Journal of Microbiology (2013) Vol. 51, No. 4, pp. 499–505
DOI 10.1007/s12275-013-3319-y
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The N3 Subdomain in A Domain of Fibronectin-Binding Protein B Isotype I Is an Independent Risk Determinant Predictive for Biofilm Formation of *Staphylococcus aureus* Clinical Isolates

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(Received June 10, 2013 / Accepted August 8, 2013)

Fibronectin-binding proteins (FnBP), FnBPA and FnBPB, are purported to be involved in biofilm formation of *Staphylococcus aureus*. This study was performed to find which of three consecutive N subdomains of the A domain in the FnBP is the key domain in FnBP. A total of 465 clinical isolates of *S. aureus* were examined for the biofilm forming capacity and the presence of N subdomains of FnBP. In the biofilm-positive strains, N2 and N3 subdomains of FnBPA, and N1 and N3 subdomains of FnBPB were significantly more prevalent. Multivariate logistic regression analysis of 246 biofilm-positive and 123 biofilm-negative strains identified only the FnBPB-N3 subdomain as an independent risk determinant predictive for biofilm-positive strains of *S. aureus* (Odds ratio [OR], 13.174; *P*<0.001). We also attempted to delete each of the *fnbA-N2* and *-N3* and *fnbB-N1* and *-N3* from *S. aureus* strain 8325-4 and examined the biofilm forming capacity in the derivative mutants. In agreement with the results of the multivariate regression analysis, deletion of either the *fnbA-N2* or *-N3*, or *fnbB-N1* did not significantly diminish the capacity of strain 8325-4 to develop a biofilm, while deletion of the *fnbB-N3* did. Therefore, it is suggested that the FnBPB-N3 subdomain of isotype I may be a key domain in FnBP which is responsible for the causing biofilm formation in *S. aureus* clinical isolates.

Keywords**:** fibronectin-binding proteins, N3 subdomains of FnBPB, biofilm, *S. aureus*

Introduction

Staphylococcus aureus is one of the most important human pathogens responsible for a variety of nosocomial infections. In addition to the several virulence factors, *S. aureus* biofilms on medical devices as well as dead tissues contribute to chronic, recurrent infections in clinical settings since biofilm-embedded *S. aureus* is protected not only from conventional chemotherapy, but also from human immune surveillance (Costerton *et al.*, 1999). The biofilm matrix produced by *S. aureus* is composed of cell surface proteins, polysaccharide intercellular adhesin (PIA) and extracellular DNAs (Cramton *et al.*, 1999; Boles *et al.*, 2010). The nature of the biofilm depends on environmental conditions; for example, the matrix predominantly consists of either PIA or protein components in the presence of sodium chloride (NaCl) or glucose, respectively (Vergara-Irigaray *et al.*, 2009).

Biofilm development by *S. aureus* can in part be attributed to the expression of cell surface proteins designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), including up to 21 different proteins containing the LPXTG motif anchored to the cell wall by sortase. MSCRAMMs as adhesins are required for primary attachment to the surface of biomedical devices which are coated with host-derived extracellular films (Mazmanian *et al.*, 2001; O'Neill *et al.*, 2008; Merino *et al.*, 2009; Vergara-Irigaray *et al.*, 2009). Fibronectin-binding proteins (FnBP), FnBPA and FnBPB of *S. aureus* which belong to a family of MSCRAMMs, are multifunctional proteins consisting of two distinct ligand binding domains. The N-terminal A region of either FnBPA or FnBPB exposed on the cell surface binds to fibrinogen and elastin, and the 11 tandem-repeated BCD region located between the A domain, consisting of three consecutive N subdomains (N1-N3), and wall-spanning W domain binds to fibronectin (Keane *et al.*, 2007). Recently, Burke *et al.* (2010) reported that in the *S. aureus* 8325-4 strain, the BCD domains of FnBPA and FnBPB are highly conserved, with 95% amino acid identity, while the A domain are more divergent. The same research group also classified seven different variants each of FnBPA and FnBPB in *S. aureus* on the basis of divergence in the A domain (Loughman *et al.*, 2008; Burke *et al.*, 2010).

