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Persistent Nasal Carriage of *Staphylococcus aureus* Is Associated with Deficient Induction of Human β-Defensin 3 after Sterile Wounding of Healthy Skin *In Vivo*▽

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Persistent nasal carriage of *Staphylococcus aureus* is the primary reservoir for this pathogen and a risk factor for infection. The nares of 12 to 30% of healthy individuals are persistently colonized with staphylococci. Elucidating the yet enigmatic determinants of this phenomenon is of major public health interest. We hypothesized that differences in the levels of antimicrobial peptides (AMPs) that are found in human skin and have pronounced antistaphylococcal activity may contribute to this phenomenon. We compared constitutive and induced mRNA levels of RNase 7 and human β-defensin 3 (HBD-3) in healthy and experimentally wounded gluteal skin of 60 volunteers after ascertaining their carrier status through repeated nasal cultures. We found that levels of HBD-3 expression in skin of persistent nasal carriers of *S. aureus* were lower: induced levels in carriers were 63% (95% confidence interval, 43 to 94%; $P = 0.02$) and constitutive levels were 76% (95% confidence interval, 52 to 110%; $P = 0.14$) of those found in noncarriers. No such associations were present for RNase 7. In conjunction with existing knowledge, these findings suggest that healthy individuals with deficient HBD-3 expression in keratinocytes are more prone to persistent nasal colonization with *S. aureus*.

Nasal carriage of *Staphylococcus aureus* is the primary reservoir for this pathogen and a risk factor for infection, explaining the public health interest in elucidating its determinants (25). Despite considerable research efforts that were able to identify a variety of associations (3, 17, 19, 23, 25), our current understanding of host characteristics that allow *S. aureus* to colonize human nares is still incomplete. The identification of additional factors is needed to define groups at increased risk for colonization and to translate this knowledge into the development of biomarkers for clinical application.

In a longitudinal study taking repeated nasal swab specimens from the anterior nares, 50 to 80% of healthy individuals have *S. aureus* in at least one culture of the nasal swab specimens, but only 12 to 30% of the population carry these bacteria constantly over years (17, 24) and are generally referred to as persistent carriers (25). Persistent nasal carriage of *S. aureus* is associated with high pathogen burden (26, 27), increased risk of infection (14), and more extensive spreading of staphylococci into the environment (27), while patterns of intermittent carriage are not (14, 25). Hence, research that ultimately aims at developing preventive interventions should focus on the study of factors promoting persistent *S. aureus* colonization of the nares.

Antimicrobial peptides (AMPs) are important components of the human's innate immune defense and are expressed in epithelial cells of various tissues, at the interface of the host-pathogen interaction. A variety of AMPs have been isolated from human skin, but evidence from *in vivo* studies demonstrating their physiological role in protecting healthy human skin from *S. aureus* colonization is scarce. One published study showed that AMP-blocking antibodies added onto skin explants lead to enhanced growth of *S. aureus* (20). While this approach is useful in demonstrating a protective role of AMPs *per se*, it cannot be used to explore their contribution toward the variation of colonization observed in the population. Earlier research compared expression of human β-defensin 2 (HBD-2)—an AMP with low *in vitro* activity against *S. aureus*—in patients with atopic dermatitis (AD) and psoriasis and concluded that the lower AMP levels found in patients with AD may explain their propensity to be colonized with *S. aureus* (16). More recently, however, the skin of AD patients was found to express higher levels of this AMP than the skin of healthy individuals (1), disproving a role of HBD-2 in controlling colonization with *S. aureus* and illustrating that determinants of colonization under physiological conditions should be studied in healthy individuals.

About half of *S. aureus* nasal carriers also show colonization of the perineum and, to a lesser extent, of other body regions (25). This is obviously a function of manual spread of staphylococci from the nasal reservoir to other sites but may, at least in part, also reflect the presence of common host determinants of colonization within a given individual. Since keratinocytes are lining those parts of the anterior nares that were found to be persistently colonized with *S. aureus* (25), one could hypothesize that, irrespective of localization, all keratinocytes within an individual are deficient in AMP expression, thus allowing staphylococci to persistently colonize the nares and, when they are spread from there, the skin of other body regions as well. However, an association of reduced AMP expression either with *S. aureus* nasal carriage or with colonization at other body sites has not been demonstrated so far (25). On the contrary, studies measuring HBD-2 and α-defensin levels in nasal secretions found higher concentrations in carriers than in noncar-

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riers (2, 22). This seemingly contradictory finding can be explained by an upregulation of inducible AMPs by a local inflammatory response against colonizing *S. aureus* that is obviously present in carriers only (2) and illustrates that the meaningful study of differences in inducible AMP expression among carriers and noncarriers of *S. aureus* has to compare induced AMP levels in both colonized and noncolonized individuals, e.g., through applying standardized stimuli in both.

The discovery of RNase 7 (7) and human β-defensin 3 (HBD-3) (5), two AMPs that are particularly active against *S. aureus in vitro* and expressed in human keratinocytes, justifies reevaluation of a potential association of AMP expression with persistent *S. aureus* nasal carriage. In healthy skin, HBD-3 is found in low concentrations only, but it can be induced in cultured keratinocytes by *S. aureus* (5) via Toll-like receptor 2 and the mitogen-activated protein kinase pathway (12). Interestingly, one study showed that HBD-3 is essential for the constitutive ability of keratinocytes to kill *S. aureus* within 1 h after contact, suggesting an underlying mechanism that is independent from induced gene expression (11). It was observed that this killing requires internalization of bacteria into the keratinocyte via phagocytosis while AMP concentrations secreted into culture medium were insufficient to inactivate *S. aureus* effectively (11). Hence, the authors suggested that locally increased HBD-3 concentrations found in phagosomes into which vesicles containing HBD-3 are mobilized are capable of killing *S. aureus* (11), a model that would resolve the inconsistency between the findings of a low overall HBD-3 protein concentration in healthy skin on the one hand and the proposed role of this AMP for the constitutive killing of *S. aureus* by keratinocytes on the other (11). Although detailed investigations on the exact intracellular localization of HBD-3 are missing, it seems plausible to hypothesize that in colonized keratinocytes with ongoing *S. aureus* phagocytosis, AMP protein content in intracellular compartments depends on the gene expression apparatus that supplies mRNA templates for the replacement of used AMP, once the proposed vesicles with preformed HBD-3 have been emptied and a steady state has been reached. Following this rationale, persistent colonization of keratinocytes could be promoted in part by lower HBD-3 gene transcription rates after contact with *S. aureus* in keratinocytes of carriers than in those of noncarriers. This concept was fostered by recent findings of our group showing that failure to induce HBD-3 mRNA is associated with the clinical severity of *S. aureus* skin infections (29) and highlighting that further experimental evaluation of a model that puts interindividual variations in HBD-3 induction in the context of *S. aureus* colonization could be a promising approach.

In contrast to HBD-3, RNase 7 is present at high levels in healthy skin (7) and is, at least on a transcriptional level, not induced in skin infections caused by Gram-positive bacteria *in vivo* (28, 29). Together, these observations have led to the general perception of RNase 7 as a constitutively expressed AMP, although studies in cultured keratinocytes after stimulation with heat-inactivated *S. aureus* and cytokines and one study quantifying RNase 7 protein in hair follicles suggest additional inducibility (7, 18). Independent from induced transcription, increased RNase 7 protein concentrations can be detected on the skin surface shortly after superficial skin injury, most likely through the release of preformed AMP (20). In contrast, RNase 7 expression after deep wounding of the skin has not been studied so far. Considering the constant rate at which human skin is replaced under physiological conditions, RNase 7 protein concentrations at the skin surface are likely to be a function of the constitutive rate at which RNase 7 gene transcription in deep vital layers of the epidermis takes place. Along this line, our group demonstrated lower constitutive RNase 7 mRNA levels in unaffected skin of individuals with *S. aureus* skin infection than in healthy skin of controls (29), suggesting the importance of RNase 7 for protecting skin from symptomatic infection. This finding and the role of RNase 7 in protecting skin from colonization with *S. aureus* proposed recently (20) allow speculation on whether interindividual differences in constitutive RNase 7 expression may contribute to the population variation of nasal *S. aureus* colonization.

In vivo experiments on the role of induced AMP expression in colonization and infection are methodologically demanding since they require a well-standardized stimulus that cannot rely on potentially harmful pathogens. Sørensen and colleagues reported that sterile wounding of healthy skin also induces the expression of HBD-3 through transactivation of the epidermal growth factor receptor (21). Although induction of HBD-3 expression after stimulation with *S. aureus* is transduced via other pathways, both signals—injury and infection—will ultimately converge in a common final path at the latest on the level of gene transcription. Hence, sterile wounding may be used as a stimulus to investigate an individual's ability to induce HBD-3 expression *in vivo* in an ethical and easy-to-standardize way.

This study aims at elucidating whether deficient HBD-3 and RNase 7 expression in keratinocytes promotes nasal carriage of *S. aureus* and contributes to the variation in nasal colonization found in the general population. To this end, we determined differences in constitutive and induced *in vivo* mRNA expression levels of these AMPs in healthy and experimentally wounded gluteal skin of persistent nasal carriers and noncarriers identified from a cohort of healthy volunteers.

MATERIALS AND METHODS

Study subjects. Healthy volunteers were attracted through public advertising, enrolled in a cohort, and screened for nasal carriage of *S. aureus* by taking four sequential swab specimens from the anterior nares with at least 6 days between each sampling. Individuals with *S. aureus* in cultures of all four swabs and a quantitation score sum of $\geq$11 over all four cultures were defined as persistent carriers; those with four negative swabs were defined as noncarriers. Whenever a carrier was identified, a noncarrier was randomly selected from the cohort. Both were asked to provide a blood sample and a sample of healthy and sterile wounded gluteal skin (4- by 7-mm biopsy punch specimen; Kai Medical). After the specimen of healthy skin was taken, the contralateral gluteal region was wounded using a 1.5- by 7-mm biopsy punch specimen. Exactly 72 $\pm$ 2 h later, this lesion was excised at the center of a second biopsy specimen measuring 4- by 7-mm. All procedures were performed under aseptic conditions. A questionnaire and figures depicting clinical presentations of pyoderma were used to collect information on potential confounders and, in particular, on the subject's history of purulent skin infections.

Laboratory procedures. Within 24 h after sampling, nasal swabs were plated onto a third of a plate of selective solid medium (mannitol salt agar; Oxoid) and fractioned into two further thirds for semiquantification. After incubation for 48 h at 37°C, plates were inspected for mannitol fermentation, and this growth was quantified by assigning a score of 0, 1, or 2 to each third showing no, $<$10, and $\geq$10 CFU that fermented mannitol, respectively. These were added, giving a quantitation score of 1 to 6 per *S. aureus*-positive plate. Plates without mannitol fermentation were further incubated at room temperature for 24 h and then analyzed accordingly. After transfer of colonies onto 5% sheep blood agar

TABLE 1. Characteristics of subjects stratified by *Staphylococcus aureus* nasal carriage status

Characteristic	Persistent carriers (n = 30)	Noncarriers (n = 30)	P value[a]
No. (%) of female gender	14 (47)	18 (60)	0.3
Mean (SD) age (yr)	26.1 (7.4)	25.0 (5.5)	0.5
Mean (SD) ht (cm)	173.2 (9.6)	172.8 (8.2)	0.8
Mean (SD) wt (kg)	71.4 (19.7)	71.2 (14.7)	1.0
No. (%) smokers	3 (10)	3 (10)	1.0
No. (%) of women using hormonal contraception	10 (71)	11 (61)	0.5
No. (%) with a history of allergy	8 (27)	8 (27)	1.0
Natural log of serum IgE concn (IU/ml)	3.2 (1.9)	2.9 (2.0)	0.5
No. (%) with a history of pyoderma	7 (23)	3 (10)	0.2
Median quantitation score sum[b] (IQR[c])	17 (14–18)	0	<0.0001[d]
Mean time from 1st to 4th swab in days (SD)	60.4 (20.3)	59.2 (15.2)	0.8

[a] Unpaired, two-sided t test or chi-square test.
[b] Obtained by adding scores from 1 to 6 of four *S. aureus* positive cultures.
[c] IQR, interquartile range.
[d] Wilcoxon rank-sum test.

(Oxoid), *S. aureus* was identified using a latex coagulation test (Staph Plus; DiaMondiaL) and, in case of a negative result, by additional tube coagulation (BBL coagulase plasma; Becton Dickinson). Skin specimens were stored for 48 h in an RNA stabilization reagent (RNAlater; Ambion) and then frozen at −80°C until analysis. RNA extraction, cDNA synthesis, and real-time PCR were performed as described elsewhere (28), with the modification that only 50 ng of solved RNA was used. In each run, samples of wounded and healthy skin specimens from one carrier and one noncarrier were simultaneously amplified in triplicate. The cycle-to-cycle fluorescence emissions were analyzed using the threshold cycle (C_T) method. The C_T value of a specific sample was determined using the comparative quantitation analysis tool (Rotor-Gene 6000 software, version 6.1; Corbett), and afterwards, ΔC_T was calculated by subtracting the β-actin C_T value from the AMP C_T value. Serum IgE levels were determined using a commercial enzyme-linked immunosorbent assay kit (IBL International).

Statistical analysis. We examined the inducibility of AMP by wounding by calculating the mean of all $\Delta\Delta C_T$ values defined for each individual as follows: $\Delta\Delta C_{T\ \text{inducibilty}} = \Delta C_{T\ \text{wounded skin}} - \Delta C_{T\ \text{healthy skin}}$.

We analyzed differences in AMP expression by *S. aureus* nasal carriage by calculating the mean of all ΔC_T values stratified by carrier status and type of skin sample separately and then by subtracting these to obtain the mean $\Delta\Delta C_T$ for carriers versus noncarriers (which is equal to mean $\Delta C_{T\ \text{all carriers}}$ − mean $\Delta C_{T\ \text{all noncarriers}}$) for induced and constitutive expression of a given AMP. We then tested differences in AMP expression for statistical significance using appropriate t tests. On the basis of the assumption of a doubling of cDNA per PCR cycle, we used a ratio of $2^{-\text{mean }\Delta\Delta CT}$ to express relative mRNA expression on a multiplicative scale. We applied logistic regression to estimate odds ratios (ORs) and to adjust for the following potential confounders (continuous or binary variable): sex, age in years, height in cm, weight in kg, smoking status, history of allergy, serum IgE level in IU/ml (as natural logarithm), and history of pyoderma. To be considered a confounder and included in the final multivariable logistic regression model, the inclusion of a covariate into the univariable model had to lead to an adjustment of at least 0.1 of the crude OR estimate. All statistical procedures were done using Stata software (version 10; StataCorp).

Ethics. The study protocol was approved by the Ethics Committee, Faculty of Medicine, University of Tübingen. All study subjects gave written informed consent.

RESULTS

Table 1 illustrates the distribution of baseline characteristics and potential confounders between persistent carriers and noncarriers of *S. aureus*. None of the study subjects experienced clinically overt infection at the site of sterile wounding.

TABLE 2. Inducibility of antimicrobial peptide expression in human skin by sterile wounding

AMP	mRNA ratio[a] (95% CI[b]) in wounded vs healthy skin	P value[c]
HBD-3	5.20 (4.25–6.37)	<0.0001
RNase 7	0.47 (0.40–0.58)	<0.0001

[a] The ratio is $2^{-\text{mean }\Delta\Delta CT}$ and compares expression levels of AMP mRNA in sterile wounded skin (induced expression) and healthy skin (constitutive expression) from 60 individuals.
[b] CI, confidence interval.
[c] Paired, two-sided t test for the null hypothesis: there is no change of AMP mRNA expression by sterile wounding.

Comparing mRNA levels in specimens of healthy and experimentally wounded skin of all 60 volunteers, irrespective of *S. aureus* nasal carrier status, showed that HBD-3 expression was about 5 times higher in specimens of skin 72 h after sterile wounding than in specimens of healthy skin taken before stimulation. A similar comparison for RNase 7, in contrast, showed that gene expression decreased in skin after sterile wounding to half the level found in healthy skin (Table 2).

Stratifying constitutive and induced mRNA levels by nasal carrier status revealed that persistent presence of *S. aureus* in the nares was associated with detection of 37% lower induced levels of HBD-3 mRNA in sterile wounded gluteal skin (P = 0.02) (Table 3). This corresponds to a 1.8 times higher odds of nasal carriage for every 50% reduction in induced HBD-3 mRNA levels. Adjusting for potential confounding by age and serum IgE levels strengthened the association somewhat (OR = 1.97; P = 0.025). Similarly, we found weak evidence (P = 0.10) that constitutive, i.e., noninduced, expression of HBD-3 was lower in carriers (Table 3). Estimates of RNase 7 mRNA levels, in contrast, were similar in nasal carriers and noncarriers in the adjusted and unadjusted analyses (Table 3).

DISCUSSION

We found that induced levels of HBD-3 expressed in sterile wounded skin were, on average, 37% lower in persistent nasal carriers of *S. aureus* than in noncarriers. This means that a 50% reduction in the levels of HBD-3 mRNA induced in sterile wounded skin is associated with approximately double the risk of being a nasal carrier. A trend in the same direction was observed for constitutive HBD-3 expression in healthy skin. These findings suggest a role of HBD-3 in controlling *S. aureus* nasal colonization. The physiological significance of our findings is supported by studies that demonstrated the ability of *S. aureus* to induce β-defensins in nasal fluid (2), the high *in vitro* activity of HBD-3 against *S. aureus* (5), and the essential role of this AMP for the constitutive killing of *S. aureus* by cultured keratinocytes (11). In conjunction, these and our observations render a causal link between deficient HBD-3 expression in keratinocytes and nasal carriage of *S. aureus* biologically plausible. One could argue that we did not investigate AMP expression in the anterior nares. We acknowledge that the magnitude of HBD-3 induction may well vary within an individual but would also like to point out that induced levels at different body sites of a given subject after comparable stimuli are very likely to be correlated due to the intraindividual similarity of known (4, 8, 9, 13) and unknown determinants. Moreover,

TABLE 3. Constitutive and induced expression of antimicrobial peptides in human skin in persistent carriers of *Staphylococcus aureus*

AMP and expression	mRNA ratio[a] (95% CI[b]) in carriers vs noncarriers	*P* value for mRNA ratio[c]	Crude OR (95% CI)[d]	*P* value for crude OR[d]	Adjusted OR (95% CI)	*P* value for adjusted OR
HBD-3						
Constitutive	0.76 (0.52–1.10)	0.14	1.47 (0.87–2.47)	0.15	1.59 (0.92–2.74)[e]	0.1[e]
Induced	0.63 (0.43–0.94)	0.02	1.83 (1.05–3.18)	0.03	1.97 (1.09–3.56)[f]	0.025[f]
Rnase 7						
Constitutive	0.84 (0.56–1.26)	0.4	1.23 (0.78–1.95)	0.4	1.29 (0.80–2.07)[e]	0.3[e]
Induced	0.84 (0.56–1.27)	0.4	1.22 (0.78–1.92)	0.4	1.22 (0.76–1.94)[f]	0.4[f]

[a] The ratio is $2^{-\text{mean } \Delta\Delta CT}$ and compares expression levels of AMP mRNA in specimens of healthy skin (constitutive expression) and experimental sterile wounded skin (induced expression) of 30 persistent *S. aureus* nasal carriers relative to 30 noncarriers.
[b] CI, confidence interval.
[c] From unpaired, two-sided *t* test for the null hypothesis: expression is the same in carriers and noncarriers.
[d] From univariable logistic regression.
[e] From multivariable logistic regression adjusting for history of pyoderma.
[f] From multivariable logistic regression adjusting for IgE serum levels and age.

deducing AMP expression and regulation in keratinocytes of the nares from observations made in gluteal skin is advantageous, as this approach allows meaningful comparison of induced levels in both carriers and noncarriers. In contrast, previous studies that directly assessed β-defensin expression in colonized and noncolonized nares found higher AMP levels in carriers (2), leading to speculations that not deficient AMP expression but rather resistance of *S. aureus* may be a key player in nasal colonization (25), a hypothesis that is still lacking proof. Based on the presented findings and in line with the reasoning of others (22), we would like to argue that increased AMP levels in nasal fluids are more likely the consequence than the cause of nasal colonization with *S. aureus*, rendering its direct study *in vivo* for the presented purpose methodologically difficult. Inadvertent contamination of the gluteal wound with *S. aureus* or other colonizing bacteria after stimulation leading to additional AMP induction would be another concern with regard to the validity of the reported main association, in particular, if it is not equally distributed between groups. However, one would expect such contamination to be more likely in persistent carriers than in noncarriers, thus leading to an attenuation of the observed effect through, on the average, increased AMP levels in carriers. Hence, elimination of this bias would not invalidate the reported main association but rather strengthen it.

We did not find an association of constitutive RNase 7 mRNA expression with persistent nasal colonization. This finding is rather unexpected, considering its marked activity against *S. aureus*, its high concentration found in human skin (7), and its suggested role in preventing *S. aureus* skin infection (29) and colonization (20). One could argue that RNase 7 is primarily located in superficial nonviable layers of the epidermis devoid of gene transcription and that mRNA levels in lower viable parts of the epidermis, as measured in the presented study, may be a poor reflection of its concentration at the interface of skin and colonizing bacteria. The only study that quantified RNase 7 protein secretion and *S. aureus* colonization intensity at the skin surface (6), however, similarly failed to demonstrate an association. Hence, our current knowledge on RNase 7 expression, despite its likely role in preventing colonization of the skin with *S. aureus per se* (20), is insufficient to support the major importance of this AMP in explaining the population variation of *S. aureus* nasal colonization. Studies with much larger sample sizes are required to further clarify whether, on average, the 16% lower estimates of constitutive and induced expression of RNase 7 in carriers presented here are a chance finding or indicate a minor contribution of this AMP toward the variation of nasal carriage in the population.

We show that experimental sterile wounding of healthy gluteal skin leads to a 5-fold increase in expression of HBD-3 mRNA. This observation confirms the results of one previous study on the effects of sterile injury on AMP expression (21) and is strikingly similar to an about 7-fold HBD-3 increase found in inflamed skin of patients with *S. aureus*-positive skin lesions (28). Sørensen and colleagues could show that upregulation of HBD-3 3 to 4 days after sterile wounding is mediated through transactivation of the epidermal growth factor receptor and that such activation induces concentrations of HBD-3 that are capable of killing *S. aureus*. Interestingly, they described that induction was much less pronounced in one-third of wounded skin explants (21). Although it is difficult to know at which point exactly the signals of wounding and *S. aureus* infection converge into a common final pathway that eventually leads to induced HBD-3 transcription, the experiments by Sørensen et al., in conjunction with our new findings, suggest that sterile wounding may be a useful proxy for studying the physiological role of HBD-3 induction in protecting human skin from infection and colonization *in vivo*.

Similar to observations made for lesions of patients with group A streptococcal skin infections (28), we found that relative RNase 7 mRNA expression in human skin is, with very little variation, 50% lower 3 days after sterile wounding than at the baseline. Considering the consistency of this finding in both infection and deep wounding and the strength of the association presented here, we believe that this observation represents a true effect and raises questions about the physiological importance and the determinants of this downregulation and thus warrants further investigation. At the same time, lower RNase 7 mRNA levels 72 h after wounding illustrate that the role of this constitutive AMP is characterized by a rapid secretion of preformed RNase 7 protein after superficial injury (6, 20) but not by a sustained upregulation of gene expression and support the proposed concept that RNase 7 is an important component of the skin's early and primary response against pathogens (29).

This study has particular strengths. To reduce bias, we defined persistent carriage through qualitative and quantitative analysis of multiple swabs, as advocated previously (15), identified study subjects prospectively from a defined cohort, and performed all procedures for carriers and noncarriers in parallel throughout the study period. Moreover, and unlike other studies in the field, we evaluated and adjusted for confounding. In fact, minor negative confounding was present by serum IgE levels, a proxy measure for a reportedly inhibitory function of Th2 cytokines on HBD-3 expression (8, 10, 13), and a history of purulent skin infections, known to be associated with both lower AMP expression (28, 29) and *S. aureus* nasal carriage (25). Most importantly, this study describes AMP expression in subjects with healthy skin, allowing direct inferences about their physiological function for colonization, a major limitation of research in subjects with atopic dermatitis and psoriasis (1, 6, 16).

Summary and conclusions. The presented results suggest a role of HBD-3 in protecting healthy humans from nasal colonization with *S. aureus*. This study is the first to strongly support the hypothesis that deficiency in AMP expression of keratinocytes promotes *S. aureus* colonization of the nares *in vivo* and provides further evidence for the prevailing notion of a central role of HBD-3 in protecting human skin from *S. aureus* colonization and infection (11, 28). Future studies may want to further explore the genetic determinants of HBD-3 expression (4, 9), the next important step toward identifying risk groups of *S. aureus* colonization and infection.

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