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***Staphylococcus epidermidis* protease EcpA can be a deleterious component of the skin microbiome in atopic dermatitis**

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Abstract

Background: *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most abundant bacteria found on the skin of patients with atopic dermatitis (AD). *S aureus* is known to exacerbate AD, whereas *S epidermidis* has been considered a beneficial commensal organism.

Objective: In this study, we hypothesized that *S epidermidis* could promote skin damage in AD by the production of a protease that damages the epidermal barrier.

Methods: The protease activity of *S epidermidis* isolates was compared with that of other staphylococcal species. The capacity of *S epidermidis* to degrade the barrier and induce inflammation was examined by using human keratinocyte tissue culture and mouse models. Skin swabs from atopic and healthy adult subjects were analyzed for the presence of *S epidermidis* genomic DNA and mRNA.

Results: *S epidermidis* strains were observed to produce strong cysteine protease activity when grown at high density. The enzyme responsible for this activity was identified as EcpA, a cysteine protease under quorum sensing control. EcpA was shown to degrade desmoglein-1 and LL-37 *in vitro*, disrupt the physical barrier, and induce skin inflammation in mice. The abundance of *S epidermidis* and expression of *ecpA* mRNA were increased on the skin of some patients with AD, and this correlated with disease severity. Another commensal skin bacterial species, *Staphylococcus hominis*, can inhibit EcpA production by *S epidermidis*.

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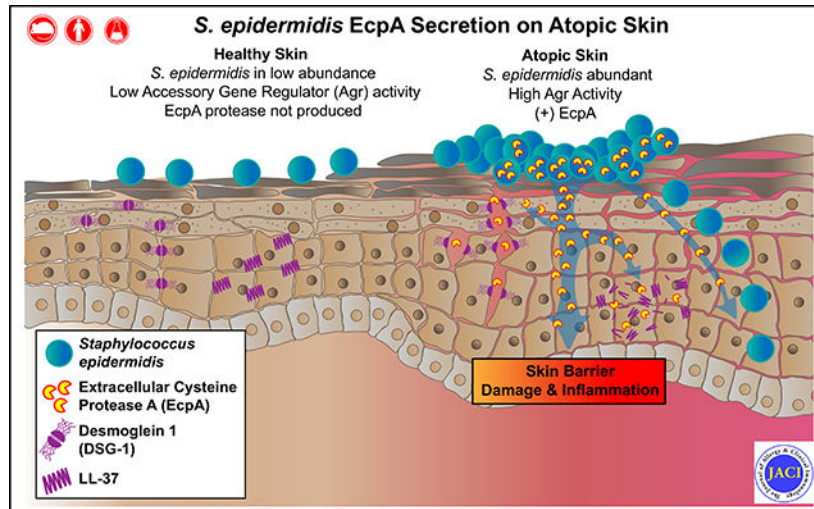
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Conclusion: *S. epidermidis* has commonly been regarded as a beneficial skin microbe, whereas *S. aureus* has been considered deleterious. This study suggests that the overabundance of *S. epidermidis* found on some atopic patients can act similarly to *S. aureus* and damage the skin by expression of a cysteine protease.

Graphical Abstract



Keywords

Atopic dermatitis; microbiome; dysbiosis; *Staphylococcus epidermidis*; protease; skin; epidermal barrier; inflammation; cytokine

Atopic dermatitis (AD) is a common inflammatory skin disease that is often associated with other allergic conditions and can affect up to 15% to 20% of children and 1% to 3% of adults worldwide.¹⁻⁴ The prevalence of AD is highest in industrialized countries and has grown over the past decades. Meta-analyses have shown that exposures to some environmental conditions, such as having 3 or more siblings, daycare, pet ownership, and farm residence during childhood, are associated with a lower risk of AD.^{5,6} Although a clear explanation for these associations remains unknown, early-life exposure to a healthy microbial community on the skin and gut have been hypothesized to protect against AD.^{7,8}

Increasingly detailed analyses have been performed to describe the composition of microbes that inhabit AD skin with the hope of better understanding how they may influence the development and progression of disease. AD skin is often characterized by an overabundance of staphylococcal bacterial species and a decrease in diversity of microbes. In particular, depending on the methods used, 30% to 100% of subjects with AD have been observed to be colonized on their skin by the common infectious pathogen *Staphylococcus aureus*.^{9,10} As a consequence of the close association between the presence of *S. aureus* and severity of skin inflammation, this bacterial species has been a main focus for study of how the skin microbiome can promote AD. Multiple experimental models have clearly shown that *S. aureus* can disrupt the epidermal barrier and alter both adaptive and innate immune responses.⁹⁻¹⁷ Several gene products of *S. aureus*, including adhesions, superantigens, toxins,

phenol-soluble modulins (PSMs), TLR ligands, and proteases, have been identified to promote these negative effects.

Although an overgrowth of *Staphylococcus epidermidis* on AD lesional skin has been reported in several studies,^{10,18–20} this common commensal bacterium is generally regarded as a key member of the healthy skin microbiota with multiple benefits for the host when present on the skin.^{21–23} For example, *S epidermidis* has been reported to protect the skin against *S aureus* colonization through the production of various antimicrobial and anti-inflammatory molecules.^{24–26} However, *S epidermidis* is also an opportunistic pathogen, especially after contamination of implanted medical devices.^{27–30} In these conditions, the overgrowth and invasion of *S epidermidis* result in serious life-threatening infections.

In this study, we hypothesized that the overabundance of *S epidermidis* colonizing some patients with AD may alter the usual commensal host-microbe relationship and result in skin disease. Because *S epidermidis* is the coagulase-negative *Staphylococcus* (CoNS) that is most closely related to *S aureus*, we investigated whether protease activity from *S epidermidis* could induce skin damage in AD by enzymatic mechanisms similar to those used by *S aureus*. We have demonstrated that some *S epidermidis* strains can cause skin barrier damage and inflammation through secretion of the cysteine protease EcpA and that this correlated with human disease severity. These findings suggest that the permissive growth conditions of AD skin enable *S epidermidis* to shift from a beneficial commensal to a deleterious pathogen similar to *S aureus*.

METHODS

Human subjects and skin swab collection

Experiments involving human subjects were done according to protocols approved by the University of California, San Diego, institutional review board (project no. 140144). Written informed consent was obtained from all subjects. Table E1 (available in this article's Online Repository at www.jacionline.org) summarizes age, sex, ethnicity, and disease severity (local Eczema Area and Severity score and Scoring Atopic Dermatitis score) of all subjects. Swabs of surface microbiota from a 5-cm² area of the antecubital fossa skin of both the left and right arms were collected from 14 healthy subjects and 13 patients with AD as previously described.²⁶ For subjects with AD, swabs were collected from both lesional and nonlesional skin.

Mouse experiments

Age-matched 8- to 10-week-old female C57BL/6J mice were used in all experiments (n = 4 per condition). The mice were cohoused at a rate of 3 to 5 mice per cage. All animal experiments were approved by the University of California, San Diego, Institutional Animal Care and Use Committee (protocol no. S09074). For both live bacteria or EcpA topical treatment, the dorsal skin of anesthetized mice (2% isoflurane) was shaved and depilated by using Nair cream followed by removal with alcohol wipes. The skin was allowed to recover from hair removal for at least 48 hours before application of bacteria. For topical bacterial exposure, *S epidermidis* or *S aureus* on agar disks (3% tryptic soy broth [TSB], 2% agar;

diameter 8 mm) at a final density of 10^6 colony-forming units (CFUs)/cm² was applied to the skin for 48 hours as previously described.¹³ An agar disk without bacteria was used as a vehicle control. For the *S epidermidis agr* inhibition experiments, live *Staphylococcus hominis* (10^6 CFUs/cm²), *S hominis* supernatant (5 μ L), or synthetic autoinducing peptide (AIP) from *S hominis* C5 (10 μ g)³¹ was loaded onto the agar disk at the same time as the *S epidermidis*. For topical application of EcpA on mouse skin, 2.5 μ g of the purified enzyme (for details about purification, see the Methods section of this article's Online Repository at www.jacionline.org) in PBS containing 5 mM L-cysteine (EcpA activator) was applied (final volume 35 μ L) for 24 hours on a 1 \times 1-cm² sterile gauze. PBS containing 5 mM L-cysteine was used as a vehicle control. The dorsal skin was then covered with wound dressing film (Tegaderm [3M]) and a bandage to hold the agar disk or gauze in place for the duration of the treatment. For the AD mouse model, age- and sex-matched BALB/c *Flg*^{-/-} (flaky tail) mouse dorsal skin was tape-stripped and treated 3 times at 2-week intervals (for 8 days with replacement every second day) with ovalbumin patches (6 weeks total) before removal of hair and application of live bacteria for 24 hours as previously published.¹³

Primary keratinocyte culture

Normal neonatal human primary epidermal keratinocytes (NHEKs) (Thermo Fisher Scientific, Waltham, Mass) were cultured in EpiLife complete medium containing 60 mM CaCl₂ (Thermo Fisher Scientific) supplemented with 1 \times human keratinocyte growth supplement (Thermo Fisher Scientific) and a 1 \times antibiotic-antimycotic (100 U/mL of penicillin, 100 U/mL of streptomycin, and 250 ng/mL of amphotericin B [Thermo Fisher Scientific]) at 37°C and 5% CO₂. NHEKs were used only for experiments between passages 3 to 5. NHEKs were grown to about 90% to 100% confluency followed by differentiation in EpiLife complete medium with 2 mM CaCl₂ for 72 hours. For bacterial supernatant treatments, differentiated NHEKs were treated with sterile-filtered bacterial supernatant at 5% (vol) in EpiLife medium for 8 hours and then harvested for RNA extraction. For treatment with EcpA, the purified enzyme (final concentration 2.5 μ g/mL) was added to the culture medium along with 5 mM of L-cysteine over the course of either 8 hours for RNA extraction or 20 hours for protein extraction. Some NHEKs were treated with cycloheximide (final concentration 20 μ g/mL) (Sigma-Aldrich, St Louis, Mo) 2 hours before treatment with EcpA and for the entire duration of the treatment.

Protease activity assays

The protease activity assays were performed by using the EnzChek Elastase Assay Kit and EnzChek Gelatinase/Collagenase Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 20 μ L of bacteria supernatant from various CoNS strains (see Table E2 in this article's Online Repository at www.jacionline.org) was incubated with 1 μ g of DQ gelatin, DQ elastin, DQ collagen I, or DQ collagen IV (Thermo Fisher Scientific) in the supplied digestion buffer in 96-well black plates (Corning, NY) for 20 hours, in the presence or absence of various protease inhibitors: E64 (Sigma-Aldrich) at 5 μ M; EDTA (Thermo Fisher Scientific) at 2 mM; 1,10-phenanthroline (Sigma-Aldrich) at 2 mM; or aprotinin (Sigma-Aldrich) at 0.5 mg/mL. Relative fluorescent intensity was analyzed with a SpectraMAX Gemini EM fluorometer (Thermo Fisher Scientific; excitation wavelength, 485 nm; emission excitation wavelength, 538 nm).

***S epidermidis agr* activity assay**

S epidermidis 12228 *agr* type I P3-GFP^{Erm^r} (AH3408), *S epidermidis* 1457 *agr* type II P3-GFP^{Erm^r} (AH273), and *S epidermidis* 8247 *agr* type III P3-GFP^{Erm^r} (AH3409) reporter strains were used to detect *S epidermidis agr* activity as previously described.³² Strains were first cultured overnight (~16 hours) in the presence of 10 µg/mL of erythromycin (plasmid antibiotic selection). The bacteria were then inoculated at 10⁷ CFUs/mL in 3% TSB (final volume 500 µL) along with CoNS sterile-filtered supernatant (10% vol/vol) or *S hominis* C5 synthetic AIP (100 nM) and shaken at 300 rpm in an incubator at 37°C. RNA was isolated after 12 hours of incubation, and the *agr* activity was measured after 24 hours. Bacteria were diluted 1:20 in PBS (final volume 200 µL) in 96-well black bottom plates (Corning), and GFP fluorescence was detected by using a SpectraMax Gemini EM fluorometer (Thermo Fisher Scientific; excitation wavelength, 485 nm; emission wavelength, 538 nm).

Quantification and statistical analysis

One-way ANOVA, 2-way ANOVA, the Student *t* test, nonparametric unpaired Kruskal-Wallis analysis, and Pearson and Spearman correlations were used throughout this study for statistical analysis, as indicated in the figure legends. All statistical analyses were performed by using GraphPad Prism, version 8.0 (GraphPad, La Jolla, Calif). All data are presented as means plus or minus SEMs, and a *P* value of .05 or lower was considered significant.

Details about the other methods used in this study are available in the Methods section of the Online Repository. For details about the specific primers and antibodies used in this study, see Tables E3 and E4 (available in this article's Online Repository at www.jacionline.org).

