************************************************************
```

5. 3L4D3

```
Mu50            AAACATTTTATCGAATCTTTCATTTTCAAATAAACCTTGGACAAATTACAAAAATTTAAC 899
3L4d3           AAACATTTTATCGAATCTTTCATTTTCAAATAAACCTTGGACAAATTTCAAAAATTTAAC 286
                ********************************************** ************

Mu50            TAGTCAAATAAAATCAGTACTGAAGCATGATAGAGGTATTAGTGAACAAGATTTAAAATA 959
3L4d3           TAGTCAAATAAAATCAGTACTGAAGCATGATAGAGGTATTAGTGAACAAGATTTAAAATA 346
                ************************************************************
```

6. 3L4D4

```
Mu50            AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 1200
3L4d4           AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 595
                ************************************************************

Mu50            TACAAATATTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 1260
3L4d4           -ACAAATTTTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 654
                 ****** ****************************************************

Mu50            CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 1320
3L4d4           CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 714
                ************************************************************
```

7. D3 mutant

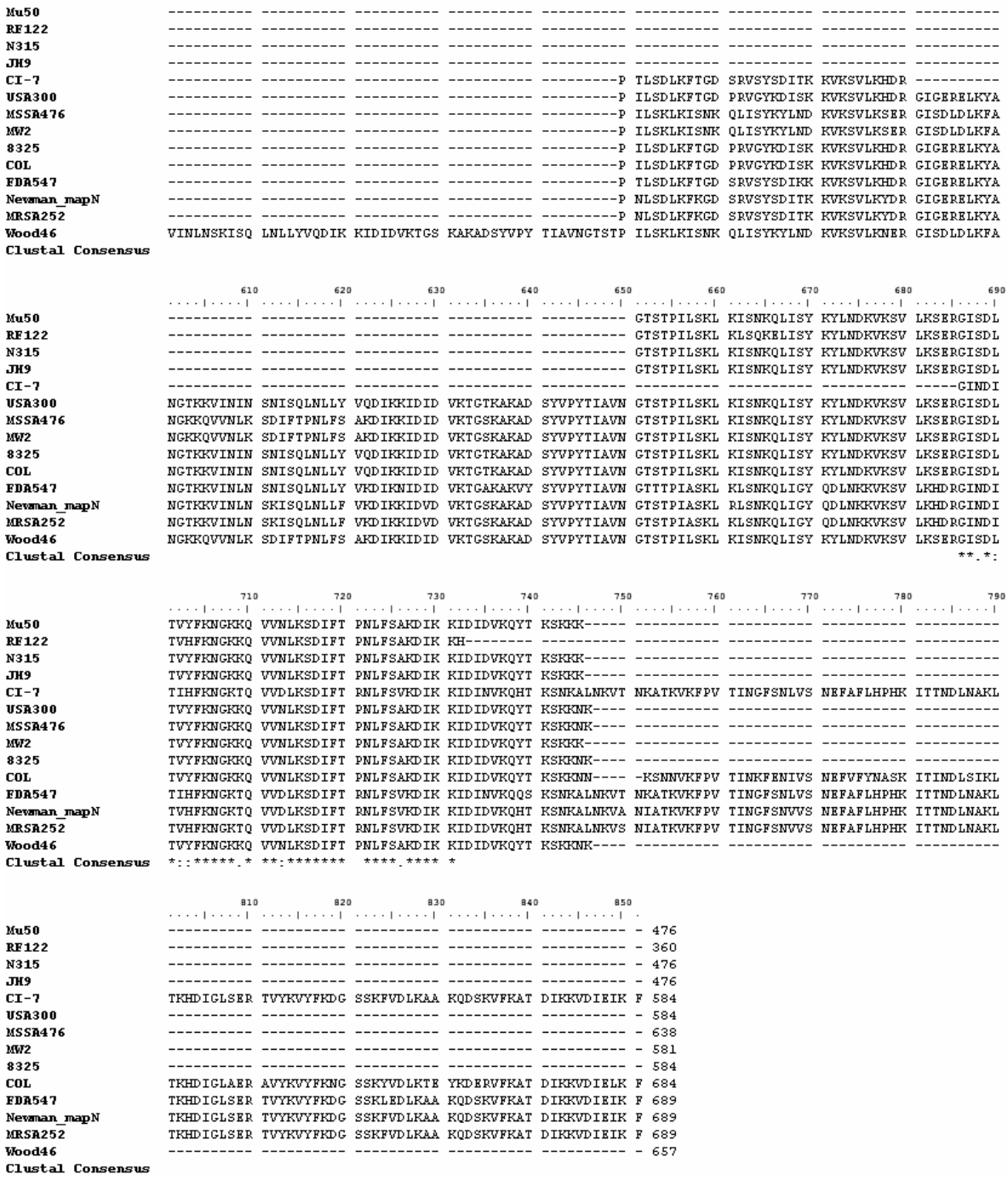
```
Mu50          TCAAGTTCCA TATTCAATTA ATCTAAATGG TACATCTACA AACATTTTAT
Domain3mut    TCAAGTTCCA TATTCAATTA ATCTAAATGG TACATCTACA AACATTTTAT
Clustal Co    ********** ********** ********** ********** **********

