A Fibrinogen-Binding Protein from *Staphylococcus epidermidis*

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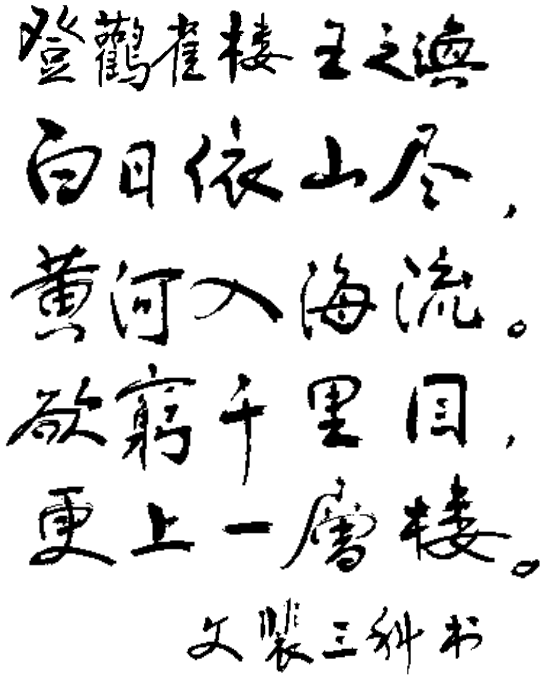
A Chinese poem by a famous poet of the Tang Dynasty



To my parents

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ABSTRACT

Staphylococcus epidermidis, a coagulase negative staphylococci (CoNS), is a major component of human flora. It is one of the leading pathogens of nosocomial infections, particularly associated with foreign body infections. The increasing rate of nosocomial epidemics caused by multiresistant staphylococci requires a better understanding of the pathogenesis of staphylococcal infections. Two steps are involved in bacterial infections on the implants: initial colonization and cell accumulation. Adherence of *S.epidermidis* to fibrinogen deposited on the surfaces of implants is important for the development of foreign body infections.

We here report our studies on a fibrinogen-binding protein from *S.epidermidis.*

A gene (*fbe*) encoding a fibrinogen-binding protein from *S.epidermidis* (Fbe) is identified by shotgun phage display. As a member of the SD-multigene family in staphylococci, Fbe shares some similar constructions with other surface proteins of staphylococci. Although most strains of *S.epidermidis* contain part of *fbe*, the binding of *S.epidermidis* to immobilized Fg *in vitro* shows a heterogeneous pattern, suggesting that other factors may influence the surface exposure of Fbe. The interactions between rFbe and Fg have been extensively studied. The Fg binding responding domain is located on a 331-amino-acid sequence of the A-region of Fbe. rFbe binds to the β-chains of Fg and this interaction can be accelerated by calcium. Such an interaction between Fbe and Fg may promote the binding of *S.epidermidis* to Fg in physiological environments. In the inhibition study, the binding between Fg and *S.epidermidis* can be blocked by rFbe and its antibodies. The antibodies against rFbe efficiently block the binding between *S.epidermidis* and surface-bound Fg. The inhibition of adherence to subcutaneously implanted catheters from rats and peripheral venous catheters from patients is less efficient than to Fg-coated ones. This suggests that other components adsorbed on the implant surfaces may contribute to bacterial adherence as well. In the study on the isogenic mutant lacking *fbe*, lower adherence to Fg exposed surfaces is found in the *fbe*-isogenic mutants in comparison to its wild type. Thus, Fbe may be a major adhesin to Fg in *S.epidermidis*. The remained adherence of the mutant to explants suggests that adhesins to other host components may strengthen the interaction between bacteria and the implants.

Furthermore, the strategies to prevent and treat *S.epidermidis*-associated foreign body infections are discussed, including infection controls, new antibiotics, new biomaterials, and immunoglobulin therapy. With more knowledge of the pathogenesis, opsonophagocytic properties of antibodies, and the role of cytokines in *S.epidermidis*-associated infections, an immunoglobulin therapy targeting Fbe may become a promising strategy in prophylaxis and therapy.

MAIN REFERENCES

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Nilsson M., Frykberg L., Flock J-I., Pei L., Lindberg M., B. Guss**
A Fibrinogen-Binding Protein of *Staphylococcus epidermidis*
Infection and Immunity 1998, Vol. 66, p.2666-2673

II. **Pei L., Palma M., Nilsson M., Guss B., J-I. Flock**
Functional Studies of a Fibrinogen-Binding Protein from *Staphylococcus epidermidis*
Infection and Immunity 1999, Vol. 67, p.4525-4530

III. **Pei L. and J-I. Flock**
Functional Study of Antibodies against a Fibrinogen-Binding Protein in *Staphylococcus epidermidis* Adherence to Polyethylene Catheters
The Journal of Infectious Diseases 2001, Vol.184, p.52-55

IV. **Pei L. and J-I. Flock**
Lack of *fbe*, the gene for a fibrinogen-binding protein from *Staphylococcus epidermidis* reduces its adherence to fibrinogen coated surfaces
Microbial Pathogenesis 2001, Vol.31, p.185-193

ABBREVIATIONS

CoNS	coagulase negative staphylococci
Fbe	fibrinogen-binding protein from *S.epidermidis*
rFbe	recombinant Fbe
GST	glutathione S-transferase
Fg	fibrinogen
FpA	fibrinopeptide A
FpB	fibrinopeptide B
Fn	fibronectin
Cn	collagen
Vn	vitronectin
vWF	von Willebrand factor
ECM	extracellular matrix
FgBPs	fibrinogen binding proteins
FnBPs	fibronectin binding proteins
SD	serine-aspartic acid
Sdr	serine-aspartate repeat
Ca^{2+}	calcium
PS/A	capsular polysaccharide/adhesin
PIA/HA	polysaccharide intercellular adhesin/hemagglutinin
agr	accessory gene regulator
sar	staphylococcal accessory regulator
ica	intercellular adhesin
CAPD	continuous ambulatory peritoneal dialysis
FgCC	fibrinogen coated catheter
FnCC	fibronectin coated catheter
SIC	subcutaneously implanted catheter
PVC	peripheral venous catheter
CVC	central venous catheter
Gm	gentamicin
Erm	erythromycin

INTRODUCTION

Genus of staphylococci

The genus of staphylococci consists of nonmotile, nonsporeforming, spherical, Gram positive cocci. These microorganisms are 0.5-1.5 μm in diameter, occurring singly, in pairs and grape-like clusters. They are facultative anaerobes which tolerate high concentration of salt. Typically, staphylococci are commensals in the normal flora of the skin and mucous membranes. Phylogenetically, this genus belongs to the low%G+C Gram positive bacteria group of the Micrococcaceae family. Traditionally, staphylococci can be divided into two groups according to their ability to clot blood plasma: coagulase positive staphylococci, mainly *Staphylococcus aureus*; and coagulase negative staphylococci (CoNS). Up to date, at least 32 species have been identified, of which 13 are indigenous in humans [Kloos 1997].

S.aureus, *S.saprophyticus* and *S.epidermidi*s are the three main pathogenic species of staphylococci. *S.aureus* can cause a wide range of diseases, such as wound infections, abscesses, septicemia, arthritis, pneumonia, endocarditis, osteomylitis, food poisoning, scalded skin syndrome, toxic shock syndrome and etc. *S.saprophyticus* is a common pathogen of urinary tract infections. *S.epidermidis*, the most important pathogen of CoNS, can cause several diseases, especially infection associated with indwelling medical devices. *S.capitis*, *S.haemolyticus*, *S.lugdunensis*, and *S.xylosus* are uncommon pathogens of CoNS in human diseases.

Colonization of staphylococci occurs commonly on mammals as transients, temporary residents or natural inhabitants. The *S.epidermidis* family makes up 65-90% of all staphylococci recovered from the resident aerobic flora. Normal individuals can harbor up to 24 strains of this species, which can survive on a dry surface for long periods [Kloos 1997]. CoNS, especially *S.epidermidis*, are the commonest isolates in a clinical microbiology laboratory. Antibiotic resistance is commonly reported in most clinical isolates. Many of them are multiresistant to the usual antibiotic agents, such as β-lactam drugs, erythromycin, clindamycin, tetracycline, and gentamicin. More than 80% of clinical isolates are resistant to methicillin and other semisynthetic penicillins, making the treatment of these infections more difficult and complicated [Archer 1995]. Vancomycin, rifampin and novobiocin are the only available agents that remain effective.

Infections caused by *S.epidermidis*

Table 1 shows some infections caused by *S.epidermidis*. These infections share some common features, e.g., most of them are nosocomial, often resistant to various antimicrobial agents, and usually involved an indwelling foreign body. To date, *S.epidermidis* is one of the commonest nosocomial pathogens together with *S.aureus*, *Escherichia coli, Pseudomonas areuginosa*, and *enterococci* [Jarvis & Martone 1992; Kloos & Bannerman 1994; Rupp & Archer 1994; Boyce 1997]. Infections caused by *S.epidermidis* can increase the risk of adverse outcome and substantially prolong the hospital stay. In some cases, the infections are difficult to treat with conventional chemotherapy and may eventually require removal of the indwelling foreign material [Rupp & Archer 1994].

Table 1. Infections associated with *S. epidermidis*

Infection	Site of infection	% of CoNS in positive culture	Reference
bacteremia/septicemia	blood (present/absent of a foreign body)	8%	[Bannerjee *et al.* 1991]
surgical site infections	superficial, deep space or organ	13%	[Boyce 1997; CDC 1997]
native valve endocarditis	native heart valve	5%	[Herwaldt *et al.* 1996]
prosthetic valve endocarditis(PVE) (other cardiac devices)	prosthetic heart valve, pacemaker, total artificial heart	30-67% (early) 20-28% (late)	[Whitener *et al.* 1993]
Endophthalmitis	intraocular lens implantation	40-60%	[Davis *et al.* 1988]
Hemodialysis infections	exit site, tubing, blood	27%	[Nafziger & Saravolatz 1999]
peritonitis (CAPD-related)	exit site, tunnel, infusion, kidney	25-50%	[Fong *et al.* 1993] [Bernardini *et al.* 1991]
Intravascular catheter infections	peripheral and central venous catheter	>50%	[Henderson 1988; Boyce 1997; Nafziger & Saravolatz 1999]
Intravascular graft infections	intravascular graft	>40%	[Boyce 1997]
prosthetic joint infections	total hip, knee, shoulder joint, elbow, etc.	30-35% (total hip)	[Boyce 1997]
central nervous system infections	cerebrospinal fluid (CSF) shunts, ventriculostomy	50% 53%	[Nafziger & Saravolatz 1999]

It is clinically important to identify true-positive results of *S.epidermidis* as prerequisite of antibiotics therapy and/or revision of the indwelling devices. The isolations and enumeration of *S.epidermidis* in clinical samples, e.g., from blood, tissue, exudate, prostheses, have been routine procedures in clinical microbial laboratories. Various methods of phenotypic and genotypic typing are of great help in determining the clinical relevance of *S.epidermidis* isolates. These include biotyping, quantitative antibiogram typing, plasmid profile, randomly amplified polymorphic DNA analysis, pulse field gel electrophoresis, and DNA fingerprinting by amplified fragment length polymorphism analysis. Despite their sophistication, interpretations of positive blood cultures involving *S.epidermidis*, remain difficult for both clinicians and microbiologists. As an inhabitant of the human skin, *S.epidermidis* may contaminate specimens during sampling. More than 50% of diagnosis results were turned out to be false positive. In most infections, phenotypic and genomic variations of *S.epidermidis* are common [Galdbart *et al.* 1999]. Reports have shown that most of them were caused by a single clone endogenous in origin, i.e., derived from the patient's indigenous flora. The microorganisms will undergo spontaneous mutations, rearrangements, and loss or transposition of DNA *in vivo*, yielding derivatives with divergent phenotypes and genotypes [Paulsen *et al.* 1997]. For most patients, multiple strains of *S.epidermidis* in specimens might be derivatives of a single clone resulting from genetic variability during the infectious episode [Galdbart *et al.* 1999]. Some cases have been ascribed to polyclonal *S.epidermidis* populations, like bacteremia and some cases of prosthetic valve endocarditis [van Wijngaerden *et al.* 1997]. However, there are also reports documenting polyclonal staphylococcal endocarditis as a result of genetic variability [van Eldere *et al.* 2000].

