LETTERS

Staphylococcus epidermidis Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization

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Commensal bacteria are known to inhibit pathogen colonization; however, complex host–microbe and microbe–microbe interactions have made it difficult to gain a detailed understanding of the mechanisms involved in the inhibition of colonization[1]. Here we show that the serine protease Esp[2,3] secreted by a subset of *Staphylococcus epidermidis*, a commensal bacterium, inhibits biofilm formation and nasal colonization by *Staphylococcus aureus*, a human pathogen[4]. Epidemiological studies have demonstrated that the presence of Esp-secreting *S. epidermidis* in the nasal cavities of human volunteers correlates with the absence of *S. aureus*. Purified Esp inhibits biofilm formation and destroys pre-existing *S. aureus* biofilms. Furthermore, Esp enhances the susceptibility of *S. aureus* in biofilms to immune system components. *In vivo* studies have shown that Esp-secreting *S. epidermidis* eliminates *S. aureus* nasal colonization. These findings indicate that Esp hinders *S. aureus* colonization *in vivo* through a novel mechanism of bacterial interference, which could lead to the development of novel therapeutics to prevent *S. aureus* colonization and infection.

Staphylococcus aureus is an important human bacterial pathogen responsible for a wide variety of conditions, ranging from subclinical inflammation to severe infections causing pneumonia, endocarditis and septicaemia[4]. Recently, emergence of multi-drug-resistant *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), has made treatment difficult and increased the rate of mortality[5,6]. The primary reservoir for *S. aureus* is the nasal cavity[7,8]. One-third of the population in the United States of America, the United Kingdom, Japan, and other countries is colonized with this pathogen asymptomatically; however, the colonization is known to be a risk factor for staphylococcal infections[9–13]. Intriguingly, there are people capable of evading *S. aureus* colonization. Elucidating this evasion mechanism may lead to a better understanding of bacterial ecology, including commensal–pathogen interactions, and provide information to develop effective drugs against *S. aureus*.

As a first step to explain the evasion mechanism, we focused on the possible inhibitory roles of *Staphylococcus epidermidis* in *S. aureus* colonization because *S. epidermidis* is the dominant commensal bacterium found in the human nasal cavity. We isolated these bacteria from the nasal cavities of 88 volunteers. Bacteriological analysis showed that *S. aureus* had colonized 28 (31.8%) subjects, of whom 26 (92.9%) were also colonized by *S. epidermidis* (Table 1). Of the 60 non-carriers of *S. aureus*, 58 (96.7%) were colonized by *S. epidermidis* (Table 1). The prevalence of *S. epidermidis* in nasal carriers and non-carriers of *S. aureus* did not differ significantly (Table 1). However, further investigation indicated that some properties of *S. epidermidis* isolated from carriers of *S. aureus* might differ from those in non-carriers. When *S. epidermidis* cells or their culture supernatants were co-cultured with *S. aureus*, 428 of 960 isolates from volunteers' nasal cavities inhibited biofilm formation by *S. aureus* in a dose-dependent manner, whereas the remaining 532 strains did not. This finding indicated that *S. epidermidis* can be divided into two types: one that inhibits biofilm formation by *S. aureus* (inhibitory type) and one that does not (non-inhibitory type) (Fig. 1a and b). Furthermore, the culture supernatants of inhibitory *S. epidermidis* destroyed pre-existing *S. aureus* biofilms, whereas those of non-inhibitory *S. epidermidis* did not (Fig. 1c).

On the basis of these findings, we conducted an epidemiological study investigating the relationship between the nasal colonization of inhibitory *S. epidermidis* and that of *S. aureus*. Univariate logistic regression analysis showed that *S. aureus* detection rate was significantly lower in the presence of inhibitory *S. epidermidis* (odds ratio (OR), 0.29; 95% confidence interval (CI), 0.11–0.75; Table 1) than in

Table 1 | Odds ratios for *S. aureus* colonization in 88 volunteers (univariate analysis)

Measurement	*S. aureus* colonization: Yes ($n = 28$)	*S. aureus* colonization: No ($n = 60$)	Odds ratio (95% CI)	*P*-value
Age (years)	22.2* (1.2)†	21.7* (1.3)†	1.10 (0.78–1.55)	0.58
Sex			2.61 (0.93–7.40)	0.07
Male	22	35		
Female	6	25		
Active smoking			0.33 (0.04–2.91)	0.32
Yes	1	6		
No	27	54		
Passive smoking			0.69 (0.26–1.83)	0.46
Yes	8	22		
No	20	38		
Allergy in nose			1.21 (0.49–3.00)	0.69
Yes	12	23		
No	16	37		
Allergy in eye			1.08 (0.30–3.95)	0.90
Yes	4	8		
No	24	52		
Allergy in skin			1.49 (0.51–4.36)	0.47
Yes	7	11		
No	21	49		
Antibiotic treatment in last month			0.69 (0.13–3.67)	0.67
Yes	2	6		
No	26	54		
Colonization of *S. epidermidis*			0.45 (0.06–3.36)	0.43
Yes	26	58		
No	2	2		
Colonization of inhibitory *S. epidermidis*			0.29 (0.11–0.76) 0.30 (0.11–0.80)‡	0.01
Yes	8	35		
No	20	25		

* Mean, †standard deviation, ‡multivariate analysis (adjusted for age, sex or smoking habits).

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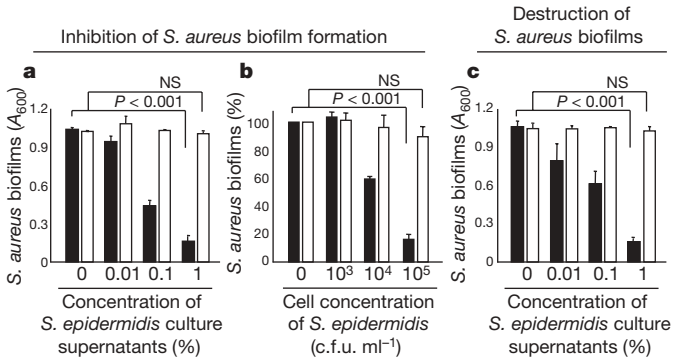

Figure 1 | Inhibition of *S. aureus* biofilm formation and destruction of *S. aureus* biofilms by *S. epidermidis*. **a**, **b**, The inhibitory effect of *S. epidermidis* culture supernatants (**a**) or cells (**b**) on *S. aureus* biofilm formation. **c**, The destructive effect of *S. epidermidis* culture supernatants on *S. aureus* biofilms. After these treatments by *S. epidermidis* culture supernatants or cells on *S. aureus*, the amount of *S. aureus* biofilms was measured. Filled bars, effect of inhibitory *S. epidermidis* (JK16 strain); open bars, effect of non-inhibitory *S. epidermidis* (JK11 strain). Bars show the mean value of the experiments ($n = 3$). Error bars show standard deviation (s.d.).

its absence. This difference remained significant (OR, 0.30; 95% CI, 0.11–0.80; Table 1) when the multivariate logistic regression model was adjusted for age, sex and smoking habit. These findings indicated that in humans, the presence of inhibitory *S. epidermidis* in the nasal cavity could be a potential determining factor for the absence of *S. aureus* colonization.

To identify the inhibitory factor, we collected the culture supernatants of inhibitory *S. epidermidis* after 16 h culture, because the biofilm-destruction activity in culture supernatants of inhibitory *S. epidermidis* increased with bacterial growth and reached its maximal level at 8–16 h (Fig. 2a). Following collection, we purified the factor from the culture supernatants by a series of fractionation procedures including salt precipitation, gel filtration and ion-exchange chromatography. The isolated protein had a single component with an apparent molecular mass of approximately 27 kDa (Fig. 2b). Amino acid sequence analysis showed that the protein factor was *S. epidermidis* serine protease (Esp[2,3]). When culture supernatants of non-inhibitory *S. epidermidis* were subjected to the same purification procedures, we could not find the protease (data not shown). In addition, immunoblotting with anti-Esp antibody showed that Esp protein was present in the culture supernatant of the inhibitory *S. epidermidis* JK16 strain, but not in that of the non-inhibitory *S. epidermidis* JK11 strain (Supplementary Fig. 1).

To confirm that Esp was responsible for the destruction of *S. aureus* biofilms, we generated an isogenic *esp*-deficient mutant from the inhibitory *S. epidermidis* strain, JK16. The culture supernatant of the mutant did not show any biofilm-destruction activity; however, this activity was restored by complementation with the *esp* gene (Fig. 2c and Supplementary Fig. 1). Because Esp shows serine-protease activity[2], we used the serine-protease inhibitor, amidinophenyl

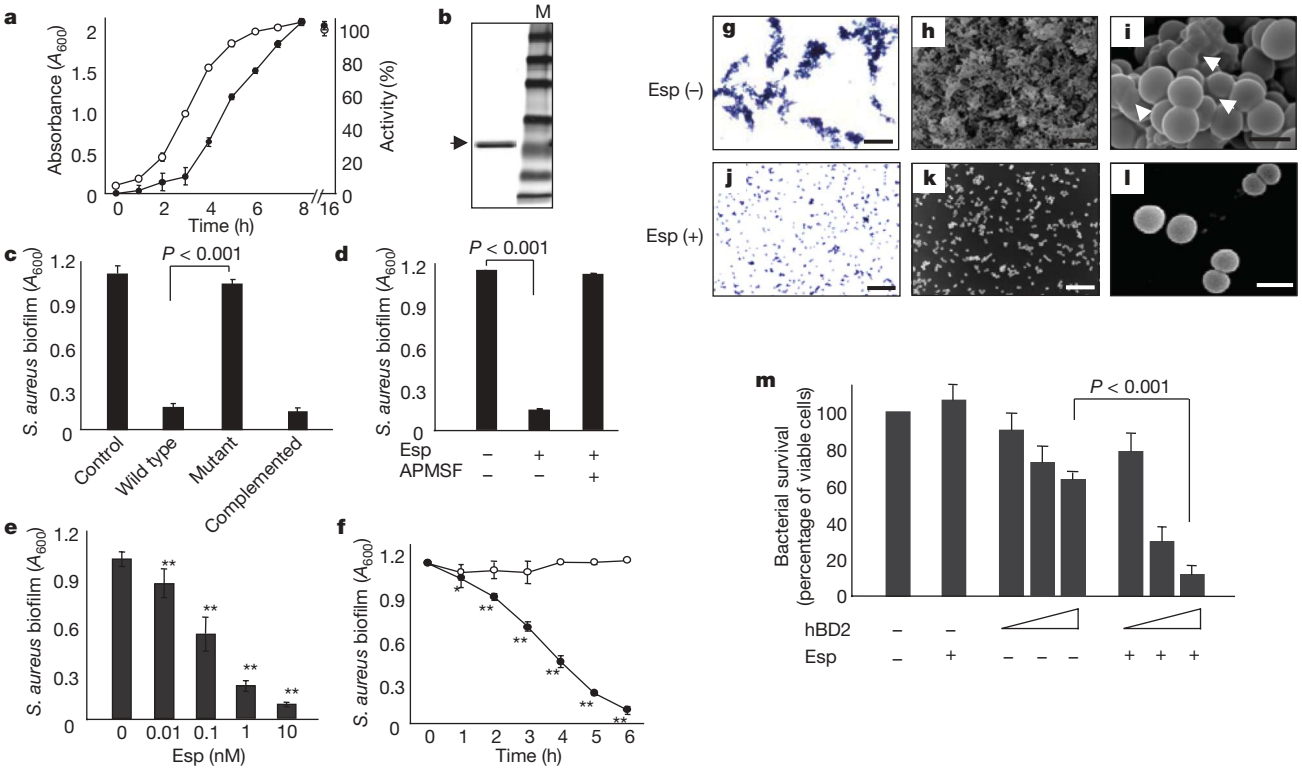

Figure 2 | Isolation and characterization of the serine protease Esp, the factor responsible for the biofilm-destruction activity, secreted by inhibitory *S. epidermidis*. **a**, Growth curve (open circles) of inhibitory *S. epidermidis* (JK16 strain) and the biofilm-destruction activity (closed circles) of the culture supernatants of the same strain. The activity of the supernatants at 8 h is shown as 100%. **b**, A protein having the biofilm-destruction activity was purified from the culture media of inhibitory *S. epidermidis* (arrow). M, molecular mass markers. **c**, Effects of the culture supernatants of inhibitory *S. epidermidis* (JK16, wild-type strain), an isogenic *esp*-deficient strain and a complemented strain on *S. aureus* biofilms. **d**, The biofilm-destruction activity of purified Esp was blocked by APMSF. (+) or (−) indicate the presence or absence of Esp and APMSF. **e**, Esp inhibited *S. aureus* biofilm formation in a dose-dependent manner. **f**, Esp destroyed *S. aureus* biofilms in a time-dependent manner. The biofilms were incubated in the presence (closed circles) or absence (open circles) of Esp for the indicated times. **g–l**, Microscopic observation of *S. aureus* biofilms incubated for 6 h in the presence (**j–l**) or absence (**g–i**) of Esp. Gram staining (**g** and **j**) and scanning electron micrographs (**h**, **i**, **k**, and **l**) of the biofilms with scale bars (10 μm in **g**, **h**, **j**, and **k**, and 1 μm in **i** and **l**). The arrows in **j** indicate the intercellular matrix. **m**, Esp enhanced the susceptibility of *S. aureus* in biofilms to hBD2. Viability of *S. aureus* cells in biofilms, which was incubated in the presence (+ or triangles) or absence (−) of Esp (1 μM) and hBD2 (1, 5 and 10 μM, from left to right in each triangle) for 6 h. The cell viability without Esp and hBD2 was set as 100%. Plots and columns show mean and s.d. ($n = 3$). Statistical significance is indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

methansulphonyl fluoride (APMSF) to test whether this protease activity was involved in biofilm destruction. The results of these tests showed that treatment with APMSF blocked biofilm destruction by Esp (Fig. 2d). Purified Esp inhibited *S. aureus* biofilm formation dose-dependently (Fig. 2e) and destroyed *S. aureus* biofilms (Fig. 2f). To investigate the destructive effect of inhibitory *S. epidermidis* on biofilms formed by various *S. aureus* strains, including MRSA and vancomycin-intermediate *S. aureus* (VISA) (Supplementary Table 1), they were subjected to the biofilm-destruction test. Biofilms formed by almost all strains were efficiently destroyed by Esp (Supplementary Fig. 2). We then investigated whether *S. aureus* could become resistant to the biofilm-destruction activity of Esp. After *S. aureus* was co-cultured with Esp for 1 year, the sensitivity of *S. aureus* to Esp was unchanged, indicating that no resistance developed during this time (data not shown).

When analysed by microscopy, Esp changed *S. aureus* from the sessile to the planktonic form (Fig. 2g and j). This same effect was also observed in MRSA and VISA biofilms (Supplementary Fig. 3). Electron microscopic analysis indicated that the intercellular matrix in *S. aureus* biofilms disappeared after Esp treatment (Fig. 2h, i, k, and l).

We further investigated the characteristics of Esp, including its effect on the host's immune system. Human beta-defensin 2 (hBD2)[14] is an antimicrobial peptide component of the human innate immune system and is secreted by keratinocytes. Esp demonstrated no bactericidal activity, and hBD2 alone demonstrated a low bactericidal activity towards *S. aureus* in biofilms (Fig. 2m). However, when combined with Esp, hBD2 effectively killed *S. aureus* in biofilms (Fig. 2m).

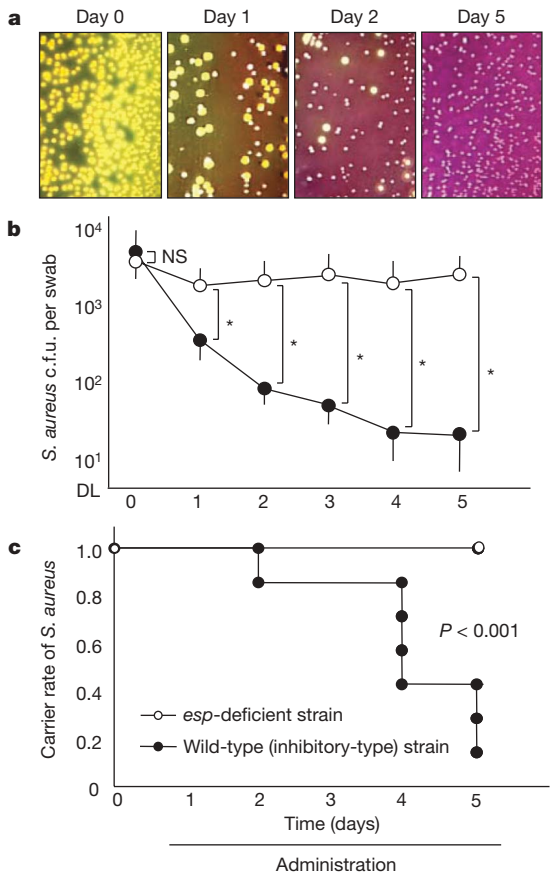

Figure 3 | Elimination effect of inhibitory *S. epidermidis* cells on *S. aureus* nasal colonization. a, Representative culture images of samples from test persons after administration of inhibitory *S. epidermidis* (JK16, wild-type strain). The nasal swabs from the volunteers were cultured on mannitol salt agar with egg yolk. **b**, The bacterial counts of *S. aureus* from the nasal swabs after the administration of *S. epidermidis* cells. JK16 strain (closed circles; $n = 7$) or an isogenic *esp*-deficient strain (open circles; $n = 6$) were placed into the nasal cavities of the volunteers. Plots show mean and s.d. Statistical significance is indicated at $*P < 0.05$. NS, not significant; DL, detection limit. **c**, Carrier rate of *S. aureus* after the administration of *S. epidermidis* wild type cells (closed circles; $n = 7$) and *esp*-deficient cells (open circles; $n = 6$). Statistical significance according to Kaplan–Meier method and log-rank test is at $*P < 0.05$.

These findings indicate that the effects of Esp secreted by inhibitory *S. epidermidis* against *S. aureus* are due to a novel mechanism distinct from all known bacterial interference mechanisms such as growth inhibition, bactericidal activity, or competition for specific attachment molecules[15–19].

To investigate the effect of the bacterial interference on *S. aureus* nasal colonization directly, *S. epidermidis* cells were introduced into the nasal cavities of volunteers who were *S. aureus* carriers. Although the wild-type *S. epidermidis* eliminated *S. aureus* colonization, its isogenic *esp* mutant did not (Fig. 3 and Supplementary Fig. 4). Moreover, purified Esp also cleared *S. aureus* (Supplementary Fig. 5). Non-inhibitory *S. epidermidis* (wild-type, inherent Esp-negative strain) did not show this eliminating effect (data not shown).

Previously, the role of *S. epidermidis* in *S. aureus* colonization has been unclear[15,20–24]. Here we demonstrate that a subset of *S. epidermidis* can inhibit *S. aureus* colonization by secreting Esp. The mechanisms regulating Esp expression in *S. epidermidis* remain to be determined. In recent years, human microbiome projects have been conducted worldwide at the species, genus, or phylum level[25–30]. Further characterization of the microbes at the strain level will help increase our understanding of microbial ecology, including the relationships between commensal bacteria and infectious pathogens, or host–microbe interactions.

METHODS SUMMARY

Eighty-eight healthy adult volunteers participated in the study, and 960 bacterial strains were identified from their nasal cavities. The isolates of *S. epidermidis* or their culture supernatants were used as the samples to assess the effects on biofilms formed by *S. aureus*. To evaluate this inhibitory effect on biofilm formation, these *S. epidermidis* samples were simultaneously incubated with *S. aureus* at 37 °C for 16 h, and to evaluate their destructive effect on biofilms they were added to the culture dishes containing *S. aureus* biofilms and incubated for 6 h. After these treatments, the amount of *S. aureus* biofilm were measured spectrophotometrically. A protein factor responsible for the biofilm destructive activity was isolated from the culture supernatants of inhibitory *S. epidermidis* using a series of chromatographic steps, and a partial amino acid sequence was determined. The relationship between colonization rates of *S. aureus* and inhibitory *S. epidermidis* in the nasal cavity was epidemiologically investigated. The inhibitory *S. epidermidis* or the protein factor obtained from it and identified as *S. epidermidis* serine protease (Esp) was placed into the nasal cavity of *S. aureus* carriers, and their effects on colonization of *S. aureus* in each cavity were analysed. Epidemiological and *in vivo* studies were approved by the ethics committees of The Jikei University School of Medicine and Kochi Medical School, respectively, and informed consent was obtained from all participants.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Epidemiological study. In January 2006, 88 healthy college students were recruited for our study. The study was approved by the ethics committees of the Jikei University School of Medicine, and informed consent was obtained from all participants.

Each participant answered a questionnaire that included questions about demographics (age and sex), antibiotic treatment received within 1 month before beginning the study, passive exposure to tobacco smoke, active smoking habits, and allergic symptoms (Table 1). Sterile 5 mm-diameter cotton swabs (Eiken Kagaku) soaked in sterile physiological saline were used to obtain nasal samples from one nostril of each subject. The swabs were streaked onto mannitol salt agar plates (Merck KGaA). *S. aureus* was identified using Gram staining and tests for DNase activity, mannitol metabolism and coagulase production. MRSA was identified using the PCR method[31] and MRSA Screen Agar (Nippon Becton Dickinson). Coagulase-negative staphylococci were identified using the PCR method[32,33], API-Staph (Japan bioMérieux), and the N-ID-Test SP-18 (Nissui Pharmaceutical).

Bacterial strains and culture conditions. The following strains were used in this study: 960 *S. epidermidis* strains isolated from the subjects (at least five from each subject, including JK16 strain and JK11 strain), *S. aureus* JCM 2874 (a methicillin-sensitive *S. aureus* (MSSA) strain), 12 laboratory-isolated MSSA strains; six clinically-isolated MRSA strains; and Mu50 (one clinically isolated VISA strain)[34], an isogenic *esp*–deficient mutant (TI1) derived from JK 16 strain; and a TI1 strain complemented by the *esp* gene (TI1/pTI1) (Supplementary Table 1). The bacteria were grown in tryptic soy broth (TSB) (Difco), on TSB agar (Difco), or on mannitol salt agar with egg yolk (Merck) at 37 °C under aerobic conditions.

Preparation of *S. epidermidis* culture supernatants. After isolating *S. epidermidis* from volunteers, the culture supernatants were immediately prepared from these fresh isolates. Culture supernatants were prepared by centrifuging cultures that had been grown overnight in TSB with shaking at 37 °C. Samples were sterilized by filtration across a 0.2-μm diameter membrane filter (Millipore).

Biofilm formation of *S. aureus*. Overnight TSB cultures of *S. aureus* were used to inoculate 35 mm-diameter polystyrene dishes (Asahi Techno Glass) filled with 2.5 ml of TSB with 0.2% glucose at cell concentrations of 10^3–10^5 colony forming units (c.f.u.) per millilitre and incubated at 37 °C for 16 h under static conditions.

Effects of culture supernatants and cells of *S. epidermidis* on *S. aureus* biofilms. To determine their effect on the formation of *S. aureus* biofilms, culture supernatants of *S. epidermidis* at various concentrations (from 0.01–10%) were simultaneously added with the *S. aureus* inoculant (10^5 c.f.u. ml^{-1}) to the test dishes. The culture media were incubated for 16 h at 37 °C under static conditions. After the incubation, the dishes were gently shaken to remove the deposited bacteria, and the culture media were discarded. The dishes were gently rinsed with phosphate-buffered saline (PBS). The biofilms were scraped out and suspended in PBS with vigorous vortex mixing for 1 min, and then the absorbance at 600 nm (A_{600}) of each suspension was measured. In addition, the *S. aureus* cell suspension at a concentration of 10^3 c.f.u. ml^{-1} was co-incubated with various concentrations of *S. epidermidis* cells (10^3, 10^4 and 10^5 c.f.u. ml^{-1}) for 16 h at 37 °C under static conditions. After the incubation, the dishes were gently shaken to remove the deposited bacteria, and the culture media were discarded. The dishes were gently rinsed with phosphate-buffered saline (PBS). The biofilms were scraped out and suspended in PBS with vigorous vortex mixing for 1 min. The number of *S. aureus* cells in the biofilms was counted by a conventional culture method using mannitol salt agar with egg yolk. To evaluate the effect of *S. epidermidis* on the stability of *S. aureus* biofilms, the culture supernatants of *S. epidermidis* at various concentrations were added to culture dishes after 16 h of *S. aureus* inoculation, and then incubated for an additional 6 h. The detached bacteria were removed, and turbidities of the biofilm suspensions were determined as described above.

Isolation and characterization of Esp, the factor responsible for biofilm destruction activity. The factor responsible for the biofilm destruction activity was purified as a protein from the culture supernatants of inhibitory *S. epidermidis*, and identified as Esp. The factor was fractionated and isolated using an indicator of the biofilm destruction activity. The 16-h culture supernatants of inhibitory *S. epidermidis* were sterilized by filtration, and salted out with 65% ammonium sulphate, the precipitates were then dissolved in 1 ml H_2O (the pH was adjusted to 7.4 with phosphate buffer). This crude material was fractionated by using a Sephadex G-200 gel filtration column (GE Healthcare UK), and by the protein purification kits comprised cation and anion exchangers (Vivapure, Vivascience) using 25 mM acetate buffer, pH 5.5, and 25 mM Tris-HCl, pH 8.0, for each equilibration, respectively. Further procedures were performed according to the manufacturer's instructions. The purified protein fraction having the activity was then subjected to native-PAGE using a 10–20% gradient acrylamide gel (Atto) and visualized with a negative staining kit (Atto); a major distinct band was spliced out and eluted by sample buffer, and its activity was confirmed. The isolated protein with biofilm destruction activity was also analysed with SDS–PAGE using a 15%-acrylamide gel and stained with a silver staining kit (Atto). In the other experiment, the isolated protein was subjected to SDS–PAGE followed by transfer to a polyvinylidene fluoride membrane (Immobilon-P^{SQ}, Millipore), and visualized with Coomassie blue staining. The corresponding band was cut out and subjected to automated Edman degradation with a protein sequencer (model 492, Applied Biosystems). An amino-terminal sequence of 15 amino acids of the obtained protein factor was determined to be VILPNNNRHQIFNTT, which corresponded to that of the *S. epidermidis* serine proteinase, Esp, which was isolated and cloned in 2001 (refs 2, 3) and characterized in detail[35]. To evaluate the effect of the Esp on the formation of *S. aureus* biofilm, Esp (1 μM) and *S. aureus* (10^5 c.f.u. ml^{-1}) were simultaneously added to the dishes filled with the test media, and biofilms were collected from the dishes 16 h after the inoculation. The effect of Esp on the stability of *S. aureus* biofilm was assessed by adding Esp to the culture dishes 16 h after the *S. aureus* inoculation, and the biofilms were then collected in a time-dependent manner. Absorbance at 600 nm was used as a quantitative measurement of the biofilms. In addition, for microscopic evaluation, the *S. aureus* biofilms were incubated with 1 μM Esp for 6 h, and the bacteria were collected and subjected to Gram staining or microscopic observations.

Effect of culture supernatant and Esp on growth of *S. aureus*. *S. aureus* was cultured in media containing the culture supernatant of *S. epidermidis* or Esp at various concentrations with shaking at 37 °C for 16 h. The A_{600} of culture media was used as a quantitative measurement of the bacteria.

Genetic evidence for the Esp activity on *S. aureus* biofilms. An *esp*-mutant derivative of the *S. epidermidis* JK16 strain was constructed by an allelic exchange method[36,37]. Briefly, the sequences flanking *esp* were PCR amplified with primers Esp_attB_F1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTAAAGCCAGCAGATAGTGCAG) and Esp_R (CCTGATATAAATATTCAGTAATTAAATTTAGTT) for the upstream region and primers Esp_F (ACATATAGATAAAAATCTCTTTTTCATATGATA) and Esp_attB_R2 (GGGGACCACTTTGTACAAGAAAGCTGGGTCGCATCGGATTGTGGTT) for the downstream region. The PCR fragments were then ligated *in vitro* and recombined with pKOR1. The pKOR1 recombinant was then introduced into the *S. epidermidis* JK16 strain by electroporation[38]. The successful deletion of the *esp* gene was verified by PCR with primers Esp_F (TTTGGAGGTTATCATATGAAAAAGAG) and Esp_R (CTGAATATTTATATCAGGTATATTGTTTC) and by the biofilm destruction activity test. The *esp*-deficient mutant of JK16 was designated TI1.

In addition, to generate a complemented strain, a DNA fragment containing the entire *esp* gene was obtained by PCR amplification with genomic DNA of strain JK16 as a template and cloned downstream of the *tetL* gene of shuttle vector pYT3[36,39,40]. Briefly, the sequences flanking *esp* were PCR-amplified with primers Esp+BamHI_F (TATGGATCCATATTTTTGGAGGTTATCATATGAAAAAGAG) and Esp+HindIII_R (CCCAAGCTTTTAGTGATGGTGATGGTGATGCTGAATATTTATATCAGGTATATTGTTTC). Esp+BamHI_F primer includes putative Shine-Dalgarno sequence and Esp+HindIII_R primer includes a His-Tag sequence. The resulting plasmid, pTI1, was introduced into the *esp*-deficient mutant strain, TI1, by electroporation[38].

The recombinant Esp protein. To obtain recombinant Esp protein with a His-Tag, we cultured the *esp*-deficient mutant strain TI1 harbouring pTI1 in TSB containing tetracycline (10 mg l^{-1}) for 16 h at 30 °C. The protein was collected using the TALON purification kit (Clontech, Takara Bio). To evaluate the activity of the recombinant protein, 1 μM of both the recombinant and native protein was used in the study.

Western blotting. Polyclonal antisera against Esp were generated in rabbits immunized with recombinant Esp protein at Operon Biotechnologies. For western blotting, protein samples were denatured and separated on 15% SDS–PAGE as described previously[41]. Briefly, blots were first incubated with antiserum against Esp followed by the secondary horseradish-peroxidase-conjugated antibody, both at 1:500 final dilution. Immunoreactive bands were visualized by ECL western blotting detection reagents (GE Healthcare UK).

Interaction between Esp and innate host immune systems. The cooperative effect of Esp and hBD2 (Peptide Institute and PeproTech) in killing *S. aureus* in biofilms was evaluated. *S. aureus* biofilms were treated with 1 μM Esp with or without hBD2 at various concentrations (1, 5 and 10 μM) in 10 mM sodium phosphate buffer for 6 h[42,43]. Bacteria were then collected and washed with physiological saline three times, and the cell viability of *S. aureus* was evaluated with a conventional culture method using TSB agar plates.

Effect of *S. epidermidis* and Esp on *S. aureus* nasal colonization. To investigate the effects of *S. epidermidis* and Esp on *S. aureus* nasal colonization, we introduced inhibitory *S. epidermidis* and Esp in the subjects' nasal cavities. Prior to this study, persistent colonization of *S. aureus* without inhibitory *S. epidermidis* was confirmed by at least three separate nasal cultures from the carriers for over three months. The 19 test subjects were randomly divided into four groups: (1)

seven subjects, including one MRSA carrier, who received daily inoculations of the inhibitory *S. epidermidis* (wild type, JK16 strain) in the anterior nares using cotton swabs, (2) six subjects who received the *esp*-deficient mutant bacteria derived from the JK16 strain, (3) three subjects who received the non-inhibitory *S. epidermidis* (JK11 strain), and (4) three subjects who received purified Esp. The sample sizes in the groups (1) and (2) were determined by the results of a pilot study according to the accepted methods for sample-size determination[44,45], which gave a statistical power of 80% with an alpha value of 0.05. For liquid formulations, the saline solutions (0.5 ml in each) containing approximately 1×10^9 c.f.u. of each bacteria[46], 500 pmol Esp or none (as control group) were administered into the nasal cavity with swabs (Eiken Kagaku).

The bacteria in the nasal cavities were collected daily with swabs by a clinical microbiologist, and the number of *S. aureus* bacteria was counted by conventional culture methods using mannitol salt agar with egg yolk for selection of *S. aureus*. Briefly, nasal swabs were vigorously vortexed for 1 min in 500 µl physiological saline, and samples were appropriately diluted. Then, the diluted samples were spread using spreaders on mannitol salt agar with egg yolk. After 24 h incubation, *S. aureus* colonies were counted. Colonies were identified as *S. aureus* if a yellow colony exhibited positive egg-yolk reaction, and if the medium surrounding the *S. aureus* colony turned yellow. Colonies were identified as *S. epidermidis* if they appeared either pink or white, and if the medium around the *S. epidermidis* colonies turned red or purple. We could not analyse samples of 3 of the 19 volunteers immediately. Therefore, the number of *S. aureus* bacteria was quantified by real-time PCR[47] instead of the conventional colony-counting method to avoid the effects of a time delay until the analysis. The samples for real-time PCR were obtained from nasal swabs kept in Tris-EDTA (TE) buffer.

Although both conventional culture and real-time PCR methods have been applied to quantify bacteria[46,47], we further checked their validity and consistency by comparing the results of both methods (see Supplementary Fig. 6). Briefly, nasal swabs obtained from the additional study ($n = 6$) were vigorously vortex-mixed in 500 µl physiological saline for 1 min, and then each sample was divided into two groups; one for real-time PCR and another for the conventional culture method. Estimation of c.f.u. values obtained by real-time PCR were calibrated by a standard curve, which was constructed with c.f.u. values of the corresponding samples counted by the conventional culture method, according to previous studies[32,33,46,47].

To confirm that the administered strains had colonized the participants' nasal cavities and to distinguish among the JK16 strain (wild type), the isogenic mutant, and pre-colonizing *S. epidermidis* strains, we tested for biofilm destruction activity of the culture supernatants of these bacteria and performed PCR using both primers designed in this study and reported previously[48] for the *esp* gene.

Before the study, we consulted several clinical microbiologists, bacteriologists, immunologists, physicians and surgeons working in the field of infection control, and statisticians regarding the design of this experiment. After considering their recommendations, we submitted our proposed experimental protocol to the ethics committee of Kochi Medical School. We were very careful to conduct the experiment according to the approved protocol after obtaining the ethics committee's approval. Nineteen healthy subjects affiliated with the university were enrolled during March 2007–July 2009. Informed consent was obtained from all participants.

Statistical analysis. Colonization by *S. aureus* was considered a dependent variable. Odds ratios (ORs) and confidence intervals (CIs) were calculated, and univariate logistic regression analysis was performed to assess the factors that might affect colonization by *S. aureus*: age, sex, passive exposure to tobacco smoke, active smoking, antibiotic treatment one month before beginning the study, allergic symptoms, presence of inhibitory *S. epidermidis*, and *S. epidermidis* bacterial count. To confirm the independence of these factors, multivariate logistic regression analysis was performed using the stepwise selection method at a significance level of 0.20. The log likelihood was used to assess goodness of fit. In the *in vitro* studies evaluating the effects on formation and stability of the *S. aureus* biofilms and on viability of the *S. aureus* cells, Student's *t* test and Dunnett's test were used. In the *in vivo* studies evaluating the effects on the *S. aureus* nasal colonization, Student's *t* test was used for each sampling day and the Kaplan–Meier method and log-rank test were used to compare the elimination effect on *S. aureus* nasal colonization by administration of the wild-type strain, JK16, and the *esp*-deficient mutant strain, TI1. A value of $P < 0.05$ was considered to indicate statistical significance. Calculations were performed using the SAS statistical program, version 9.1 (SAS Institute) and Excel software (Microsoft).

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