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Identification of *fusB*-Mediated Fusidic Acid Resistance Islands in *Staphylococcus epidermidis* Isolates▽

Hsiao-Jan Chen,[1] Jui-Chang Tsai,[2,3] Wei-Chun Hung,[1] Sung-Pin Tseng,[1] Po-Ren Hsueh,[4,5] and Lee-Jene Teng[1,4]*

Department of Clinical Laboratory Sciences and Medical Biotechnology[1] and Center for Optoelectronic Biomedicine,[2] National Taiwan University College of Medicine, and Department of Surgery, Division of Neurosurgery,[3] Department of Laboratory Medicine,[4] and Department of Internal Medicine,[5] National Taiwan University Hospital, Taipei, Taiwan

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To understand the high prevalence of *fusB* genes in fusidic acid-resistant *Staphylococcus epidermidis*, analysis of resistance elements in 34 isolates was performed. First, sequence analysis of the *aj1*-LP-*fusB* region indicated that at least three types were present. Type I contained full-length *aj1*, type II contained a partial *aj1* truncated from nucleotide position 93 to 421, and type III contained a more truncated *aj1* that retained only the last 37 bp. Isolates with type I or type II *aj1* displayed slightly higher levels of resistance to fusidic acid (MICs, 8 to 32 μg/ml) than did those with type III *aj1* (MICs, 4 to 16 μg/ml). Subsequent sequencing of the flanking regions of *fusB* from four selected isolates carrying different types of *aj1*-LP-*fusB* regions revealed that the *fusB* genes were all located on phage-related resistance islands (RIs), referred to as SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, SeRI$_{fusB\text{-}5907}$, and SeRI$_{fusB\text{-}7778}$, respectively. Among them, three islands (SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$) were located downstream of *groEL* (corresponding to the 44-min position based on *Staphylococcus aureus* whole genomic sequences), and one (SeRI$_{fusB\text{-}7778}$) was located downstream of *rpsR* (corresponding to the 8-min position). All of the RIs were inserted into integrase-recognized *att* sites. Among 34 isolates, the insertion sites of *fusB* RIs were mostly (28/34, 82%) located downstream of *groEL* and two were located downstream of *rpsR*, but four remained unidentified. The pulsotype distribution indicated that *fusB*-containing *S. epidermidis* isolates were heterogeneous. In conclusion, the *fusB* resistance determinant in *S. epidermidis* was highly associated with phage-related RIs. This is the first report of *fusB* RI in *S. epidermidis*.

Staphylococcus epidermidis, a commensal organism, may cause infection in patients whose immune systems are compromised or who have catheters (32). This bacterium is considered a reservoir of antibiotic resistance genes (1). Fusidic acid, the product of *Fusidium coccineum*, is a steroid antibiotic that is used to treat skin and systemic staphylococcal infections (7). Fusidic acid is usually used as an agent of combination therapy for treating deep-seated infections such as osteomyelitis, septic arthritis, and infective endocarditis caused by staphylococci in our hospital. The combination agents are usually vancomycin, teicoplanin, and linezolid. If the staphylococci are resistant to fusidic acid, the combination agent may be changed to rifampin or fluoroquinolones. Fusidic acid inhibits bacterial protein synthesis by interacting with elongation factor G (EF-G) to sterically influence the release of the EF-G/GDP complex from the ribosome (3, 11, 14). Two major fusidic acid resistance mechanisms have been reported: one is the alternation of the drug target site (*fusA* and *fusE*) (2, 17, 18) and the other is the protection of the drug target site by FusB family proteins (*fusB*, *fusC*, and *fusD*) (22, 24).

Acquired fusidic acid resistance genes found in *Staphylococcus* spp. include *fusB*, *fusC*, and *fusD*. The genes *fusB* and *fusC* were found in *Staphylococcus aureus* and coagulase-negative staphylococci (15, 22, 24, 33), and *fusD* is an intrinsic factor causing fusidic acid resistance in *Staphylococcus saprophyticus* (24). The *fusB* determinant, encoding a 25-kDa protein, was originally found on a transposon-like element of plasmid pUB101 or in a pathogenicity island (PI) in *Staphylococcus aureus* (21, 23). The FusB protein resulted in resistance to fusidic acid by directly binding to EF-G (the target of fusidic acid) and protecting translation in the presence of fusidic acid *in vitro*.

A high prevalence of resistance to fusidic acid has been reported for *S. epidermidis*. Resistance to fusidic acid in *S. epidermidis* is mostly associated with the *fusB* determinant (15). The proportion of fusidic acid-resistant *S. epidermidis* isolates each year ranged from 39% to 46%, higher than that in *S. aureus*, at the National Taiwan University Hospital (NTUH), Taiwan (4). Our previous study of fusidic acid resistance determinants in *S. aureus* revealed that most methicillin-susceptible *S. aureus* isolates carried *fusB*-containing plasmids. In contrast to the case of *S. aureus*, our preliminary data indicated that *fusB* was not located on a plasmid in *S. epidermidis*. Because *fusB* is widely distributed in different species, we wanted to determine if there was any correlation between *S. aureus* and *S. epidermidis* with respect to fusidic acid resistance. In this study, we identified three types of *aj1*-leader peptide (LP)-*fusB* fragments with identical LP and *fusB* sequences but different sizes of *aj1* regions: type I, full-length *aj1*; type II, truncated *aj1* that was translated into a smaller putative protein; and type III, truncated *aj1* retaining only the last 37 bp. We further analyzed the genetic structures of *fusB* resistance islands in four representative *S. epidermidis* isolates with different *aj1*-LP-*fusB* types. Sequence analysis indicated that *fusB* determinants in

* Corresponding author. Mailing address: Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, 1 Chang-Te Street, Taipei 100, Taiwan. Phone: 886-2-23123456, ext. 66918. Fax: 886-2-23711574. E-mail: ljteng@ntu.edu.tw.