Less is known about the host defense against infections caused by *S.epidermidis*. The skin and mucous membranes are the first barriers against invasion by microorganisms. However, once these barriers have been breached, e.g., by a wound, an operation, via an intrinsic contamination of infusate, or hematogeneous seeding, phagocytes play important roles in inhibiting staphylococcal infections. Innate immunity, defined as immediate nonspecific immunity, is important in host defense against microbial infection. Toll-like receptor 2 (TLR2), a well characterized receptor involved in innate immunity to Gram-positive bacterial components, recognizes peptidoglycan of *S.aureus*, while TLR4 recognizes Gram-negative bacterial lipopolysaccharide (LPS). A cytoplasmic adapter – MyD88, is crucial for signaling of IL-1R/TLRs. The cytokine response to *S.aureus* infection in MyD88-deficient mice showed that not only TLR2 but also other TLR family members, except TLR4, were involved in the early elimination of invading staphylococci [Takeuchi *et al.* 2000].

Polymorphonuclear leukocytes (PMN) originated in the bone marrow, play a primary immune response against pathogenic staphylococci [Verhoef 1997]. The fact that patients who are neutropenic or have congenital or acquired defects in PMN function are more susceptible to staphylococcal infections supports the essential role of PMN in host defense against staphylococci. However, a recent study also showed that PMN could have both a protective and deleterious role in *S.aureus* infections. Neutrophils isolated from the site of infection contained viable intracellular organisms which were sufficient to establish infection in a naïve host [Gresham *et al.* 2000]. Opsonophagocytosis is considered as the essential host defense against staphylococci, with IgG antibodies as major opsonins. The major PMN receptor for *S.epidermidis* opsonin is the FcRIII receptor [Schutze *et al.* 1991]. Administration of intravenous immunoglobulin against staphylococci has been used as alternative therapy and prophylaxis in neonatal sepsis caused by *S.epidermidis* [Fischer 1994; Fischer *et al.* 1994; Lamari *et al.* 2000]. However, antibodies alone are not sufficient for opsonization. It is speculated that antibodies dependent opsonization can be facilitated in presence of complement components - C3b or iC3b [Giese *et al.* 1994].

Pathogenesis of staphylococcal infections

Staphylococci are known as pathogen in humans. The emergence of antibiotic resistance and the increase in the prevalence of nosocomial infections with these microorganisms have urged a better understanding of the pathogenesis of life-threatening staphylococcal infections. *S. aureus*, one of the most virulent species of staphylococci, has been studied extensively. The whole genome of *S. aureus*, a circular chromosome of approximately 2.8 Mb, has been completely sequenced by a Japanese group based on a recent Japanese – derived methicillin-resistant *S.aureus* (MRSA, N315) and a closely related vancomycin-resistant strain (Mu50). There are several complete sequences of *S.aureus* available in TIGR Microbial Database (i.e., sequences from strains COL and 8325 by NIAID/MGRI) and Sanger Center (i.e., sequences from strains MRSA252 and MSSA476 by Beowulf genomics). The genome of *S.epidermidis* is almost complete, in status of closing gaps. Thus, the genetic background of these pathogens is better known than before.

Studies have been focused on the virulence factors of staphylococci. Virulence factors of *S.aureus* have been classified as adhesins, toxins, and invasins, as listed in Table 2A, 2B and 2C respectively.

In general, once a pathogen gains access to the host, microbial adhesins play an essential role in initiating an infection. *S. aureus* produces a numerous such adhesins. As an example, fibrinogen (Fg), a dominant glycoprotein abundant in blood and extracellular matrix (ECM), can be recognized by several staphylococcal adhesins. These Fg binding proteins (FgBPs) include surface bound adhesins, like ClfA [McDevitt *et al.* 1994], ClfB[Ni Eidhin *et al.* 1998], FnbpA, FnbpB, FbpA (also part of a surface-bound coagulase) [Cheung *et al.* 1995] and Map/Eap [McGavin *et al.* 1993; Palma *et al.* 1999], or secretory proteins, like Efb, Coa (soluble-form coagulase) and Eap [Boden & Flock 1989; Bodén & Flock 1992; Boden & Flock 1994]. Studies of the mutants lacking some of these adhesins showed lower virulence compared the isogenic mutants to the wild types in adherence assays [Greene *et al.* 1995] and animal models of endocarditis [Moreillon *et al.* 1995], or wound healing [Palma *et al.* 1996]. Regarding to affinity to Fibronectin (Fn), another glycoprotein in ECM, two adhesins have been identified from *S.aureus*, termed FnbpA and FnbpB [Jonsson *et al*. 1991]. Apart from a component in ECM, Fn also plays a role in mediating bacterial internalization in the endothelium and opsonization in phagocytosis. Fn binding of *S.aureus* may have a more complicated role than function as adhesin. The dual binding of FnBPs, both to Fg and Fn, makes it necessary to re-evaluate these adhesins in infections. Staphylococcal protein A (Spa), a well-characterized virulence factor, is known for its binding to Fc fragment of IgG as one of the anti-phagocytic properties to evade host defense. Likewise, a second binding function of Spa has recently been identified to von Willebrand factor (vWF) [Hartleib *et al.* 2000], suggesting its potential role in endovascular disease. Other adhesins, like Cna, which bind to collagen (Cn), acts as a virulence factor in osteomyelitis [Hienz *et al.* 1996a], endocarditis [Hienz *et al.* 1996b] and keratitis [Rhem *et al.* 2000].

The subsequent task of the colonized microorganisms is to invade tissue and spread the infection. In *S.aureus*, several toxins and exoenzymes serve this purpose. Exotoxins, enterotoxins, hemolysins, proteinases, nucleases, and lipases are involved in tissue invasion, whereby they destroy blood and tissue cells, or degrade nucleic acid and lipids, or induce an inflammatory response, or serve as superantigens neutralizing phagocytosis [Projan & Novick 1997; Lowy 1998; Dinges *et al.* 2000; Kuroda *et al.* 2001]. Several staphylococcal toxins function as superantigens, such as TSST-1, exotoxins and enterotoxins by binding to the major histocompatibility complex (MHC) class II proteins and inducing T-cells proliferation. The expansion of T-cells in turn results in the massive release of cytokines.

Table 2. defined and potential virulence factors of *S. aureus*

A. adhesins

Gene	defined or potential virulence	Ligands / functions	Reference
clfA	clumping factor A (ClfA)	Fg (C-terminal of γ-chains), calcium, platelets	[McDevitt *et al.* 1994; Hartford *et al.* 2001; Siboo *et al.* 2001]
clfB	clumping factor B (ClfB)	Fg (α-, β-chains)	[Ni Eidhin *et al.* 1998]
sdrC	SdrC	unknown	[Josefsson *et al.* 1998]
sdrD	SdrD	calcium	[Josefsson *et al.* 1998]
sdrE/bbp	SdrE/ Bbp bone sialoprotein - binding protein	bone sialoprotein	[Josefsson *et al.* 1998; Tung *et al.* 2000]
pls	plasmin sensitive protein (Pls)	reduce adherence	[Savolainen *et al.* 2001]
fnA, fnB	FnBP-A, FnBP-B	Fn, Fg (γ-chains) α5β1(epithelial cells)	[Jonsson *et al.* 1991; Miyamoto *et al.* 2001]
cna	collagen-binding protein (Cna)	collagen	[Patti *et al.* 1992; Patti *et al.* 1994]
spa	staphylococcal protein A	IgG (Fc fragment), vWF	[Uhlen *et al.* 1984a; Uhlen *et al.* 1984b; Hartleib *et al.* 2000]
ebps	elastin-binding protein	elastin	[Park *et al.* 1991; Park *et al.* 1996; Park *et al.* 1999]
fbp	fibrinogen-binding protein (FbpA)	Fg	[Cheung *et al.* 1995]
	major histocompatibility complex class II analogous protein (Map) /extracellular adherence protein (Eap)	Fg, Fn, bone sialoprotein, Vn, thrombospondin, prothrombin, internalization	[McGavin *et al.* 1993] [Palma *et al.* 1999]
vWp	vWF-binding protein (vWbp)	vWF	[Ahlen *et al.* 2001]
efb	extracellular fibrinogen-binding protein (Efb)	Fg (α-chains)	[Bodén & Flock 1992; Palma *et al.* 1998]
coa	Coagulase	Fg, prothrombin	[Boden & Flock 1989; Bodén & Flock 1992]
	thrombospondin-binding protein	thrombospondin	[Herrmann *et al.* 1991]
	laminin-binding protein	laminin	[Lopes *et al.* 1985]
icaADBC	intercellular adhesin poly-N-siccinyl-beta-1,6-glucosamine	biofilm formation	[Cramton *et al.* 1999] [Cramton *et al.* 2001]
sbi	Sbi	IgG, apolipoprotein	[Zhang *et al.* 1998; Zhang *et al.* 1999; Zhang *et al.* 2000]

B. Toxins [Projan & Novick 1997; Dinges *et al.* 2000; Kuroda *et al.* 2001]

Pathogenicity islands	Gene	Toxins	Related diseases/ functions
toxic shock syndrome	*tst*	TSST-1	toxic shock syndrome superantigen (bind to Vβ-chain of TCR)
exotoxin	*set1-5*	staphylococcal exotoxin-like proteins	susperantigens induce proinflammatory cytokine production
	set6-15		
	lpl1-9	probable lipoproteins	
enterotoxin	*egc, sea, sep, seo, sem, sei, sek, seg*	enterotoxins A-E	superantigens, food poison toxic shock syndrome unrelated to menstruation
	splA-F	secretory serine protease	
	lukD, lukE	leukotoxins	destroy white blood cells

C. Invasins [Projan & Novick 1997; Dinges *et al.* 2000; Kuroda *et al.* 2001]

Gene	Virulence factors	Functions
hla	α-hemolysin	destroys blood and tissue cells
hlb	β-hemolysin	destroys blood cells
hlgA, hlgC, hlgB	γ- hemolysin	destroys blood cells
hld	δ- hemolysin	destroys blood and tissue cells
plc	phospholipase C	hydrolyzes phosphatidylinositol
geh	lipase	degrades lipids
sak	staphylokinase	nonphysiologyical activator of fibrinolysis (activating plasminogen)
eta, etb	exfoliative toxin A, B	destroys tissue, relative to SSSS (staphylococcal scalded skin syndrome)
sspA, sspB	proteases	destroys tissue
nuc	thermonuclease	degrades nucleic acid