                   860        870        880        890        900
Mu50          CGAATCTTTC ATTTTCAAAT AAACCTTGGA CAAATTACAA AAATTTAACT
Domain3mut    CGAATCTTTC ATTTTCAAAT AAACCTTGGA CAAATTTCAA AAATTTAACT
Clustal Co    ********** ********** ********** ****** *** **********

                   910        920        930        940        950
Mu50          AGTCAAATAA AATCAGTACT GAAGCATGAT AGAGGTATTA GTGAACAAGA
Domain3mut    AGTCAAATAA AATCAGTACT GAAGCATGAT AGAGGTATTA GTGAACAAGA
Clustal Co    ********** ********** ********** ********** **********
```

8. D4 mutant

```
Mu50          AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 1200
d4mutant      AATGGCACATCAACACCAATTTTATCAAAACTAAAAATTTCGAATAAACAATTAATTAGT 267
              ************************************************************

Mu50          TACAAATATTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 1260
d4mutant      TACAAATTTTTAAACGACAAAGTGAAATCTGTATTAAAAAGTGAAAGAGGTATCAGTGAT 327
              ******* ****************************************************

Mu50          CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 1320
d4mutant      CTTGACTTAAAATTTGCGAAACAAGCAAAATATACAGTATATTTCAAAAATGGAAAGAAA 387
              ************************************************************
```

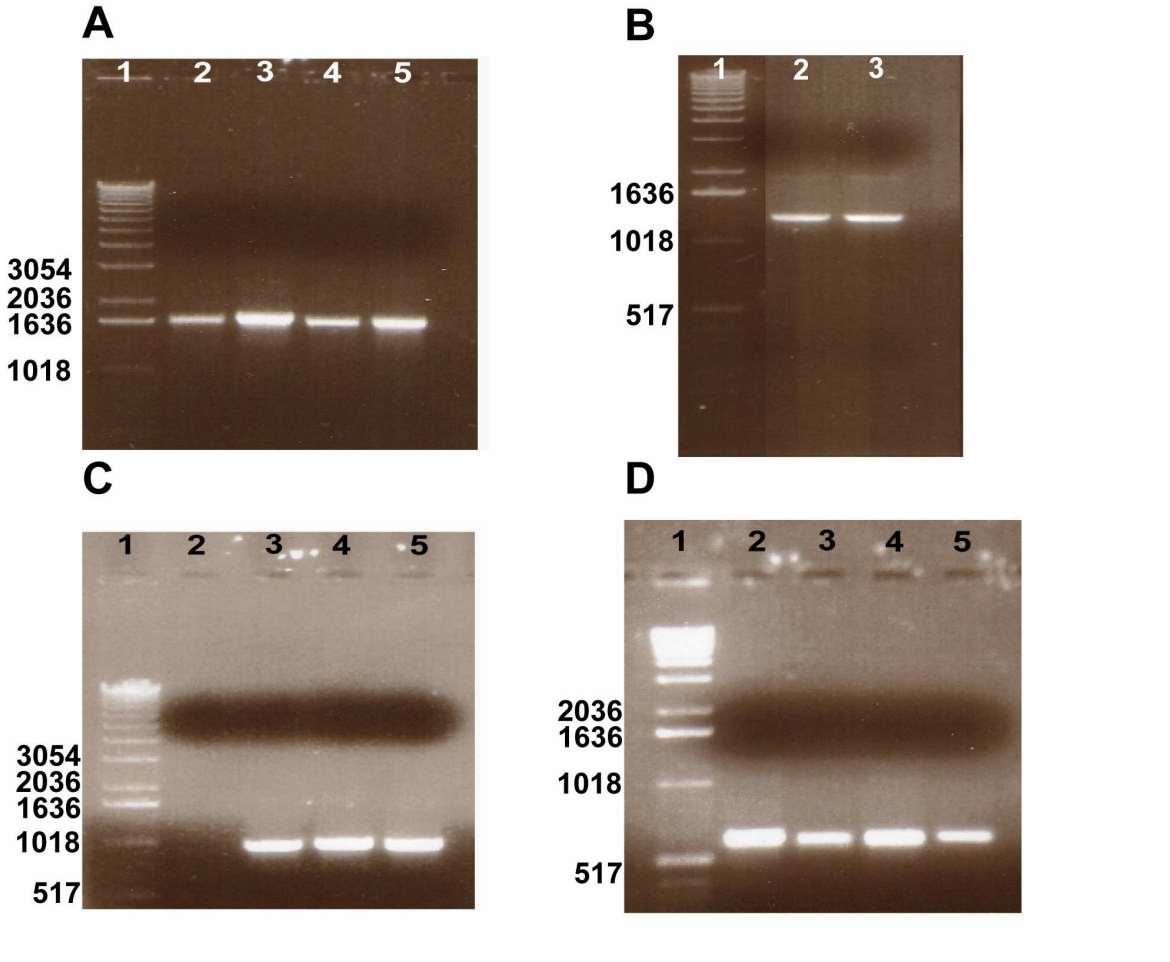
APPENDIX I . 3