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TABLE 1. Primers used in this study

Description	Primer name	Sequence (5′ to 3′)[a]	Position	Reference
aj1-LP-*fusB* detection	ri18 250-269F	GTTGCTAAATCTCCTCACGG	ri18 250-269	This study
	7778 LA 410-390R	GGGTAAATCCAGAGTTAATCG	*fusB* −232 to −252	
	aj1 606-577R	AGTAAAGAATAAGTTTTTAATCGTTAATGC	*aj1* 606-577	
	fusB 389-361R	TTCCGATTTGATGCAAGTTCATTCCATCC	*fusB* 389-361	
LA-PCR for HindIII fragments	fusB 437-465F	GAGAAATTTCTAATCAGGTTGTAAAGGGG	*fusB* 437-465	This study
	fusB 530-558F	CGGATGGTCAATATGTAAAAAAAGGTGAC	*fusB* 530-558	
	fusB 553-580F	GGTGACTATATATGTCGAGATAGCATTC	*fusB* 553-580	
	fusB 282-253R	AAGTTTTTGCGGACTAGGTAGTTCAAAAGG	*fusB* 282-253	
LA-PCR for PstI fragments	7778 LA 755-736R	CGATTGAATAACTTTGACGG	*fusB* 114-95	This study
7778 upstream detection	S. epi rpsR 6-24F	AGGTGGACCAAGAAGAGGC	*rpsR* 6-24	This study
2793 upstream detection	S. epi groEL 1213-1232F	GTKGAAGAAGGTATYGTTGC	*groEL* 1213-1232	This study
Prediction of insertion sites	int(I) 109-128F	CGTAAATCAGACGCTAAACA	SeRI$_{fusB\text{-}2793}$ *int* 109-128	This study
	int(I) 1139-1122R	CTAAACTTGTGGGAAGCG	SeRI$_{fusB\text{-}2793}$ *int* 1139-1122	
	int(II) 541-565F	GCTAAACGTAATAACTATTTAGAAG	SeRI$_{fusB\text{-}7778}$ *int* 541-565	
	int 1061-1042R	GTGTGACGTAATGTGTGCGT	SeRI$_{fusB\text{-}7778}$ *int* 1061-1042	
	S. epi groEL 1582-1599F	GAACAACCTGGAATGGGT	*groEL* 1582-1599	
	185 LA 3R	CTCACAGAGGTTCTATAATGTTGG	SeRI$_{fusB\text{-}2793}$ ORF19 419-396	
	fusB LA-2R	AATACTCCTGGATGGCGT	SeRI$_{fusB\text{-}7778}$ ORF19 −216 to −233	

[a] K = G or T; Y = C or T.

the test *S. epidermidis* isolates were all carried by phage-related resistance islands (RIs). Thus, the *fusB*-containing RIs were highly associated with the dissemination of fusidic acid resistance in *S. epidermidis*.

MATERIALS AND METHODS

Bacterial strains. Thirty-six fusidic acid-resistant *S. epidermidis* isolates were used from a collection made between January 2003 and January 2007 in the Bacteriology Laboratory of the National Taiwan University Hospital, a 2,500-bed teaching hospital in northern Taiwan. *S. epidermidis* was initially identified by the Phoenix Automated System and further confirmed by 16S rRNA gene sequencing. The sources of the 36 isolates included blood samples ($n = 35$) and a central venous catheter ($n = 1$). Among these isolates, four (NTUH-2793, NTUH-704, NTUH-5907, and NTUH-7778) were used to determine the full-length sequences of the *fusB* element.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed by standard agar dilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (5). Bacterial inocula were prepared by direct colony suspension to a turbidity of a 0.5 McFarland standard. A bacterial density of 10^4 CFU/spot was inoculated on Mueller-Hinton agar containing various concentrations of fusidic acid (0.03 to 128 μg/ml) using a Steers replicator, and the plates were incubated at 33 to 35°C for 16 to 20 h. *S. aureus* ATCC 29213 was used as the reference organism. The breakpoint used to indicate fusidic acid resistance was 2 μg/ml (6).

Detection of fusidic acid resistance determinants by PCR. For detection of fusidic acid resistance determinants, the DNA of the isolates was amplified with primers specific for *fusB*, *fusC*, or *fusD*, as previously described (4). For isolates showing negative results for *fusB*, *fusC*, and *fusD*, sequencing of *fusA* was also performed (4).

***aj1*-LP-*fusB* detection.** To detect the *aj1*-LP-*fusB* fragments among the *S. epidermidis* clinical isolates, DNA was amplified with the primers ri18 250-269F and 7778 LA 410-390R (Table 1). Because this pair of primers could not produce PCR products for two isolates, another pair of primers, aj1 606-577R and fusB 389-361(R) (Table 1), was used. PCRs were carried out with 30 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 1.5 min), followed by a final extension step (72°C, 10 min). PCR products were subsequently sequenced.

Southern hybridization. To clone and sequence the *fusB* and flanking fragment in *S. epidermidis*, Southern hybridization was used to estimate the fragment sizes after digestion with restriction enzymes and to choose the optimal restriction enzyme. Southern blot analysis was performed with the DNA of NTUH-2793 and NTUH-7778, which were digested with a series of restriction enzymes (BamHI, EcoRI, HindIII, PstI, SalI, and XbaI; New England BioLabs) and detected with a digoxigenin (DIG)-labeled *fusB*-specific probe prepared by PCR amplification (4). Probe labeling and detection were performed using a commercial kit (Roche Diagnostics GmbH, Penzberg, Germany).

Cloning and sequencing of *fusB* and flanking regions. To determine the sequence of the entire *fusB* gene and its flanking regions, an LA-PCR *in vitro* cloning kit (Takara Shuzo Co. Ltd., Japan) was used. For *S. epidermidis* NTUH-2793, the LA-PCR was carried out with the HindIII fragments. After ligating the HindIII-digested DNA fragments with cassette adapters, the amplification was performed with the cassette primers (C1 for the first PCR and C2 for the nested PCR) supplied by the manufacturer and target gene-specific primers (fusB 437-465F paired with C1 and fusB 530-558F paired with C2, Table 1). After determining the downstream sequence of *fusB* fragments, the insertion site of the island was predicted. The upstream sequence was amplified by the designed primers S. epi groEL 1213-1232F and fusB 282-253R (Table 1). For *S. epidermidis* NTUH-7778, the LA-PCR was carried out with the HindIII- and PstI-digested DNA fragments hybridized with a *fusB*-specific probe. After ligating the HindIII- or PstI-digested DNA fragments with cassette adapters, the amplification of the HindIII-digested DNA was performed with four pairs of primers, including C1 and fusB 389-361R, C1 and fusB 282-253R, C2 and fusB 530-558F, and C2 and fusB 553-580F (Table 1). The amplification of PstI-digested DNA fragments was performed by two pairs of primers (C1 and 7778 LA 755-736R, C2 and 7778 LA 410-390R; Table 1). After determining the downstream sequences of the *fusB* fragments, the insertion site of the island was predicted. The upstream sequence was amplified by the designed primers 7778 LA 410-390R and S. epi rpsR 6-24F (Table 1). The amplified fragments were subsequently sequenced on an Applied Biosystems model 3100 DNA sequencer (Applied Biosystems, Foster City, CA) using the *Taq* BigDye-Deoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequences of two other *S. epidermidis* isolates, NTUH-5907 and NTUH-704, were determined by PCR amplification based on the sequence of NTUH-2793.

Phylogenetic analysis of integrases. The phylogenetic relationships among integrases, which are associated with pathogenicity islands found in staphylococci, were analyzed by the neighbor-joining (NJ) method described in the MEGA4 (molecular evolutionary genetic analysis) analytical package (27). For the NJ analysis, the distance between the sequences was calculated using Kimura's two-parameter model. The levels of similarity among species were determined. Bootstrap values were obtained using 500 randomly generated trees.

Prediction of insertion sites in other *S. epidermidis* isolates. We designed eight pairs of primers to detect the insertion sites (downstream of *groEL* or *rpsR*) in *S. epidermidis* clinical isolates. The primer pairs int(I) 109-128F/int(I) 1139-1122R and int(II) 541-565F/int 1061-1042R (Table 1) were used to detect the integrase genes recognizing the sites of *groEL* or *rpsR*, respectively. The primer pairs S. epi groEL 1582-1599F/int(I) 109-128F and S. epi rpsR 6-24F/int(II) 541-565F were used to amplify the junction of the chromosomal genes and the integrase genes, *groEL*-integrase and *rpsR*-integrase, respectively. The junctions of the *fusB* gene and the downstream chromosomal genes were detected by fusB 530-558F/185 LA 3R and fusB 530-558F/fusB LA-2R. The junctions of two chromosomal genes without the insertion of resistance islands were amplified using S. epi groEL 1582-1599F/185 LA 3R and S. epi rpsR 6-24F/fusB LA-2R. The primers used in this study are listed in Table 1.

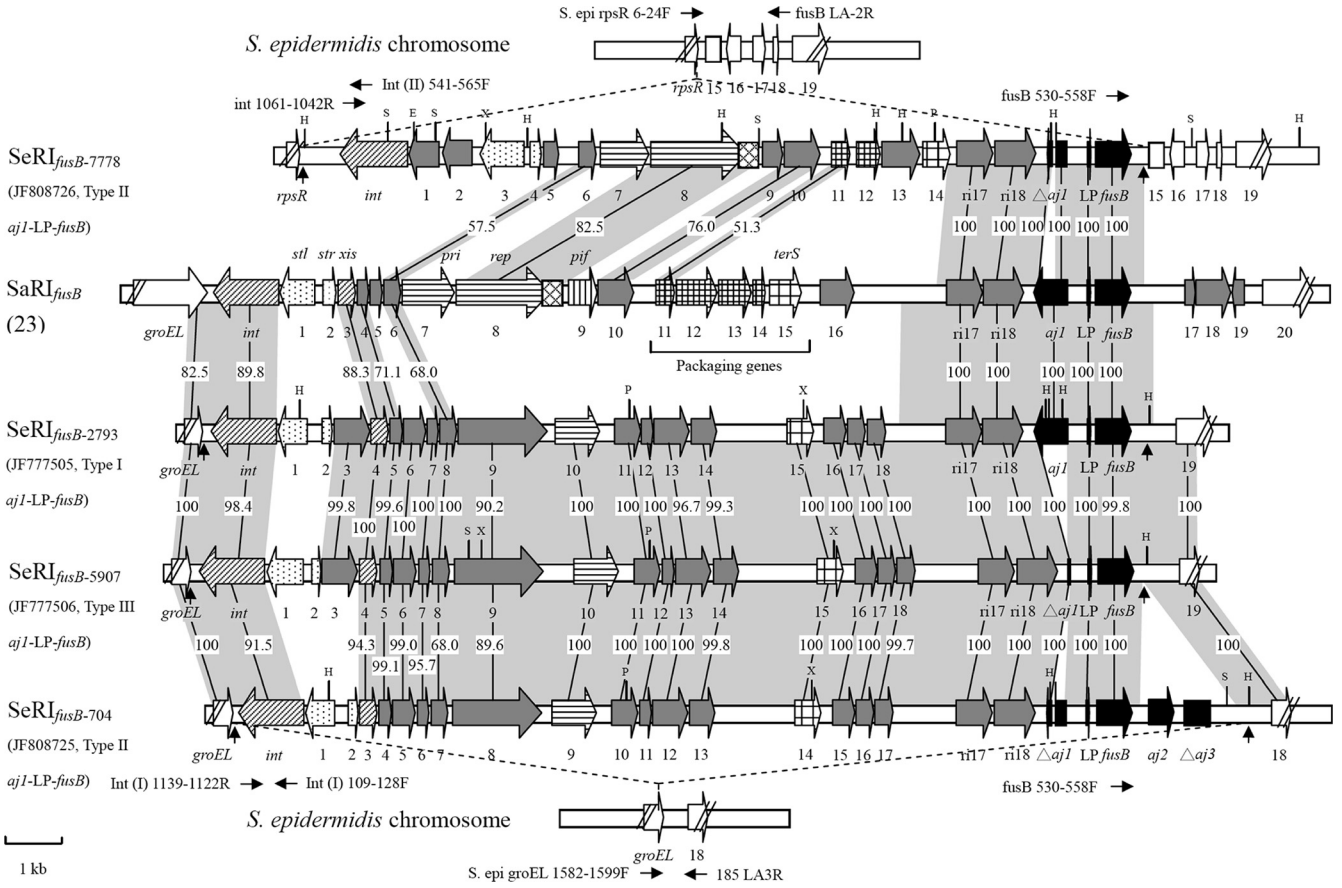

FIG. 1. Genetic organization of SeRI$_{fusB\text{-}2793}$ (GenBank accession no. JF777505), SeRI$_{fusB\text{-}704}$ (GenBank accession no. JF808725), SeRI$_{fusB\text{-}5907}$ (GenBank accession no. JF777506), and SeRI$_{fusB\text{-}7778}$ (GenBank accession no. JF808726) compared with SaRI$_{fusB}$ (GenBank accession no. AM292600). Genes are drawn according to their sequences and function: *int* and *xis* are ▨; transcription regulators are ⬚; replication genes (including the primase gene [*pri*] and the replication initiator gene [*rep*]) are ▤; the replication origin (ori) is ▩; of encapsidation genes, the terminase small subunit gene (*terS*) is ⊞ and other genes are ▦; *pif* (phage interference) is ▥; *aj1*-LP-*fusB* regions are ■; chromosome genes adjacent to SeRI$_{fusB}$ are □; other genes encoding hypothetical proteins are ▢. The predicted direct repeats are indicated by vertical arrows. The horizontal arrows represent the PCR primers used to determine the insertion sites. Regions homologous between resistance islands are shown in shadow, and the numbers in shadow show percent homology between the corresponding sequences. The restriction sites are also shown: H, HindIII; P, PstI; X, XbaI; E, EcoRI; S, SpeI.

PFGE. The genotypes of the fusidic acid-resistant *S. epidermidis* isolates were determined by pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared as described previously (16). The DNA was digested with SmaI (New England BioLabs, Ipswich, MA) and then separated in a CHEF-DRII apparatus (Bio-Rad Laboratories). PFGE was carried out at 200 V and 13°C for 18.5 h, with pulse times ranging from 5 to 60 s. The pulsotypes were analyzed by BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). The dendrogram of pulsotype relationships was produced by the unweighted pair group method using arithmetic averages (UPGMA) based on Dice similarity indices.

Nucleotide sequence accession numbers. The SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, SeRI$_{fusB\text{-}5907}$, and SeRI$_{fusB\text{-}7778}$ sequences from the *S. epidermidis* clinical isolates NTUH-2793, NTUH-704, NTUH-5907, and NTUH-7778, respectively, were deposited in GenBank under accession numbers JF777505, JF808725, JF777506, and JF808726, respectively.

RESULTS

Fusidic acid resistance determinants. FusB family resistance determinants were detected by PCR. Amplification with primers specific for *fusB*, *fusC*, and *fusD* revealed that 33 of the 36 *S. epidermidis* isolates possessed *fusB* (92%), one isolate carried *fusC*, one isolate carried both *fusB* and *fusC*, and one isolate carried none of the FusB family resistance determinants but contained a *fusA* point mutation (V90I). The presence of *fusB* was also confirmed by Southern hybridization with a *fusB*-specific probe (data not shown).

Variation of *aj1*-LP-*fusB* fragments. To test the correlation between *aj1*-LP-*fusB* structures and the levels of resistance, the *aj1*-LP-*fusB* fragments of clinical isolates were amplified and sequenced. The results revealed that at least three different types of *aj1*-LP-*fusB* fragments were present. Different sizes of the *aj1* regions were detected among the *fusB*-carrying isolates (Fig. 1). Structures with full-length *aj1* were classified as type I. The type II *aj1* region had a partial *aj1* gene truncated from nucleotide (nt) 93 to 421 that was translated into a putative protein smaller than full-length *aj1*. The type III fragment possessed a more truncated *aj1* gene that retained only the last 37 bp. Among 34 isolates (33 clinical isolates carrying *fusB* alone and one isolate carrying both *fusB* and *fusC*), 4 type I, 11 type II, and 17 type III isolates were identified, but two remained unidentified. The correlation between the different structures of the *aj1*-LP-*fusB* fragment and the resistance level is shown in Table 2. Low-level resistance (MIC < 8 μg/ml) was

TABLE 2. Distribution of fusidic acid resistance determinants and MICs among fusidic acid-resistant *S. epidermidis* isolates

Resistance determinant	*aj1*-LP-*fusB* type	No. of bacterial isolates	No. of isolates with different MICs (μg/ml):			
			4	8	16	32
fusB	I	4	0	0	2	2
	II	11	0	1	7	3
	III	17	1	5	11	0
	U[a]	1	0	1	0	0
fusC		1	0	0	1	0
fusB and *fusC*	U[a]	1	0	0	0	1
fusA point mutation		1	1	0	0	0

[a] U, undetermined *aj1*-LP-*fusB* type.

detected mostly in isolates with the type III *aj1*-LP-*fusB* structure. Most isolates carrying type I or type II *aj1*-LP-*fusB* showed relatively higher levels of resistance (MIC = 16 or 32 μg/ml). One isolate with an unidentified *aj1*-LP-*fusB* fragment also had an MIC of 32 μg/ml, possibly due to the presence of both the *fusB* and *fusC* determinants and/or unknown mechanisms.

Sequence analysis of *fusB* and flanking regions. Based on *aj1*-LP-*fusB* types and Southern blot hybridization patterns, we chose four isolates to further analyze the flanking regions of *fusB* elements. Sequence data revealed that the *fusB* elements of four representative isolates were all located on phage-related resistance islands (RIs) (Table 3 and Fig. 1). The sizes of the RIs ranged from 15 kb to 17.5 kb, which were shown as follows. The sizes of the RIs in NTUH-2793 (type I *aj1*-LP-*fusB*), referred to as SeRI$_{fusB\text{-}2793}$ (*S. epidermidis* resistance island carrying *fusB* in NTUH-2793); NTUH-704 (type II *aj1*-LP-*fusB*), referred to as SeRI$_{fusB\text{-}704}$; NTUH-5907 (type III *aj1*-LP-*fusB*), referred to as SeRI$_{fusB\text{-}5907}$; and NTUH-7778 (type II *aj1*-LP-*fusB*), referred to as SeRI$_{fusB\text{-}7778}$, were 16,913 bp, 17,554 bp, 16,695 bp, and 15,307 bp, respectively. SeRI$_{fusB\text{-}2793}$ was composed of 24 putative open reading frames (ORFs), SeRI$_{fusB\text{-}5907}$ had 23 putative ORFs and one truncated gene (*aj1*), SeRI$_{fusB\text{-}704}$ was made up of 23 putative ORFs and two truncated genes (*aj1* and *aj3*), and SeRI$_{fusB\text{-}7778}$ was composed

TABLE 3. Comparison of genetic content of resistance islands carrying *fusB*

Annotation or function	ORF no. or gene name of RI[a]					Orientation
	SeRI$_{fusB\text{-}7778}$	SaRI$_{fusB}$	SeRI$_{fusB\text{-}2793}$	SeRI$_{fusB\text{-}5907}$	SeRI$_{fusB\text{-}704}$	
Integrase	*int*	*int*	*int*	*int*	*int*	−
Hypothetical protein	1, 2	Absent	Absent	Absent	Absent	−
PI master repressor	3	1	1	1	1	−
Regulatory protein	4	2	2	2	2	+
Prophage antirepressor	Absent	Absent	3	3	Absent	+
Excisionase	Absent	3	4	4	3	+
Hypothetical protein	Absent	4	5	5	4	+
Hypothetical protein	Absent	5	Absent	Absent	Absent	+
Hypothetical protein	5	Absent	Absent	Absent	Absent	+
SaPIbov ORF-17-like protein	Absent	Absent	6	6	5	+
Hypothetical protein	Absent	Absent	7	7	6	+
Hypothetical protein	6	6	8	8	7	+
Bacteriophage resistance protein	Absent	Absent	9	9	8	+
Similar to DNA primase	7	7	Absent	Absent	Absent	+
Primase-polymerase	Absent	Absent	10	10	9	+
Replication initiator	8 (*rep*)	8 (*rep*)	Absent	Absent	Absent	+
Replication origin	ori	ori	Absent	Absent	Absent	
Phage interference	Absent	9	Absent	Absent	Absent	+
Hypothetical protein	9	Absent	Absent	Absent	Absent	+
Hypothetical protein	10	10	Absent	Absent	Absent	+
Capsid size determinant	11	11	Absent	Absent	Absent	+
Capsid size determinant	12	12–14	Absent	Absent	Absent	+
Hypothetical protein	Absent	Absent	11–14	11–14	10–13	+
Hypothetical protein	13	Absent	Absent	Absent	Absent	+
Bacteriophage terminase small subunit	14 (*terS*)	15 (*terS*)	15 (*terS*)	15 (*terS*)	14 (*terS*)	+
Hypothetical protein	Absent	16	Absent	Absent	Absent	+
Hypothetical protein	Absent	Absent	16, 17	16, 17	15, 16	+
Pathogenicity island protein	Absent	Absent	18	18	17	+
Hypothetical protein	ri17	ri17	ri17	ri17	ri17	+
Hypothetical protein	ri18	ri18	ri18	ri18	ri18	+
Hypothetical protein	Δ*aj1*	*aj1*	*aj1*	Δ*aj1*	Δ*aj1*	−
fusB leader peptide	LP	LP	LP	LP	LP	+
Fusidic acid resistance gene	*fusB*	*fusB*	*fusB*	*fusB*	*fusB*	+
Hypothetical protein	Absent	17	Absent	Absent	Absent	+
Hypothetical protein	Absent	18	Absent	Absent	Absent	+
Transposase	Absent	19	Absent	Absent	Absent	−
Hypothetical protein	Absent	Absent	Absent	Absent	*aj2*	+
Hypothetical protein	Absent	Absent	Absent	Absent	Δ*aj3*	+

[a] SaRI, *Staphylococcus aureus* resistance island (23); SeRI, *Staphylococcus epidermidis* resistance island.

of 19 putative ORFs and truncated *aj1*, as shown in Fig. 1. The G+C contents of SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, SeRI$_{fusB\text{-}5907}$, and SeRI$_{fusB\text{-}7778}$ were 29.9%, 29.3%, 30.2%, and 29.5%, respectively. Three islands, SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$, were flanked by *groEL*, which encodes a heat shock protein on the left side, and another chromosomal gene encoding a cell wall surface anchor family protein on the right side. SeRI$_{fusB\text{-}7778}$ was flanked by *rpsR*, which encodes the 30S ribosomal protein S18, and another chromosomal gene encoding a hypothetical protein, followed by three other chromosomal genes encoding two hypothetical proteins and *lysE*, which encodes an efflux protein. The chromosomal genes flanking the resistance islands were arranged in the same order as in *S. epidermidis* ATCC 12228 (GenBank accession no. AE015929) and RP62A (GenBank accession no. CP000029). Taken together, the resistance islands containing *fusB* determinants were inserted into at least two different sites of the chromosome.

Comparison of the sequences of SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, SeRI$_{fusB\text{-}5907}$, SeRI$_{fusB\text{-}7778}$, and SaRI$_{fusB}$ (GenBank accession no. AM292600) showed that SeRI$_{fusB\text{-}7778}$ was more similar to SaRI$_{fusB}$ found in *S. aureus* than to the other three islands in *S. epidermidis*, but the other three islands were similar to each other (Table 3 and Fig. 1). Three genes near the *fusB* gene (ri17, ri18, and LP) were very conserved in the above five islands. Besides, all five islands carried genes encoding integrase (*int*) at the left end, two divergently oriented putative transcriptional regulators, primase, and terminase small subunit (Table 3 and Fig. 1). Four islands (SaRI$_{fusB}$, SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$) had genes encoding excisionase. SeRI$_{fusB\text{-}7778}$ and SaRI$_{fusB}$ carried the *rep* encoding replication initiator (ORF 8) and replication origin (ori), which could not be found within SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$ (Table 3 and Fig. 1). Comparison of packaging genes showed that all had the terminase small subunit. But unlike SaRI$_{fusB}$, carrying four other genes associated with capsid size determinant, SeRI$_{fusB\text{-}7778}$ carried two capsid size determinant genes, and no putative capsid size determinants were detected in SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$.

***att$_c$* site sequences and locations.** The 21-nt direct repeats flanking SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$, known as *att$_c$* core sites, ATGCCAGGTATGATGTAAAAA and ATGCCAGG(T/A)ATGATGTAAATA, were located downstream of the *groEL* gene. The *att$_c$* site was located at 44 min based on the whole genomic sequences of *S. aureus* (Fig. 2). SeRI$_{fusB\text{-}7778}$ was inserted 99 nt downstream of the *rpsR* gene, and the first 18 nt of the sequence (AAAGAAGAACAATAATAT) were predicted to be an *att$_c$* core site located at 8 min related to the sequence of the *S. aureus* genome (Fig. 2).

Phylogenetic relationships among integrases. The insertion sites of the pathogenicity islands in the bacterial chromosome are recognized by integrases. An unrooted phylogenetic tree constructed from the amino acid sequences of integrases collected from GenBank and determined in this study is presented in Fig. 3. The phylogenetic analysis revealed that the amino acid sequences of the integrases were divided into six major clusters consistent with the insertion sites of the pathogenicity islands (Fig. 2). These results indicate that the integrases recognize the *att$_c$* sites specifically.

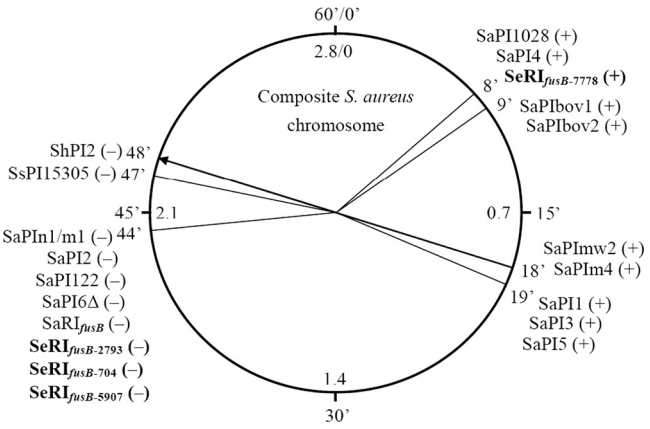

FIG. 2. Locations of PI *att$_c$* core sites based on *S. aureus* whole genomic sequence. The circle presents the composite of the staphylococcal genome with average locations of 60 min (outer scale) to assign 2.8-Mb sequences (inner scale) (modified from reference 20 with permission). Plus or minus in the parentheses indicates the insertion orientation of PIs in clockwise or counterclockwise orientation with respect to the chromosome, respectively.

Determination of insertion sites in the remaining *S. epidermidis* isolates. Based on the obtained sequences from four representative *fusB* resistance islands, we designed eight pairs of primers to determine the insertion sites of resistance islands in the remaining 30 *fusB*-carrying isolates. For isolates with a resistance island inserted downstream of the *groEL* gene, the left junction (*groEL*-integrase) and the right junction (*fusB* and a downstream chromosomal gene such as ORF19 in SeRI$_{fusB\text{-}2793}$ [Fig. 1]) could be amplified by different pairs of the primers listed in Materials and Methods. Moreover, the chromosomal genes could not be amplified due to the insertion of the resistance island (Fig. 1). Among the isolates tested, the majority (28/34, 82%) had insertions downstream of *groEL*, regardless of the *aj1*-LP-*fusB* types. Resistance islands in two isolates with type II *aj1*-LP-*fusB* were inserted downstream of *rpsR*, and the insertion sites in the remaining four isolates (two carrying type I *aj1*-LP-*fusB* and two carrying undetermined *aj1*-LP-*fusB*) were unknown (Table 4).

PFGE analysis. PFGE analysis was used to determine the genetic relatedness among the 34 *fusB*-containing *S. epidermidis* isolates, and the pulsotypes were analyzed by BioNumerics software version 4.0. The 34 isolates were divided into 19 clusters with >80% similarity based on the Dice similarity index in the dendrogram created by the UPGMA algorithm (Fig. 4). Isolates with type II or type III *aj1*-LP-*fusB* regions were distributed in different pulsotypes. Two isolates (NTUH-8108 and NTUH-7778) with *rpsR* insertion were closely related. No particular pulsotype was predominant among these isolates.

DISCUSSION

In agreement with previous studies, the predominant fusidic acid resistance determinant in our *S. epidermidis* isolates was the *fusB* gene, which has been reported in Leeds General Infirmary in the United Kingdom and around Europe (15). In the present study, among the 34 *fusB*-positive *S. epidermidis*

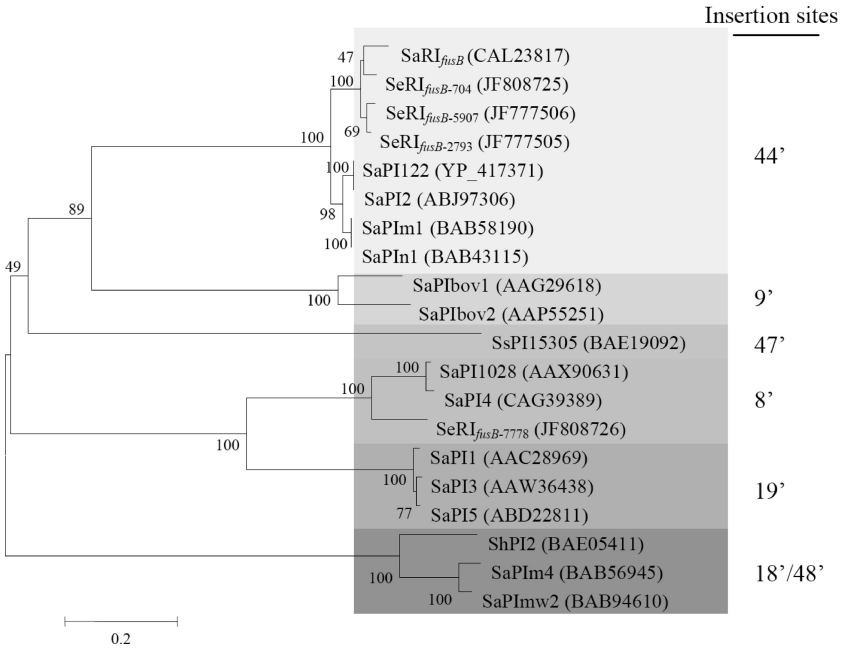

FIG. 3. Phylogenetic relationships among integrases based on amino acid sequences collected from PIs. The phylogenetic tree was generated by using the neighbor-joining method in the MEGA4 package. Numbers at nodes are confidence levels expressed as percentages of occurrence in 500 bootstrapped resamplings. The scale bar indicates the evolutionary distance between sequences determined by measuring the lengths of the horizontal lines connecting two organisms. GenBank accession numbers for integrases are shown in the parentheses. Different degrees of gray indicate PI insertions into different sites based on the *S. aureus* chromosome (Fig. 2).

clinical isolates, 33 carried *fusB* only and 1 carried both *fusB* and *fusC*. All *fusB*-positive isolates contained the *aj1*-LP-*fusB* fragments but with different sizes of the *aj1* region, which can be divided into at least three types. In a previous study in *S. aureus*, *fusB* constructs carrying partial *aj1* fragments of different sizes conferred different levels of resistance to fusidic acid (22). In this study, a slightly higher level of resistance (MICs, 16 to 32 μg/ml) to fusidic acid was found for isolates carrying type I (full-length *aj1*) and type II (truncated *aj1* gene from 93 to 421 nt) *aj1*-LP-*fusB* fragments than for isolates carrying the type III fragment (truncated *aj1*, only the last 37 bp retained) (MICs, 4 to 16 μg/ml). Although it has been demonstrated that the *fusB* gene alone can mediate full resistance to fusidic acid (22), the role of *aj1* in fusidic acid resistance is still unknown.

Sequence analysis of flanking regions of *fusB* in four representative *S. epidermidis* isolates revealed that the resistance elements containing *fusB* were PI-like structures (9). The sizes of the four RIs (SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, SeRI$_{fusB\text{-}5907}$, and SeRI$_{fusB\text{-}7778}$) ranging from 15,307 to 17,554 bp fit the criteria of a PI. PIs in staphylococci usually comprise genomic regions of approximately 10 to 200 kb. The G+C contents of the four RIs ranged from 29.3% to 30.2%, which were lower than that of the published whole genome of *S. epidermidis* (~32%). The four resistance islands were all flanked by directed repeats and carried conserved core genes, including integrase at one end and other proteins associated with staphylococcal PIs. Several PIs in *S. aureus*, including SaPI1 to SaPI4 (12, 26), SaPIn1 to SaPIn3 (10), and SaPIbov1 to SaPIbov3 (30, 31), usually carry genes encoding toxic shock syndrome toxin 1 (TSST-1) and other superantigen toxins. Phage-related chromosomal islands containing antibiotic resistance genes, including *aad*, *fosB*, or *fusB*, have been reported in *Staphylococcus* (19). SaRI$_{fusB}$, containing the *fusB* resistance determinant, was identified from a European fusidic acid-resistant impetigo clone (EEFIC) of *S. aureus* that did not harbor any virulence gene but only the *fusB* resistance gene (23). Mobile genetic elements (MGEs), including plasmids, transposons, phages, and pathogenicity islands (PIs), have been reported to be mobile carriers of antibiotic resistance or virulence factors. We believe that RIs of *S. epidermidis* should play an important role in horizontal gene transfer for fusidic acid resistance within species and/or between species (9).

TABLE 4. Insertion sites of *fusB* elements in *S. epidermidis* clinical isolates

Type of *aj1*-LP-*fusB* (no. of isolates)	No. of isolates with insertion site:		
	groEL	*rpsR*	Unknown
I (4)	2	0	2
II (11)	9	2	0
III (17)	17	0	0
U[a] (2)	0	0	2
Total (34)	28	2	4

[a] U, undetermined *aj1*-LP-*fusB* type.

Two major insertion sites of SeRI$_{fusB}$ in the chromosome were found: one (from the majority of isolates) was downstream of *groEL* (for SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$) and the other one was at *rpsR* (SeRI$_{fusB\text{-}7778}$). The same site of insertion downstream of *groEL* (44 min based on the whole genomic sequences of *S. aureus*) was also found in SaRI$_{fusB}$ (21). However, the direct repeats at the right end of