Comparatively less is known about the virulence of *S.epidermidis* (Table 3). Fewer adhesins have been identified in *S.epidermidis* than in *S.aureus*, although reports showed that plasma and connective tissue proteins are involved in *S.epidermidis* associated foreign body infections, such as Fg, Fn, Vn (vitronectin), Cn, vWF, thrombospondin, and laminin [Herrmann *et al.* 1988; Herrmann *et al.* 1997]. Autolysin E (AtlE) was identified to bind to Fn and Vn [Heilmann *et al.* 1997; Hussain *et al.* 1999]. The Fg affinity of Fbe/SdrG was characterized in this study (**Paper I &II**) and others [McCrea *et al.* 2000]. The corresponding adhesins for vWF, Cn, thrombospondin and laminin remain undefined. In addition, a potential heparin binding protein has been described. As a virulence factor long defined, the composition of bacterial extracellular slime substance have been studied extensively, especially capsular polysaccharide adhesin (PS/A) [Muller *et al.* 1993a; Muller *et al.* 1993b; Shiro *et al.* 1995; McKenney *et al.* 1998] and polysaccharide intercellular adhesin /hemagglutinin (PIA/HA) [Mack *et al.* 1996; Fey *et al.* 1999; Mack *et al.* 1999b; Rupp *et al.* 1999a; Rupp *et al.* 1999b]. Other slime substances were also reported, such as extracellular slime substance (ESS) [Ludwicka *et al.* 1984], sulfated slime polysaccharide [Arvaniti *et al.* 1994], slime-associated antigen (SAA) [Christensen *et al.* 1990; Baldassarri *et al.* 1996], 20kD polysaccharide (PS), 80kD PS, and teichoic acid (TA)-like substance [Kolonitsiou *et al.* 2001]. Mack, D. et al have shown that PIA function as hemagglutinin or part of it. PS/A and PIA/HA are recently found to be encoded by the same gene loci – *icaADBC*, suggesting that they are structurally related or even identical [Mack *et al.* 2000]. The composition of slime substance is culture medium and growth phase dependent. Thus the compositions of slime substance are diverse. However, a linear β-1,6-linked glucosamine is commonly found in polysaccharides of staphylococcal glycocalyx [Mack *et al.* 1999a]. Biofilm formation on the implant surface was viewed as a major strategy of *S.epidermidis* to induce an infection in a susceptible host. The biofilm of *S.epidermidis* consists of clusters of multilayered cells embedded in extracellular slime substance, up to 160 μm thick, exceeding 50 cells. Such biofilm acts as a diffusion or penetration barrier to antibiotics and host defense [Rodgers *et al.* 1994; Proctor 2000]. Other virulence factors, such as toxins, are not well defined in *S.epidermidis*. Some toxin homologues to those of *S.aureus* are found in *S.epidermidis*, like δ-haemolysin homologue [Hebert & Hancock 1985], exotoxin C-like component [Schlievert & Kelly 1982] and some heat-stable cytotoxins [Solymossy *et al.* 1982]. Invasins such as lipases, nucleases and proteinases, are also found in *S.epidermidis* as in *S.aureus*. Database search of the nucleotide in PubMed revealed several potential open reading frames of lipases and proteinases, like elastase, cystein proteinases, and serine proteinases.

Table 3.Defined and potential virulence factors (adhesins) in *S.epidermidis*

Gene	virulence factor (defined or potential)	Location	Ligand / function	Reference
icaADBC	PS/A (capsular polysaccharide /adhesin)	surface	promotes adherence	[Tojo *et al.* 1988; Kojima *et al.* 1990; Muller *et al.* 1993a; Muller *et al.* 1993b; Shiro *et al.* 1995; McKenney *et al.* 1998]
icaADBC	PIA / HA (polysaccharide intracellular adhesin /hemagglutinin)	surface	biofilm formation, hemoagglutination, promotes adherence	[Mack *et al.* 1996; Fey *et al.* 1999; Mack *et al.* 1999b; Rupp *et al.* 1999a; Rupp *et al.* 1999b]
	teichoic acid	surface/ secretory	promotes adherence, Fn	[Hussain *et al.* 1992]
atlE	AtlE (autolysin E)	surface	Fg, Fn, Vn promotes adherence	[Heilmann *et al.* 1997; Hussain *et al.* 1999]
	Ssp-1 , Ssp-2 (staphylococcal surface protein 1 and 2)	surface	mediates adherence	[Veenstra *et al.* 1996]
	AAP (accumulation associated protein)	secretory	cell accumulation on polymer surface	[Hussain *et al.* 1997]
fbe/sdrG	Fbe	surface	Fg (N-terminal of β-chains)	[Nilsson *et al.* 1998; Pei *et al.* 1999; McCrea *et al.* 2000; Davis *et al.* 2001]
sdrF	SdrF	surface	unknown	[McCrea *et al.* 2000]
sdrH	SdrH	surface	unknown	[McCrea *et al.* 2000]
	Tpn (transferrin-binding protein)	surface	transferrin, glyceraldehyde-3-phosphate dehydrogenase	[Modun *et al.* 1998a; Modun *et al.* 1998b; Modun & Williams 1999]
	ABC transporter (ATP-binding cassette)		antibiotic resistance, uptake of ATP	[Otto & Gotz 2001]
ssaA	SsaA (staphylococcal secretory antigen)	secretory	antigenic protein endocarditis-related	[Lang *et al.* 2000]

The genes encoding bacterial virulence factors are tightly controlled. Studies on *S.aureus* have identified at least two essential global regulatory systems, *agr* (accessory gene regulator) [Peng *et al.* 1988] and *sar* (staphylococcal accessory regulator) [Cheung *et al.* 1992]. The *agr* locus consist of five genes, *agr*A, *agr*C, *agr*B, *agr*D and *hld* [Ji *et al.* 1995]. It is involved in regulating synthesis of both exoproteins (TSST-1, α-toxin, and some extracellular enzyme) and cell wall proteins (Spa,

Fnbps, and Coa) in a growth phase dependent manner [Novick *et al.* 1993; Morfeldt *et al.* 1996; Wolz *et al.* 1996; Ji *et al.* 1997]. On transcriptional level, *agr* consists of two divergent units, RNAII and RNAIII. RNAII is transcribed from promoter P2 encoding for four open reading frames, AgrA (response regulator), AgrC (sensor), AgrD (precursor of an octapeptide pheromone) and AgrB (AgrD processor). RNAIII is transcribed from the promoter P3 and codes for Hld (δ haemolysin). The *sar* locus is also involved in the regulation of exoproteins and surface proteins [Cheung *et al.* 1997b]. Both *agr*-independent and -dependent manner might be involved in gene regulation of *sar* [Cheung *et al.* 1997a; Chien & Cheung 1998]. Studies on single or double mutants of *agr* and *sar*, have shown significant reduction of the virulence of *S.aureus* in animal models [Abdelnour *et al.* 1993; Cheung *et al.* 1994; Gillaspy *et al.* 1995]. Both *agr* and *sar* homologues are found in *S.epidermidis* [Fluckiger *et al.* 1998; Otto *et al.* 1998]. The comparisons were done on these homologous genes. The *agr* loci of *S.epidermidis* comprise *agr*A, *agr*C, *agr*B, *agr*D as those of *S.aureus*. Study on an isogenic mutant of *agr* in *S.epidermidis* indicated a defect in lipase processing as well as an impact on biofilm formation [Vuong *et al.* 2000]. A major open reading frame of a *sar* homologue was found in *S.epidermidis* of 84% identity to that of *sar* in *S.aureus* [Fluckiger *et al.* 1998]. A recent study shows that RNAIII homologues from *S.epidermidis* can regulate *agr*-dependent virulence genes in *S.aureus*, suggesting that such virulence factors may also exist in *S.epidermidis* [Tegmark *et al.* 1998].

Foreign body infection associated with *S.epidermidis*

I. Conditioning on the implanted foreign body

The medical devices used extensively in health care, have been regarded as a great breakthrough in modern medicine. The biomaterials have many applications, from a simple intravenous catheter to a more complex artificial organ. They consist of various materials: polymers, homologous- or heterologous-modified tissue, and metals. They may be implanted in patients temporarily or permanently. The patients receiving a foreign body, have various degrees of immunocompetence, who may be fairly healthy or seriously ill. In those subpopulations of immunocompromised, chronically ill and at the extremes of life, the presence of a foreign body increase the risk of infection [Hubner & Kropec 1995].

Due to the nature of the biomaterial, initial implantation of a medical device is associated with a rapid covering of the implant by human serum albumin (HSA), Fg, Fn, Cn, vWF, Vn and platelets. The adsorbance of these components occurs in a specific order: first by HAS, which is most

abundant in plasma, then replaced by Fg, Fn and others. The final composition of the conditioning film may differ according to the type of material, insertion site and duration of the implant. Generally speaking, to short-term implants, Fg is constantly present, whereas Fn and vWF are present occasionally [Francois *et al.* 2000], while to long-term implants, Fn is more commonly found. As a subsequent processing, host cells, such as platelets, endothelial cells, fibroblasts, and bone cells, may attract to bind to the implant by the newly-formed conditioning film [Gristina 1987]. To the pathogens, such a layer of conditioning film, or adherent host cells may provide potential sites to initiate colonization.

It is currently believed that two steps are involved in *S.epidermidis* associated foreign body infections: primary attachment and cell accumulation on the polymer surface. Studies have shown that not only hydrophobic interactions, but also specific interactions between matrix proteins and their adhesins on the bacterial surface contributed to microbial adhesion on biomaterials. PS/A and antiserum against it show inhibition in bacterial attachment [Kojima *et al.* 1990; Shiro *et al.* 1995]. Biofilm formation, mediated by PIA/HA, is important in cell accumulation, and thus contributes to its virulence [Mack *et al.* 1996; Rupp *et al.* 1999a]. However, one third of clinical isolates of *S.epidermidis* are biofilm-negative. Biofilm formation is a late event in bacterial growth. Other factor, like adhesin(s) to ECM, may be a prerequisite for microorganisms to persist on the foreign body and cause infection.

II. Fibrinogen

Fg, a dominant glycoprotein in plasma and extracellular matrix, is composed by three pairs of polypeptide chains, $A\alpha B\beta\gamma_2$. The molecule is a covalent dimer linked by 29 disulfide bonds. The structure of Fg has been well studied. The Fg chain amino termini are clustered in a central E-domain from which two coiled-coil regions end in a distal carboxyl terminal D-domain containing predominantly the C-terminal of β and γ globular domains (as shown in Figure 1). The overall molecular weight of this dimeric molecule is 340 kDa – 67 kDa for α-chain (α1-610), 55 kDa for β-chain (β1-461) and 48 kDa for γ-chain (γ1-411).

Clot formation, a primary function of Fg, has been extensively studied for decades [Doolittle 1984; Blomback 1996]. A series of reactions lead to thrombin cleavage of Fg, which releases fibrinopeptides A (FpA) and B (FpB) from the amino termini of the α- and β-chains. Thus fibrin monomers polymerize spontaneously. By releasing FpA, the interaction between the newly-

exposed α-chain N-terminus (Gly-Pro-Arg-Pro, GPRP), also called A-knob, and the complementary polymerization pockets located in the carboxyl-terminal domains of the γ-chains (a-site), provide the main driving force for the fibrin monomer association. This noncovalent end-to-end association brings together each central amino terminal E-domain with the distal carboxyl terminal D-domains of two other fibrinogen molecules, and results in a double array of protofibrils. The FpB is released later than FpA. The newly-exposed β-chain N-terminus (Gly-His-Arg-Pro, GHRP), also called B-knob, binds to the complementary pockets located on its C-terminal (b-site), which leads to lateral growth of the fiber [Everse *et al.* 1998]. The polymer is further stabilized by factor XIIIa-catalyzed covalent ε-amino-(γ-glutamyl)-lysine cross-links.

In a recent study of clotting in whole blood, fibrin I formation in whole blood is found to start with a low level of thrombin. The sites exposed in the E-domain after release of FpA are overlapped by the D-domain to form protofibrils. Cross-link forms between intact Fg/Fg, Fg/fibrin, or soluble cross-linked fibrin/fibrin. Thrombin continues to remove FpAs and activate factor XIII, yielding an initial clot composed by fibrin I and Fg with γ-γ cross-links. Only in the late stage of clotting, does thrombin remove some of the FpBs to produce the final clot. The newly-released FpB is degraded by a carboxypeptidase-B-like enzyme to des-Arg FpB. Only 30% of FpB is released, while most Bβ-chains remain intact in whole blood clotting [Brummel *et al.* 1999].