```
                                10        20        30        40        50        60        70        80        90
                                |....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Mu50                            ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VIASTGANFN TNEASAAAKP
RF122                           ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VLASTGANFN NNEASAAAKP
N315                            ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VIASTGANFN TNEASAAAKP
JH9                             ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VIASTGANFN TNEASAAAKP
CI-7                            ---------- ---------- ---------- ---------- ---------- ------MKFH GSRATTLALG VIASTGANFN TNEASAAAKQ
USA300                          ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VLASTGANFN NNEASAAAKP
MSSA476                         LDLKFAKQAK YTVYFKNGKK QVVNLKSDIF TPNLFSAKDI KKIDIDVKQY TKSKKKMKFK SLITTTLALG VIASTGANFN TNEASAAAKQ
MW2                             ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VIASTGANFN TNEASAAAKP
8325                            ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VLASTGANFN NNEASAAAKP
COL                             ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VLASTGANFN NNEASAAAKP
FDA547                          ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VIASTGANLD TNEASAAAKQ
Newman_mapN                     ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VLASTGANFN TNEASAAAKQ
MRSA252                         ---------- ---------- ---------- ---------- ---------- ------MKFK SLITTTLALG VLASTGANFN TNEASAAAKQ
Wood46                          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------AAKP
Clustal Consensus                                                                                                             ***