RESULTS

***S epidermidis* produces proteolytic activity through expression of EcpA**

The production of proteases by *S aureus* promotes damage to the epidermis in subjects with AD,^{13,33} but whether other bacterial species that reside on skin may also display proteolytic activity that could damage the skin is not well understood. To test this hypothesis, CoNS was collected from healthy human skin and lesional skin of subjects with AD and then assayed *in vitro* for proteolytic activity against a gelatin substrate. Among the initial CoNS strains tested, some *S epidermidis* isolates showed gelatinase activity and the level of enzymatic activity varied greatly between strains of the species (Fig 1, A and see Table E5 in this article's Online Repository at www.jacionline.org). The activity produced by *S epidermidis* 05-A5 was identified as likely to be a cysteine protease, as it was inhibited only by the cysteine protease inhibitor E64, whereas other classes of protease inhibitors had no effect (Fig 1, B). *S epidermidis* was known to only produce 1 cysteine protease (termed *extracellular cysteine protease A* [EcpA]); the expression of EcpA is under the control of the accessory gene regulator (*agr*) quorum sensing system.^{32,34} We therefore next sought to test whether EcpA was responsible for the proteolytic activity that we observed in *S epidermidis*.

To determine whether EcpA was responsible for the enzymatic activity observed in the *S epidermidis* strains, we next measured *ecpA* mRNA levels. *S epidermidis* strains such as the

clinical isolate 05-A5 that we observed with maximal activity in Fig 1, A also had increased *ecpA* transcript levels (Fig 1, C and D). Use of a specific EcpA substrate on *S epidermidis* isolates from healthy and AD skin revealed that multiple strains from both populations had the capacity to produce active enzyme (Fig 1, E). Furthermore, targeted deletion of the *ecpA* gene (*ecpA*)³² in the strain *S epidermidis* 1457 eliminated specific EcpA substrate activity as well as gelatinase activity (Fig 1, F and see also Fig E1, A and B and Table E6 in this article's Online Repository at www.jacionline.org). The expression of other known *S epidermidis*-secreted proteases, *sepA* and *esp*, was not affected by *ecpA* deletion (Fig E1, C and D), and in addition to gelatin, EcpA also degraded elastin, collagen I, and collagen IV *in vitro* (Fig 1, F and see Fig E2 in this article's Online Repository at www.jacionline.org). These results therefore showed that the gene responsible for proteolytic activity from *S epidermidis* was EcpA and that this protease was actively secreted by multiple clinical isolates. Notably, EcpA was also recently shown to be associated with skin damage in patients with Netherton syndrome, who possess a mutation in the human protease inhibitor *SPINK5*.³⁵

***S epidermidis* cysteine protease EcpA disrupts the skin barrier**

The potential of the *S epidermidis* protease to influence the skin barrier in AD was next evaluated by comparing *S epidermidis* strains that express EcpA with *S aureus*, *S epidermidis* 1457, and the clinical isolate *S epidermidis* 05-A5 from AD skin, which have a high EcpA expression (Fig 1). Both *S epidermidis* 1457 and 05-A5 induced disruption of the epidermal barrier as shown by an increase in transepidermal water loss (TEWL). *S epidermidis* 1457 *ecpA* and the AD skin isolate 18-F3 (which has low EcpA expression) did not (Fig 2, A and B). The difference in activity between the strains was not due to different amounts of live bacteria colonizing the skin (see Fig E3 in this article's Online Repository at www.jacionline.org). The severity of skin damage and epidermal barrier disruption correlated with the amount of *S epidermidis* 1457 wild-type (WT) when applied on the skin at a physiologically relevant bacterial density between 10³ and 10⁶ CFUs/cm² (Fig 2, C and D). Furthermore, using a mouse model of AD, we also observed that *S epidermidis* 1457 increased TEWL and that this effect was dependent on EcpA (Fig 2, E). Additionally, EcpA expression was necessary for *S epidermidis* to penetrate the skin (Fig 2, F), and purified EcpA was able to increase TEWL even without application of live bacteria (Fig 2, G and H). These observations support the potential of *S epidermidis* EcpA to damage the epidermal barrier and exacerbate AD through a mechanism similar to that of *S aureus*.

EcpA degrades DSG-1 and the antimicrobial peptide LL-37

To better understand how EcpA may damage the epidermis, human keratinocytes in culture were exposed to purified EcpA. This treatment was not cytolytic to keratinocytes and did not result in lactate dehydrogenase release (see Fig E4, A in this article's Online Repository at www.jacionline.org). However, Western blot analysis showed that desmoglein-1 (DSG-1), which is a key component of the corneodesmosome, was cleaved by EcpA. In contrast, other important epidermal barrier components, including corneodesmosin (CDSN) and involucrin (IVL), were not degraded and showed increased expression by treatment with EcpA (Fig 2, I and J). Treatment of keratinocytes with cycloheximide to inhibit new protein synthesis inhibited the ability of EcpA to induce CDSN and IVL (Fig 2, I) and increased the reduction

in full-length DSG-1 (Fig 2, I and J). Interestingly, transcripts for DSG-1 (*Dsg1*), CDSN (*Cdsn*), IVL (*Ivl*), and filaggrin (*Flg*) were all increased by EcpA exposure (see Fig E4, B). Overall, these data suggest that EcpA can degrade an essential barrier protein such as DSG-1 and that human keratinocytes attempt to compensate for this by increased synthesis of several barrier proteins. The loss of full-length DSG-1, and potentially other substrates not measured here, can explain the loss of barrier function of the stratum corneum after exposure to *S epidermidis* EcpA.

To identify additional potential substrates for EcpA, we next evaluated its capacity to degrade an antimicrobial peptide (AMP) that is partly responsible for controlling the composition of the skin microbiome.^{36–40} SspB is a cysteine protease produced by *S aureus*; it has been previously shown to degrade the cathelicidin AMP LL-37 and share strong homology with EcpA.^{35,41} Incubation of synthetic LL-37 with purified EcpA showed that this enzyme degraded LL-37 and decreased the capacity of this AMP to inhibit *S aureus* bacterial growth (Fig 2, K and L). This illustrates an additional potential substrate for EcpA that would result in a deleterious effect on cutaneous defense function.

EcpA induces skin inflammation

To further assess the potential for EcpA to influence AD, the ability of *S epidermidis* strains that express EcpA to promote an inflammatory response was directly tested on human keratinocytes and on mouse back skin. The WT *S epidermidis* 1457 with intact EcpA activity, but not the *S epidermidis ecpA* mutant strain, induced skin inflammation characterized by epidermal thickening, immune cell infiltration, and increased expression of cytokines, including IL-6, IL-4, and IL-17 α (Fig 3, A and B). EcpA-induced inflammation was also observed by using an AD mouse model colonized with either WT *S epidermidis* 1457 or the *S epidermidis ecpA* mutant strain. Notably, the expression of EcpA resulted in a significant increase in T_H2 cytokines (Fig 3, C) in addition to the disruption of barrier function previously shown in Fig 2, E.

Treatment of mouse skin with purified EcpA resulted in a response different from that to treatment with the live bacteria. Purified EcpA induced skin inflammation but only *Il-8* appeared to be significantly increased with application of the enzyme alone (Fig 3, A and D). Similarly, cultured human keratinocytes treated with purified EcpA showed an increase in levels of IL-6, IL-8, TLSP, IL-1 α , and IL-1 β (Fig 3, E). Interestingly, exposure to crude supernatant from *S epidermidis* 1457 without EcpA (*ecpA*) also induced a strong cytokine response in cultured human keratinocytes—even more than the *S epidermidis* 1457 WT supernatant did (Fig 3, E). These results suggest that the activity of EcpA is important for *S epidermidis* to induce skin inflammation on live skin, where an intact stratum corneum protects underlying live cells. However, without an established physical barrier, other bacterial components can also contribute to stimulation of the inflammatory response.

EcpA expression is increased in AD

Having established that some *S epidermidis* strains can cause damage and inflammation to mice in an EcpA-dependent manner, we next conducted an observational study to evaluate *S epidermidis* abundance and *ecpA* mRNA on subjects with AD. A cohort of 13 subjects with

AD and 14 healthy subjects (see Table E1) was sampled by skin surface swabs from the antecubital crease to evaluate *S aureus* and *S epidermidis* genomic DNA (gDNA) absolute abundance by real-time quantitative PCR (qPCR) with species-specific primers.⁴² *S epidermidis* gDNA abundance was significantly increased on AD lesional skin compared with on healthy control skin, with no difference based on sex of the subjects assessed (Fig 4, A and see also Fig E5 in this article's Online Repository at www.jacionline.org). Two populations were identified within the AD cohort: 1 group with *S epidermidis* colonization similar to that on healthy skin (around 10³ CFUs/cm²) and a second group with high to very high *S epidermidis* abundance (between 10⁴ and 10⁶ CFUs/cm²). This result is consistent with that in prior reports and demonstrates that *S epidermidis* overgrowth, similar to *S aureus* overgrowth, occurs in only some patients with AD.^{10,18–20} The abundance of gDNA from *S epidermidis* correlated with *S aureus* abundance (Fig 4, B and see also Fig E6, A in this article's Online Repository at www.jacionline.org).

Next, the expression of *ecpA* mRNA by *S epidermidis* on AD skin was measured by qPCR analysis of *ecpA* mRNA extracted from skin swabs. Expression of mRNA for *ecpA* demonstrates the presence of live bacteria. Furthermore, because this gene is under control of the *agr* quorum sensing system, the expression of *ecpA* indicates that the density of *S epidermidis* is sufficiently high to permit expression. Analysis of RNA from AD skin swabs showed a high abundance of *ecpA* mRNA on the lesional skin of some subjects with AD (Fig 4, C). The abundance of *ecpA* mRNA correlated with the absolute abundance of *S epidermidis* gDNA (Fig 4, D). Subjects with the highest *S epidermidis* and *S aureus* absolute abundance, as well as *ecpA* mRNA relative expression, had the most severe disease as assessed by local Eczema Area and Severity Index score (Fig 4, E and F and see Fig E6, B).

EcpA activity is pH dependent, and its expression by *S epidermidis* is downregulated by CoNS AIPs

We next examined factors that could influence the capacity of *S epidermidis* to contribute to disease in AD. Inflamed skin in subjects with AD has increased surface pH compared with that in healthy subjects.⁴³ Analysis of EcpA activity at a range of physiologic pH values showed that EcpA enzymatic activity is low at the pH of normal skin (pH 4.5–6) but becomes active at higher pH values (6.5–8) consistent with the pH of lesional AD skin (see Fig E7 in this article's Online Repository at www.jacionline.org). Therefore, the environmental conditions of AD skin are supportive of EcpA enzymatic activity.⁴⁴

Finally, we sought to better understand how other members of the surface microbial community could influence *S epidermidis* EcpA expression. As discussed previously, *S epidermidis* protease activity is dependent on cell density, an observation that is consistent with regulation of EcpA by the *agr* quorum sensing system.³² We have previously shown that AIPs from certain *S hominis* strains can inhibit the *S aureus agr* quorum sensing system and thus inhibit the production of virulence factors under *agr* control.^{31,45,46} To investigate whether *S hominis* could also inhibit *S epidermidis* EcpA production, an *S epidermidis* 1457 *agr* type II reporter strain that contains an *agr* activity fluorescent reporter was cultured in the presence of either *S hominis* A9 supernatant or the *S hominis* C5 synthetic AIP peptide. Both *S hominis* A9 and C5 were able to inhibit *S epidermidis* 1457 *agr* reporter activity.

Furthermore, *S hominis* A9 supernatant and the *S hominis* C5 synthetic AIP peptide inhibited *ecpA* mRNA expression and EcpA protease activity (Fig 5, B and D and see Fig E8, C in this article's Online Repository at www.jacionline.org). *S hominis* A9 supernatant and the *S hominis* C5 synthetic AIP were also able to inhibit both the *agr* activity and EcpA activity of the 2 other known *S epidermidis agr* types (see Fig E8, A and B). Importantly all of these *agr* inhibition and reduced EcpA expression phenotypes were independent of a reduction in *S epidermidis* growth rate as assessed through OD measurements at 600 nm, indicating that this is specifically due to quorum sensing inhibition and not due to inhibition of growth. Finally, we demonstrated that this quorum sensing interaction between *S hominis* and *S epidermidis* can occur when *in vivo* murine skin models are used. Application of live *S hominis* A9 or C5, *S hominis* A9 supernatant, or *S hominis* C5 synthetic AIP alongside *S epidermidis* 1457 WT resulted in decreases in *S epidermidis*-induced skin barrier damage without a decrease in *S epidermidis* abundance (Fig 5, E and F and see Fig E8, D). Taken together, these data show how the local skin microenvironment and microbial community will influence the activity of *S epidermidis*.

DISCUSSION

Current understanding of the pathophysiology of AD suggests that the skin microbiome can strongly influence this disease.⁴⁷⁻⁵⁰ The presence of *S aureus* in AD and the negative consequences of colonization by this major human pathogen have long been known and extensively described.⁹⁻¹³ One mechanism by which *S aureus* has been shown to damage the skin and exacerbate AD is through its capacity to increase proteolysis of the stratum corneum.^{31,33,51,52} This increased proteolytic activity can combine with a wide range of inherited mutations in human genes of the epidermal differentiation complex⁵³⁻⁵⁶ to result in disruption of the epidermal barrier and worsening of disease symptoms.¹³ However, despite the presence of many other species of *Staphylococcus* in AD besides *S aureus* and the large fraction of patients with AD who are not culture positive for *S aureus*, the potential harmful effect of other species of *Staphylococcus* has been poorly explored. In this study we have shown that *S epidermidis*, a common species of commensal bacteria found on healthy skin, can also drive skin injury through secretion of the cysteine protease EcpA.

S epidermidis has often been regarded as a key member of the healthy skin microbiota with multiple benefits for the host.²¹⁻²³ For example, various *S epidermidis* strains have been shown to limit pathogen infections, tune skin immune development, promote wound repair, and protect against skin cancer.^{26,57-61} However, *S epidermidis* is also known to behave as an opportunistic pathogen, especially following contamination of implanted medical devices.²⁷⁻³⁰ Despite the infectious potential of *S epidermidis*, its frequency on healthy human skin has led to the conclusion that it is harmless when colonizing the skin surface. Indeed, even though *S epidermidis* overgrowth on lesional skin has been reported in several studies,^{10,18-20} its capacity to damage skin and contribute to AD has not been clearly addressed. A previous study reported that the abundance of *S epidermidis* on AD lesions correlated with disease severity,¹⁹ but a mechanism that could suggest causation was not clear. In the present work, we have confirmed that *S epidermidis* overgrowth occurs on the lesional skin of some patients with AD and that *S epidermidis* abundance is correlated with local disease severity. Interestingly, in this cohort, we observed a correlation between *S aureus* and *S epidermidis*

abundance on AD lesional skin, suggesting that the 2 species may both contribute in some patients. Furthermore, we showed that the capacity of *S epidermidis* to promote skin barrier damage is associated with its abundance. This finding is in agreement with the findings of 2 recent studies suggesting that *S epidermidis* overgrowth could contribute to lowered skin barrier function in facial seborrheic dermatitis⁶² and in Netherton syndrome.³⁵

We have now identified the proteolytic activity of the cysteine protease EcpA as a mechanism through which *S epidermidis* can induce skin damage. Several observations support this conclusion, as the deletion of *ecpA* in *S epidermidis* 1457 eliminated the *in vitro* proteolytic effect and prevented the capacity of the bacteria to disrupt the barrier and increase TEWL. We further showed that purified EcpA can disrupt both physical and antimicrobial barriers of the skin through the proteolysis of DSG-1 and the cathelicidin AMP LL-37. Furthermore, EcpA appeared essential to the enabling of *S epidermidis* to induce inflammation *in vivo*, where an intact stratum corneum protects underlying skin. *In vitro* however, EcpA did not seem to be the major factor responsible for *S epidermidis*-induced keratinocyte immune response. Other factors such as TLR ligands or toxins produced by *S epidermidis* may promote inflammation after EcpA has enhanced penetration past the stratum corneum. This would be similar to that shown for *S aureus*, in which case toxins such as PSM α or δ -toxin can induce a strong proinflammatory immune response if the barrier is first disrupted.^{14–17} *S epidermidis* also produces PSMs, and some of them have been shown to induce release of proinflammatory cytokines and cytolysis in neutrophils.^{63–65} We speculate that *S epidermidis* may use a similar 2-step process in which a protease first disrupts the barrier and other factors then trigger inflammation. Further investigations of the contribution of *S epidermidis* PSMs to skin inflammation are needed.

It is unlikely that the capacity of *S epidermidis* to induce skin injury is specific to the number of strains isolated from subjects with AD, as we have observed that isolates from both healthy and AD skin are able to produce EcpA. Indeed, both the population with AD and the healthy population may have a similar frequency of strains within the CoNS community that produce EcpA. This does not argue against the importance of EcpA in promoting the pathogenesis of some patients with AD. Rather than the presence or absence of the strain, our results suggest that it is the absolute abundance (in CFUs/cm²) that is important. Skin damage by EcpA is dependent on bacterial density on the skin, pH, and lack of other CoNS strains that could inhibit the *S epidermidis agr* system. On healthy skin, the activity of *S epidermidis* EcpA would be low owing to a low abundance of the bacteria and a high relative abundance of inhibitory CoNS strains. Furthermore, if EcpA were expressed, the enzymatic activity of the protein would be minimal as a result of the low pH of healthy skin. This could explain why, on the lesional skin of some patients with AD, *S epidermidis* may outcompete the other CoNS strains and increase production and activity of EcpA. This in turn would increase skin barrier damage and further exacerbate disease.

Why some *S epidermidis* strains appear to have lost the capacity to express EcpA is not clear. Studying the regulatory mechanisms of EcpA expression in *S epidermidis* will provide a more complete understanding of the factors predicting the ability of *S epidermidis* to become pathogenic on skin. In addition, the overgrowth of *S epidermidis* on the skin of some patients with AD remains unexplained, and the abundance of *S epidermidis* and *S*

aureus appears to evolve in parallel on the skin of such patients. It is therefore difficult to determine whether 1 of the bacteria has a greater deleterious effect than the other. Also unclear is how endogenous cysteine protease inhibitors or even expression of the well-characterized staphostatin A (*ecpB*) secreted by *S epidermidis* itself can inhibit EcpA activity in AD.⁶⁶ Thus, further work is needed to ultimately understand how *S epidermidis* and *S aureus* are coregulated and to explore the interaction between the 2 bacteria on AD skin.

We show that some unique CoNS strains, and *S hominis* A9 and C5 in particular, are able to inhibit the *S epidermidis agr* system and thus prevent *S epidermidis*-induced skin damage. These 2 strains are of interest as *S hominis* A9 was previously shown to have the capacity to kill *S aureus* through the production of a lantibiotic, whereas *S hominis* C5 AIP was shown to inhibit the *S aureus agr* system.^{16,31} Therefore, such commensal CoNS strains could constitute potential therapeutic tools to fight *S aureus* colonization and both *S aureus* and *S epidermidis* toxin production in AD. Clinical trials to evaluate the benefit of *S hominis* in AD are currently under way.

In conclusion, this study adds an extra level of complexity to our understanding of the interspecies and interkingdom communication that occurs between bacteria and host on the skin in AD. Our data suggest that *S epidermidis* can promote disease and identify a specific mechanism that is consistent with current understanding of the pathogenesis of AD.^{1,67,68} It is possible that other microbial species, including fungi such as *Malassezia furfur*, could have a similar detrimental effect.^{49,69–72} Such observations emphasize that AD should be considered a complex multifactorial disease that is influenced not only by multiple host genes but also by multiple microbial genes from multiple species. This highlights the need for multitargeted and more personalized therapeutic strategies in AD.

METHODS

Bacterial preparation

All bacteria strains used in this study are listed in Table E2 (in this article's Online Repository at www.jacionline.org). For preparation of CoNS supernatant, all strains were grown for 24 hours in 3% tryptic soy broth (TSB) at 300 rpm in an incubator at 37°C. The OD at 600 nm was read to evaluate bacterial concentration, and cultured bacteria were pelleted (for 15 minutes at 4,000 rpm and room temperature) followed by filter sterilization of the supernatant (0.22 µm). For RNA extraction, bacteria were cultured for 12 hours in the conditions described earlier. After the OD measurements, the bacteria suspension was mixed with 2 volumes of RNProtect Reagent (Qiagen, Hilden, Germany) for 10 minutes before centrifugation (for 10 minutes at 13,000 rpm and room temperature) and storage of pellet at –80°C for further analysis. For mouse experiments with live bacteria colonization, bacteria were grown overnight (~16 hours), and the number of CFUs was approximated by measuring OD at 600 nm before application to mouse back skin followed by confirmation of the actual number of CFUs the next day.

Purification of the protease EcpA from *S epidermidis* supernatant

EcpA was purified from *S epidermidis* supernatant by cation exchange chromatography using a protocol based on the purification published by Dubin et al.^{E1} Specifically, 3% TSB(300 mL) was inoculated with a colony of *S epidermidis* strain 05-A5 and incubated at 37°C with shaking (220 rpm) for 24 hours. Bacteria were removed by centrifugation (2,900 *g* for 45 minutes at 4°C) followed by filtration (0.45 mm). Ammonium sulfate was added to 85% saturation, and the filtered supernatant was stirred gently at 4°C for 1 hour before the precipitated protein was pelleted by centrifugation (20,000 *g* for 45 minutes at 4°C). The resulting protein pellet was dissolved in 20 mM sodium phosphate (pH 6.0) containing 5 mM NaCl and then dialyzed extensively (4 × 4.5 L) at 4°C against this same buffer using Spectra/Por dialysis tubing (3.5-kDa cutoff). The protein solution was concentrated to 1.5 mL by using Amicon Ultra-15 concentrators (3-kDa cutoff) and then loaded on a HiScreen CaptoS column (3 columns in series) that had been equilibrated with 20 mM sodium phosphate (pH 6.0) containing 5 mM NaCl. The column was washed with 7 column volumes of equilibration buffer before the protein was eluted with use of a linear gradient of 5 to 500 mM NaCl (in 20 mM sodium phosphate [pH 6.0]) over 27 column volumes. Elution fractions containing active EcpA were identified by using a FRET-based, EcpA-specific protease assay (described later)^{E2} and analyzed for purity by SDS-PAGE. Those fractions estimated to contain EcpA (approximately 90% purity) were pooled and dialyzed against PBS. The resulting protein was confirmed to be EcpA by mass spectrophotometric analysis of tryptic peptides conducted at the University of Colorado School of Medicine Biological Mass Spectrometry Facility. The final recombinant EcpA used in this study was more than 95% pure with a molecular weight slightly greater than 20 kDa, as indicated by SDS-PAGE. This molecular weight is a little higher than previously reported as 19.8 kDa for a mature protease.^{E1} Because EcpA activates by autoprocessing, there can sometimes be slight differences in the final mature protease depending on the cleavage site. The remaining processed fragments of the preprotease are removed during the purification steps. Aliquots containing either 10 or 20 mg of purified EcpA, as determined by Bradford protein concentration assay, were lyophilized and stored at –80°C until they were used in various assays.

EcpA-specific protease assay

The measurement of EcpA activity was performed by using a specific FRET substrate with the sequence (5-FAM)-Lys-Leu-Leu-Asp-Ala-Ala-Pro-Lys-(QXL520)-OH (AnaSpec, Fremont, Calif) in 96-well black bottom plates (Corning) as previously described.^{E2} For measurement of EcpA activity in *S epidermidis* supernatant, 20 µL of supernatant was incubated with 250 nM of EcpA FRET substrate in Tris-HCl (pH 7.8) in a final concentration of 10 mM (total volume 100 µL). To test the effect of pH on EcpA activity, 2× sweat-like buffer (80 mM NaCl, 20 mM KCl, 2 mM sodium dihydrogen phosphate, and 10 mM L-cysteine) was prepared at different pH values (4 to 8). Then, 100 ng of EcpA was incubated with 250 nM EcpA FRET substrate with a 1× final concentration of sweat-like buffer in a total volume of 100 µL. Relative fluorescent intensity was measured with a SpectraMAX Gemini EM fluorometer (Thermo Fisher Scientific) (excitation wavelength, 485 nm; emission wavelength, 538 nm) after incubation at 37°C for 1 hour.

Keratinocyte protein extraction and Western blot analysis

For cell lysis, 200 μ L of cold 1 \times RIPA buffer (Sigma-Aldrich) containing 1X protease inhibitor cocktail (cOmplete [Roche, Basel, Switzerland]) was applied to NHEKs (in a 12-well plate) followed by scraping. The cell lysates were incubated on ice and vortexed for 30 seconds every 15 minutes during a total of 1 hour and then centrifuged (at 14000 g for 20 minutes at 4°C) to remove debris. Protein concentration was determined by using a Pierce BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (12 μ g) were run on 4% to 20% Tris-Glycine precast TGX gels (Bio-Rad, Hercules, Calif) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Neb), incubated overnight at 4°C with primary antibodies, and finally incubated for 2 hours at room temperature with Odyssey (LI-COR Biosciences) fluorescent secondary antibodies (for details about the antibodies used in this study, see Table E4 in this article's Online Repository at www.jacionline.org). Images were acquired with an Odyssey infrared imaging system (LI-COR Biosciences). ImageJ software was used to quantify immunoreactive bands. Signals were normalized to actin immunodetection.

Lactate dehydrogenase cytotoxicity assay

The Pierce Lactate Dehydrogenase Cytotoxicity Assay Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions to test whether EcpA was inducing cytotoxicity in NHEKs.

Quantification of live staphylococci from mouse skin swabs

After live bacteria treatment of mouse skin, the dressing film and agar disk were removed and surface bacteria were collected by using a swab soaked in TSB-glycerol solution. The swab head was then placed in 1 mL of TSB-glycerol solution, vortexed (for ~1 minute), serial-diluted, and plated onto mannitol salt agar plates supplemented with 3% egg yolk. After overnight incubation at 37°C, the number of CFUs was determined.

TEWL measurement

To determine damage to the epidermal skin barrier, TEWL of murine skin treated for 48 hours with *S aureus* or *S epidermidis* was measured by using a TEWAMETER TM300 (Courage + Khazaka Electronic, Köln, Germany).

Histologic staining

Full-thickness murine skin samples were collected, fixed in paraformaldehyde (4%), and embedded in paraffin. Sections (5- μ m) were mounted onto Superfrost Plus glass slides (Thermo Fisher Scientific). Hematoxylin and eosin staining was performed by the University of California, San Diego, Dermatopathology Core. Gram staining was performed by a using Richard-Allan Scientific Chromaview Gram Stain Tissue kit (Thermo Fisher Scientific), which includes Crystal Violet to stain gram-positive bacteria and tartrazine (yellow dye) to counterstain the tissue elements. Pictures were taken on an Olympus BX51 fluorescent microscope.

LL-37 *in vitro* proteolysis and killing assay

A quantity of 20 μM of LL-37 dissolved in H_2O (Genomemed; LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES) was incubated with 10 $\mu\text{g}/\text{mL}$ of EcpA (or with PBS at equal volume) in the presence of 5 mM of L-cysteine for 18 hours at 37°C. Then, 10 μL of the reaction mix was mixed with Laemmli buffer (Bio-Rad; 1 \times final concentration), incubated at 95°C for 5 minutes, run on 4% to 20% Tris-glycine precast TGX gels (BioRad), and stained with Simply Blue Safe Stain (Thermo Fisher Scientific) following the manufacturer's instructions. For the *S aureus* killing assay, *S aureus* 113 was inoculated at a concentration of 10^5 CFUs/mL in 0.6% TSB in Dulbecco PBS (Life Technologies, Carlsbad, Calif) with the LL-37 solution pretreated with either EcpA or PBS vehicle (with a final concentration of LL-37 of 16, 8, 4, 2, or 0 μM) and incubated at 30°C for 20 hours. OD was measured at 600 nm to evaluate *S aureus* growth.

Skin swab collection from human subjects

For collection of skin microbiome DNA, a swab head was soaked in molecular biology-grade TE buffer (Invitrogen, Carlsbad, Calif) containing 0.1% TritonX100 and 0.05% Tween-20 before rubbing the skin, after which it was placed in a clean microcentrifuge tube. For collection of skin microbiome RNA, the swab head was soaked in a 3% TSB and 16.67% glycerol solution (TSB-glycerol solution) before rubbing the skin; it was then placed in 1 mL of the same solution. The skin of the subjects was finally cleaned with an alcohol swab, and the samples were stored at -80°C for further analysis.

RNA isolation and real-time qPCR

All RNA was isolated by using the Purelink RNA isolation kit according to manufacturer's instructions (Thermo Fisher Scientific). For isolation of human skin microbiome RNA, the swab head in TSB-glycerol solution was first vortexed for 1 minute. Then, 500 μL of bacteria suspension was incubated with 2 volumes of RNAprotect reagent (Qiagen) for 10 minutes before centrifugation (10 minutes at 13,000 rpm and room temperature), resuspension in 700 μL of RNA lysis buffer, bead beating (two 1-minute cycles with 5 minutes on ice after each) using lysing matrix B tubes, and centrifugation again. For isolation of RNA from the bacterial suspension, bacteria pellets were resuspended in 700 μL of RNA lysis buffer, bead-beated (two 1-minute cycles with 5 minutes on ice after each sequence) by using lysing matrix B tubes, and centrifuged (10 minutes at 13,000 rpm and room temperature). For cultured keratinocytes, 350 μL of RNA lysis buffer was directly added to the well (of a 24-well plate) after the cells had been washed with 1 Dulbecco PBS and incubated for 2 minutes at room temperature. For mouse tissue, full-thickness skin was preserved in RNAlater (Qiagen) at 4°C before bead beating in 700 μL of RNA lysis buffer (two 30-second cycles with 5 minutes on ice after each; 2.0-mm zirconia bead). The tissue was then centrifuged (for 10 minutes at 13,000 rpm and 4°C). For all samples, 350 μL of clear lysate was then added to 350 μL of 70% EtOH and column-based isolation of RNA. After RNA isolation, an equal volume of human skin swab microbiome RNA elution (11 μL) or equal quantity of bacterial, mouse, or keratinocyte RNA (500 ng) was reverse-transcribed by using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). Oligodeoxythymidines were not added to the reverse transcription mix for skin swab

microbiome RNA samples to limit amplification of contaminant human DNA. qPCR assays were run on a CFX96 Real-Time Detection System (Bio-Rad) by using SYBR Green qPCR Master Mix (Biotool, Stratech, Ely, United Kingdom) along with specific primers (for details about primers, see Table E3 in this article's Online Repository at www.jacionline.org). The housekeeping genes used were *Hprt/HPRT* for both mouse and keratinocyte samples and *gyrB* for *S epidermidis* cultures. *ecpA* mRNA abundance from the skin swabs was normalized on skin area.

Microbial DNA extraction from human skin swabs and quantification of staphylococcal gDNA

Microbial DNA was extracted from skin swabs by using the PureLink Microbiome DNA Purification Kit according to the manufacturer's instructions (Thermo Fisher Scientific). The absolute abundance of *S aureus* and *S epidermidis* gDNA in the microbial DNA elution was determined by qPCR as previously described.^{E3,E4} Briefly, qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) by using *S epidermidis*- and *S aureus*-specific primers, targeting the *S epidermidis sodA* gene and *S aureus femA* genes, respectively. To determine the relative CFUs of *S aureus*- or *S epidermidis*-specific DNA, a standard curve was generated with gDNA extracted from known CFUs of *S aureus* (ATCC113) or *S epidermidis* (ATCC12228), respectively. The specificity of all primer pairs was confirmed by melting curve analysis and comparison with standard curves.

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Abbreviations used

AD	Atopic dermatitis
<i>Agr</i>	Accessory gene regulator
AIP	Autoinducing peptide
AMP	Antimicrobial peptide
CFU	Colony-forming unit
CoNS	Coagulase-negative <i>Staphylococcus</i>
DSG-1	Desmoglein-1
EcpA	Extracellular cysteine protease A
IVL	Involucrin
HEK	Neonatal human primary epidermal keratinocyte

PSM	Phenol-soluble modulin
qPCR	Quantitative PCR
TEWL	Transepidermal water loss
TSB	Tryptic soy broth
WT	Wild-type

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Key messages

- *S epidermidis* produces the protease EcpA under quorum sensing control.
- EcpA can damage the skin barrier and promote inflammation in mouse skin.
- The abundance of *S epidermidis ecpA* correlates with disease severity in some subjects with atopic dermatitis.

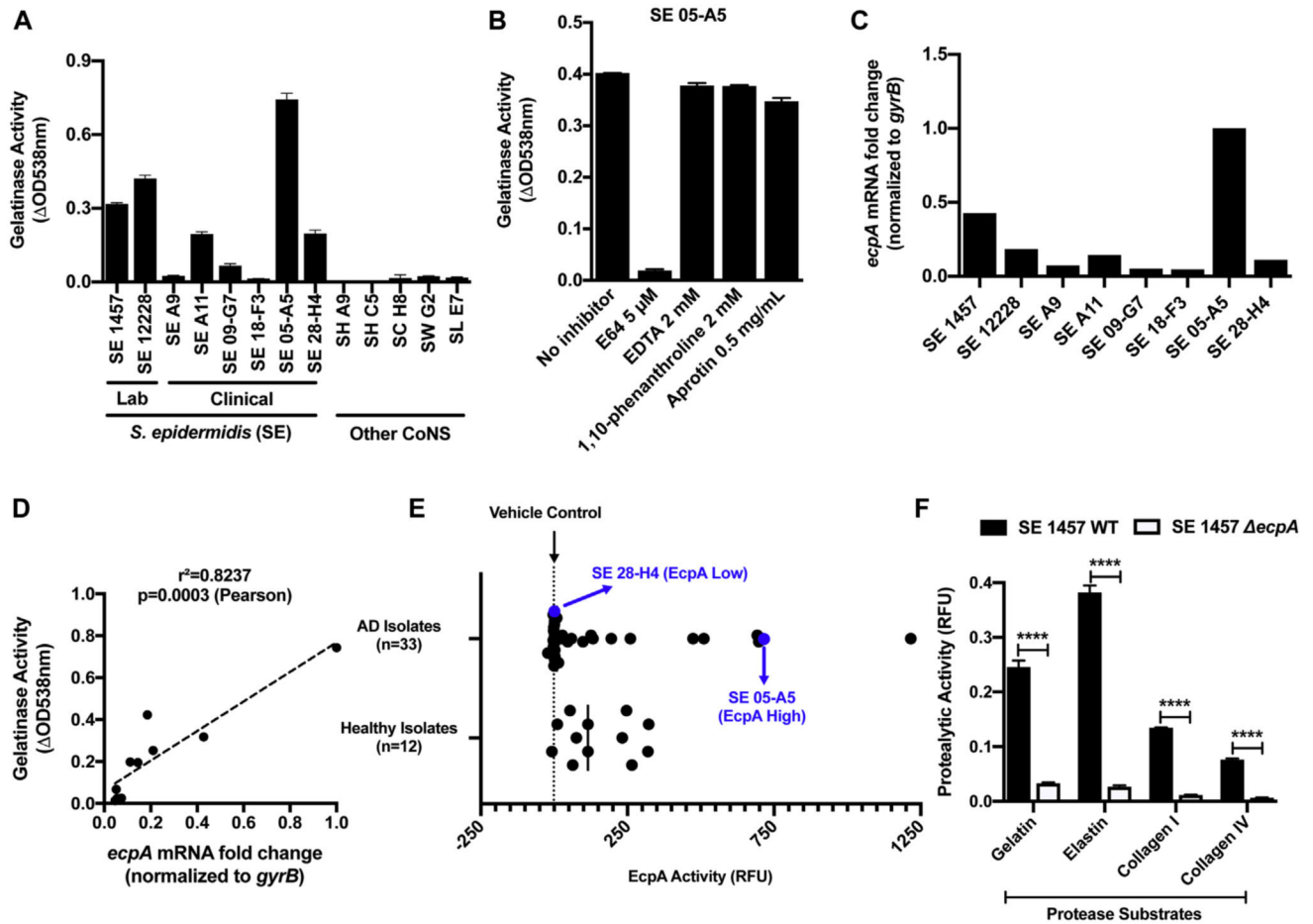


FIG 1.

The cysteine protease EcpA secreted by *S. epidermidis* (SE) presents a unique proteolytic activity. **A**, Protease activity against gelatin measured in the supernatant of CoNS strains cultured for 24 hours: SE, *S. hominis* (SH), *S. capitis* (SC), *S. warneri* (SW), and *S. lugdunensis* (SL) (n = 3). **B**, Protease activity against gelatin measured in the supernatant of SE 05-A5 cultured for 24 hours in the presence of various protease inhibitors: E64 (a cysteine protease inhibitor), EDTA, 1,10-phenanthroline (a metalloprotease inhibitor), and aprotinin (a serine protease inhibitor) (n = 3). **C** and **D**, *ecpA* mRNA levels across SE strains and Pearson correlation to gelatinase activity. **E**, Assessment of EcpA specific activity (FRET substrate) in supernatant of SE isolates from both healthy (n = 12) and atopic (n = 33) individuals after the isolates had been cultured for 24 hours (n = 3). **F**, Protease activity against gelatin, elastin, collagen I, and collagen IV substrates measured in the supernatant of SE 1457 WT or Δ ecpA strains cultured for 24 hours. All data are representative of at least 2 independent experiments, and the results are means \pm SEMs. **F**, Student *t* tests were used to determine statistical significance: **P* < .05; ***P* < .01; ****P* < .001; **** *P* < .0001. RFU, Relative fluorescence unit.

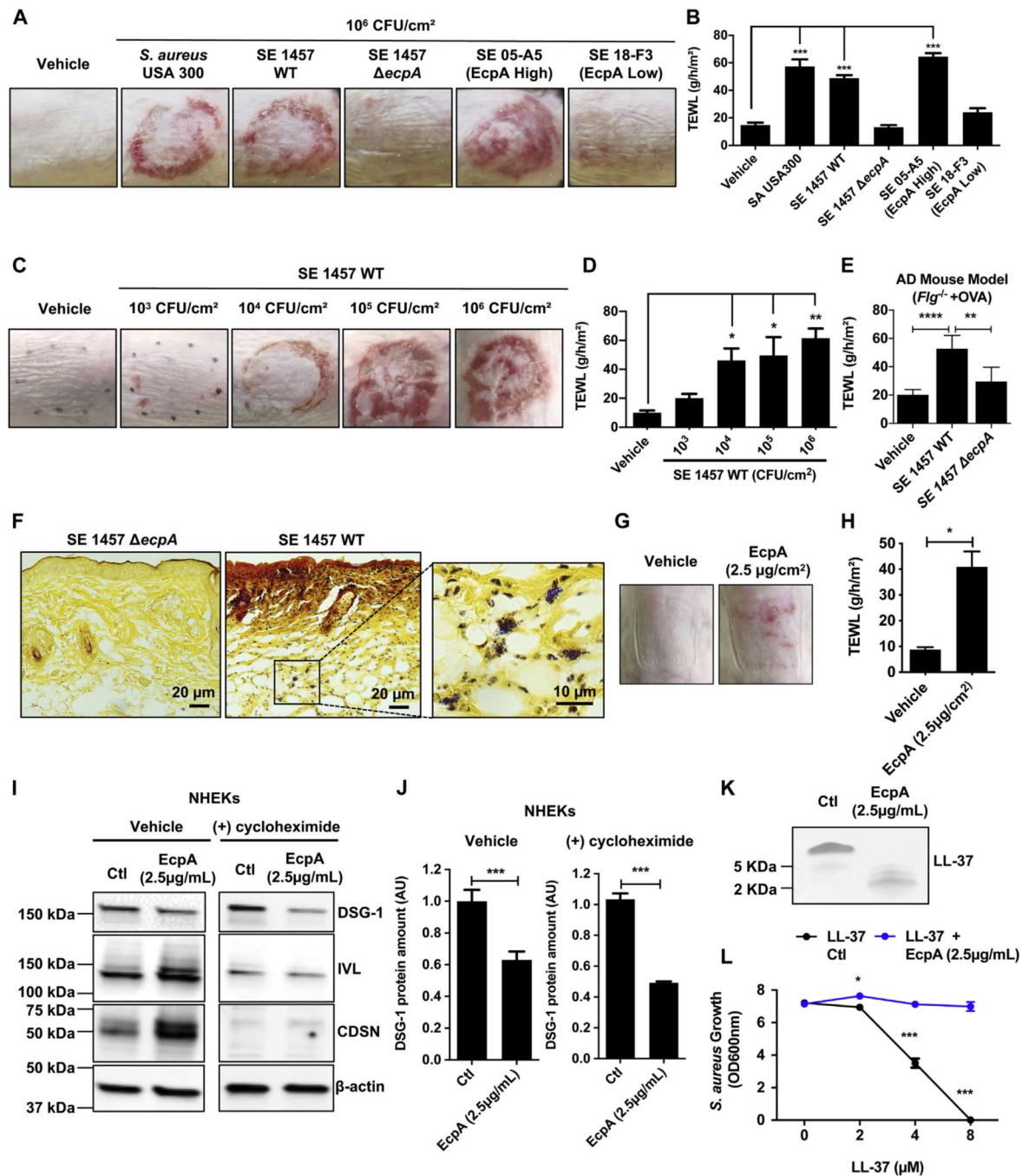


FIG 2. *S. epidermidis* (SE) cysteine protease EcpA disrupts the skin barrier and degrades DSG-1 and LL-37. **A** and **B**, Representative pictures of the murine back skin treated with 10^6 CFUs/cm² SA or SE isolates for 48 hours and TEWL measurements ($n = 5$). **C** and **D**, Representative pictures of the back skin after colonization with 10^3 to 10^6 CFUs/cm² SE 1457 WT for 48 hours and TEWL measurements ($n = 4$). **E**, TEWL measurements of AD mouse model back skin (Balb/c *Flg*^{-/-} + ovalbumin [OVA]) colonized for 24 hours with live 10^6 CFUs/cm² SE 1457 WT or SE 1457 *ecpA* ($n = 5$ or 6). **F**, Gram-positive bacteria

staining (*in purple*) of skin sections from mice treated with either 10^6 CFUs/cm² of SE 1457 WT or SE 1457 *ecpA* (n = 5). **G** and **H**, Assessment of C57BL/6 murine back skin and TEWL measurements after treatment with 2.5 µg/cm² of EcpA or vehicle for 24 hours (n = 3). **I** and **J**, Differentiated NHEKs were treated for 20 hours with 2.5 µg/mL of EcpA ± 20 µg/mL of cycloheximide (protein synthesis inhibitor). Immunoblotting for DSG-1, involucrin (IVL), corneodesmosin (CDSN), and β-actin. Quantification of DSG-1 was normalized on β-actin (n = 3) (see Table E4). **K** and **L**, Coomassie blue staining of *in vitro* proteolysis of LL-37 by EcpA and *S aureus* (strain 113) growth inhibition assay (n = 3). All data are representative of at least 2 independent experiments, and results are means ± SEMs. One-way ANOVAs (**B**, **D**, and **E**), Student *t* tests (**H** and **J**), and 2-way ANOVA (**L**) were used to determine statistical significance: **P* < .05; ***P* < .01; ****P* < .001, *****P* < .0001. *Ctl*, Control.

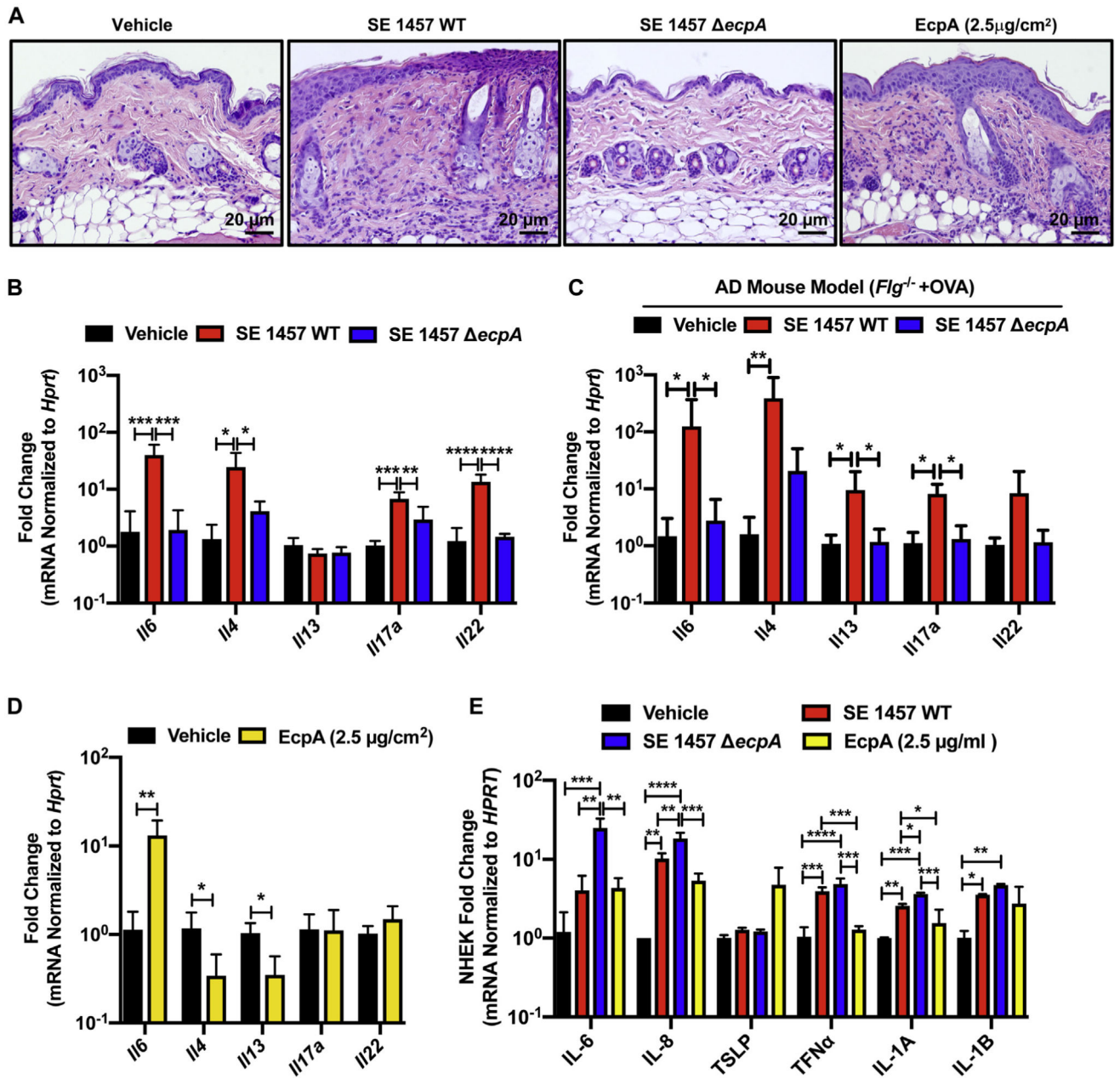


FIG 3. EcpA induces skin inflammation in combination with other *S. epidermidis* toxins. **A**, Representative hematoxylin and eosin staining of mouse skin sections across treatments. **B** and **C**, Analysis of the mRNA level of various cytokines after treatment of murine back skin with 10⁶ CFUs/cm² of *S. epidermidis* (SE) 1457 WT or SE 1457 Δ ecpA for 48 hours in the C57BL/6 mouse model (**B**) or AD mouse model (BALB/c *Flg*^{-/-} 1 ovalbumin [OVA]) (**C**), respectively (n = 4–6). mRNA levels normalized by using the housekeeping gene *Hprt*. **D**, Analysis of the mRNA level of various cytokines after treatment of C57BL/6 murine back skin with vehicle or 2.5 μ g/cm² of EcpA for 24 hours. **E**, Differentiated NHEK treated for 8 hours with 2.5 μ g/mL of EcpA or with 5% bacterial supernatant followed by analysis of the

mRNA level of various cytokines normalized to the housekeeping gene *HPRT* (n = 3). All data are representative of at least 2 independent experiments, and results are means \pm SEMs. One-way ANOVAs (**B**, **C**, and **E**) and Student *t* tests (**D**) were used to determine statistical significance: **P* < .05; ***P* < .01; ****P* < .001; **** *P* < .0001.

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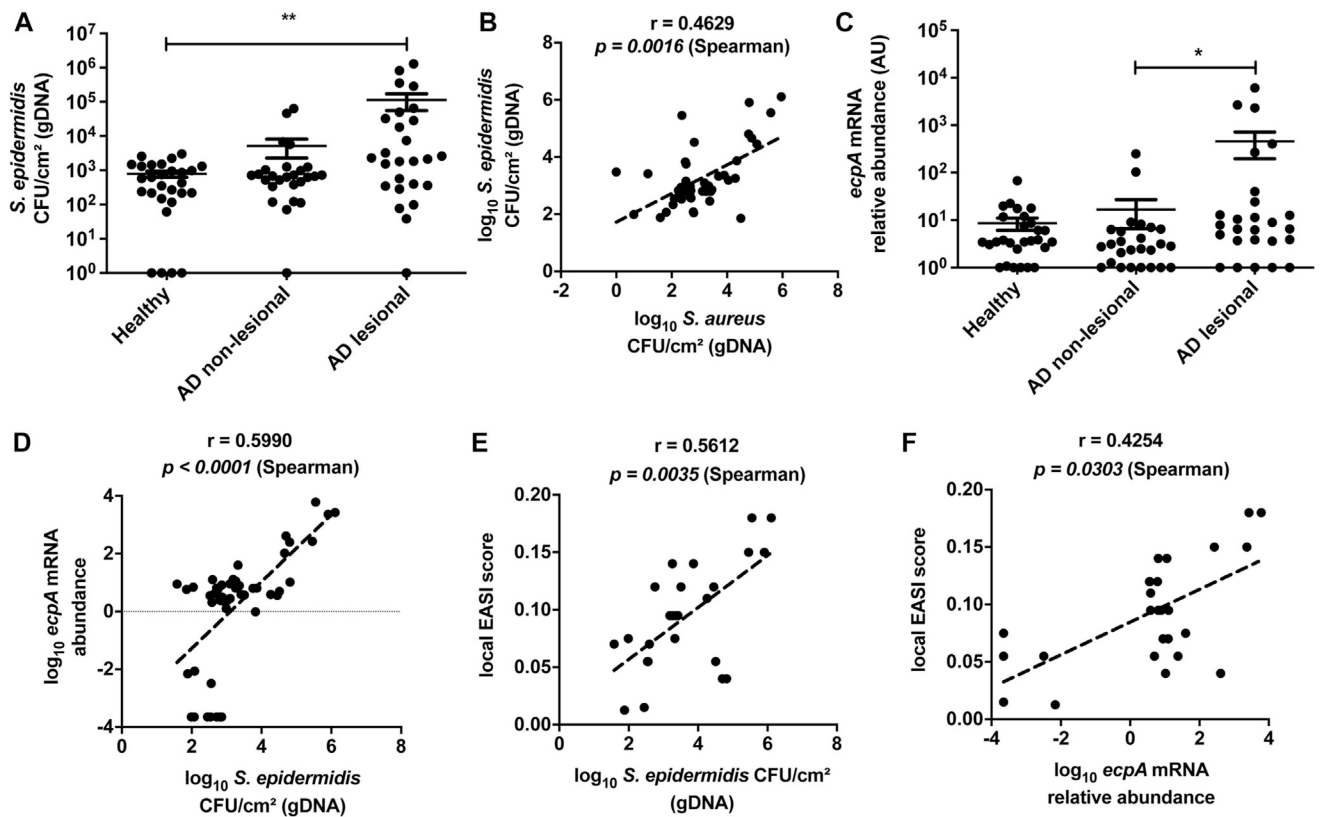


FIG 4.