Calcium plays an important role in clotting by promoting formation and cross-linking of fibrin polymers. Several Ca^{2+} binding sites have been identified in Fg with a widely varying affinity, i.e., a strong Ca^{2+} binding site composed of $Asp^{\gamma 318}$, $Asp^{\gamma 320}$, $Phe^{\gamma 322}$ and $Gly^{\gamma 324}$, four weak binding sites of $Asp^{\beta 381}$-$Asp^{\beta 383}$-$Trp^{\beta 385}$, $Glu^{\gamma 132}$-$Asp^{\beta 261}$-$Glu^{\beta 397}$-$Asp^{\beta 398}$, $Glu^{\gamma 132}$-$Asp^{\beta 261}$-$Gly^{\beta 263}$ and $Asp^{\gamma 294}$-$Gly^{\gamma 296}$-$Asp^{\gamma 298}$-$Asp^{\gamma 301}$. It is believed that binding of Ca^{2+} leads to conformation changes and rearrangement of the amino acid side-chains participating in fibrin formation [Everse *et al.* 1999]. Furthermore, Ca^{2+} binding is known to stabilize Fg against heat denaturation, and reduces fibrin (ogen)'s susceptibility to proteases, such as plasmin and trysin, thus strengthen the clots.

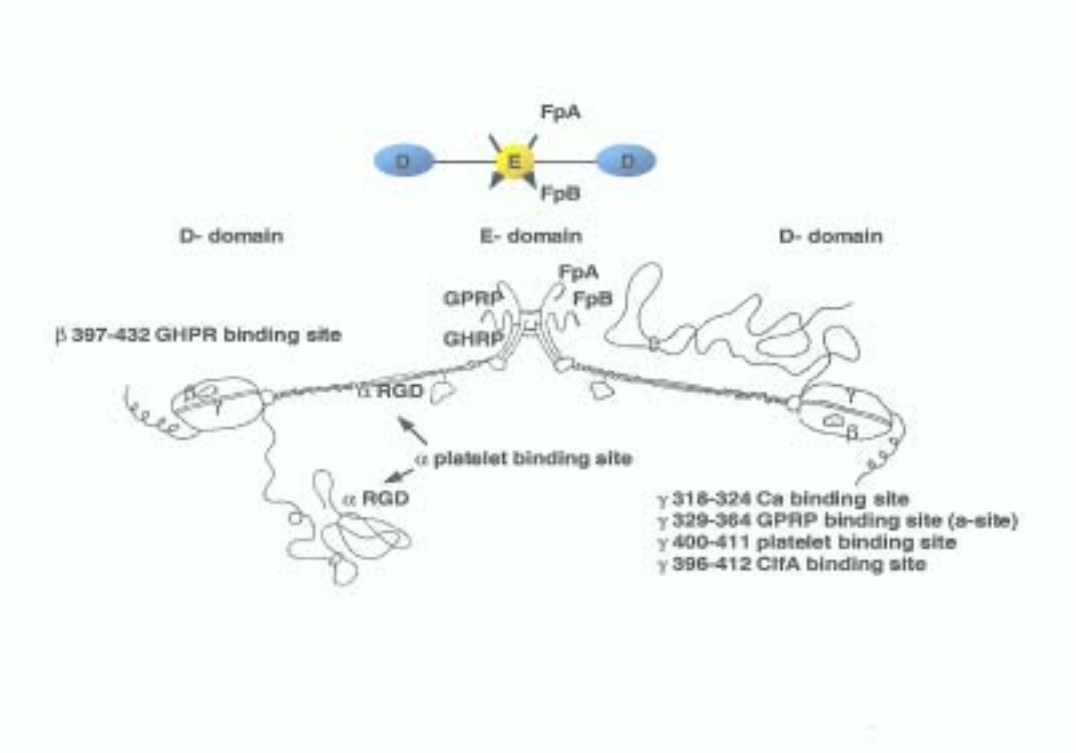

Figure 1. Schematic drawing of fibrinogen. Modified from Doolittle R., 1984, with permission.
D for distal domain, E for central domain, FpA for fibrinopeptide A, FpB for fibrinopeptide B, GPRP for Gly-Pro-Arg-Pro, the A-knob on the N-terminal of α-chain after releasing FpA, GHRP for Gly-His-Arg-Pro, the B-knob on the N-terminal of β-chain after releasing FpB, RGD for Arg-Gly-Asp, and Ca for calcium.

Other host factors also interact with fibrin(ogen) [Blomback 1996]. Fn forms complexes with fibrin(ogen) via covalent bonds between ε-amino groups of lysine in the α-chains of Fg and glutamine residues of fibronectin. Platelets, another important factor in hemostasis and wound healing, interact with fibrin(ogen) via two glycoprotein complexes GPIIb/IIIa, which is activated by various agonists. Approximately 40,000 such receptors are present on the surface of a platelet. At least three binding sites on Fg for platelets have been identified. One is located on the C-terminal of γ-chains (γ400-411), and the other two are located on α-chains with identified sequence Arg-Gly-Asp (RGD) at residues Aα95-97 and Aα572-574. Thrombospondin, present on platelets and endothelial cells, forms a complex with Fg via α - and β-chains. Some data show that Fg binds to blood cells and endothelial cells via different ligands, i.e., integrin MAC-1 mediates binding of Fg to leukocytes [Altieri *et al.* 1988] and intercellular adhesion molecule-1 (ICAM-1) mediated binding of Fg to endothelium [van de Stolpe *et al.* 1996].

AIMS OF STUDY

The main aim was to study a fibrinogen-binding protein (Fbe) from *S. epidermidis*.

The more detailed aims were:

- To identify fibrinogen-binding protein (FgBP) of *S.epidermidis*
- To characterize FgBP of *S.epidermidis*
- To evaluate the role of antibodies against FgBP of *S.epidermidis* in adherence of *S.epidermidis* to biomaterials
- To evaluate the role of FgBP of *S.epidermidis* by constructing an isogenic mutant deficient in the corresponding gene.

METHODS

Phage display to identify interactions between fibrinogen and *S. epidermidis*

Chromosome DNA of *S. epidermidis* strain HB was prepared and fragmented by sonication. A sample, with most DNA fragments about the size of 500 bp, was made into a blunt end by using T4 DNA polymerase. These fragments were ligated into SmaI-digested and dephosphorylated pG8H6 vector and then electrotransferred into *E.coli* strain TG1. The phage particles, which represented phagemid libraries of *S. epidermidis*, were eluted from the soft agar and the suspension was collected by centrifugation and filtration to obtain phagemid libraries of *S. epidermidis*. These constructed phagemid libraries were subjected to human Fg-coated microtiter wells. The bound phage was eluted and enriched by another round of panning. Sequences were determined on phagemid DNA of the individual clones obtained from the second round of panning.

Construction of recombinant Fbe

A DNA fragment of *fbe* gene (encoding A region of Fbe) was amplified by PCR and cloned into a glutathione S-transferase (GST) fusion vector pGEX-4T3 (Pharmacia, Uppsala, Sweden). This fusion expression system utilizes an affinity tag consisting of GST at the N-terminal end of the recombinant protein, which can bind to Glutathione-Sepharose 4B. This plasmid encodes a thrombin protease recognition site between glutathione S-transferase of 29 kDa and the C-terminal fused protein.

Extraction of proteins

To extract proteins from bacterial surfaces, washed bacterial cells were incubated with 1 M LiCl together with cocktail protease inhibitors, DNaseI and RNaseA, at 37°C for 1 hour. The supernatant of LiCl-treated bacteria was desalted by dialysis. In another experiment, bacterial surface components were extracted with 2% SDS at 95°C for 10 minutes. The whole cell components were obtained with cells lysed by Lysostaphin in addition of cocktail protease inhibitors, DNaseI and RNaseA [Hussain *et al.* 1999].

To extract proteins from biomaterials, polyethylene catheters coated with Fg *in vitro* (FgCC), subcutaneously implanted in rats (SIC), and intravascularly in patients (PVC and CVC), were extracted with 2% SDS at 95°C for 10 minutes as described by Francois [Francois *et al.* 2000].

Purification of proteins

I. Purification of recombinant Fbe by affinity to Glutathione-Sepharose

Recombinant Fbe, previously appeared as GST-Fbe, is now renamed as rFbe, referring either GST-Fbe or thrombin-cleavage GST-Fbe, was expressed by isopropyl thio galactoside (IPTG) induced culture of *E. coli* strain TG1 harboring recombinant plasmid. The lysed bacterial cells were applied to Glutathione-Sepharose 4B column. The bound rFbe was eluded by affinity elution of reduced glutathione.

II. Purification of recombinant Fbe by affinity to Fibrinogen-Sepharose

Fg-Sepharose was prepared by coupling 70 mg human Fg (Sigma) to 3.5 g CNBr-activated Sepharose 4B (Pharmacia) using the procedure recommended by the manufacturer. Bacterial lysate was applied to Fg-Sepharose 4B, washed and eluted by high concentration of salt.

III. Purification of recombinant Fbe by Fast Protein Liquid Chromatography (FPLC)

Proteins purified by affinity chromatography, or further by thrombin digestion, were purified by FPLC on the MonoQ HR5/5 column (Pharmacia). Elution was done by salt gradient.

IV. Generation and purification of antibodies

Rats were immunized with 3 x 20 μg of rFbe, or GST as a control, with 2-week's interval. Serum samples were taken two weeks after the last immunization. Freund´s complete adjuvant was used at the first time and Freund's incomplete ajuvant the second and third times. Antibodies were purified by affinity to Protein G Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden).

V. Isolation of α -, β - and γ -chains of fibrinogen

The chains of the reduced Fg were purified by running overnight at 200V a preparative 8% SDS-PAGE on a 491 Prep Cell (BioRad).

VI. Isolation of fibrinogen digested by trypsin

Proteolytic fragmentation of Fg with trypsin was done to identify the binding region of β-chains of Fg to Fbe. Fg fragments D and E were prepared from trypsin-digested human Fg. In brief, human Fg (7.5 mg/ml) was digested with 0.05 of the original volume of trypsin (1 mg/ml) for four hours at room temperature, then 0.05 of the original volume of soybean trypsin inhibitor (3 mg/ml) was added to stop further digestion. The fragmented Fg was applied to the MonoQ HR5/5 column to

separate fragment D (mainly distal domain of Fg) and fragment E (mainly central domain of Fg) by FPLC.

Interaction between recombinant Fbe and fibrinogen

Microtiter wells were coated with serially-diluted human Fg (Sigma), or isolated chains of Fg (α-, β-, or γ-) at room temperature overnight. The nonspecific binding sites were reduced by 2% bovine serum albumin (BSA). After thorough washing, rFbe was added to the immobilized Fg. Capture of the rFbe was detected with antiserum from rats. In another study, microtiter wells were coated with serially-diluted rFbe, and then soluble Fg was added. Captured Fg was detected by horseradish peroxidase-conjugated rabbit antibodies against human Fg (Fg-HRP, Dakopatts, Denmark).

The role of Ca^{2+} in rFbe-Fg interaction was studied by dialyzing all proteins against 10mM EDTA and then 10 mM Tris + 100mM NaCl to remove Ca^{2+} trapped with the proteins. The protocol mentioned above was followed to study the effect of additional Ca^{2+}.

To study the cross-interaction of competitive components in rFbe-Fg, various proteins in stepwise dilution (such as rFbe, GST, or ClfA) together with a constant concentration of Fg, was added to rFbe-coated microtitier wells. The bound Fg to immobilized rFbe in the presence of competitive components, was detected by Fg-HRP.

Function of recombinant Fbe and its antibodies in the interaction between *S. epidermidis* and polymer surfaces

I. Function of rFbe and its antibodies in adherence of *S. epidermidis* to fibrinogen

Radioactive-labeled *S. epidermidis* cells, which were pre-incubated with competitive components (rFbe or GST) in serially-diluted concentrations, were added to Fg-coated microtiter wells. After incubation, unbound bacteria were rinsed and the adherent bacteria were released by adding 2% SDS. The bacteria released were determined by their radioactivity. Radiolabeled bacteria were also pretreated with various dilutions of antibodies against rFbe or GST. The antibody-treated bacteria were transferred to Fg-coated microtiter wells and adherence was determined, as described above.

II. Function of rFbe and its antibodies on adherence of *S. epidermidis* to catheters

Polyethylene catheters (I.D. 2.92 mm, O.D. 3.73 mm, Becton Dickinson, Sparks, MD, USA) were conditioned by coating with Fg (FgCC) or Fn (FnCC), or by subcutaneous implantation in rats

(SIC). Ex vivo materials - peripheral venous catheters (PVC) were collected from patients who had undergone intravenous injections. While subjected to adherence assay, catheters were cut into halves and analyzed in pairs. Bacteria pretreated with various purified antibodies, anti-rFbe, or as a control anti-GST, in the concentrations indicated were applied to the conditioned catheters. After rinsing, the bacteria bound to these catheters were released by sonication, and determined by viable counting on blood agar plates.

Inactivation of *fbe* by allelic replacement

Allelic replacement has been applied to inactive *fbe*. In brief, two fragments composed of *fbe*1 and *fbe*2, upper and lower parts of *fbe* respectively, with an inserted fragment (*gmr*) in-between, were amplified by PCR and cloned into a temperature-sensitive shuttle vector plasmid carrying ErmR marker. Chromosomal DNA from strain HB was used as template. The constructed plasmid (*fbe*::*gmr*) was first transformed into restriction-deficient *S. aureus* strain by electroporation, and then plasmid purified from the transformed strain was introduced into *S. epidermidis* strain HB. The correct chromosomal integration of the transformed plasmid was selected, following the protocol described by Palma [Palma *et al.* 1996]. In brief, wild type strain harbored the constructed plasmid was grown at its permissive temperature (32°C) together with Erm+Gm at their subinhibition concentration. The culture was also grown continuously at nonpermissive temperature (42°C) of the plasmid, with Gm alone. In the latter cultivation, the nonpermissive temperature favored the strains of which harbored plasmid had inter-molecularly recombined with the homologous part of *fbe* in its chromosomal DNA. After dilution, the culture continued to grow at 42°C but with no antibiotics. During this period, a second recombination may have happened intra-molecularly between inserted plasmid DNA and *fbe* in bacterial chromosome in certain strains. This culture was plated on agar plates with Gm alone. The colonies that grew only on Gm plates, but not Gm+Erm, were selected for further tests. PCR amplification, using different pairs of primers was done to identify mutants. Direct sequencing on fragments of mutant chromosomal DNA was done by primer walking, using reverse primers.

RESULTS

Identification of *fbe*, the gene encoding a Fibrinogen-binding protein of *S.epidermidis* (Fbe)

I. *fbe* is identified by shotgun phage display

A clone of phagemid, termed pSEFG1 was identified by panning a *S.epidermidis* phage display library (derived from strain HB) against immobilized Fg. Purified protein derived from pSEFG1 could block the adherence of *S.epidermidis* to Fg-coated polymer surfaces, which implied that part of a Fg adhesin of *S.epidermidis* had been identified. The complete sequence then resulted in an open reading frame of 3276 nucleotides, called *fbe* (fibrinogen binding protein from *S.epidermidis*). Preliminary analysis showed that several domains were encoded by *fbe*, including an A-domain following a signal sequence (S) at the N-terminal, an R-domain composed of dipeptide serine-aspartate repeats (Sdr), a cell wall anchoring motif (W) and a membrane spanning region (M) (**Paper I**). Such composition was commonly found in other surface proteins of Gram positive bacteria [Navarre & Schneewind 1999]. Fbe and other members of the Sdr multigene family are compared in Figure 2. To date, at least eleven of them have been identified. Three (Fbe/SdrG, SdrF, SdrH) are from *S.epidermidis*, six (ClfA, ClfB, SdrC, SdrD, SdrE/Bbp, Pls) from *S.aureus*, and two (SdrY and SdrZ) from *S.caprae* [Aubert *et al.* 2000].

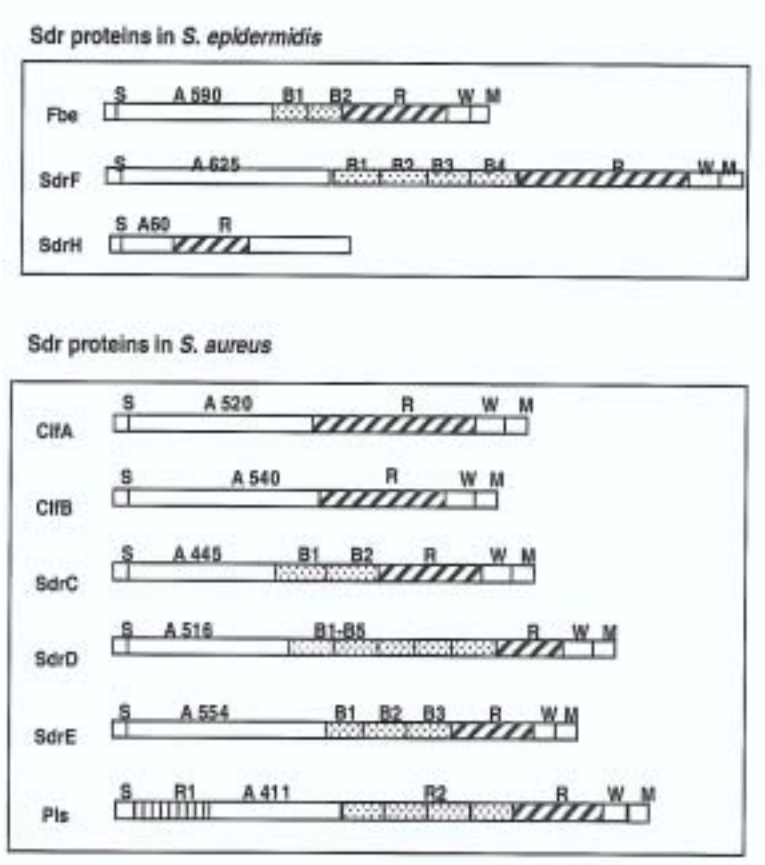

Figure 2.
Diagram of structures of several staphylococcal proteins from the Sdr multigene family.
S stands for signal sequence, A for ligand binding region, B for 110-residue repeat, R for Ser-Asp dipeptide repeat, R1 for 14-residue repeat, R2 for 128-residue repeat, W for wall-spanning region, and M for membrane-spanning region. Adopted from Josefsson *et al.* 1998, McCrea *et al.* 2000, and Savolainen *et al.* 2001.

II. *fbe* is common and conserved in isolates of *S.epidermidis*

Heterogeneous adhesion to immobilized Fg was found among *S.epidermidis* isolates, as shown in Figure 3A (**Paper I**). They could be classified as strong adherence strains (like strain No.2 and No.19), moderate adherence strains (like HB and No.269) or weak adherence strains (like No.333). Forty-three isolates of *S.epidermidis* were studied by PCR, using primers derived from the A-region of *fbe*. This part of *fbe* was commonly found among them. The amplified fragments from strains No.269, JW14 and KJ4 were sequence determined. Alignments of them showed that part of *fbe* was conserved, with identity over 80% of their amino sequences (Figure 3C). However, variations in the length of Sdr were found in PCR amplifications using primers located in the B2-region and cell wall anchoring motif in seven strains (HB, No.269, No.19, No.2, O47, JW14 and KJ4, as shown in Figure 3B). Homologous search in the database using *fbe* as query identified another two complete sequences of *fbe* from *S.epidermidis* strains – RP62A and K28. Fbe homologue of RP62A, a strain well characterized for its ability to form biofilm, has a very short SD repeat (50 amino acids) with a truncated signal sequence at its N-terminal. Fbe homologue of K28 (also called SdrG [McCrea *et al.* 2000]), contains short SD repeats of 56 amino acids as well, while Fbe of HB contains 216 amino acids in SD repeats. The alignments among these Fbe proteins showed high homologies, with 94-100% identity, as shown in Figure 3C.

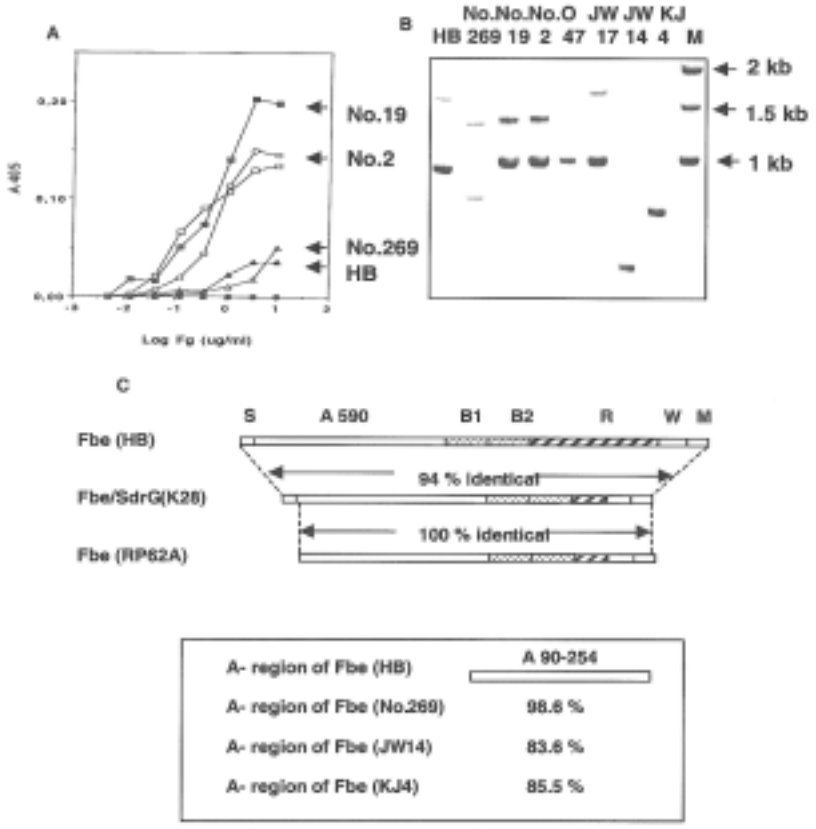

Figure 3.
A. Adhesion of *S.epidermidis* to immobilized Fg on the microtiter plates.
B. PCR results showing the variation in the length of SD-repeats from eight different strains of *S.epidermidis*.
C. Alignment of sequences of Fbe from three strains (HB, K28 and RP62A) of *S.epidermidis*, as well as the comparisons of part of the A-regions from four strains (HB, No.269, JW14 and KJ4).

Functional studies of recombinant Fbe

I. Binding site of rFbe to fibrinogen is located in its A-domain.

A more extensive *S.epidermidis* phage display library was constructed based on the genome of a peritonitis isolate strain No.19, which could bind strongly to Fg. Nine phagemids were enriched by panning against Fg. Alignment of the insertion in these phagemids with Fbe revealed an overlap within residues 269 and 599. Thus, these 331 amino acids in the A-region of Fbe corresponded to its Fg binding (**Paper I**).

Part of *fbe* was then cloned into a glutathione S-transferase (GST) fusion vector. Such portion of Fbe (amino acid residues 87 to 646) covered the putative Fg binding sites and part of the B1-repeat. It was expressed in its fusion form with GST, which functioned as an affinity tag for chromatography. Characterization studies showed that (1) rFbe could be purified by Fg-Sepharose; (2) rFbe could bind to immobilized Fg on polymer surfaces of microtiter plates; and (3) rFbe could be recognized by soluble Fg in captured ELISA assays. As a control in these assays, GST could not interact with Fg. Thus, the affinity of rFbe to Fg was further confirmed (**Paper II**).

II. The binding sites of fibrinogen to rFbe are located in its β–chains

Chains derived from Fg, the α–, β– and γ– chains, were separated by SDS-PAGE, transferred to nitrocellular membranes and probed by rFbe. Results showed that rFbe bound to the β–chains of Fg, while in controls, ClfA bound to the γ–chains. The α–, β– and γ–chains were further separated by gel filtration. Either α–, β– or γ–chains were immobilized on microtiter plates. rFbe only bound to β–chains of Fg in a dose dependent manner. Thus, the binding sites of Fg to rFbe are located in its β–chains (**Paper II**).

Limited enzyme digestion of Fg by trypsin was done to generate fragment D and E of Fg. Under the designed condition of enzyme digestion, N-termini of Fg, FpA and FpB, were degraded. E fragment contained the remaining N-termini of Fg, and D fragment contained most of the C-terminal of β– and γ–chains together with a small part of the α–chains. Among the trypsin-digested derivatives of Fg, none of them were recognized by rFbe in the Western immunoblotting assay compared to the intact β–chains of Fg. This suggests that the binding site for rFbe in β–chains may be in the N-termini and sensitive to trypsin digestion.

III. Calcium can promote the interaction between rFbe and fibrinogen

The interaction between EDTA pretreated Fg and rFbe was promoted in a dose dependent manner by adding Ca^{2+}. The increase in adherence between immobilized rFbe and soluble Fg is greater than the increase in adherence of immobilized Fg and soluble rFbe (**Paper II**).

IV. rFbe can reduce the adherence of *S.epidermidis* to immobilized fibrinogen

When radioactive-labeled *S.epidermidis* was pre-incubated with rFbe, a significant reduction in bacterial adherence to immobilized Fg was found. Such adherence was inhibited almost completely by rFbe, but not by the control proteins either GST or ClfA (**Paper II**).

Functional studies of antibodies against recombinant Fbe

Antibodies raised against rFbe could inhibit the adherence of *S. epidermidis* to Fg-coated microtiter plates. The reduction in bacterial adherence was dose-dependent in response to antibodies against rFbe (**Paper II**). Anti-rFbe were also applied to adhesion of *S. epidermidis* to three types of catheters: coated with Fg *in vitro* (FgCC), implanted subcutaneously in rats for 24 hours (SIC), and peripheral venous catheters taken from patients (PVC). Fg or fragments of Fibrin(ogen) was detectable from all these types of catheters, despite difference in Fg density (Figure 4. A1-A3). In all cases, antibodies against rFbe reduced the adherence of *S. epidermidis* compared to pretreatment with antibodies against GST. An 80% reduction was obtained with FgCC (p<0,001), 50% with SIC (p=0,05) and 60% with PVC (p<0,01). Analysis on components adsorbed on these catheters showed that the ability of anti-rFbe to inhibit bacterial adherence was not only relative to the amount of Fg, but also to the other adsorbed components. The reduction was most pronounced on surfaces coated solely with Fg, like FgCC, while reduction obtained on SIC and PVC where Fg co-presented with other undefined host components showed to of a lower magnitude. In other adherence assays, neither adherence of *S.epidermidis* to Fn-coated surfaces (FnCC) nor adherence of *S.aureus* to Fg-coated surfaces could be blocked by anti-rFbe, suggesting affinity and species specificity of anti-rFbe (**Paper III**).

Role of fibrinogen in adherence of *S.epidermidis* to polymer surfaces

The adherence of *S.epidermidis* to the surface occurred rapidly, reaching 50% of the maximal levels within 15 minutes and it was completed within 150 minutes. The bacteria bound to the Fg-coated surfaces in a dose-dependent manner. The subcutaneously implanted catheters in rats were left for 2 hours, 24 hours, 2 days or 4 days and then explanted to assess bacterial adherence. The maximal

number of bacteria recovered from catheters was found on catheters implanted for one day. Bacterial adherence to SIC was only one third of that to FgCC, when the semi-quantitative comparison by Western immunoblotting showed that the amount of Fg adsorbed on SIC was less than that of FgCC.

Paralleled comparison was also done on catheters coated with 0.2 mg/ml Fg or Fn, 20mg/ml (2% w/v) BSA or left uncoated. Adherence was highest on the uncoated ones, while those coated with BSA showed the lowest adherence. Comparing to the BSA-coated surfaces, Fg- and Fn- coated increased bacterial adherence. Such promotion in adherence was to the same extent by either Fg- or Fn-coated. On the other hand, while comparing to the uncoated surfaces, Fg- and Fn-coated showed a reducing effect.

Three types of explanted materials have been subjected to studies on the components adsorbed– SIC from rats, PVC and central venous catheters (CVC) from patients. Silver staining revealed more than 30 bands from these materials representing host components – extracellular matrix (ECM) and/or host cells adsorbed on polymer surfaces. Derivatives of fibrin(ogen) were found in all extracts of these samples, particularly its β–chains. Further study on PVC showed that only one of these fibrin(ogen) derivations, its β-chains, could be recognized by rFbe (Figure 4. B1 and B2). No band was detected by probing these materials with anti-Fn.

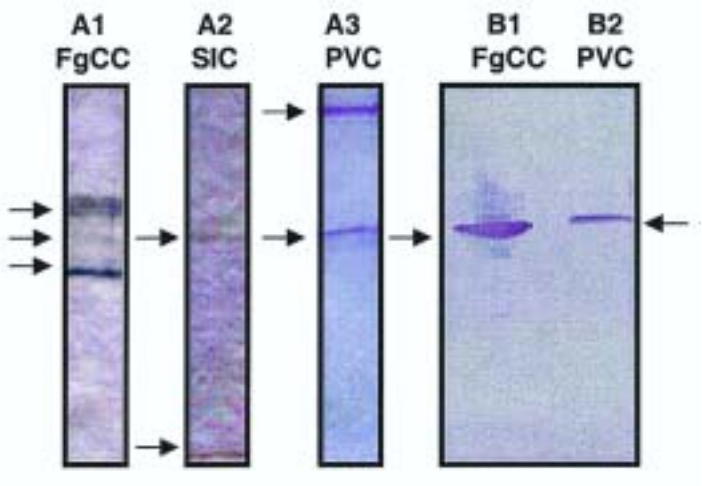

Figure 4. Western immunoblotting results of extracts of different catheters. Panel A1-3, extracts of FgCC, SIC from rats and PVC from patients respectively, probed by anti-Fg-HRP; panel B1-2, extracts of FgCC and PVC from patients, probed by rFbe, then rat-anti-rFbe, and detected by sheep-anti-rat-HRP. The bands detected are indicated by arrows.

Role of *fbe* in adherence of *S.epidermidis* to fibrinogen-related biomaterials

I. Isogenic mutant lacking *fbe*, STO56 (*fbe*::*gmr*), is constructed in *S.epidermidis*

A strain called STO56 (*fbe::gmr*) derived from strain HB with an allele replacement of the *fbe* gene was constructed. PCR analysis with various primer combinations verified the substitution of a 1.4 kb fragment of the central part of the *fbe* gene replaced with a 2.0 kb fragment of *gmr*. A difference in size of approximate 600 bp was found between the amplified DNA from wild type strain HB and its isogenic strain STO56 as expected from the designation. Integration of *fbe::gmr* by intermolecular and intramolecular recombination was evident also by the fact that primers not annealing to the recombinant plasmid pPL 3.7 (HB-26, HB3218 and HB3473) gave PCR amplification. Correct integration of *gmr* was further shown by primer walking on the PCR-amplified fragment generated by primers HB-37+GmR5. The sequence obtained from the PCR fragment was compared to the wild type sequence of *fbe*. The alignment showed that the sequence obtained from STO56 contained the *gmr* sequence inserted and the restriction enzyme sequence (KpnI, GGTACC) from primer P1L had been replaced by the wild type sequence. These findings indicated that allelic replacement had taken place and the *gmr* fragment thus replaced the part of *fbe* between *fbe1* and *fbe2*. Since *gmr* fragment, containing transcriptional and translational stop codons on both termini, had been introduced into *fbe*, the native Fbe was interrupted (**Paper IV**).

II. Mutant STO56 (*fbe*::*gmr*) binds less to fibrinogen than its parental strain HB

Strain STO56 (*fbe*::*gmr*) and its parental strain HB were tested for their adherence to Fg. Strain HB adhered to the immobilized Fg on microtiter plates in a dose dependent manner compared to strain STO56. When coated by serially-diluted Fn, strains HB and STO56 showed a clear tendency to dose response. On a fixed coating concentration (200 μg/ml) of Fg or Fn, the binding of strain STO56 to Fg was significantly lower than that of strain HB ($p<0.001$), whereas the binding to Fn, remained the same in these two strains ($p>0.05$). Strain STO56 bound 75% less to FgCC ($p<0.0001$), and 52% less to PVC ($p<0.05$) than its wild type strain HB (**Paper IV**).

DISCUSSION

Serine-aspartate repeat protein multigene family of staphylococci

Adhesins of microorganisms have been considered as virulence factors of microbial- associated infectious diseases. They serve as mediators to initiate the colonization of the pathogens. Adhesins of staphylococci, mainly from *S.aureus*, have been studied extensively. Some of them contain serine-aspartate repeat region (Sdr), which are also cell wall-associated proteins with specific affinity to extracellular matrix proteins. Such groups of proteins are termed Sdr multigene family [Josefsson *et al.* 1998]. At least six members have been identified in *S.aureus*, i.e., ClfA, ClfB, SdrC, SdrD, SdrE/Bbp and Pls. In our study (**Paper I**), a new adhesin of *S.epidermidis*, termed Fbe with affinity to Fg, was identified and characterized. Subsequently, two more Sdr members (SdrF and SdrH) together with a homologue of Fbe (SdrG) were identified by others [McCrea *et al.* 2000]. The homologous gene of *fbe* can be found in a common laboratory strain of *S.epidermidis*, RP62A. In addition, Sdr proteins found in the two virulent species of staphylococci, two more Sdr proteins (SdrY and SdrZ) were identified in an avirulent species of CoNS, *S.caprea* [Aubert *et al.* 2000]. Despite the species specificity of these Sdr proteins, they share some common features. Most are composed of a putative secretory signal sequence at the N-terminal, followed by an A-region of specific affinity, R-region of serine-aspartate dipeptide repeats, cell wall spanning sequence, an LPXTG motif and a hydrophobic amino acid sequence at the C-terminal. Some members have B-repeats between the A- and the R-region, i.e., SdrC, SdrD, SdrE/Bbp, Fbe/SdrG and SdrF, and some have a long residue region dividing the LPXTG motif from the hydrophobic amino acid sequence, i.e., SdrH and SdrZ [Aubert *et al.* 2000; McCrea *et al.* 2000].

The properties of some A-regions have been identified. Three of them have the affinity to Fg, like the A-regions of ClfA, ClfB and Fbe/SdrG. These regions share similarity approximately 25%, for instance, 26% of ClfA and ClfB, 25% of Fbe and ClfB, 24% of Fbe and ClfA. Despite their similarities, Fbe binds to the N-termini of β-chains of Fg, while ClfA binds to the C-termini of γ-chains and ClfB binds to both α- and β-chains [Ni Eidhin *et al.* 1998]. Bbp/SdrE is identified by its affinity to bone sialoprotein. The A-region of Fbe is highly homologous to that of Bbp/SdrE, up to 45%. Despite the similarities of other proteins to Fbe, like ClfA, ClfB and Bbp/SdrE, the affinity ligands were different among them. Even for the similar ligand, the dissociation constant was different, e.g., K_d=1.4x10^{-7}M for Fg-Fbe [Davis *et al.* 2001] and K_d=2x10^{-5}M for Fg-ClfA [O'Connell *et al.* 1998]. Our results indicated the Fg binding region of Fbe was located on amino acid 269-599 (**Paper I**). Based on the high affinity of Fbe to Fg, the interaction between Fbe and Fg

may be structural dependent. Only certain residues distributed in the wide region of 331 amino acids of Fbe may contribute to its affinity. It is of note that a recent study has identified the affinity determining residues in ClfA. Residues 500-559 of ClfA are involved in Fg affinity. Site-directed mutagenesis showed that two adjacent residues, Glu^{526} and Val^{527}, were important for the activity of ClfA. The substitution of Glu^{526} and/or Val^{527} showed no alternation in the secondary structure [Hartford *et al.* 2001]. Alignment of this region of ClfA to Fbe resulted in a 21-residue overlap with 47% identity (residues 574-594). Such a sequence is included in the Fg affinity region of Fbe residue 269-599. In this sequence, residues Thr^{582} and Ile^{583} of Fbe was aligned with Glu^{526} and Val^{527} of ClfA, which were not identical. This implies that Thr^{582} and Ile^{583} of Fbe might be involved in its affinity to the β-chains of Fg. The other 21 residues might help to project these two adjacent residues for ligand recognition.

The B-repeats consist of approximately 110 residues, which are rather conserved with over 40% identical. They are common not only in Sdr multigene family, like Fbe/SdrG, SdrF, SdrC, SdrD, SdrE/Bbp, but also in other surface proteins, like Cna of *S.aureus*, which binds to Cn. Study on the B-repeats of SdrD, has shown that they contain EF-hand-like motifs, which can bind to calcium with high affinity [Josefsson *et al.* 1998]. A mutant of *cna* lacking B-repeats showed no effect on the Cn binding ability. Another study on a chimeric protein, the A-region of ClfA and B-repeats of Cna hybrid failed to support Fg binding by *S.aureus* [Hartford *et al.* 1999]. It suggested that B-repeat of the surface protein is not necessary for ligand presentation on the cell wall, but might be involved in the cation-induced regulation of ligand-receptor affinity. In a cation poor environment, B-repeats might shed the A-region, while in a cation rich environment (e.g., accumulation of calcium in blood clots), conformation change might take place, resulting in a complete exposure of A-region for ligand recognition.

The SD-repeats are the commonest feature of all Sdr proteins. It is known that this dipeptide repeat region is required for functional expression of the A-region on the cell surface. The minimum length necessary for surface display of the biological active region is 60-90 residues of the repeats [Hartford *et al.* 1997; Hartford *et al.* 1999]. In Fbe of strain HB, the SD-repeat region is composed of 216 amino acids, which is long enough to expose the A-region on the cell surface. However, in Fbe homologues in strains RP62A and K28, only 50 and 56 residues comprise of the R-region. Both barely reach the minimal length needed to expose the ligand-binding region on the surface. Variations in SD repeat are common among *S.epidermidis* isolates as evidenced by PCR results obtained from 7 other strains using the primers located in the beginning of the second B-repeat and

the end of the LPXTG motif (Figure.3B). Such variants in length may affect the ligand binding ability of the adhesins, which may be used as a virulence marker of isolates of *S.epidermidis*.

LPXTG motif is another common feature found in Sdr proteins, as well as some other surface proteins of Gram-positive bacteria, such as FnBPs, Cna, Spa of *S.aureus*, M proteins of *Streptococcus pyogenes* (GBS), Protein G, FnBA and FnBB of *Streptococcus spp*. (GSC and GGS). Sorting signal is composed of LPXTG motif together with W- and M-regions. Four steps are involved in sorting surface protein to the cell wall. These include: (1) exporting signal peptide of the precursor protein through the secretory pathway of the cell membrane; (2) retention of M-region in the cytoplasmic membrane; (3) cleavage of LPXTG by sortases and (4) cell wall linkage of LPXT to glycine of glycopeptide in the cell wall [Aly *et al.* 1981; Wilkinson 1997; Mazmanian *et al.* 2001]. Sortase, a membrane protein of *S.aureus* encoded by *srtA* gene, cleaves polypeptides between T and G of the LPXTG motif and catalyzes the formation of an amide bond between the carboxyl-group of peptidoglycan cross-bridges. Sortase homologues, at least up to 92, were found in almost all of the Gram- positive bacterial genomes, e.g., *Bacillus halodurans, B.subtilis, Enterococcus faecalis, Streptococcus mutans, S.pneumoniae, S.equi,*and *S.pyogenes*. Other potential substrates of sortase were found in bacterial genomes besides LPXTG [Pallen *et al.* 2001]. Mutant lacking *srtA* gene can not cleave surface proteins at the LPXTG motif and their precursors accumulated in the cytoplasm, membrane and cell wall fractions (missorted phenotype). Such a mutant of *srtA* is less virulent than the wild type in a murine renal abscess model [Mazmanian *et al.* 2000]. The Sortases of *S.epidermidis* are less known. It can be predicated that such genes are exist in *S.epidermidis* as that in *S.aureus*. Mutations of sorting signal and *srtA* may not be uncommon in *S.epidermidis* as well.

Fbe and fibrinogen

Most of the strains of *S. epidermidis* possess the *fbe* gene (**Paper I**). According to the alignments between sequences from HB and two other strains (JW14 and No.269), part of *fbe* was conserved within different strains of *S. epidermidis* with over 80 % identity (Figure.3C). Result from a study of markers to *S.epidermidis*, all of the strains tested possess *fbe* as evidenced by southern blot using part of *fbe* as probe [Galdbart *et al.* 2000]. Therefore, it is further confirmed that *fbe* is common in *S.epidermidis*. The conserved part of *fbe* can be used as a marker to identify *S.epidermidis* at the species level. On the other hand, *S. epidermidis* was found to bind to immobilized Fg in a

heterogeneous manner. Our finding here implies that although most of the strains possess the gene which is conserved, other factors might effect the exposure of Fbe on the surface, such as protease activity, sortase cleavage on LPXTG motif [Mazmanian *et al.* 1999], variation in the length of the SD-repeat region [Hartford *et al.* 1997; Hartford *et al.* 1999] and capsular formation.

In the functional studies of Fbe, we have shown that Fbe finds to the β–chains of Fg (**Paper II**). As a dominant component in plasma, Fg was found present not only constantly but also actively promoted bacterial adherence in explanted materials [Vaudaux *et al.* 1995; Francois *et al.* 2000]. β–chains of Fg are found more resistant to enzyme degradation [Doolittle 1984]. Recent report shows that 70% of the FpB remains intact in the clots of the whole blood [Brummel *et al.* 1999]. Study of SdrG, homologue of Fbe in strain K28, provided further evident that the recognized sites located on the N-termini of β–chains overlapped with its thrombin cleavage site in FpB [Davis *et al.* 2001]. Our study on explanted materials from patients showed that only one of the two bands of the adsorbed components recognized by Fg followed by anti-Fg (Figure 4, panel A3), can be recognized by rFbe followed by anti-rFbe, which were its β–chains (Figure.4, panel B). As an adhesin to Fg found in *S. epidermidis*, Fbe might play an important role *in vivo* in the early stage of a bacterial infection, by mediating bacterial attachment to the conditioning film on the implant surface via its high affinity to the remaining-intact N-termini of β–chains of fibrin(ogen). This specific ligand-receptor interaction may be constant in the dynamic environment, thus superceding the non-specific interaction mediated by hydrophobic force [Kloos & Bannerman 1994].

In the functional studies of rFbe, it is also evident that the interaction between rFbe and Fg can be promoted by calcium. Calcium has a well defined and important role in fibrin formation. It not only facilitates the clotting but also strengthens the clot by preventing further enzyme degradation. Until now, the Ca^{2+} binding sites identified in Fg do not overlap with its binding site to Fbe/SdrG, unlike the case of the binding site to ClfA, which overlaps with a Ca^{2+} binding site in the C-terminus of γ-chains. The Ca^{2+} binding site(s) has not been identified directly on Fbe, but a homologous structure of B-repeats in SdrD was shown to have affinity for Ca^{2+} [Josefsson *et al.* 1998]. It is possible that the corresponding structure of Fbe may play the same role. Binding to Ca^{2+} and the subsequent change in conformation induced by Ca^{2+} binding might regulate, promote and strengthen the interaction between *S.epidermidis* and fibrin(ogen) on the surface of an implant. By contrast, Ca^{2+} has a dual functional effect on the interaction between *S.aureus* and Fg mediated by ClfA. Ca^{2+} in a certain concentration can facilitate the interaction, which may increase the bacterial colonization.

While the Ca^{2+} concentration keeps raising, binding of Ca^{2+} reduces the interaction of ClfA-Fg, which helps to spread the infection from the initially colonized sites. The linear dose-response manner we observed on the effect of Ca^{2+} on the binding of rFbe-Fg suggesting another type of regulation differing from that of Ca^{2+} in ClfA-Fg (**Paper II**). In a wounded tissue and vessel, an implant surface or a clot, the local concentration of Ca^{2+} may be comparatively high. In most of these cases, Fg, or fibrin, or its derivative is present constantly. On one hand, the already-bound Ca^{2+} favors the conformation change on Fg to expose the Fbe recognized site(s). On the other hand, Ca^{2+}-binding to the B-repeats of Fbe favors the exposure of the A-region of Fbe for Fg binding. In both cases, the bacterial colonization to implants via Fg is facilitated.

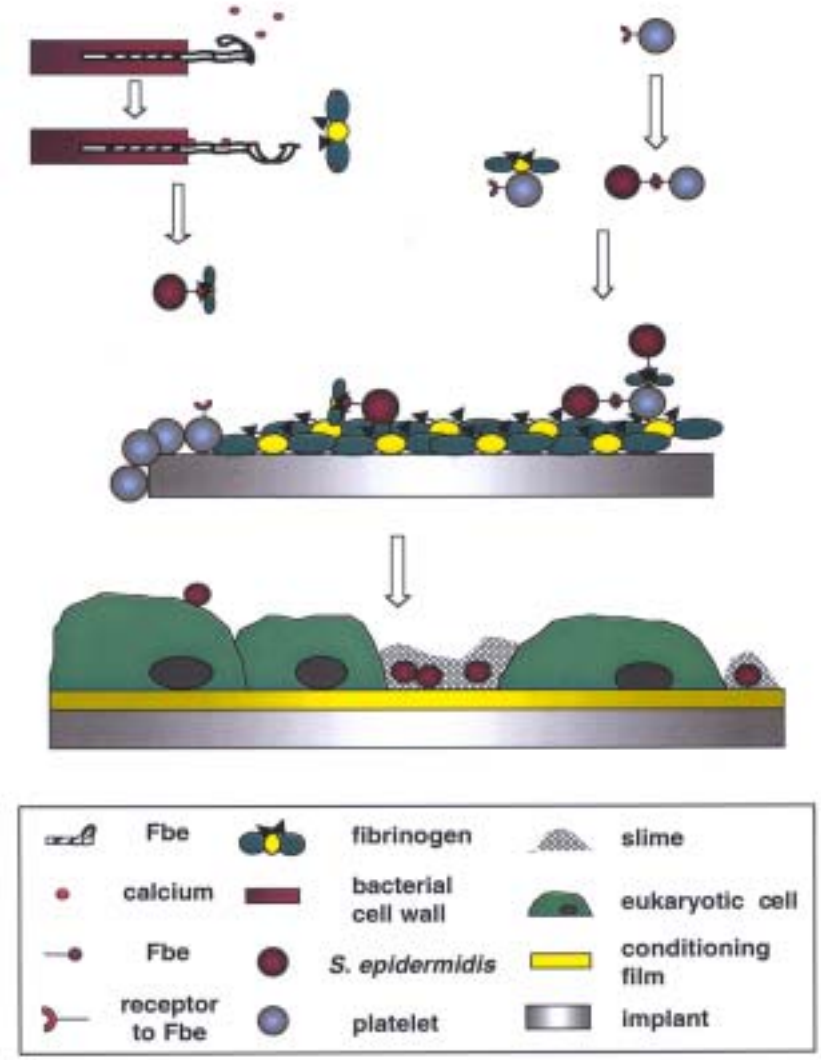

Figure 5. Schematic drawing of the interaction of Fbe-Fg-platelet in foreign body infection

On the top of the left side, induced by calcium binding, the A-region of Fbe exposed on the bacterial surface binds to Fg via its N-terminal of the β-chain. On the top of the right side, the possible interaction between Fbe and platelet via its putative receptor to Fbe, or via bound Fg on the platelet surface are shown. The formation of the conditioning film on the implant surface by Fibrin(ogen), or in part, directly or indirectly, by platelets, initiates the colonization of *S.epidermidis*. Once colonized, biofilm is formed on the surface of the implant.

In addition to its function as a direct affinity ligand to *S.epidermidis* on the implant surfaces, Fg may play other roles. The interaction between Fg and platelets is important in hemostasis and wound healing. It was found that 50% of adherent *S.epidermidis* were bound to contact-activated

platelets, which occupied only 4% of the surface area. This suggests that platelets can promote bacterial adherence to the implants better than plasma proteins [Wang *et al.* 1993]. To date, no adhesin to platelet has been found in *S.epidermidis*. As an adhesin to Fg, Fbe may also act as a bridge molecule mediating bacterial adhesion to platelets via affinity to the bound Fg on platelet surfaces. Platelets are known for their interaction with ECM and host cells mediated by different glycoproteins. The β1 integrins are receptors for Cn, Fn and laminin. The β2 integrins are receptors for immune complexes (via Fc receptor leading to aggregation) and inflammatory cells [Weksler 1992], such as monocytes, neutrophils (P-selectin) and lymphocytes [Nagata *et al.* 1993]. The β3 integrins are receptors for Fg (GPIIb/IIIa) and Vn [White 1994]. Therefore, the interaction of *S.epidermidis* to ECM and other host cells may be mediated indirectly via platelets in a Fg-dependent or -independent manner. The virulent role of Fbe in foreign body infections caused by *S.epidermidis* may be more complicated than that of an adhesin to Fg. As the affinity ligand for Fbe, Fg may also play a more complex role than a constantly-present component on implant surfaces. The role of Fbe, Fg, platelets, and calcium in *S.epidermidis* associated foreign body infection is illustrated in Figure 5.

S.epidermidis and biomaterials

Implanted biomaterials are covered by plasma components from the host fluids, in which Fg seems to be abundant. This is supported by our findings with explanted materials (e.g., PVC, CVC, and SIC, **Paper IV**) and by other researchers [Vaudaux *et al.* 1995; Francois *et al.* 1996; Francois *et al.* 2000]. Study on adherence of *S.epidermidis* to FgCC, shows a dose dependent response to Fg. In another comparison, adherence of *S.epidermidis* to FgCC and to FnCC seemed to be similar (**Paper III**). Therefore, binding to Fg contributes to the adherence of *S.epidermidis* to implants *in vivo*.

Most strains of *S.epidermidis* have a marked ability to bind nonspecifically to naked polymer surfaces. This binding can be blocked by coating the surface with various proteins, including Fg. Our studies on the adherence of *S. epidermidis* also show that microorganisms bind strongly to the uncoated surfaces, compared with a conditioned polymer surface. However, the situation *in vivo* differs from that *in vitro*. The conditioning film is formed rapidly by components of the host fluid and tissue, and the nonconditioned surface of the implants may not exist for long. The proper infection control makes it less likely that the medical devices might become contaminated before implantation. Furthermore, the binding of *S.epidermidis* to biomaterials is stimulated more by Fg,

than that to BSA-coated surfaces, and thereby overrule the blocking by Fg, as compared to uncoated surfaces. Thus, Fg seems to have two effects: blocking of nonspecific binding to hydrophobic surface and promotion of specific binding mediated by ligand-receptor interactions.

Adherence of *S.epidermidis* to Fg was shown to be dependent on a Fg binding protein, Fbe. Our studies indicate that both rFbe and its antibodies can block the bacterial adherence to Fg-coated surfaces efficiently (**Paper II**). In the study on antibodies against rFbe, we evaluated the ability of anti-rFbe to block adherence of *S.epidermidis* to Fg-coated polyethylene catheters, catheters placed subcutaneously in rats and peripheral venous catheters (**Paper III**). In all cases, the antibodies against rFbe had a greater blocking effect than the control antibodies against GST. On the other hand, the anti-Fbe antibodies did not block bacterial adherence to Fn-coated surfaces, demonstrating the specificity of anti-Fbe. The blocking effect was most pronounced, ca 80%, on catheters coated solely with Fg (e.g., FgCC and Fg-coated wells in microtiter plates) than those coated with various components *in vivo* (e.g., SIC and PVC).

In **Paper IV**, an isogenic mutant of *fbe*, strain STO56 (*fbe*::*gmr*), is derived from strain HB by allelic replacement. The adherence to Fg was significantly reduced in the mutant compared to the wild type. Such reduction was particularly marked on surfaces coated *in vitro* with Fg alone (e.g. FgCC and Fg-coated wells on the microtiter plates). The bacterial adherence on explanted materials like PVC, was also reduced when comparing the *fbe* isogenic mutant with its wild type strain. Meanwhile, the adherence to immobilized Fn between these two strains was similar. This suggests that the replacement of *fbe* mainly affects its Fg affinity, while the binding to Fn and/or other binding properties remain unchanged. In addition to Fg found on PVC from patients, many other components were present. Therefore, the lower reduction in adherence observed with PVC than with FgCC (50% vs. 75%) suggests that components other than Fg, adsorbed on PVC, may also contribute to the bacterial adherence. Therefore, the deletion of *fbe* did not completely eliminate adherence. Adherence of *S.epidermidis* to implants may be a multiple-factors outcome.

Prophylaxis and therapy of *S.epidermidis* infections

Since most *S.epidermidis* associated-foreign body infections are difficult to treat, and may have serious complications, it is important to prevent them at an early stage. Proper infection control is crucial [Eggimann & Pittet 2000]. Most infections associated with *S.epidermidis* are of nosocomial origin, arise from the patient's indigenous flora, or from the hands of hospital staff [Boyce *et al.*

1990; Huebner *et al.* 1994]. The guidelines of infections control should be strictly followed in pre-, peri- and post-surgical procedures [Eggimann & Pittet 2000].

Antibiotic prophylaxis and therapy are important in foreign body infections caused by *S.epidermidis*. Since most infections are hospital-acquired, it is common that the pathogens are multi-resistant to antibiotics available. Some data showed that bacterial growth on implant surfaces differs from that of the planktonic ones. The generation time of the adherent bacteria is shorter. Their metabolic rate is low, limiting the efficacy of the common antibiotics which target active cell division and metabolism [Hoyle & Costerton 1991]. The bacteria may persist on the implant by forming biofilm to prevent antibiotic action. Thus, antibiotics based on other mechanisms are needed, e.g., inhibitors of sortases, or homologous substance to sortases, which reduce the sorting of surface proteins on the bacterial cell wall; antibiotics with specific ability to penetrate the biofilm on implant surface; inhibitors of global regulation system (such as inhibitors of *agr*, *sar*, *ica,* etc.).

In addition to new antibiotics, development of other types of biomaterials is another approach to prevent *S.epidermidis*-associated foreign body infection. Materials of less hydrophobic may help to reduce the nonspecific adhesion mediated by hydrophobic interaction. Coating materials with antibiotics and/or antiseptic drugs may prevent biofilm formation on the surface [Darouiche *et al.* 1999; Veenstra *et al.* 1999]. Biologically-modified or -derived materials may help to reduce the bacterial adherence. This has been supported by the findings of lower infection rates on tunneling catheters than the nontunneling ones [Eggimann & Pittet 2000], as well as biomodified heart valves are less infected than the polymer ones.

Vaccine is a powerful approach for control and eradication of diseases. With the emergence of widespread antibiotic resistance among pathogens, new vaccines are needed. Staphylococci, particularly *S.aureus*, are well known for their abilities to escape the host immune defense, thus remain a challenge to the development of vaccine because of our limited understanding of the pathogenesis. Polyclonal or monoclonal antibodies against a certain virulence factors of staphylococci, have been studied by clinical trials and animals via either active or passive immunization [Yoshida & Ichiman 1978; Ichiman *et al.* 1989; Poole-Warren *et al.* 1991; Lee 1996; Balaban *et al.* 1998; Kronvall & Jonsson 1999; McKenney *et al.* 1999; Tarkowski 2000]. Some of them have shown protection and/or reduction in bacterial adherence. Immunoglobulin treatment of *S.epidermidis*-associated infections is of particular interest. This has been applied as an alternative treatment for peritonitis and neonatal sepsis. Slime-related components, such as PS/A ,PIA/HA, 20

kDa PS, and teichoic acid, have been used as vaccine targets [Kojima *et al.* 1990; Ichiman *et al.* 1991a; Takeda *et al.* 1991; Karamanos *et al.* 1997; Rupp *et al.* 1999a; Rupp *et al.* 1999b; Lamari *et al.* 2000; Kolonitsiou *et al.* 2001]. The specific antibodies generated have been studied regarding to their protective effects in animal models with various outcome. Our studies on rFbe and its antibodies have shown that this surface adhesin to Fg may be suitable as a vaccine candidate. Antibodies against Fbe may be applied passively to the high-risk group with *S.epidermidis* infections, such as premature neonate, patients undergoing immunosuppressive therapy, patients with a foreign body or undergoing continuous ambulatory peritoneal dialysis (CAPD). The anti-Fbe immunoglobulins can be given locally or systemically. The homologue of Fbe may be applied locally to the implant, or as an anti-adhesion component in an infusate, to antagonize colonization by endogenous staphylococci. Passive immunity is a practical therapy in neonatal sepsis. It is presumed that specific antibodies to *S.epidermidis* may enhance immunity to this important neonatal pathogen by facilitating opsonophagocytosis [Fischer 1994]. Since the opsonization of antibodies against Fbe remains unclear, Fbe as a vaccine target can be evaluated more exactly once we learn more about this aspect. Immunoglobulin IgM is found to be more effective in opsonophagocytosis [Yoshida & Ichiman 1978; Ichiman *et al.* 1991a; Ichiman *et al.* 1991b]. It may improve the efficacy of immune therapy by using specific IgM against Fbe. In addition, cytokines, which play important roles in immunity and inflammatory response in bacterial infections, should take into consideration. In intraperitoneal immunoglobulin therapy of CAPD patients, poor responders to intraperitoneal interferon were found to be associated with a high incidence of peritonitis [Carozzi & Lamperi 1988; Carozzi *et al.* 1988; Lamperi & Carozzi 1988]. Therefore, combined antibody therapy with cytokines may further improve the treatment.

In conclusion, a novel adhesin for Fg, Fbe of *S.epidermidis*, has been identified and characterized in this thesis. Functional studies of Fbe and its antibodies have been done. By generating an isogenic mutant of *fbe*, we show that Fbe is important for the interaction between Fg and *S.epidermidis*.

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