                                110       120       130       140       150       160       170       180       190
                                |....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Mu50                            GHSNIQIPYT ITVNGTSQNI LSSLTFNKNQ NISYKDIENK VKSVLYFNRG ISDIDLRLSK QAEYTVHFKN GTKRVIDLKS GIYTADLINT
RF122                           GYSKVHVPYA ITVNGTSQNI LSSLTFNKNQ NISYKDLEDR VKSVLKSDRG ISDIDLRLSK QAKYTVYFKN GAKRVIDLKA GIYTADLINT
N315                            GHSNIQIPYT ITVNGTSQNI LSSLTFNKNQ NISYKDIENK VKSVLYFNRG ISDIDLRLSK QAEYTVHFKN GTKRVIDLKS GIYTADLINT
JH9                             GHSNIQIPYT ITVNGTSQNI LSSLTFNKNQ NISYKDIENK VKSVLYFNRG ISDIDLRLSK QAEYTVHFKN GTKRVIDLKS GIYTADLINT
CI-7                            GYSKIQIPYT ITVNGTSQNI LSSLTFNKNQ QISYKDIENK VKSVLYFNRG ISDIDLRLSK QAKYTVHFKN GTKRVVDLKA GIHTADLINT
USA300                          GYSKVHVPYA ITVNGTSQNI LSSLTFNKNQ NISYKDLEDR VKSVLKSDRG ISDIDLRLSK QAKYTVYFKN GTKKVIDLKA GIYTADLINT
MSSA476                         GHSNIQIPYT ITVNGTSQNI LSSLTFNKNQ NISYKDIENK VKSVLYFNRG ISDIDLRLSK QAEYTVHFKN GTKRVIDLKS GIYTADLINT
MW2                             GHSNIQIPYT ITVNGTSQNI LSSLTFNKNQ NISYKDIENK VKSVLYFNRG ISDIDLRLSK QAEYTVHFKN GTKKVIDLKS GIYTADLINT
8325                            GYSKVHVPYA ITVNGTSQNI LSSLTFNKNQ NISYKDLEDR VKSVLKSDRG ISDIDLRLSK QAKYTVYFKN GTKKVIDLKA GIYTADLINT
COL                             GYSKVHVPYA ITVNGTSQNI LSSLTFNKNQ NISYKDLEDR VKSVLKSDRG ISDIDLRLSK QAKYTVYFKN GTKKVIDLKA GIYTADLINT
FDA547                          GYSKIQIPYT ITVNGTSQNI LSSLTFNKNQ QISYKDIENK VKSVLYFNRG ISDIDLRLSK QAKYTVHFKN GTKRVVDLKA GIHTADLINT
Newman_mapN                     GYSKIQIPYT ITVNGTSQNI LSSLTFNKNQ QISYKDIENK VKSVLYFNRG ISDIDLRLSK QAKYTVHFKN GTKRVVDLKA GIHTADLINT
MRSA252                         GYSKIQIPYT ITVNGTSQNI LSSLTFNKNQ QISYKDIENK VKSVLYFNRG ISDIDLRLSK QAKYTVHFKN GTKRVVDLKA GIHTADLINT
Wood46                          GHSNTQIPYT ITVNGTSQNI LSSLTFNKNQ NISYKDIENK VKSVLYFNRG ISDIDLRLSK QAEYTVHFKN GTKRVIDLKS GIYTADLINT
Clustal Consensus               *:*:: ::**: ********** ********** :*****:*:: *****  :** ********** **:***:*** *:*:*:***: * :*******

                                210       220       230       240       250       260       270       280       290
                                |....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Mu50                            DTKKQPKDK- -AKANVQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLEG KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
RF122                           DTKKQVEDKK KDKANYQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLEG KVKSVLESNR GIIDVDLRLS KQAKYTVNFK NGTKKVIDLK
N315                            DTKKQPKDK- -AKANVQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLEG KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
JH9                             DTKKQPKDK- -AKANVQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLEG KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
CI-7                            DTKKQVKDKE -AKANVQVPY TITVNGTSQN ILSNLTFKKI SKLVIKILEN NVKSVLKSNR GITDVDLRLS KQAKFTVNFK NGTKKVIDLK
USA300                          DTKKQVEDKK KDKANYQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLED KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
MSSA476                         DTKKQPKDK- -AKANVQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLEG KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
MW2                             DTKKQPKDK- -AKANVQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLEG KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
8325                            DTKKQVEDKK KDKANYQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLED KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
COL                             DTKKQVEDKK KDKANYQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLED KVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
FDA547                          DTKKQVKDKE -AKANVQVPY TITVNGTSQN ILSNLTFKKN QQISYKDLEN NVKSVLKSNR GITDVDLRLS KQAKFTVNFK NGTKKVIDLK
Newman_mapN                     DTKKQVKDKE -AKANVQVPY TITVNGTSQN ILSNLTFKKN QQISYKDLEN NVKSVLKSNR GITDVDLRLS KQAKFTVNFK NGTKKVIDLK
MRSA252                         DTKKQVKDKE -AKANVQVPY TITVNGTSQN ILSNLTFKKN QQISYKDLEN NVKSVLKSNR GITDVDLRLS KQAKFTVNFK NGTKKVIDLK
Wood46                          DTKKQPKDK- -AKANVQVPY TITVNGTSQN ILSNLTFNKN QNISYKDLED RVKSVLESNR GITDVDLRLS KQAKYTVNFK NGTKKVIDLK
Clustal Consensus               ***** :**    *** **** ********** *******:*  .::  * **. .*****:*** ** ******* ****:***** **********