S. epidermidis colonization and *ecpA* expression are increased on some subjects with AD. **A**, Measurement of gDNA absolute abundance of *S. epidermidis* CFUs/cm² from swabs of healthy control and AD nonlesional and lesional skin normalized to skin area. **B**, Spearman correlation between the gDNA absolute abundance of *S. epidermidis* and *S. aureus* from skin swabs. **C**, Relative abundance of *S. epidermidis ecpA* mRNA isolated from swabs of healthy control and AD nonlesional and lesional skin normalized to skin area. **D**, Spearman correlation between *S. epidermidis EcpA* mRNA relative abundance and *S. epidermidis* CFUs/cm² from swabs of AD skin. **E**, Spearman correlation between the local Eczema Area and Severity Index (EASI) score and *S. epidermidis* CFUs/cm² from swabs of AD lesional skin. **F**, Spearman correlation between the local EASI score and *ecpA* mRNA relative abundance from swabs of AD lesional skin. Each dot represents a single swab, and the bars represent means \pm SEMs. A and C, A nonparametric unpaired Kruskal-Wallis analysis was used to determine statistical significance. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

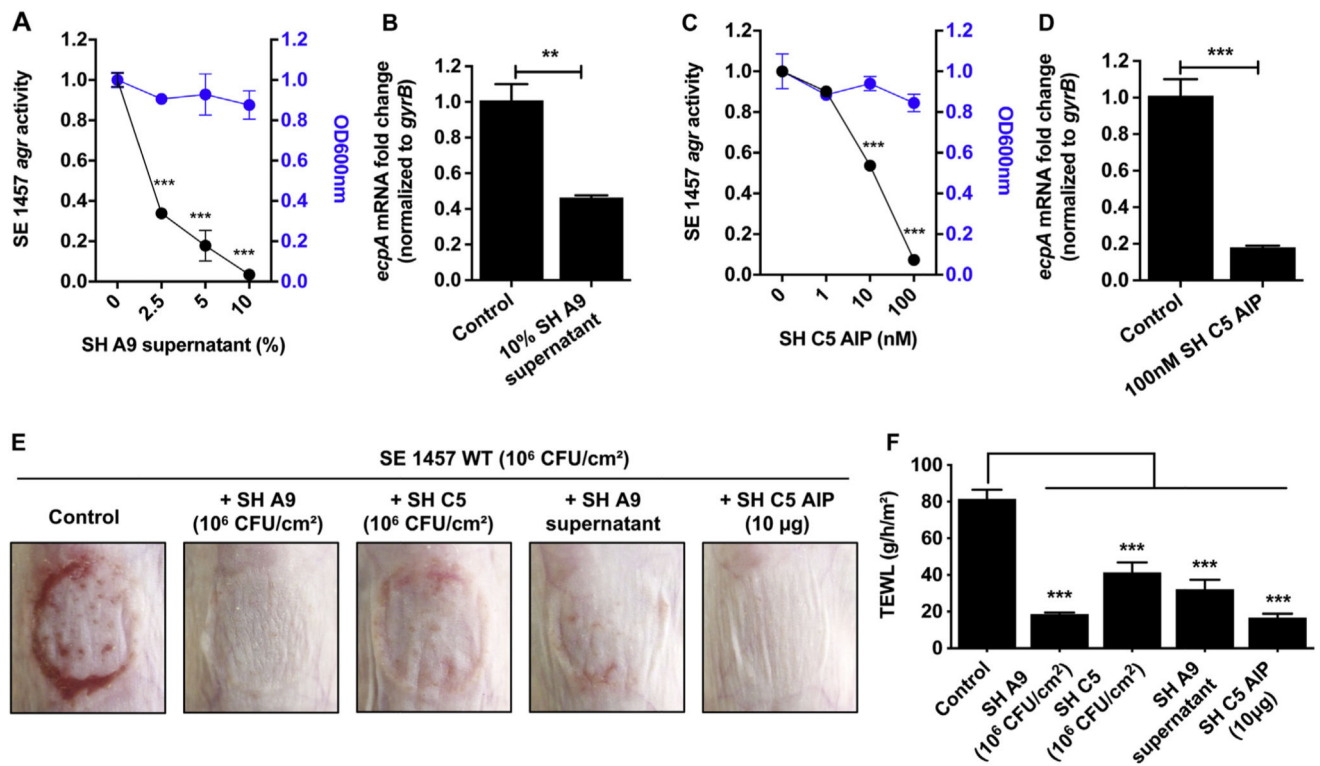
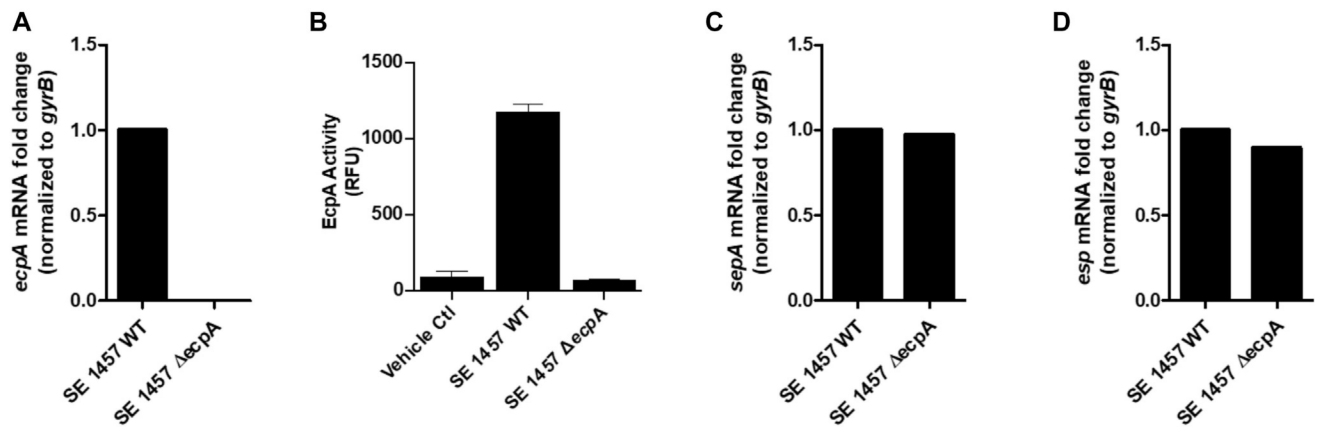


FIG 5.

EcpA production is downregulated by CoNS AIPs. **A** and **C**, Modulation of the *S. epidermidis* (SE) 1457 agr type II P3-GFP reporter strain activity by *S. hominis* (SH) A9 supernatant (**A**) or synthetic SH C5 AIP (**C**) after 24 hours of culturing (n = 3). OD values at 600 nm used to assess bacterial growth at the hour 24 time point. **B** and **D**, *ecpA* mRNA levels after 12 hours of culturing of SE 1457 agr reporter strain with SH A9 supernatant (10%) (**B**) or SH C5 AIP (100 nM) (n = 3) (**D**). **E** and **F**, C57BL/6 murine back skin was topically treated with 10⁶ CFUs/cm² of SE 1457 for 48 hours either alone (control) or with 10⁶ CFUs/cm² of *S. hominis* (SH) A9, 10⁶ CFUs/cm² of SH C5, SH A9 supernatant (5 μL), or SH C5 synthetic AIP (10 μg). **E**, Representative pictures of the treated back skin and measurement of TEWL (**F**) (n = 4). All data are representative of at least 2 independent experiments, and the results are means ± SEMs. One-way ANOVAs (**A**, **C**, and **F**) and Student *t* tests (**B** and **D**) were used to determine statistical significance: **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

**FIG E1.**

Characterization of *S. epidermidis* (SE) 1457 *ecpA*. **A**, Analysis of the mRNA levels of *ecpA* in both the SE 1457 WT or *ecpA* strain after 12 hours of culturing. **B**, EcpA activity with use of a specific FRET substrate measured in the sterile-filtered supernatant of SE 1457 WT or *ecpA* after 24 hours of culturing (n = 3). **C** and **D**, Analysis of mRNA levels for 2 other SE-secreted protease genes, *sepA* and *esp*, in both SE 1457 WT or *ecpA* after 12 hours of culturing. Data are representative of at least 2 independent experiments, and the results are represented as means \pm SEMs. *RFU*, Relative fluorescence unit.

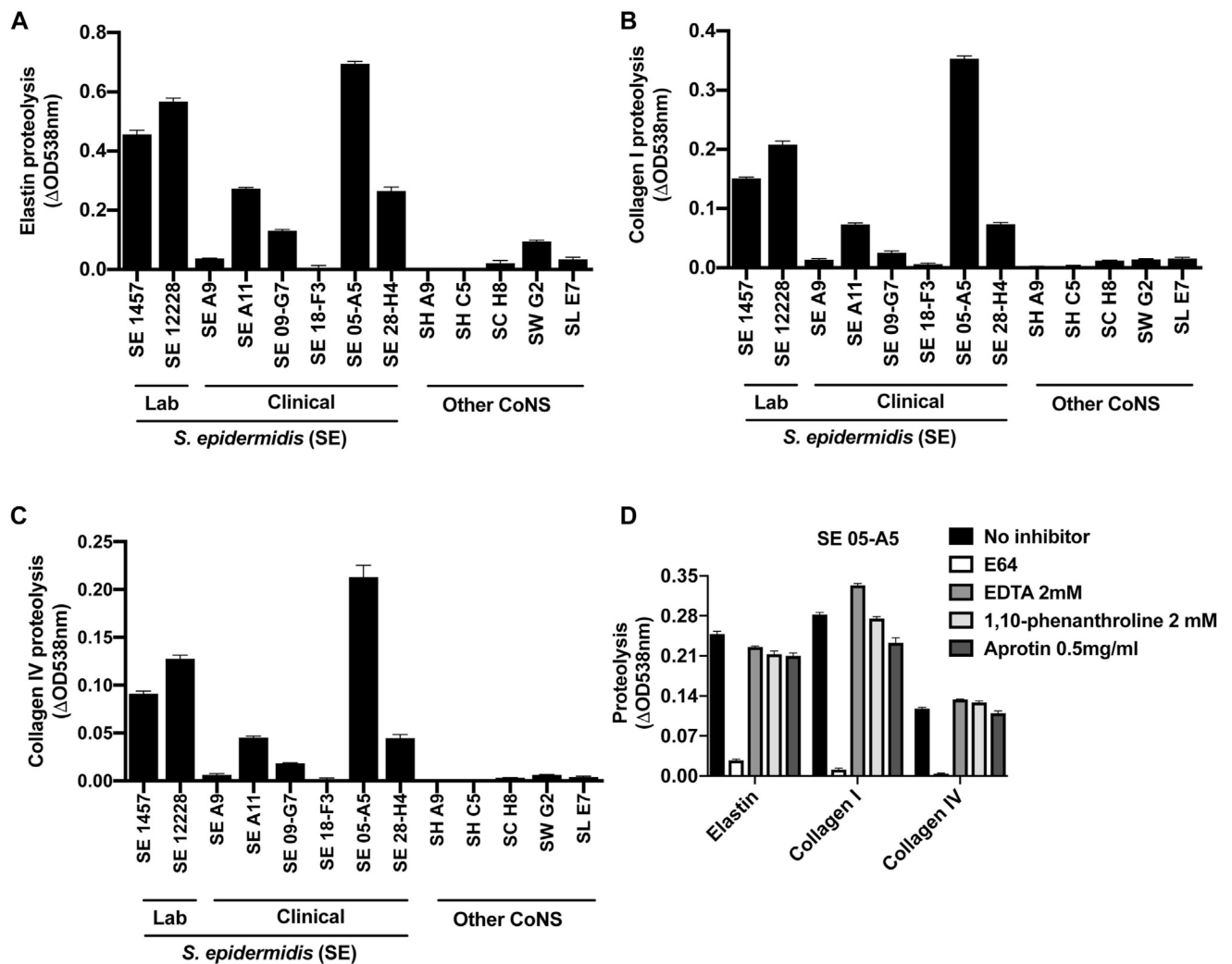


FIG E2.

Characterization of *S. epidermidis* (SE) protease activity. **A-C**, Measurement of protease activity against the substrates elastin (**A**), collagen I (**B**), and collagen IV (**C**) with CoNS sterile-filtered supernatants that had been cultured for 24 hours ($n = 3$). **D**, Protease activity against the substrates elastin, collagen I, and collagen IV measured in the sterile-filtered supernatant of SE 05-A5 (after 24 hours of culturing) in the presence of various protease inhibitors: E64 (a cysteine protease inhibitor), EDTA, and 1,10-phenanthroline (a metalloprotease inhibitor), and aprotinin (a serine protease inhibitor) ($n = 3$). Data are representative of at least 2 independent experiments, and results are represented as means \pm SEMs. *SH*, *S. hominis*; *SC*, *Staphylococcus capitis*; *SW*, *Staphylococcus warneri*; *SL*, *Staphylococcus lugdunensis*.

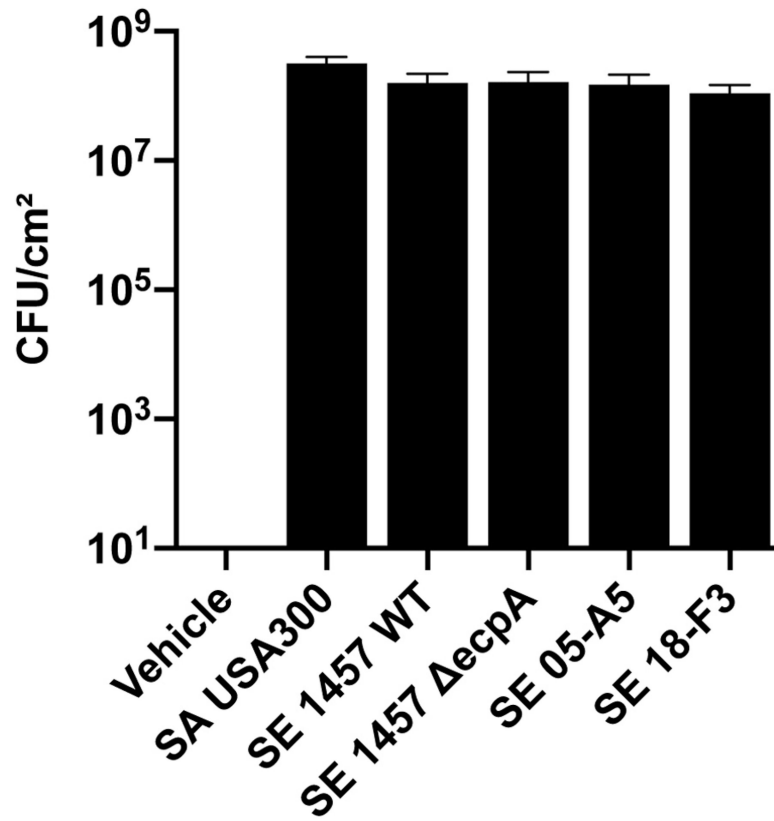
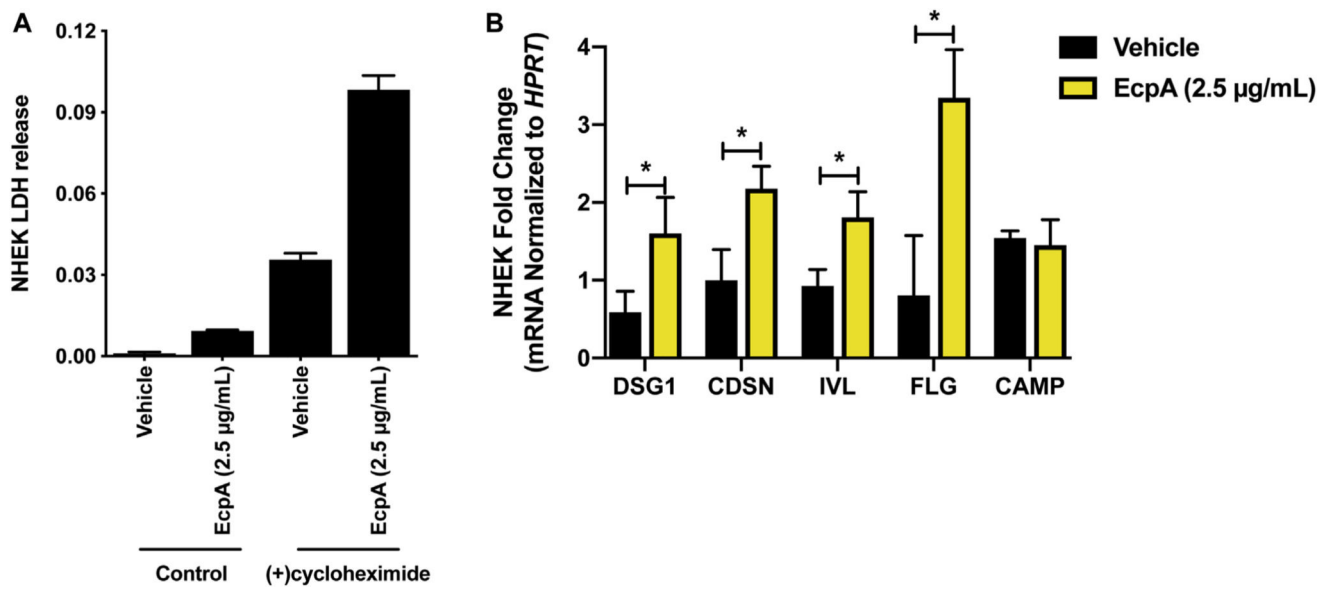
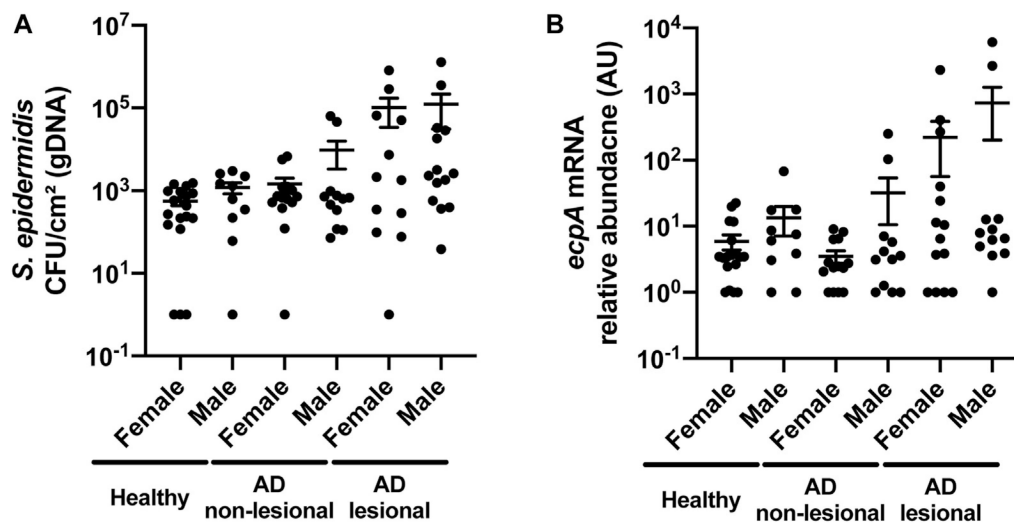


FIG E3. Quantification of live staphylococci on 8- to 10-week-old female C57BL/6 mice skin after topical treatment with *S aureus* (SA) or *S epidermidis* (SE) live bacteria for 48 hours.

**FIG E4.**

Effect of EcpA on cultured keratinocytes. **A**, Evaluation of the cytotoxicity of EcpA on differentiated NHEKs by measuring lactate dehydrogenase (LDH) release in the culture medium after 20 hours of treatment alone or in the presence of 20 µg/mL of cycloheximide. **B**, Differentiated NHEKs were treated for 8 hours with 2.5 µg/mL of EcpA followed by analysis of the mRNA level of several epidermal differentiation markers and *CAMP* by qPCR ($n = 3$). Data are representative of at least 2 independent experiments, and results are represented as means \pm SEMs. **B**, Student *t* tests were used to determine statistical significance: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

**FIG E5.**

Sex differences in *S. epidermidis* colonization and EcpA expression on AD skin. **A** and **B**, Measurement of gDNA absolute abundance of *S. epidermidis* CFUs/cm² or *ecpA* mRNA levels from swabs of healthy control and AD nonlesional and lesional skin normalized to skin area. Data are represented as means \pm SEMs. **A** and **B**, Nonparametric unpaired Kruskal-Wallis analysis and Spearman correlation were used to determine statistical significance.

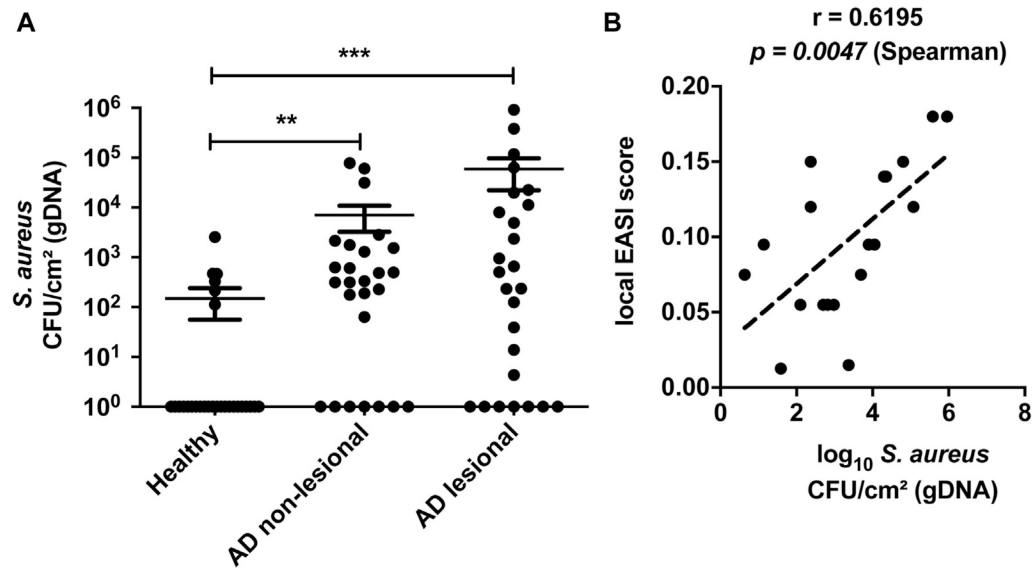


FIG E6.

Colonization of AD skin by *S. aureus* and correlation to disease severity. **A**, Measurement of gDNA absolute abundance of *S. aureus* CFUs/cm² from swabs of healthy control and AD nonlesional and lesional skin normalized to skin area. **B**, Spearman correlation between the local Eczema Area and Severity Index (EASI) score and *S. aureus* CFUs/cm² from swabs of AD lesional skin. Each dot represents a single swab and the bars represent means \pm SEMs. **A**, Nonparametric unpaired Kruskal-Wallis analysis was used to determine statistical significance: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

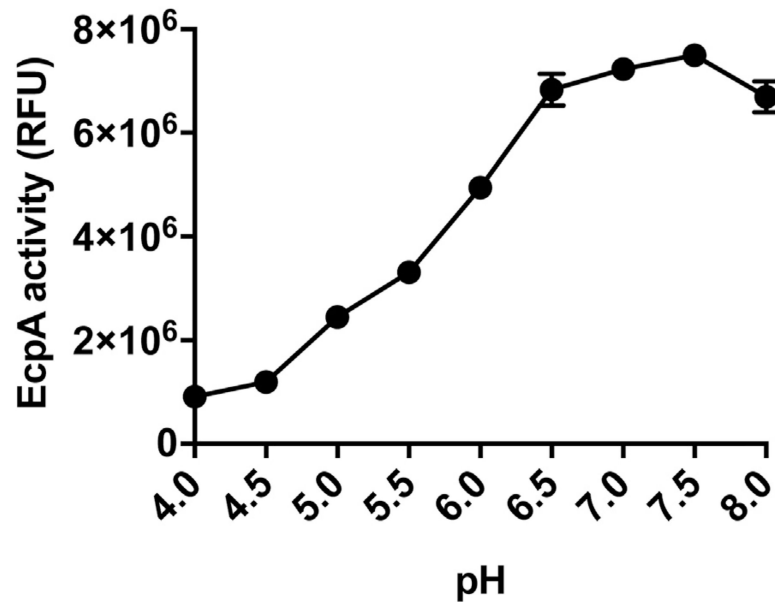


FIG E7. pH-dependent activity of *S. epidermidis* EcpA. EcpA (100 ng) was incubated with EcpA FRET substrate (250 nM) in sweat-like buffer (40 mM NaCl, 10 mM KCl, 1 mM sodium dihydrogen phosphate, 5 mM *L*-cysteine) at different pH ranges (4–8) for 1 hour at 37°C (n = 3). Data are representative of at least 2 independent experiments, and results are represented as means ± SEMs.

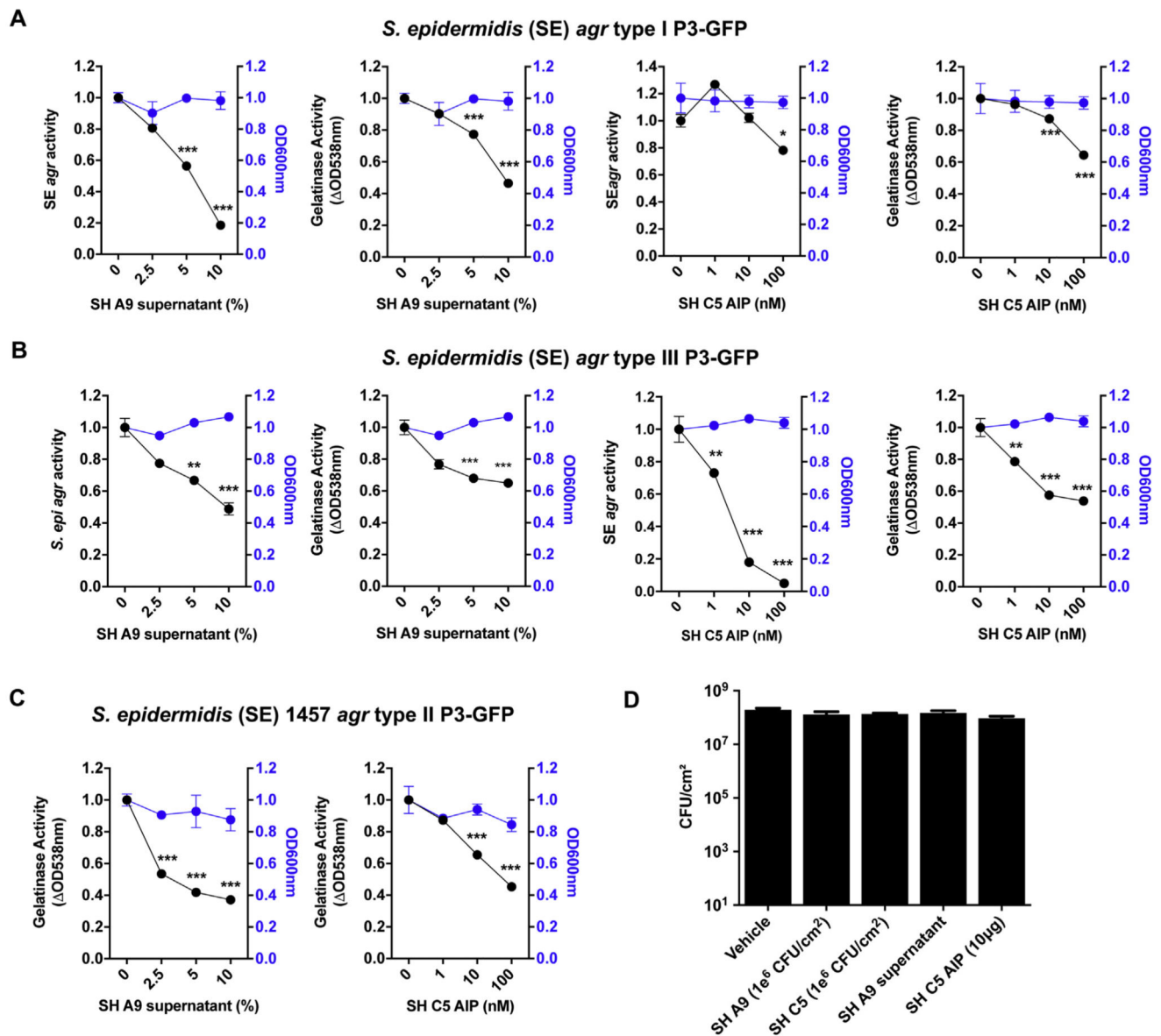


FIG E8.

Modulation of *S. epidermidis* (SE) agr activity by some *S. hominis* (SH) strains. **A**, Modulation of the agr activity and gelatinase activity of SH 12228 agr type I P3-GFP reporter strain by either SH A9 supernatant or SH C5 synthetic AIP (n = 3). **B**, Modulation of the agr activity and gelatinase activity of SE 8247 agr type III P3-GFP reporter strain by either SH A9 supernatant or SH C5 synthetic AIP (n = 3). **C**, Modulation of the gelatinase activity of SE 1457 agr type II P3-GFP reporter strain by either SH A9 supernatant or SH C5 synthetic AIP (n = 3). **A-C**, OD values recorded at 600 nm to approximate bacterial growth overtime. **D**, Quantification of live staphylococci on 8-to 10-week-old female C57BL/6 mice skin after topical treatment with SE 1457 live bacteria for 48 hours either alone (vehicle) or along with SH A9, C5 live bacteria, SH A9 supernatant (5 μ L), or SH C5 AIP (n = 4). Data are representative of at least 2 independent experiments, and results are represented as

means \pm SEMs. **A-C**, One-way ANOVAs were used to determine statistical significance: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

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TABLE E1.

Human subjects involved in this study

Group	Subject no.	Date of collection	Sex	Age (y)	Ethnicity	Local EASI score		oSCORAD disease score
						Right arm	Left arm	
Healthy subjects	H01	15/07/2019	F	51	Hispanic or Latino/ White	N/A	N/A	N/A
	H02	15/07/2019	F	22	Hispanic or Latino/ White	N/A	N/A	N/A
	H03	15/07/2019	F	40	Hispanic or Latino/ White	N/A	N/A	N/A
	H04	15/07/2019	F	45	White	N/A	N/A	N/A
	H05	15/07/2019	M	27	White	N/A	N/A	N/A
	H06	17/07/2019	F	23	Asian	N/A	N/A	N/A
	H07	19/07/2019	F	23	Hispanic or Latino/ White	N/A	N/A	N/A
	H08	22/07/2019	F	31	Black/White	N/A	N/A	N/A
	H09	30/07/2019	F	28	White	N/A	N/A	N/A
	H10	14/08/2019	M	40	Asian	N/A	N/A	N/A
	H11	16/09/2019	F	55	Asian	N/A	N/A	N/A
	H12	16/09/2019	M	58	Asian	N/A	N/A	N/A
	H13	18/09/2019	M	21	White	N/A	N/A	N/A
	H14	01/10/2019	M	27	Hispanic or Latino/ White	N/A	N/A	N/A
Subjects with atopic dermatitis	AD01	16/07/2019	M	44	Black	0.095	0.095	66
	AD02	18/07/2019	F	26	Asian	0.075	0.055	54.3
	AD03	25/07/2019	M	53	White	0.14	0.14	55.9
	AD04	25/07/2019	M	23	Asian	0.12	0.12	34.2
	AD05	25/07/2019	F	21	Asian	0.11	0.12	34.9
	AD06	27/08/2019	M	40	White	0.055	0.075	67.2
	AD07	19/09/2019	F	20	Asian	0.07	0.07	64.3
	AD08	20/09/2019	M	20	White	0.055	0.055	24.6
	AD09	30/09/2019	F	36	Hispanic or Latino/ White	0.015	0.0125	22.68
	AD10	04/10/2019	F	51	White	0.095	0.095	55.5
	AD11	04/10/2019	F	21	White	0.04	0.04	33.4
	AD12	08/10/2019	M	18	Asian	0.18	0.18	53.2
	AD13	08/10/2019	F	18	Asian	0.15	0.15	44.8

EASI, Eczema Area and Severity Index; F, female; M, male; N/A, nonapplicable; oSCORAD, Objective Scoring Atopic Dermatitis.

All samples were collected on the antecubital fossa skin. None of the subjects were undergoing treatment at the time of collection.

TABLE E2.

Bacteria strains used in this study

Name	Source	Reference
<i>Staphylococcus epidermidis</i> 1457 (AH2490)	Horswill (UC Denver)	Olson et al, 2014 ^{E2}
<i>Staphylococcus epidermidis</i> 1457 <i>ecpA</i> (AH2924)	Horswill (UC Denver)	Olson et al, 2014 ^{E2}
<i>Staphylococcus epidermidis</i> 12228 <i>agr</i> type I <i>P3-GFP Erm^r</i> (AH3408)	Horswill (UC Denver)	Olson et al, 2014 ^{E2}
<i>Staphylococcus epidermidis</i> 1457 <i>agr</i> type II <i>P3-GFP Erm^r</i> (AH273)	Horswill (UC Denver)	Olson et al, 2014 ^{E2}
<i>Staphylococcus epidermidis</i> 8247 <i>agr</i> type III <i>P3-GFP Erm^r</i> (AH3409)	Horswill (UC Denver)	Olson et al, 2014 ^{E2}
<i>Staphylococcus epidermidis</i> (SE) ATCC12228	Gallo (UCSD)	Zhang et al, 2003 ^{E5}
<i>Staphylococcus epidermidis</i> (SE) A9	Gallo (UCSD)	This study
<i>Staphylococcus epidermidis</i> (SE) A11	Gallo (UCSD)	This study
<i>Staphylococcus epidermidis</i> (SE) 09-G7	Gallo (UCSD)	This study
<i>Staphylococcus epidermidis</i> (SE) 18-F3	Gallo (UCSD)	This study
<i>Staphylococcus epidermidis</i> (SE) 05-A5	Gallo (UCSD)	This study
<i>Staphylococcus epidermidis</i> (SE) 28-H4	Gallo (UCSD)	This study
<i>Staphylococcus hominis</i> (SH) A9	Gallo (UCSD)	Nakatsuji et al, 2017 ^{E6}
<i>Staphylococcus hominis</i> (SH) C5	Gallo (UCSD)	Williams et al, 2019 ^{E7}
<i>Staphylococcus capitis</i> (SC) H8	Gallo (UCSD)	This study
<i>Staphylococcus warneri</i> (SW)G2	Gallo (UCSD)	This study
<i>Staphylococcus lugdunensis</i> (SL) E7	Gallo (UCSD)	This study
<i>Staphylococcus aureus</i> USA300 (AH1263)	Horswill (UC Denver)	Mootz et al, 2015 ^{E8}

UC, University of Colorado; UCSD, University of California, San Diego.

TABLE E3.

qPCR primers used in this study

Gene target	Forward primer	Reverse primer	Reference
SE <i>ecpA</i>	TGTGCTTAAAACGCCACGTA	GTATAGCCGGCACACCAACT	Olson et al, 2014 ^{E2}
SE <i>sepA</i>	TGGTGTAACACAGCAAAGTGC	ACTTCTTAAGGCATCTCCGCC	This study
SE <i>esp</i>	ACGTTGTTAATGGAGCTAAGGGT	TGCAGGTTTAACTACTTGACCA	This study
SE <i>sodA</i>	TCAGCAGTTGAAGGGACAGAT	CCAGAACAATGAATGGTTAAG	Nakatsuji et al, 2013 ^{E4}
SA <i>femA</i>	AACTGTTGGCCACTATGAGT	CCAGCATACCTGTAATCTCG	Nakatsuji et al, 2013 ^{E4}
h <i>HPRT</i>	CCTGGCGTCGTGATTAGTGAT	AGACGTTTCAGTCTGTCCATAA	Zhang et al, 2016 ^{E9}
h <i>IL-6</i>	AATTCGGTACATCCTCGACGG	TTGGAAGGTTTCCAGGTTGTTTTCT	Zhang et al, 2016 ^{E9}
h <i>IL-8</i>	TCCAAGCTGGCCGTGGCTCT	CTGTGTTGGCGCAGTGTGGTCC	Zhang et al, 2016 ^{E9}
h <i>TSLP</i>	ATGTTCCGCATGAAAATAAGGC	GCGACGCCACAATCCTTGTA	This study
h <i>TNFα</i>	GCTGCACTTTGGAGTGATCG	GGGTTTGCTACAACATGGGC	Zhang et al, 2016 ^{E9}
h <i>IL-1α</i>	TGGTAGTAGCAACCAACGGGA	ACTTTGATTGAGGGCGTCATTC	This study
h <i>IL-1β</i>	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA	This study
h <i>DSG-1</i>	AACCCAATCGCCAAAATCACT	ACCTCTCGATCAACTATGGATGT	This study
h <i>CDSN</i>	ACTGCTGCTGGCTGGTCT	AGAGCTTCTGGCACTGGAAA	Cau et al, 2017 ^{E10}
h <i>JVL</i>	GCCAGGTCCAAGACATTCAAC	GGGTGGTTATTTAIGTTTGGGTGG	Cau et al, 2017 ^{E10}
h <i>FLG</i>	CCCTCGGTTTCCACTGTCTC	CCCTCGGTTTCCACTGTCTC	Cau et al, 2017 ^{E10}
h <i>CAMP</i>	AGGTCCTCAGCTACAAGGAAG	TCTTGAAGTCACAATCCTCTGGT	Liggins et al, 2019 ^{E11}
m <i>Hprt</i>	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT	Zhang et al, 2016 ^{E9}
m <i>Il6</i>	ACAAAGCCAGAGTCCTCAGAGAGA	AGCCACTCCTTCTGTGACTCCAG	Liggins et al, 2019 ^{E11}
m <i>Il4</i>	GAGCCATATCCACGGATGCGAC	ATGCGAAGCACCTTGAAGCCC	This study
m <i>Il13</i>	TGCTTGCTTGGTGGTCTCGC	GCGGCCAGGTCCACACTCCA	This study
m <i>Il17α</i>	ACGCGCAAACATGAGTCCAGGG	TGAGGGATGATCGCTGCTGCCT	This study
m <i>Il22</i>	CCTACATGCAGGAGGTGGTG	AAACAGCAGGTCCAGTTCCT	This study

h, Human; m, mouse; SA, *S. aureus*. SE, *S. epidermidis*.

TABLE E4.

Antibodies used in this study

Antigen	Antibody description	Source	Dilution used for WB
Human DSG-1	Mouse monoclonal IgG2b B-11 (sc-137164)	Santa Cruz Biotechnology, Santa Cruz, Calif	1:100
Human corneodesmosin (CDSN)	Rabbit polyclonal IgG H-80 (sc-134451)		1:100
Human involucrin (IVL)	Goat polyclonal IgG N-17 (sc-15223)		1:100
Human β -actin	Goat polyclonal IgG I-19 (sc-1616)		1:500

WB, Western blot.

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TABLE E5.

Growth of the various CoNS cultures (24-hour) used to prepare supernatants for protease assays

Strain	OD 600 nm	Equivalent CFU/mL
<i>Staphylococcus epidermidis</i> (SE) 1457	12.91	3.87E+09
<i>Staphylococcus epidermidis</i> (SE) ATCC12228	10.68	3.21E+09
<i>Staphylococcus epidermidis</i> (SE) A9	11.03	3.31E+09
<i>Staphylococcus epidermidis</i> (SE) A11	6.34	1.90E+09
<i>Staphylococcus epidermidis</i> (SE) 09-G7	7.19	2.16E+09
<i>Staphylococcus epidermidis</i> (SE) 18-F3	10.17	3.05E+09
<i>Staphylococcus epidermidis</i> (SE) 05-A5	9.22	2.77E+09
<i>Staphylococcus epidermidis</i> (SE) 28-H4	4.28	1.29E+09
<i>Staphylococcus hominis</i> (SH) A9	6.48	1.94E+09
<i>Staphylococcus hominis</i> (SH) C5	10.13	3.04E+09
<i>Staphylococcus capitis</i> (SC) H8	14.26	4.28E+09
<i>Staphylococcus warneri</i> (SW) G2	13.02	3.90E+09
<i>Staphylococcus lugdunensis</i> (SL) E7	9.29	2.79E+09

TABLE E6.

Growth of *S epidermidis* 1457 WT and *ecpA* cultures (24-hour) used to prepare supernatants for protease assays

Strain	OD 600 nm	Equivalent in CFU/mL
<i>Staphylococcus epidermidis</i> (SE) 1457 WT	14.05	4.21E+09
<i>Staphylococcus epidermidis</i> (SE) 1457 <i>ecpA</i>	13.99	4.20E+09

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