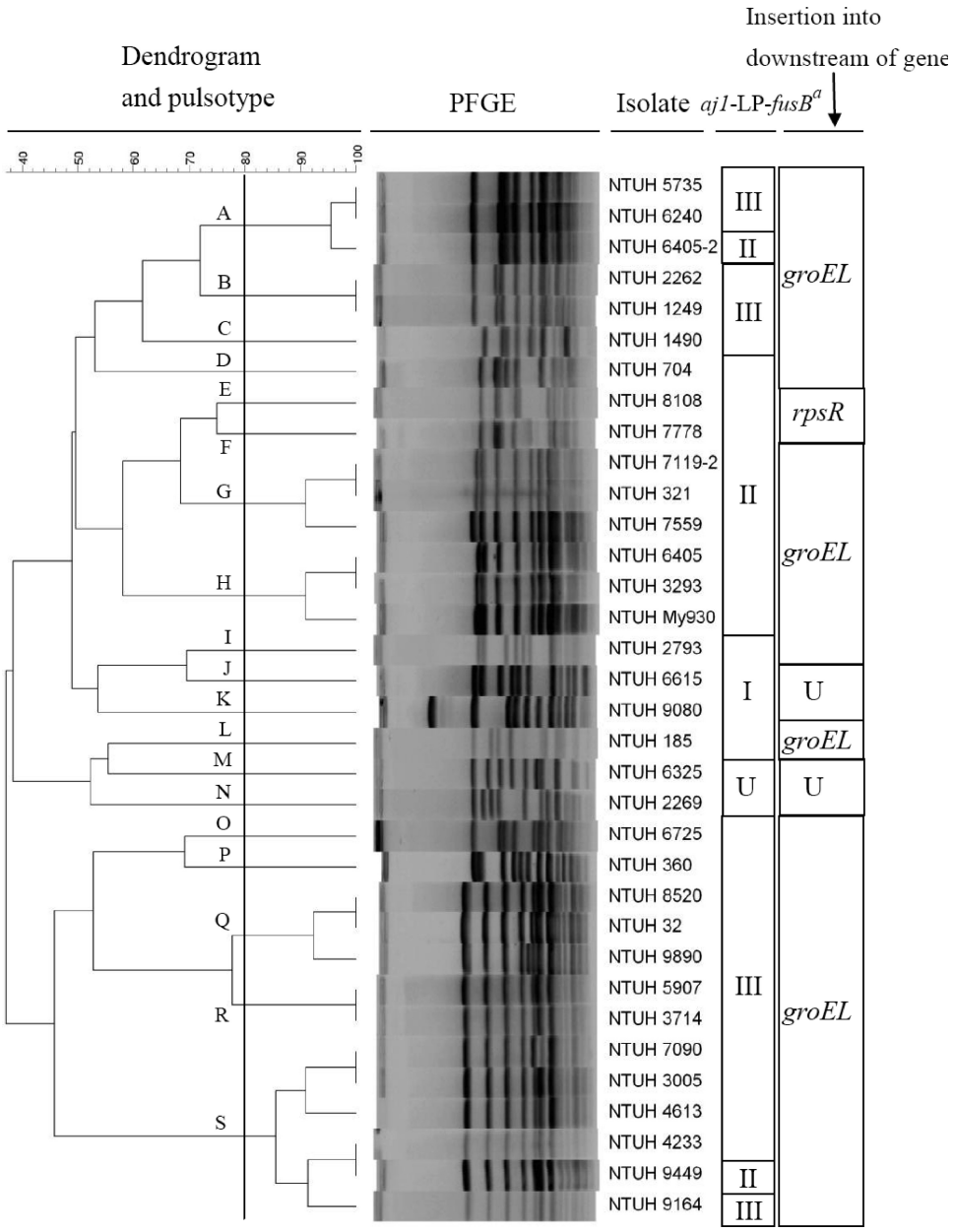

FIG. 4. Dendrogram produced by BioNumerics software, showing distances calculated by Dice similarity index of SmaI-digested DNA fragments among 34 *fusB*-carrying *S. epidermidis* isolates. The degree of similarity is shown in the scale. Different degrees of gray indicate *aj1*-LP-*fusB* types. a, *aj1*-LP-*fusB* types; b, insertion sites of resistance islands; U, undetermined.

the flanking resistance islands were slightly different, ATGCC AGG(T/A)ATGATGTAAATA in SeRI$_{fusB\text{-}2793}$, SeRI$_{fusB\text{-}704}$, and SeRI$_{fusB\text{-}5907}$ and ATGCCAGGTATGATGTAAAAC in SaRI$_{fusB}$ (23). The other insertion site, downstream of the *rpsR* gene, found in SeRI$_{fusB\text{-}7778}$, was located at the 8-min position of the *S. aureus* genome, the same site as that in SaPI4 and SaPI1028 (19). The integration sites, known as the *att* sites of the classical temperate phage, contain core sequence (15 to 22 nt), plus essential sequences depending on the PIs and the chromosome (8, 31). In the present study, SeRI$_{fusB\text{-}7778}$ was flanked by 99-nt directed repeats comprised of an 18-nt putative conserved core sequence, which was identical to that in SaPI4 and SaPI1028, and an 81-nt essential flanking sequence.

Integrase is responsible for the insertion of PIs into specific sites. Phylogenetic analysis of integrases in staphylococcal PIs and RIs revealed that those islands that were inserted into the same sites grouped together. Accordingly, PIs with closely related integrases were located at integrase-recognized specific sites. For example, SeRI$_{fusB\text{-}2793}$, SaRI$_{fusB}$ (23), and SaPI2 (25) with the same integrases were all inserted at the same chromosome site, the 44-min site.

Although we did not test the transferability of SeRI$_{fusB}$, the

mobility of PIs in *S. aureus* has been studied (28, 29). The excision and replication of SaPIbov1 were induced after SOS induction in the presence of temperate phage, and then SaPIbov1 was packed into phage-like particles to infect more bacteria (30). In the present study, resistance islands can be found in different clones and the pulsotypes of *fusB*-containing *S. epidermidis* isolates were very heterogeneous, suggesting the possibility that RIs are easily transferred. In addition, some integrases can be found in *S. epidermidis* and *S. aureus*, which means that they recognize the same core *att* sequences. Thus, it is possible that the RIs have the ability to spread among different species (13). Since *S. epidermidis* has been considered to be a reservoir of antibiotic resistance genes (1), the high prevalence of *fusB* in *S. epidermidis* may raise the chance for spreading fusidic acid resistance to other species.

The four different types of *fusB*-containing resistance islands ($SeRI_{fusB\text{-}2793}$, $SeRI_{fusB\text{-}704}$, $SeRI_{fusB\text{-}5907}$, and $SeRI_{fusB\text{-}7778}$) identified in *S. epidermidis* isolates showed many similarities but with some variations. Preliminary identification of $SeRI_{fusB\text{-}2793}$, $SeRI_{fusB\text{-}704}$, $SeRI_{fusB\text{-}5907}$, or $SeRI_{fusB\text{-}7778}$ can be determined based on the *aj1*-LP-*fusB* type, restriction hybridization with *fusB* probes, and the insertion sites.

In summary, the *fusB* genes in *S. epidermidis* isolates were mostly located in phage-related resistance islands. The resistance islands may be responsible for the dissemination of fusidic acid resistance in *S. epidermidis* through horizontal gene transfer. This is the first report that *fusB* resistance islands are found in *S. epidermidis*.

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