                                310       320       330       340       350       360       370       380       390
                                |....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Mu50                            SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
RF122                           SSDIKSIDIN VDTKKHVENK -AKRNYQVPY SINLN----- ---------- ---------- ---------- ---------- ----------
N315                            SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
JH9                             SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
CI-7                            TGDIKNININ VETKKQAKDK EAKVNNQVPY SINLNGTTTN IQSNLAFSNK PWTNYKNLTT KVKSVLKSDR GVSERDLKHA KKAYYTVYFK
USA300                          SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
MSSA476                         SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
MW2                             SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
8325                            SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
COL                             SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
FDA547                          TGGIKNININ VETKKQAKDK EAKVNNQVPY SINLNGTTTN IQSNLAFSNK PWTNYKNLTT KVKSVLKSDR GVSERDLKHA KKAYYTVYFK
Newman_mapN                     TGDIKNININ VETKKQAKDK EAKANNQVPY SINLNGTTTN IQSNLAFSNK PWTNYKNLTA KVKSVLKSDR GVSERDLKHA KKAYYTVYFK
MRSA252                         TGDIKNININ VETKKQAKDK EAKANNQVPY SINLNGTTTN IQSNLAFSNK PWTNYKNLTA KVKSVLKSDR GVSERDLKHA KKAYYTVYFK
Wood46                          SSDIKSININ VDTKKHIENK -AKRNYQVPY SINLNGTSTN ILSNLSFSNK PWTNYKNLTS QIKSVLKHDR GISEQDLKYA KKAYYTVYFK
Clustal Consensus               :..**.*:** *:***: ::*  ** * **** *****