Since *S. aureus* is capable of binding to glycoprotein-coated films by means of expressing either one or two FnBPs on the cell surface (Menzies, 2003), FnBPA and FnBPB of *S. aureus* are purported to be involved in staphylococcal biofilm development. O'Neill *et al.* (2008) reported that only deletion of *fnbAB* genes, not *fnbA* or *fnbB* alone, abolished biofilm formation in *S. aureus* and the biofilm development of the *fnbAB* mutant was not complemented by a plasmid containing the fibronectin-binding BCD domain but by a plasmid containing the A domain of FnBPA. Furthermore

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Table 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
S. aureus strains		
8325-4	Prophage-free laboratory strain	J. Iandolo
RN4220	8325-4r⁻ (restriction deficient)	
YL50	A clinical isolate with FnBPs isotype III of strain N315	This study
YL75	8325-4 (pLI50)	This study
YL32	8325-4 △*fnbB* (pLI50)	This study
YL409	8325-4 △*fnbB_N1* (pLI50)	This study
YL260	8325-4 △*fnbB_N3* (pLI50)	This study
YL356	8325-4 △*fnbA_N2* (pLI50)	This study
YL344	8325-4 △*fnbA_N3* (pLI50)	This study
E. coli strain		
DH5α	*fhuA2△(argF-lacZ)U169 phoA glnV44 Φ80△(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
Plasmids		
pKOR1	*E. coli-S. aureus* thermosensitive shuttle vector for allele replacement; Ap^r (*E. coli*) Cm^r (*Staphylococcus*)	
pLI50	*E. coli-S. aureus* shuttle cloning vector	
pFnbB	pKOR1 with deleted *fnbB* ORF in RN4220	This study
pFnbB_N1	pKOR1 with deleted *fnbB_N1* ORF in RN4220	This study
pFnbB_N3	pKOR1 with deleted *fnbB_N3* ORF in RN4220	This study
pFnbA_N2	pKOR1 with deleted *fnbA_N2* ORF in RN4220	This study
pFnbA_N3	pKOR1 with deleted *fnbA_N3* ORF in RN4220	This study

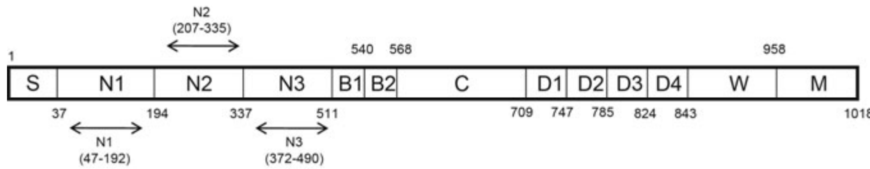
it is revealed that the major autolysin, Atl, promotes primary attachment and extracellular DNA release during the early stage of *ica*-independent, FnBP-mediated biofilm development (Houston *et al.*, 2010). Another research group has reported that among 20 genes encoding LPXTG proteins only deletion of the *fnbB* gene diminished biofilm formation in TSB-glucose medium (Vergara-Irigaray *et al.*, 2009). Nevertheless, little is known about which of the three consecutive N subdomains of the A domain in either FnBPA or FnBPB are involved in biofilm formation of *S. aureus* clinical isolates.

In this study, we wanted to find which of three consecutive N subdomains of the A domain in the FnBP is important in biofilm formation by clinical isolates of *S. aureus*. A total of 465 clinical isolates of *S. aureus* were studied for the ability to form biofilm and the presence of N subdomains of FnBP. Then the relationship between biofilm forming ability and the presence of N subdomains of FnBP in *S. aureus* was evaluated. We also attempted to delete each of the *fnbA-N2* and *-N3* and *fnbB-N1* and *-N3* from *S. aureus* strain 8325-4 and examined the biofilm forming capacity in the derivative mutants. Based on our results, we suggest that N3 subdomain of FnBPB isotype I, rather than the isotype III, is involved in biofilm formation by *S. aureus*.

Table 2. PCR primers used for detecting each of the subdomains in this study

PCR product (strain name)	Name of primer	Primer sequences[a]	Product length (bp)
fnbA-N1 (8325-4)	fnbA-N1-F	AACGCGAAAGTGGAAACG	436
	fnbA-N1-R	TATCAGTAGCTGAATTCCCA	
fnbA-N2 (8325-4)	fnbA-N2-F	TTCAAAGGAATTAGATGTTAAA	380
	fnbA-N2-R	ACTTTATTTGTATTGTTATGCC	
fnbA-N3 (8325-4)	fnbA-N3-F	ATTATGATAGAGGATATACC	355
	fnbA-N3-R	CAGTCACACTTGTCGTTTTA	
fnbB-N1(8325-4)	fnbB-N1-F	CTTCGCGAGTTGATTTGC	225
	fnbB-N1-R	AGTATTGTTTTGTTCCGATG	
fnbB-N2 (8325-4)	fnbB-N2-F	GAGACTACGGTTAGCAAAAT	396
	fnbB-N2-R	TACAATTTCACTACCTTCTTCT	
fnbB-N3 (8325-4)	fnbB-N3-F	GTAACAGCTAATGGTCGAATTGATACT	270
	fnbB-N3-R	AGTATCAAAATCTAAACTCATA	
fnbB-N3 (N315)	fnbB-N315-F	GTTACTGTTAATGGTCGTATTGATACT	270
	fnbB-N315-R	ACTTGTGTAAGATAGATTTACT	
fnbB (8325-4)	fnbB-F	GTAACAGCTAATGGTCGAATTGATACT	523
	fnbB-R	CAAGTTCGATAGGAGTACTATGTTC	

[a] Sequence of primers were derived from *fnbA* and *fnbB* sequence data (GenBank accession no. SAOUHSC 02803 and SAOUHSC 02802, respectively) to specifically amplify the genes encoding each of the N1, N2, and N3 subdomains of either FnBPA or FnBPB of strain 8325-4.



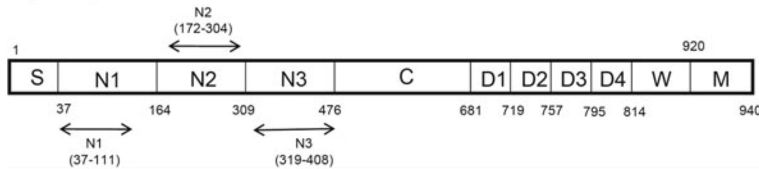


Fig. 1. Structural organization of the fibronectin-binding protein A and B from *S. aureus* 8325-4 (Keane *et al.*, 2007) and each region (↔) of N1, N2, and N3 subdomains of the A domain detected by each of the six sets of corresponding primers is depicted. Secretory signal sequence (S), A region (consisting N1, N2, and N3), B, C, and D region, cell wall-spanning domain (W) and membrane-spanning domain (M) are indicated.

Materials and Methods

Bacterial isolates and growth conditions

The relevant bacterial strains and plasmids used in this study are listed in Table 1. A total of 465 clinical isolates of *S. aureus* were included: 413 strains recovered from university hospitals in South Korea and 52 strains from 10 South-East Asian countries as follows: six each from China and Indonesia, and five each from India, the Philippines, Saudi Arabia, Singapore, Sri Lanka, Thailand, Taiwan, and Vietnam. These 52 strains were supplied by the Asian-Pacific Research Foundation for Infectious Diseases (ARFID). Strains tested were isolated from individuals over seven years (1998–2004) under various clinical settings: 108 from wounds and pus, 77 from blood, 57 from catheter-related urine, 47 from sputum, 34 from the anterior nasal cavity or hands of medical staff, 31 from body fluids, 24 from hands of outpatients, 15 from dermatologic lesions, 14 from ENT lesions, 1 from vaginal discharge and 57 from unknown sites. All isolates were identified using the API-Staph system and confirmed by detection of the *nuc* gene using polymerase chain reaction (PCR) (Brakstad *et al.*, 1992). *Escherichia coli* DH5α cells were grown in Luria-Bertani (LB) (Difco, USA) broth or on LB agar plates. *S. aureus* 8325-4 strain and its derivative strains were cultured on trypticase soy agar (TSA) (Difco) or in TSB (Difco) supplemented with 0.5% glucose. Media were supplemented with either anhydrotetracycline (100 ng/ml) or chloramphenicol (5 μg/ml).

PCR analysis for the presence of N subdomains of FnBP

Genomic DNA was purified by the method of Reischl *et al.* (1994). PCR was performed in a reaction mixture containing genomic DNA, Tris-HCl (pH 9.0), 20 mM $MgCl_2$, $(NH_2)_4SO_4$, 10 mM dTNPs mixture and 0.5 U of *Taq* polymerase (GENET BIO, Korea). Six sets of primers were derived from *fnbA* and *fnbB* sequence data (GenBank accession no. SAOUHSC 02803 and SAOUHSC 02802, respectively) to specifically amplify the genes encoding each of the N1, N2, and N3 subdomains of either FnBPA or FnBPB of strain 8325-4 as listed in Table 2. Each region of N1, N2, and N3 subdomains of the A domain detected by each of the six sets of corresponding primers is depicted in Fig. 1.

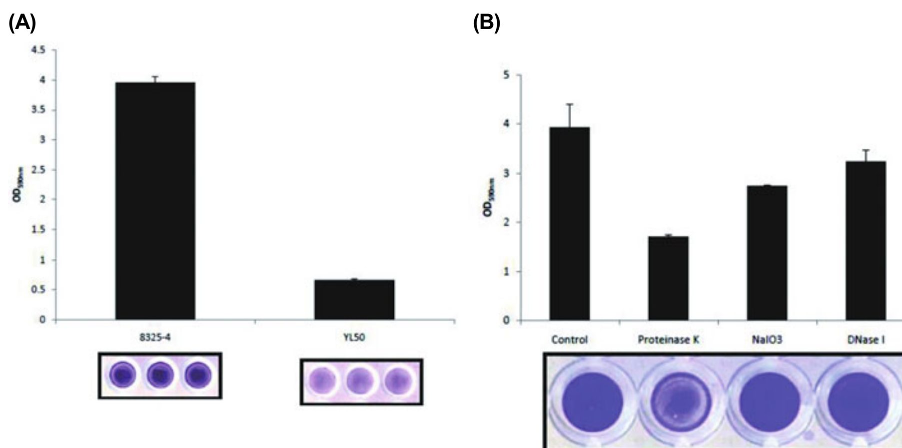


Fig. 2. (A) Biofilm formation of *S. aureus* strain 8325-4 and YL50 grown on microtiter plates overnight under static conditions at 37°C in TSB supplemented by 0.5% glucose. The biofilm formed was stained with crystal violet for 5 min, and air-dried for 1 h. The absorbance was determined at 590 nm. The results are representative of three independent experiments performed in triplicate. Error bars indicate the standard deviations. (B) Biofilm detachment assays. Biofilms formed by *S. aureus* strain 8325-4 grown in TSB supplemented by 0.5% glucose overnight were treated for 2 h at 37°C with 10 mM sodium metaperiodate ($NaIO_3$) or with 100 μg of proteinase K or with 0.7 u DNase I. The control was the biofilm of strain 8325-4 without any treatment. The quantification of the nondetached biofilm was performed as described for panel (A).

Allelic replacement of *fnbB-N1* and *-N3*, *fnbA-N2* and *-N3* and whole *fnbB* genes

For deletion of the *fnbB* gene in *S. aureus* strain 8325-4 using the temperature-sensitive plasmid pKOR1, we amplified two fragments by PCR that flanked the left side (primer attB1-fnbB-F and fnbB-*Sac*II-R listed in Table 3) and the right side (primer fnbB-*Sac*II-F and attB2-fnbB-R listed in Table 3) of the 8325-4 *fnbB* sequence. The resulting PCR products were subsequently restricted with *Sac*II and ligated in equimolar amounts. The resulting ligation products were integrated into pKOR1 via *in vitro* recombination (clonase mix from Invitrogen) and *E. coli* DH5α was transformed with the recombination mix. RN4220 was transformed with a plasmid from a positive clone by electroporation. *S. aureus* 8325-4 strain was subsequently transduced from RN4220 using phage 52A. The successful deletion of the *fnbB* gene was verified by PCR with primers fnbB-F and fnbB-R listed in Table 2. Deletion of each *fnbB-N1* and *-N3* and *fnbA-N2* and *-N3* loci was accomplished in the same manner as that used for the *fnbB* gene.

Biofilm assay

A biofilm assay in microtiter plates was quantitatively determined for 465 clinical isolates using the method developed by Heilmann *et al.* (1996). Briefly, overnight cultures were adjusted to an optical density (OD) of 600 nm at 4.0 and diluted 200-fold in TSB supplemented with 0.5% glucose. A 200 μl aliquot of the cell suspension was then inoculated into each well of a 96-well flat-bottom polystyrene microtiter plate (Corning Inc., USA). Following 24 h of incubation at 37°C, wells were rinsed twice with 200 μl of phosphate buffered saline (PBS), air-dried, and stained for 1 min with 0.1% safranin. Absorbance was measured at 590 nm with a microELISA reader, SUNRISE™ (Tecan Co., Austria). For three independent biofilm assay experiments performed in triplicate, the average was 0.15 at OD_{590}. *S. aureus* isolates producing biofilms with an OD≥0.15 were defined as biofilm-positive strains, and those producing biofilms with and OD<0.1 as biofilm-negative strains. For *S. aureus* strain 8325-4 and its derivative mutants, a biofilm assay was performed in the same manner and as many times as the 465 clinical strains, except that after air-dried, the biofilm-formed wells were stained for 5 min with 0.25% crystal violet, rinsed twice with 200 μl of PBS, and air-dried for 1 h. The absorbance was measured at 590 nm with a microELISA reader (Peeters *et al.*, 2008). A biofilm detachment assay was performed as follows: biofilms formed by *S. aureus* strain 8325-4 grown in TSB supplemented with 0.5% glucose overnight were treated for 2 h at 37°C with 10 mM sodium metaperiodate ($NaIO_3$), or with 100 μg of proteinase K, or with 0.7 u DNase I. Quantification of the non-detached biofilm was performed as described for the biofilm assay for strain 8325-4 and its derivative mutants.

Statistical analysis

Statistical analysis was conducted using SPSS version 12.0 (SPSS, USA). Univariate comparisons were analyzed with chi-square or Fisher's exact test, and the results expressed as odds ratios (ORs) and associated 95% confidence intervals (CIs) to illustrate the amount of risk associated with the assumed risk factors. Statistically significant risk factors in the univariate analysis were introduced to a multivariate logistic regression analysis to adjust the estimated effect of each of risk factors for biofilm-positive strains. The statistical significance was set a priori at $P<0.05$. The non-para-

Table 3. Oligonucleotides used for deleting the targeted gene in this study

Targeted gene for deletion	Primer name	Sequence (5′→3′)
fnbB	attB1-fnbB-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAATTTAGTTCACCATTTC
	fnbB-SacII-R	GGACCTCCGCGGTCAATCCAAAATTGACAGGTT
	attB2-fnbB-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAGTAAGCGGCCAAAATGAA
	fnbB-SacII-F	GGACCTCCGCGGTGTTCCTAAGAATACTGAGG
fnbB-N1	attB1-fnbB-N1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTTGGATTATTAACCCCTGGT
	fnbB-N1-SacII-R	GGACCTCCGCGGGCGACAGGTACAGATGTAAC
	attB2-fnbB-N1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAATGTCGGCTTGAAATACGG
	fnbB-N1-SacII-F	GGACCTCCGCGGTTTTTCTTGTCCCATTCCAA
fnbB-N3	attB1-fnbB-N3-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTACCTTCGTAACCTAATTCAC
	fnbB-N3-SacII-R	GGACCTCCGCGGTTTAAATCAGAGCCGCCAGT
	attB2-fnbB-N3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGAAATTAAACCAAGAAT
	fnbB-N3-SacII-F	GGACCTCCGCGGTCCCCAATTATCTCTAACTC
fnbA-N2	attB1-fnbA-N2-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCTCATTTCCGTTCGCTTT
	fnbA-N2-SacII-R	GGACCTCCGCGGGGTATTGGGAATTATTATGC
	attB2-fnbA-N2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTGGGACAAGACAAAGAAGCT
	fnbA-N2-sacII-F	GGACCTCCGCGGTGTACCCGTTTCCACTTTCG
fnbA-N3	attB1-fnbA-N3-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCTCTTCAGGTAATTCAT
	fnbA-N3-SacII-R	GGACCTCCGCGGTCCGATTATTCAAAATAATA
	attB2-fnbA-N3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGGCACAGCCAAGAACGGCAT
	fnbA-N3-SacII-F	GGACCTCCGCGGATCTTTATATTTAACATCTA

The attB1 or attB2 site is underlined and the italic (*CCGCGG*) indicates *Sac*II digestion site.

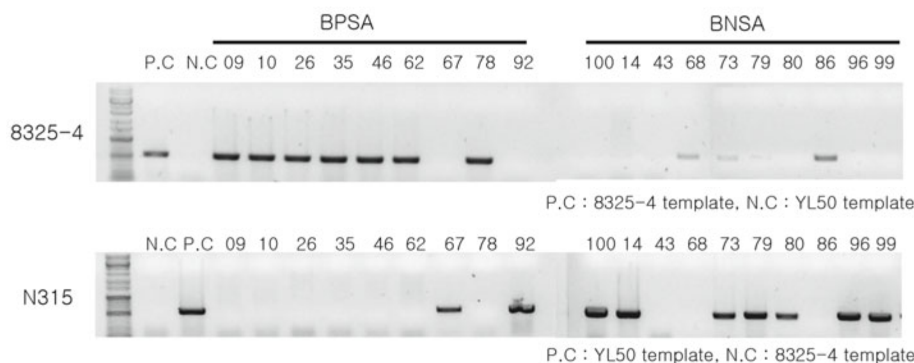


Fig. 3. The detection of N3 subdomain by PCR using each of the primers specific for *S. aureus* strain 8325-4 (upper) and N315 (lower), respectively. P.C and N.C mean positive and negative control, respectively. BPSA and BNSA mean biofilm-positive *S. aureus* strains (from left to right: 09 to 92) and biofilm-negative *S. aureus* strains (from left to right: 100 to 99), respectively.

metric Mann-Whitney *U* test was used to compare the differences in biofilm activity between strain 8325-4 and each of the five deleted mutants as listed in Table 1.

Results and Discussion

Relationship between isotype of FnBPs in *S. aureus* and biofilm forming capacity

A drastic difference in biofilm formation between *S. aureus* 8325-4 and YL50 was observed in the assay of biofilm forming capacity (Fig. 2A). Compared to strain YL50, strain 8325-4 showed more than 4-fold higher ability to form biofilm. Upon examining the major matrix components of biofilms produced by strain 8325-4 using a detachment assay, we realized that biofilms formed by strain 8325-4 in TSB supplemented with 0.5% glucose were not detached by treatment with $NaIO_3$ as well as DNase I, but were by treatment with proteinase K (Fig. 2B), indicating that biofilms developed by strain 8325-4 under these assay conditions mainly consisted of a protein-based matrix. Based on a previous report, which stated that decreased biofilm formation was found in only *fnbB* deleted mutant following systemic mutation of each of the 20 LPXTG proteins (Vergara-Irigaray *et al.*, 2009), we tried to determine whether FnBPs of *S. aureus* are involved in the observed difference in biofilm formation between 8325-4 and YL50. The FnBPs of *S. aureus* strain 8325-4 are classified into the isotype I of seven different isotypes based on divergence in the A domains of FnBPs from several *S. aureus* strains (Loughman *et al.*, 2008; Burke *et al.*, 2010). To determine which isotype of the FnBPs of *S. aureus* strain YL50 belong to, we sequenced genes encoding the A domains of FnBPA and FnBPB of YL50. Sequences analysis showed that the genes of strain YL50 tested were highly homologous to those of strain N315 (GenBank accession no. SA 2290 for *fnbB* gene and SA 2291 for *fnbA* gene) that belongs to isotype III (Loughman *et al.*, 2008; Burke *et al.*, 2010). Therefore, we hypothesized that distinct nucleotide alleles encoding the A domains between isotype I FnBPs and isotype III FnBPs are responsible for the observed differences in biofilm formation.

To test this hypothesis, we first designed two sets of primers which specifically detect N3 subdomains of FnBPB isotype I and isotype III. Using type-specific DNA probes comprising nucleotides which encode the highly divergent N3 subdomain, we examined the prevalence of isotype I and III of FnBPB in nine biofilm-positive and ten biofilm-negative strains. As expected, we found that prevalence of the N3 subdomain of FnBPB isotype I was higher in the nine biofilm-positive isolates, while that of isotype III was higher in the ten biofilm-negative strains (Fig. 3). This result suggested that FnBPB isotype I is more closely related to biofilm-positive *S. aureus* clinical isolates compared to isotype III which showed a higher prevalence in biofilm-negative strains. Moreover, it is highly likely that biofilm formation of *S. aureus* would occur differently depending on the isotype of FnBPs.

Role of FnBPB-N3 subdomain of FnBP isotype I of strain 8325-4 in biofilm formation

To examine the relatedness of the other N subdomains of the A domain of isotype I to biofilm-positivity under the biofilm assay condition, we designed primers to specifically detect not only each N1, and N2 subdomain of FnBPB isotype I, but also N1, N2, and N3 subdomain of FnBPA isotype I, in addition to the N3 subdomain of FnBPB isotype I as described in 'Materials and Methods'. We examined the prevalence of each of the N subdomains of FnBP isotype I

Table 4. Univariate and multivariate logistic regression analysis of N subdomains as risk factors predictive for biofilm-positive strains

N subdomains	No. of clinical isolates		Univariate analysis		Multivariate analysis	
	Biofilm-positive (n=246)	Biofilm-negative (n=123)	OR* [95% CI]	*P* value	OR [95% CI]	*P* value
FnBPB-N1	167 (67.9%)	61 (49.6%)	**2.149 [1.379–3.347]**	**0.001**	0.710 [0.377–1.336]	0.288
FnBPA-N2	133 (54.1%)	34 (27.6%)	**2.959 [1.859–4.712]**	**<0.001**	0.251 [0.054–1.172]	0.079
FnBPA-N3	131 (53.3%)	30 (24.4%)	**3.381 [2.096–5.452]**	**<0.001**	3.444 [0.800–14.826]	0.097
FnBPB-N3	127 (51.6%)	13 (10.6%)	**9.030 [4.825–16.902]**	**<0.001**	**13.174 [5.787–29.993]**	**<0.001**

* Odds ratio. *P* values less than 0.05 are in boldface.

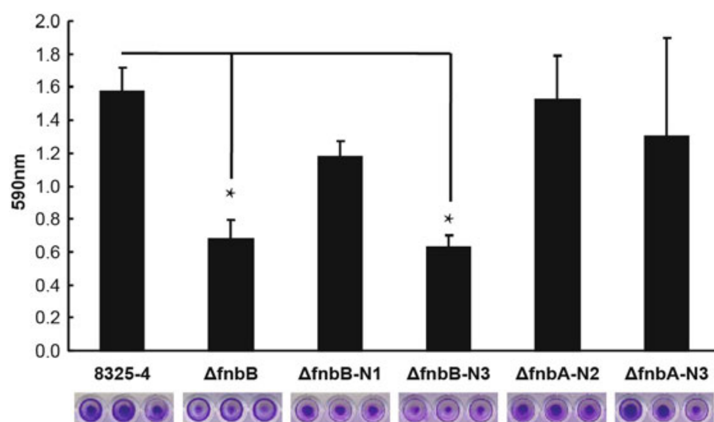


Fig. 4. Contribution of N3 subdomains of FnBPB to biofilm formation in *S. aureus* strain 8325-4. 8325-4 strain and its derivatives including *fnbB* deleted (ΔfnbB), *fnbB-N1* deleted (ΔfnbB-N1), *fnbB-N3* deleted (ΔfnbB-N3), *fnbA-N2* deleted (ΔfnbA-N2), and *fnbA-N3* deleted (ΔfnbA-N3) mutants were grown on microtiter plates overnight under static conditions at 37°C in TSB supplemented by 0.5% glucose. The biofilm formed was stained by crystal violet and dissolved by the addition of 200 μl of ethanol/acetone. The absorbance was determined at 590 nm. The results are representative of three independent experiments performed in triplicate. Error bars indicate the standard deviations. Significant differences are detected when either *fnbB* deleted mutant or *fnbB-N3* deleted mutant is compared to the parent strain 8325-4 (*$P<0.01$; Mann-Whitney test).

in the same representative strains as we used to observe the prevalence of the N3 subdomain of FnBPB isotype I. Following further testing, we observed that of the three consecutive N subdomains (N1-N3) of FnBP isotype I, the prevalence of each of the N2, and N3 subdomains of FnBPA (FnBPA-N2 and -N3) and the N3 subdomain of FnBPB (FnBPB-N3) was higher in representative biofilm-positive than biofilm-negative strains, while the prevalence of the N1 subdomain of FnBP isotype I was not significantly different between the two (data not shown). This result may be attributed to the fact that the N1 subdomain with less diversity is not required for ligand binding, whereas the N2 and N3 subdomains with more diversity are involved in ligand binding as described in previous reports (Loughman *et al.*, 2008; Burke *et al.*, 2010).

To extend our observations to more strains, we tried to examine not only the prevalence of each FnBPA-N2 and N3 and FnBPB-N3 subdomain, but also that of FnBPB-N1 subdomain as a negative control, with 465 clinical isolates of *S. aureus*. In the biofilm assays, 246 of 465 strains were defined as as biofilm-positive strains (OD≥0.15) and 123 of 465 strains were defined as as biofilm-negative strains (OD<0.1). Ninety-seven of 456 strains (0.1<OD<0.15) were excluded for further experiments. The prevalence of each FnBPA-N2, -N3, and FnBP-N3 subdomain of FnBP isotype 1 was higher in 246 biofilm-positive strains than in 123 biofilm-negative strains. Unexpectedly, the prevalence of FnBPB-N1 subdomain of FnBP isotype 1 was also higher in 167 (67.9%) of the 246 biofilm-positive strains than in 61 (49.6%) of the 123 biofilm-negative strains (Table 4). Moreover, in univariate logistic regression analysis to explore if each of the four N subdomains was associated with biofilm formation between the two groups, the four N subdomains were found to be significantly related to biofilm formation (Table 4). This result was consistent with the previous report, which mentioned that both FnBPA and FnBPB cooperate to produce a biofilm in TSB supplemented with glucose (Vergara-Irigaray *et al.*, 2009). However, upon multivariate logistic regression analysis to elucidate which of the four N subdomains of FnBP isotype I is a key region responsible for biofilm formation, only the FnBPB-N3 subdomain was identified as an independent risk determinant predictive for biofilm-positive strains of *S. aureus* (OR, 13.174; $P<0.001$) (Table 4). This result suggested that the N3 subdomain of the A domain in FnBPB isotype I of strain 8325-4 is a leading region accounting for biofilm development by *S. aureus* clinical isolates.

To confirm our results, we attempted to delete each of the *fnbA-N2* and *-N3* and *fnbB-N1* and *-N3* loci from strain 8325-4 by means of allelic replacement as described in 'Materials and Methods'. In agreement with the results of the multivariate regression analysis, the deletion of either the *fnbA-N2* or *-N3* or *fnbB-N1* locus did not diminish the capacity of strain 8325-4 to develop biofilm compared to the diminished biofilm formation of the *fnbB-N3* locus-deleted mutant as well as the entire *fnbB* gene deleted mutant (Fig. 4). Results related to mutation of the FnBPA-N2 subdomain were consistent with that of a previous report, which stated that the substitution of A domain N304A corresponding to the N2 domain of FnBPA did not affect biofilm formation, but did abolish the fibrinogen-binding activity (O'Neill *et al.*, 2008).

In conclusion, strains of *S. aureus* harboring FnBP isotype I of strain 8325-4 were more biofilm-positive than those harboring FnBP isotype III of the strain N315. Among the three consecutive N subdomains in the A domain of FnBP isotype I, the N3 subdomain was an independent risk determinant predictive for biofilm formation of *S. aureus* clinical isolates.

Acknowledgements

We thank Dr. K.S. Ko for 52 strains recovered from 10 other South-East Asian countries. This study was supported by a grant of the Korean Health Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A084442) and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) through the Research Center for Resistant Cells (R13-2003-009) (No. 2009-0091400).

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