                                410       420       430       440       450       460       470       480       490
                                |....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Mu50                            SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN ---------- ---------- ---------- ---------- ----------
RF122                           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
N315                            SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN ---------- ---------- ---------- ---------- ----------
JH9                             SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN ---------- ---------- ---------- ---------- ----------
CI-7                            SNIYTANLVH AKDVKRIEVT VKTVSKVKAE RYVPYTIAVN GASN------ ---------- ---------- ---------- ----------
USA300                          SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
MSSA476                         SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
MW2                             SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
8325                            SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
COL                             SKNYTANLVH AKDVKRIEIT VKTGTKAKAD RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
FDA547                          SNIYTANLVH AKDVKRIEVT VKTVSKVKAE RYVPYTIAVN GASN------ ---------- ---------- ---------- ----------
Newman_mapN                     SNIYTANLVH AKDIKKIEVT VKTGSKANAE RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
MRSA252                         SNIYTANLVH AKDIKKIEVT VKTGSKANAE RYVPYTIAVN GTST------ ---------- ---------- ---------- ----------
Wood46                          SKNYTANLVH VKDVKRIEIT VKTGTKAKAD RYVPYTIAVN GTSTPILSDL KFTGDPRVGY KDITKKVKSV LKHDRGIGER ELKYAKKATY
Clustal Consensus
```



The Clustal W2 alignment of the amino acids sequences of Eap from fourteen different strains of *Staphylococcus aureus* species. The aminoacid sequences were retrieved from EMBL data base (http://www.ebi.ac.uk/embl/) and alignment was performed by the Clustal W. The 14 different strains of *S. aureus* Eap includes Mu50 (SAV1938), RF122 (SAB1873c), N315 (SA1751), JH9 (A5IUA7), CI-7 (AJ243790), USA300 (ABX29943), MSSA476 (SAS1861), MW2 (EFK80959.1) , 8325 (ABD31208), COL (AAW36971), FDA547 (Q53599), Newman mapN (AJ132841), MRSA252 (SAR2030) and Wood46 map-W (AJ245439).

APPENDIX I . 4



Agarose gel electrophoresis of the insert of Mu50 Eap. The amplification of positive clones using PCR during the mutagenesis is shown. **A. Amplification of Mu50 full length (FL) Eap and its mutants:** lane 1- marker, lane 2- FLWT, lane 3 - FLD3, lane 4 - FLWT, lane 5 - FLD4; **B. Amplification of Mu50 three domain Eap (2L4):** lane 1- marker, lane 2 - 2L4D3, lane 3 - 2L4D4; **C. Amplification of Mu50 two domain Eap (3L4):** lane 1- marker, lane 3 - 3L4WT, lane 4 - 3L4D3, lane 5 - 3L4D4; **D. Amplification of Mu50 single domain Eap:** lane 1- marker, lane 2 - D3WT, lane 3 - D3M, lane 4 - D4WT, lane 5 - D4M**.**

APPENDIX II

A. INSTRUMENTS

Instrument	Characteristics	Company
FPLC		Bio-Rad
Electrophoresis unit	Trans-blot SD, Semi-dry transfer cell	Bio-Rad
Balance	Mettler PC-4400, Delta range	
Centrifuge	Eppendorf, 5810R	Eppendorf
	Eppendorf, 5417R	Eppendorf
Electroporator		Bio-Rad
Gel dryer		Bio-Rad
Culture Bench/Laminar flow		Heraeus Safe
pH meter	PHM220 lab pH meter	Radiometer, Meterlab
Power supply unit	Electrophoresis power ECPS 301	Amersham Bioscience
Water Bath		GFL
Incubator	Multitron	Infors-HT
Spectrophotometer	Helios	Heraeus Electronic Gene-Quantpro Unicam
Vortex	VORTE Gene-2	Scientific Industries
Plate reader	Wallace Victor2	Perkin Elmer Life Science
Nitrocellulose transfer membrane		Whatmann Protran®
Room shaker/Agitator		IKA-VIBRAX-VXR
Magnetic stirrer		IKA-COMBIMAG-RET

B. CHEMICALS

All commercially obtained materials used in this work were of the finest quality available purchased from the companies listed below:

C. ENZYMES/MARKER

Proteinase K, Qiagen

Prestained Protein ladder, Fermentas life science

Stratagene Ltd, Cambridge, UK

New England Biolabs

CLONTECH Laboratories

MBI Fermentas

Promega Corporation

C. KITS

Purpose	Company
Plasmid DNA purification	Qiagen GmbH
Quick Change Site directed mutagenesis kit	Stratagene
Endotoxin detection	Lonza
Protein quantification BC Assay Kit	Thermo Scientific
Click-iT EdU Microplate Assay	Invitogen

D. COLUMN MATERIALS FOR PROTIEN PURIFICATION

Affinity column: Ni-NTA GE Healthcare

Size exclusion chromatography: Superdex-75, GE Healthcare.

E. CONSUMABLES

Amersham Pharmacia Biotech

Endotrap, hyglos

Fisher Scientific

Tissue culture discs, Falcon, Becton Dickinson labware

MicroTest^{TM} 96, Falcon, Becton Dickinson labware

24 well -, 6 well - cell culture plate, Cellstar

Tissue culture disc 100 x 20 mm, Sarstedt

Millipore

APPENDIX III

1. Culture media

i. TB broth

A	Bacto tryptone	12 g	**B**	KH_2PO_4	2.31 g
	Bacto yeast extract	24 g		K_2HPO_4	12.54 g
	Glycerin	4 ml		Add d/w up to 100 ml and autoclave it	
	Add d/w up to 900 ml and autoclave it				
	Mix solution A and B for use				

ii. Medium A

Bacto Tryptone	10 g
Yeast Extract	2.5
5M Nacl	1.00 ml
1M KCl	1.25 ml
1 M $MgCl_2$	5.00 ml
1M $MgSO_4$	5.00 ml

Add d/w up to 500 ml and autoclave it

iii. Medium B

0.2 M PIPES (10 mM, pH 6.4)	12.50 ml
1.0 M $MnCl_2$ (55 mM)	13. 75 ml

1.0 M $CaCl_2$ (15 mM)	3.75 ml
1.0 M KCl (250 mM)	62.50 ml

Add d/w up to 250 ml and autoclave it

iv. SOC Medium

Trypton	2%
Yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM

Adjust pH 7.0, autoclave, add 20 mM glucose and filter sterilize by 0.22-micron filter.

2. ANTIBIOTICS AND OTHERS

Antibiotics	Stock solution	Solvent	Working conc.
Kanamycin	50 mg/ml	water	50 µg/ml

3. BUFFERS AND COMMONLY USED SOLUTIONS

i. Common Buffers

a. 10 X TSB

Tris	30 g
NaCl	80 g
KCl	2 g

pH 7.4, HCl, add d/w up to 1000 ml

b. 10 X PBS

NaCl	81.8 g
KCl	2 g
$Na_2HPO_4.2H_2O$	16 g
KH_2PO_4	2 g

pH 7.4, HCl, add d/w up to 1000 ml

c. Potassium phosphate buffer (10 mM, pH 7.4)

1M K_2HPO_4	8.4 ml
1M KH_2PO_4	1.6 ml add d/w up to 1000 ml

ii. BUFFERS AND SOLUTIONS FOR AGAROSE GEL ELECTROPHORESIS

10 x TAE Buffer

Tris base	242.28 g
EDTA-Na	18.61 g
Acetic acid	60.05 ml
	pH 8.5 ± 0.2 Add d/w up to 1000 ml

10 x Loading buffer

Bromophenol blue	2.5 mg
Xylencyanole	2.5 mg
Glycerol	300 µl
	add d/w up to 1 ml

iii. Buffers and solutions for protein purification

A. Denaturing cell lysis buffer

0.1 M Tris, pH 8.0

6 M Guanidine-HCl

B. Buffer for affinity chromatography

i. Denaturing wash buffer

20 mM Sodium phosphate buffer, pH 6.0

500 mM NaCl

10 mM imidazole

8 M Urea,

add d/w up to 500 ml

ii. Denaturing elution buffer

20 mM Sodium phosphate buffer, pH 6.0

500 mM NaCl

200 mM imidazole

8 M Urea

add d/w up to 200 ml

iii. Native buffer

20 mM Tris buffer, pH 8.0
500 mM NaCl
add d/w up to 500 ml

iv. Native wash buffer

20 mM Tris buffer, pH 8.0
500 mM NaCl
10 mM imidazole
add d/w up to 500 ml

v. Native elution buffer

20 mM Tris buffer, pH 8.0
500 mM NaCl
500 mM imidazole
add d/w up to 200 ml

iv. Buffer for size exclusion chromatography (Buffer D)

Potassium phosphate buffer (10 mM, pH 7.4).

APPENDIX IV

1. Buffers and solutions for polyacrylamide gel electrophoresis

separating gel

Stock solutions	**Final acrylamide concentration**		
	10%	**12%**	**15%**
4 x LT	3.75 ml	3.75 ml	3.75 ml
10% APS	75 µl	75 µl	75 µl
dest.H_2O	ad 15 ml	ad 15 ml	ad 15 ml
30% AA/Bis	5.0 ml	6.0 ml	7.5 ml
TEMED	7.5 µl	7.5 µl	10 µl

stacking gel

Stock solutions	**5% acrylamide concentration**
4 x UT	2.5 ml
10% APS	50 µl
Dest.H_2O	ad 10 ml
30% AA/Bis	1.6 ml
TEMED	5 µl

4 x Buffer for separating gel (LT)	1.5 M Tris/Cl, pH 8.8 0.4% SDS
4 x Buffer for stacking gel (UT)	0.5 M Tris/Cl, pH 6.8 0.4% SDS
2 x SDS loading buffer	125 mM Tris/Cl, pH 6.8 20% Glycerol 4% SDS 10% β-mercaptoethanol 0.004% Bromphenol blue

Staining solution	0.1% Coomassie Brilliant Blue G-250 40% Methanol 10% Acetic acid
Destaining solution	25% Methanol 10% Acetic acid

APPENDIX V

Radio-Immunoprecipitation assay (RIPA) buffer

50 mM Tris-HCl, pH 7.4,

1% w/v Igepal™,

0.25% w/v desoxycholate,

150 mM NaCl,

1 mM EGTA,

1 mM NaF,

0.5 mM sodium orthovanadate,

1 mM sodium pyrophosphate,

2 µg/ml aprotinin,

5 µg/ml leupeptin,

10 µg/ml pepstatin A,

0.2 mg/ml PMSF

13 Acknowledgments

My duration at Department of Institute of Microbiology and Hygiene, Saarland University has been wonderful and rewarding experience. I am forever indebted to the department and the people for the contributions to my scientific carrier over the years.

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I would like to thank our research technician Karin Hilgert for her help and sharing my personal feeling during my stay. I would like to thank all my colleagues in this groups which have made my PhD enjoyable throughout these years.

My loving thank is always for my husband Yogan for his encouragement, support and suggestions and always standing by my side. I am indebt and thankful to parents Raj Gopal and Pabita, and my dear brothers Ritesh and Aashish. During this time, though I was far away from them, there was no distance in between. Sweet remembrance and everlasting memory was always in front and be ever! I love you a lot for your blessings and everlasting support!

14 Declaration

I hereby declare that this thesis is my original work and I have prepared it independently, except where specific references are made to other sources. It has not been submitted in part either in Germany or in another country for any other degree.

Rajita Rajbhandari

Place/date:

Signature: