nature portfolio

Peer Review File

An antimicrobial daptide from human skin commensal *Staphylococcus hominis* protects against skin pathogens

Corresponding Author: Dr Alexander Horswill

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Manuscript Review: The antimicrobial daptide from human skin commensal Staphylococcus hominis protects against skin pathogens
Summary
The manuscript investigates the role of a daptide bacteriocin produced by a skin commensal Staphylococcus hominis strain D11 in antagonizing other skin commensals and pathogens. The authors utilize a combination of microbiology, genetics, bioinformatics, biochemistry, and biophysics to elucidate the machinery and mode of action of the S. hominis daptide. The authors have also identified a novel gene encoding for an immunity protein that confers resistance to daptide-mediated killing. Lastly, the authors have demonstrated the therapeutic capability of daptide in an epicutaneous infection with S. aureus in a murine model. Thus, this study highlights the importance of exploring commensal bacteria to identify novel bioactive molecules as antimicrobial agents. The most noteworthy results are the discovery of a novel antimicrobial and the demonstration that this peptide has potential for treatment of S. aureus infections. Overall, the manuscript is presented with rigorous experiments and is well-written. There are only a few suggestions offered.
Specific comments/suggestions
The work is of high significance to the field and related fields and compares very favorably with the established literature.
The rigor of the study is apparent and support the conclusions and claims made in the manuscript.
The methods are sound and rigorously performed. Sufficient detail is provided in the methods for the work to be reproduced.
L22, 67: Suggest adding a few words to highlight the biological relevance of the N2-N2-dimethyl-1,2-propanediamine C-terminus feature. This becomes more apparent later in the manuscript but is not clear when introduced early on.
L68-70: Please provide references for these studies.
L102,115: Please provide a rationale for why S. hominis AH4553 was the strain of choice/ control strain to demonstrate daptide-mediated killing if in Fig. 1B, there were several S. hominis strains that were also sensitive to conditioned media from S. hominis AH5011 strain.
L141-153. Is the reaction mechanism for the fully modified daptide described here supported by data provided in the manuscript or is this a proposed mechanism? There were few references provided to support this.
L172: Please provide a reference for the sentence that states “daptide-resistant S. aureus strain RN4220”. Also, why is this strain resistant and what is the mechanism underlying disparate sensitivities to the daptide (Fig. 1B). Do these encode homI?
L172 and Discussion/Fig. S14. There was little discussion/speculation as to the mechanism underlying how HomI provides resistance, other than it may be membrane spanning. Is there any evidence for HomI and daptide direct interactions?
Line 256, Table S3, Fig. S11A. Are these gene clusters also located on plasmids or are some of these chromosomally encoded?
L289-290: Suggest clarifying that the S. aureus strain expressing homABCDIJM1M2PlanMD is the RN4220 strain expressing hom BGC.

L303: Please specify the WT strain for this experiment.

Lines 327-335. It’s surprising that the 2-log reduction in AH5011 upon hominicin treatment, results in such profound effects on skin barrier function and disease scores. There was no inclusion of a control with hominicin treatment alone in the absence of infection to determine if there is some benefit to overall healing but it’s unclear if this model would address the question. Perhaps an incisional wound model would be better here.

L895: The data in Fig. 1 A, D, and F are represented as OD600 and CFUs where either set of data conveys the same result. Since the experiments in the subsequent figures (Figs. 1E,G, 4C, 6A,B, S2 A-D) are all represented as OD600, consider representing Fig. 1A, D, and F as OD600 only for consistency. Alternatively, provide a rationale for why both OD600 and CFU were chosen to be displayed.
Fig. 1C: Please change the label from Spot Strain to Lawn Strain.
Fig. 1F: Please remove the misplaced text box with “D” within the graph.
Fig. 7 and methods: Please report the sex of the mice used in these studies and the rationale for this choice. The methods section should include whether sex and/or gender were considered in the study design and whether sex and/or gender of participants was determined based on self-report or assigned (and methodology used).

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Daptides are a small, relatively new family of RiPPs characterized by the presence of an unusual (S)-N2-N2-dimethyl-1,2 propanediamine (Dmp)-modified C-terminus. Homincin is retrospectively considered as the inaugural compound of the daptide RiPP family, as it had previously been isolated and structurally characterized from Staphylococcus hominis MBBL by Pyoung Il Kim and co-workers (Biochemical and Biophysical Research Communications 2010, 399, 133–138). However, nothing was known about its biosynthesis. Recently, a seminal paper on daptides established most of the structural and biosynthetic aspects of a family of daptide compounds isolated from Microbacterium paraoxydans (Ren H. et al., Nat Commun 2023, 14, 1624).

In this work, the authors report the discovery of the plasmid-encoded biosynthetic gene cluster (BGC) of hominicin from a human skin isolate of Staphylococcus hominis. The authors demonstrate the link between (the known) hominicin and the (unknown) hom BGC by heterologous expression and propose a plausible and logical biosynthetic pathway to the daptide compound. However, the role or the output of some key enzymes in the BGC are just assumed but not proved. Of note is the role of HomI in the self-resistance of the producing strain, the first time this mechanism of immunity is reported in daptide-producing strains.
The authors clearly showcase the narrow-spectrum antibacterial activity of hominicin against a set of skin commensal Staphylococcus strains (and other genera of commensal bacteria), which aligns perfectly aligns with the ecological niche roles of bacteriocins. They further elaborate on the mechanism of action and successfully establish the pore-forming activity of hominicin. Furthermore, they demonstrate that hominicin is able to prevent S. aureus infection in vivo (mouse skin).

Overall, the results are comprehensive, well-substantiated and very well presented. This work represents an advance in the knowledge of the biosynthesis, biological activity and mechanism of action of daptides, especially considering that daptides remain an understudied and slowly growing family of RiPPs.

Before publication, some points need to be addressed:

A. Major comments:

1) My main concern relates to the N,N-dimethyl Ile (Dmlle1) at the N-terminus of homincin. Considering that this post translational modification (PTM) is so far restricted to hominicin but it is absent in the other few daptide compounds characterized so far (Ren H. et al., Nat Commun 2023, 14, 1624), I believe that author should provide experimental evidences about this N,N-dimethylation.
They propose that HomM1 is likely responsible for this methylation, but nothing has been done to prove it. I think they should conduct some experiments on it, as they have done for other enzymes in the BGC. Also, this experiment could give valuable insight into the importance or not of this N-terminal dimethylation on the bioactivity or even the mechanism of action of homincin. Of note, the N-terminal methylation has been proved to be essential in the bioactivity of cypemycin and other linaridin RiPPs (e.g.; Claesen J, Bibb M. Proc Natl Acad Sci USA 2010, 107:16297–16302).

2) Regarding the stereochemistry of hominicin, I greatly miss experimental confirmation. Hominicin is a previously known compound, but the stereochemistry remains a knowledge gap in its structure.
Of course, the authors know that being a RiPP, most of the constituent amino acids will be L. However, they claim that genetically encoded Ser-10 and Ser-17 are converted to Dha by dehydratase HomMD and then reduced to D-Ala residues by oxidoreductase HomJ. This explanation is perfectly consistent with the biosynthetic logic of lantipeptides, but I think it should be empirically tested by Marfey´s analysis. Moreover, this experiment would make it possible to completely rule out

the presence of cryptic epimerases acting on the precursor peptide.

Instead of tedious partial hydrolysis and derivatizations, a full hydrolysis and determination of the L-Ala to D-Ala ratio would suffice, as has been done in previous works (e.g.; Ortiz-López et al. Angewandte Chemie International Edition, 2020, 59(31), 12654-12658). In addition, this experiment would further confirm the presence of Glu-13 (of which most NMR signals are not observed) in the structure.

As for the stereochemistry of the Dmp residue at the C-terminus, I think it is quite reasonable to assume the absolute (S) configuration based on biosynthetic arguments, but the authors should make this argument more clearly explicit in the hominicin structure characterization part of the manuscript.

B. Apart from the above, some minor questions on the bioactivity and mechanism of action of hominicin:

3) The authors provide valuable insights into the mechanism of action of hominicin. However, I am not sure about the extrapolation of these results to daptides in general, as the authors state in the introduction and conclusions (last sentence of the last paragraph of the manuscript). As they themselves discuss in the manuscript, there is some genetic diversity within the daptide BGCs found in staphylococcal strains, and these differences include the presence or not of PTM enzymes such as LanMD dehydratases and/or HomJ oxidoireductases. It is possible that other novel daptides with additional structural features will later be discovered that modify the molecules to the extent that the mechanism of action changes (but they will still be daptides).

4) Although the authors showcase the in vivo activity of hominicin (mouse skin infection model), I think the therapeutic applicability remains doubtful unless additional experiments such as cytotoxicity and hemolytic activity are done. Do the authors have data on this?

5) Hominicin does not seem to inhibit S. capitis, any plausible explanation?

6) I have not been able to find any information on the S. aureus strain AH6350, which was used in the work to show bioactivity in vitro and in vivo (mouse skin infection model). The authors should add an appropriate reference(s) on this pathogenic strain, particularly those related to antimicrobial resistance mechanisms and/or membrane-level characterization.

C. Finally, there are other very minor comments:

i) Line 139: This part of the sentence is a bit confusing; the reader could understand that there are some structural differences between hominicin and the compound identified in this work (which is actually the same). Please rewrite it.

ii) Line 168: I would not say that NMR was just an orthogonal approach to confirm the structure, but that it was applied to unequivocally confirm the structure.

iii) Line 179: I miss the data of the amount of compound isolated, and I could not find this data in the supporting information. Please include this data.

iv) Line 207: The reading of abbreviation for the mutant HomACIM1PlanMD was a bit confusing to me. I would change it to HomACIM1PLanMD in line 207 and the successive times it appears (at least in the manuscript text).

v) Line 216: Actually, the first ion of the MS cluster should be the previous m/z (1026.0642, calculated with Bruker Isotope Pattern tool). Please reflect this also in Figure S9C

vi) Line 339: I would write “understudied” instead of “unstudied”.

vii) Line 394: I agree that the differences between Mpa daptides and hominicin regarding their bioactivity profiles are probaly due to the presence of dehydroamino acids in the latter, but the authors should not forget that the other key difference is the N-terminal dimethylation. This kind of modification has been proved essential for the bioactivity of cypemycin and other linaridins, as commented above (comment 1)

viii) Line 553: The NMR spectra were recorded in methanol-d3 (CD3OH). Were the experiments carried out under a solvent-presaturation scheme? If so, please indicate it in the experimental section.

ix) Figure 7C: Images are not clear. They appear blurry or pixelated. I think they should be improved.

x) Supporting Information, Figure S3: I´m a bit surprised by the impressive accuracy of the LC-MS1 data shown across different samples. The retention time and the m/z are completely identical for both hominicin AH5011(wt) and homABCDIJM1M2PlanMD mutant. Perhaps the authors have inadvertently duplicated the same figure.

xi) Supporting Information, Figure S9C: (see also comment v above).

--At entry 1 of the table, ion y9 is not correct. It should be m/z 868.xxx instead 852.4864. In the corresponding peptide

sequence, there is another typo: it reads Glu-Ile-Dhb-Dha-Ala-Val-Ile-Ala-Thr, but it should be Glu-Ile-Dhb-Ala-Dha-Val-Ile-Ala-Thr.

--From entry 7 (ion b4) to entry 12 (ion b11) there are some erroneous annotations of m/z values and/or aa sequence. Please check it before publishing.

xii) Supporting Information, Table S4: The coupling constant (J) value for γ´-CH3 signal of Val-18 is not feasible. Please revise this probable typo.

Reviewer #4

(Remarks to the Author)
Review of Nguyen et al
This manuscript is an activity-based natural product isolation story focused on a molecule made by members of the skin microbiome. The molecule in question is a RiPP (ribosomal natural product) called a daptide. These RiPPs are distinguished by the presence of methylated amine groups found at both the N- and C-termini. Previous work on daptides clearly laid out the biosynthesis of these molecules but showed only minimal characterization of bioactivity. The current manuscript convincingly shows that daptides originating from Staph. are membrane active and depolarize the membrane. In this current work the authors also discover an immunity factor for daptides, a YidC homolog. Finally, the authors also show that the daptide, named hominicin, is effective at combatting Staph aureus skin infections in mice. If there is any substantial criticism of the work it is lack of novelty of the hominicin compound, but in my opinion this weakness is overcome by the new insights into the structure-activity relationship, the discovery of a highly unusual immunity factor, the clear experiments into the mechanism of action, and the demonstration of efficacy in a mouse skin infection model. I am highly supportive of publication pending some clarifications/edits delineated below.
1) Line 60, make sure to include the AD abbreviation when atopic dermatitis is first mentioned
2) Line 139, this sentence should be altered a bit to make it clear that the disagreement is at positions 10 and 17 of the core peptide, not the precursor. This is only clear when looking at Figure 2. Moreover, it is still unclear to me after reading the paper if there is any difference between the homincin in ref 17 and what is reported here. Finally, and related to this, can the authors include a comparison or sequence alignment of hominicin(s) (if they are different) and the daptide from ref 12?
3) Line 147, related to the above point, how does the hominicin BGC compare to the daptide BGC in ref 12?
4) Line 172, is the RN4220 strain resistant because it is a hominicin producer? Or does it just harbor the gene encoding HomI?
5) Line 179, can the authors provide the peptide yield after purification to homogeneity?
6) Line 183: the reference to Figure S5 is incorrect, this is a standard curve
7) Line 203: too many significant figures on the report zone sizes
8) Line 207-217: this section was hard for me to follow because it is difficult to keep track of what all of the genes do. This may be improved when the Figure 4 is put near this text, but I might still suggest that the authors explain briefly what each variants is omitting
9) Line 222: ref 12 did show that the original daptides had hemolysis activity. I agree that this isn't the same as forming pores, but the hemolysis activity should probably be mentioned here
10) Line 283: this section discusses the presence of homI in the Staph BGCs, but what about the set of ~500 daptides predicted in ref 12? Is the presence of homI more general or is it Staph-specific?
11) Line 286: when I see the phrase "homI mutant" I think of a deletion mutant, but this is an overexpression mutant. I suggest the authors make this clear and mention whether the homI expression plasmid is multicopy
12) Line 326: express should be expresses
13) Line 328: I find the statement that "infection with S. aureus was augmented by the topical addition…" confusing. The word augment could suggest that homincin was helping colonization of S. aureus
14) Line 339: I think the authors mean understudied
15) Figure 1: there is an extra letter D floating in panel F
16) Figure 1B caption: Table S1 in the SI does not seem to include information about all of the strains used for susceptibility testing. This information is important to include if others want to follow up on the work

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done an excellent job of responding to the critiques raised in the prior review and there are no additional concerns.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Dear corresponding author,

I appreciate the additional experimental work and thank the detailed responses to all my questions

Reviewer #4

(Remarks to the Author)
The authors have satisfactorily addressed my concerns

REVIEWER COMMENTS

Reviewer #1

The manuscript investigates the role of a daptide bacteriocin produced by a skin commensal Staphylococcus hominis strain D11 in antagonizing other skin commensals and pathogens. The authors utilize a combination of microbiology, genetics, bioinformatics, biochemistry, and biophysics to elucidate the machinery and mode of action of the S. hominis daptide. The authors have also identified a novel gene encoding for an immunity protein that confers resistance to daptide-mediated killing. Lastly, the authors have demonstrated the therapeutic capability of daptide in an epicutaneous infection with S. aureus in a murine model. Thus, this study highlights the importance of exploring commensal bacteria to identify novel bioactive molecules as antimicrobial agents. The most noteworthy results are the discovery of a novel antimicrobial and the demonstration that this peptide has potential for treatment of S. aureus infections. Overall, the manuscript is presented with rigorous experiments and is well-written. There are only a few suggestions offered. The work is of high significance to the field and related fields and compares very favorably with the established literature. The rigor of the study is apparent and support the conclusions and claims made in the manuscript. The methods are sound and rigorously performed. Sufficient detail is provided in the methods for the work to be reproduced.

Response: We appreciate the positive comments from the reviewer.

L22, 67: Suggest adding a few words to highlight the biological relevance of the N2-N2-dimethyl-1,2-propanediamine C-terminus feature. This becomes more apparent later in the manuscript but is not clear when introduced early on.
Response: To the best of our knowledge, the seminal work by Ren *et al.* (DOI: 10.1007/s12223-013-0259-1) has thoroughly investigated the biochemical and functional aspect of the N_2-N_2-dimethyl-1,2-propanediamine (Dmp)-modified C-terminus. No other works have examined the Dmp feature. To date, the identified Dmp-containing peptides include MpaA1, MpaA2, and MpaA3 from *M. paraoxydans* and hominicin; however, the genetic and biosynthetic origin of the latter, as a RiPP, remains undetermined. We now provide this information in the text with works cited (Line 68-76).

L68-70: Please provide references for these studies.
Response: We have added references citing these studies (Line 68).

L102,115: Please provide a rationale for why S. hominis AH4553 was the strain of choice/ control strain to demonstrate daptide-mediated killing if in Fig. 1B, there were several S. hominis strains that were also sensitive to conditioned media from S. hominis AH5011 strain.
Response: We selected *S. hominis* AH4553 as a sensitive control strain based on several factors: it is a well-established reference strain from the American Type Culture Collection (also referred to as ATCC 27844) that was isolated from human skin, is commercially available, and has a publicly available genome in the National Center for Biotechnology Information database through the National Institute of Health.

This information is provided in the text and Supplementary Table 1. “We observed that the antimicrobial function inhibits the *S. hominis* reference strain ATCC 27844 (AH4553) from the American Type Culture Collection in a concentration-dependent manner (Fig. 1 c, d, e). This strain was adopted as a sensitive control strain for subsequent experiments.”

L141-153. Is the reaction mechanism for the fully modified daptide described here supported by data provided in the manuscript or is this a proposed mechanism? There were few references provided to support this.
Response: The description of the post-translational modifications described in this section represent a proposed biosynthetic model based on the genetic data and is later supported by cloning experiments, MS, NMR, and Marfey's analysis. Furthermore, the chemical modification of the C-terminal threonine into Dmp has been extensively investigated in previous work by Ren *et al*. (DOI: 10.1007/s12223-013-0259-1). The dehydration of serines and threonines, as well as the oxidoreduction of dehydroalanine intermediates, are well documented in RiPP biosynthesis. Additional references have been added to the text of this section to support the biosynthetic model.

L172: Please provide a reference for the sentence that states "daptide-resistant S. aureus strain RN4220". Also, why is this strain resistant and what is the mechanism underlying disparate sensitivities to the daptide (Fig. 1B). Do these encode homI?
Response: After careful review, we believe it is not appropriate to describe *S. aureus* strain RN4220 as "daptide-resistant" because we did not perform an antimicrobial MIC test to establish its resistance and have relied on the conditioned media growth experiment as an indicator of susceptibility. We have now revised the text to read, "We cloned the 9.6-kb BGC on the plasmid pCM28 and heterologously expressed the construct in a restriction-deficient cloning host *S. aureus* strain RN4220."

We confirmed that RN4220 (GenBank Accession CP076105) does not encode either a biosynthetic gene cluster for hominicin or harbor the *homI* gene. RN4220 does encode two putative *yidC_1* and *yidC_2* genes, which share structural similarity with HomI and may contribute to the observed resistance phenotype. With limited number of sequencing data, we cannot verify with certainty that all resistant strains encode *homI* and/or homologs of HomI. With that being said, we also cannot rule out other potential mechanism(s). The disparate sensitivities to hominicin could be attributed to canonical transporter systems for bacteriocins (e.g., ATP-binding cassette transporters), which can confer cross-resistance to other bacteriocins. Another plausible explanation is that the concentration of hominicin in the CM may be insufficient to elicit observable sensitivity and could vary between batches. We have included additional text addressing these alternative explanations in the Discussion section.

L172 and Discussion/Fig. S14. There was little discussion/speculation as to the mechanism underlying how HomI provides resistance, other than it may be membrane spanning. Is there any evidence for HomI and daptide direct interactions?
Response: To the best of our knowledge, there is little to no literature reporting mechanisms of daptide resistance and/or HomI-specific resistance. To investigate potential interactions, we performed protein-ligand modelling using Boltz-1x with HomI protein sequence and hominicin ligand SMILES as inputs (see Fig. 6, Supplementary Fig. 15). Our structural modeling shows hominicin docks within HomI's charged core with high predictability. While YidC facilitates protein insertion into the membrane, HomI may sequester hominicin within its core or participate in the transport of hominicin. Other studies also suggest that YidC is involved in immunity against bacteriocin and lantibiotics. We have provided these speculations and the related references in the Discussion section.

Line 256, Table S3, Fig. S11A. Are these gene clusters also located on plasmids or are some of these chromosomally encoded?
Response: In our initial analysis of fourteen staphylococcal assemblies, we identified one closed plasmid sequence from a fully assembled genome of *S. epidermidis* strain ADQBK (Accession

CP134841) that encodes a daptide gene cluster. The remaining thirteen other assemblies are shotgun sequences and cannot be verified with certainty whether the daptide BGCs they encode are chromosomally or plasmid-derived. We have adjusted the text accordingly to clarify that the genome assemblies used for comparison were sourced from shotgun sequencing data.

L289-290: Suggest clarifying that the S. aureus strain expressing homABCDIJM1M2PlanMD is the RN4220 strain expressing hom BGC.
Response: We thank the reviewer for their suggestion. We have revised the text to read, "the *S. aureus* strain RN4220 expressing *hom* BGC," which improves clarity.

L303: Please specify the WT strain for this experiment.
Response: We thank the reviewer for their comment. We have revised the text to specify the strain that now reads, "the wild-type strain *S. aureus* Newman," which indicates that the strain Newman was used as the parental strain in this experiment.

Lines 327-335. It's surprising that the 2-log reduction in AH5011 upon hominicin treatment, results in such profound effects on skin barrier function and disease scores. There was no inclusion of a control with hominicin treatment alone in the absence of infection to determine if there is some benefit to overall healing but it's unclear if this model would address the question. Perhaps an incisional wound model would be better here.
Response: We have now included *in vivo* data showing hominicin treatment alone in the absence of *S. aureus* infection (see Fig. 7 and Supplementary Fig. 16). We believe that the improvements in skin barrier function and disease scores are attributed to the reduction or absence of *S. aureus* burden rather than to hominicin promoting skin healing. *S. aureus* is known to secrete various virulence factors (e.g., exoenzymes, pore-forming toxins, superantigens) that contribute to skin barrier disruption and inflammation. We have revised the text to clarify this.

L895: The data in Fig. 1 A, D, and F are represented as OD600 and CFUs where either set of data conveys the same result. Since the experiments in the subsequent figures (Figs. 1E,G, 4C, 6A,B, S2 A-D) are all represented as OD600, consider representing Fig. 1A, D, and F as OD600 only for consistency. Alternatively, provide a rationale for why both OD600 and CFU were chosen to be displayed.
Response: We appreciate the reviewer's suggestion and agree that the growth curve represented as OD_{600} conveys the same results as CFUs. We believe that displaying the CFU data strengthens the accuracy and biological relevance of the OD_{600} measurements. While OD_{600} provides information on culture turbidity and allows quick real-time monitoring of growth, it does not distinguish cell viability. CFU counts reflect actual viability, cytotoxicity, and antimicrobial effects, thus serve as a standard validation metric for growth curve experiments. Having validated our growth experiments using CFU counts, we rationalized that OD_{600} measurements can be used as indicators of inhibition in subsequent experiments.

Fig. 1C: Please change the label from Spot Strain to Lawn Strain.
Response: We thank the reviewer for the comment and fixed the typo for Figure 1c.

Fig. 1F: Please remove the misplaced text box with "D" within the graph.
Response: We thank the reviewer for the comment and removed the misplaced label.

Fig. 7 and methods: Please report the sex of the mice used in these studies and the rationale for this choice. The methods section should include whether sex and/or gender were considered in the study design and whether sex and/or gender of participants was determined based on self-report or assigned (and methodology used).

Response: We used five male and five female 8-week-old C57BL6/J mice per condition for the epicutaneous infection experiment. The sex of mice is mentioned in the Result, and the rationale has been added in the Methods section, along with relevant literature citing the use of this model.

For the isolation of bacteria from skin swabs, we recruited human participants of both sexes and all ethnicities. Each bacterial isolate is linked to anonymized, self-reported information about the age and sex of sampled volunteer. This information is now explicitly stated in the Methods section.

Reviewer #2

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Response: We appreciate the reviewer's evaluation of this manuscript.

Reviewer #3

Daptides are a small, relatively new family of RiPPs characterized by the presence of an unusual (S)-N2-N2-dimethyl-1,2 propanediamine (Dmp)-modified C-terminus. Hominicin is retrospectively considered as the inaugural compound of the daptide RiPP family, as it had previously been isolated and structurally characterized from Staphylococcus hominis MBBL by Pyoung Il Kim and co-workers (Biochemical and Biophysical Research Communications 2010, 399, 133–138). However, nothing was known about its biosynthesis. Recently, a seminal paper on daptides established most of the structural and biosynthetic aspects of a family of daptide compounds isolated from Microbacterium paraoxydans (Ren H. et al., Nat Commun 2023, 14, 1624).

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Overall, the results are comprehensive, well-substantiated and very well presented. This work represents an advance in the knowledge of the biosynthesis, biological activity and mechanism of action of daptides, especially considering that daptides remain an understudied and slowly growing family of RiPPs.

Before publication, some points need to be addressed:

A. Major comments:

1) My main concern relates to the N,N-dimethyl Ile (DmIle1) at the N-terminus of hominicin. Considering that this post translational modification (PTM) is so far restricted to hominicin but it is absent in the other few daptide compounds characterized so far (Ren H. et al., Nat Commun 2023, 14, 1624), I believe that author should provide experimental evidence about this N,N-dimethylation. They propose that HomM1 is likely responsible for this methylation, but nothing has been done to prove it. I think they should conduct some experiments on it, as they have done for other enzymes in the BGC. Also, this experiment could give valuable insight into the importance or not of this N-terminal dimethylation on the bioactivity or even the mechanism of action of hominicin. Of note, the N-terminal methylation has been proved to be essential in the bioactivity of cypemycin and other linaridin RiPPs (e.g.; Claesen J, Bibb M. Proc Natl Acad Sci USA 2010, 107:16297–16302).

Response: In response to this point, we made an expression strain lacking the *homM1* gene and observed a pronounced loss of antimicrobial activity (Fig. 4). We performed mass spectrometric analysis of the spent media from the mutant and detected the proposed molecule displayed in Supplementary Fig. 11b, which is supported by MS/MS analysis. As expected, this peptide lacks the N-terminal methylations while retaining formation of D-Ala from L-Ser. Surprisingly, the observed product also lacked the Dmp modification on the C-terminus. The biosynthetic rationale for this is unclear as the installation of the C-terminal modifications should precede removal of the leader peptide needed for N-terminal methylation (Ren H. et al., Nat Commun 2023, 14, 1624). We speculate that perhaps the removal of *homM1* impacted expression of the daptide specific genes or that the Dmp biosynthetic enzymes are not leader dependent. To evaluate this further, we attempted to generate a knockout of the *homM2* but was unsuccessful. We next produced a *homD* knockout. As shown in Supplementary Fig. 11c, we observed formation of a hominicin analogue with the expected N-terminal methylations and D-Alas, but a C-terminal ketone instead of the fully modified Dmp. This indicates that the N-terminal methylations can be installed even without formation of the Dmp motif.

2) Regarding the stereochemistry of hominicin, I greatly miss experimental confirmation. Hominicin is a previously known compound, but the stereochemistry remains a knowledge gap in its structure. Of course, the authors know that being a RiPP, most of the constituent amino acids will be L. However, they claim that genetically encoded Ser-10 and Ser-17 are converted to Dha by dehydratase HomMD and then reduced to D-Ala residues by oxidoreductase HomJ. This explanation is perfectly consistent with the biosynthetic logic of lantipeptides, but I think it should be empirically tested by Marfey´s analysis. Moreover, this experiment would make it possible to completely rule out the presence of cryptic epimerases acting on the precursor peptide.

Instead of tedious partial hydrolysis and derivatizations, a full hydrolysis and determination of the L-Ala to D-Ala ratio would suffice, as has been done in previous works (e.g.; Ortiz-López et al. Angewandte Chemie International Edition, 2020, 59(31), 12654-12658). In addition, this experiment would further confirm the presence of Glu-13 (of which most NMR signals are not observed) in the structure.

As for the stereochemistry of the Dmp residue at the C-terminus, I think it is quite reasonable to assume the absolute (S) configuration based on biosynthetic arguments, but the authors should make this argument more clearly explicit in the hominicin structure characterization part of the manuscript.

Response: In response to these important points, we have now performed a full hydrolysis of hominicin followed by Marfey's derivatization and LC-MS analysis to determine the ratio of L-Ala to D-Ala residues. The results confirmed the presence of D-Ala in hominicin, consistent with the

biosynthetic model and supporting the stereochemical assignment. These new data have been incorporated into the Supporting Information (refer to Supplementary Fig. 7) and summarized in the manuscript text (Line 173-181). Moreover, we have quantified the ratio of L:D Ala and found it to be 1.2:1. This is consistent with our hypothesis of three L-Alas and two D-Alas (1.5:1).

Regarding the Dmp residue, we agree that the (*S*) configuration is most likely because published work (DOI: 10.1007/s12223-013-0259-1) confirmed the (*S*) configuration for other daptides. We have added a statement to this effect in the structure elucidation section of the manuscript, as follows:

"The configuration of the stereocenter of the Dmp group on the C-terminus was not confirmed, although previous work has shown this functional group to have an (*S*) configuration in other daptides."

B. Apart from the above, some minor questions on the bioactivity and mechanism of action of hominicin:

3) The authors provide valuable insights into the mechanism of action of hominicin. However, I am not sure about the extrapolation of these results to daptides in general, as the authors state in the introduction and conclusions (last sentence of the last paragraph of the manuscript). As they themselves discuss in the manuscript, there is some genetic diversity within the daptide BGCs found in staphylococcal strains, and these differences include the presence or not of PTM enzymes such as LanMD dehydratases and/or HomJ oxidoreductases. It is possible that other novel daptides with additional structural features will later be discovered that modify the molecules to the extent that the mechanism of action changes (but they will still be daptides).

Response: We agree with the reviewer that the mechanism of action for hominicin–specifically pore formation–may not apply to all daptides. Ren *et al*. (DOI: 10.1007/s12223-013-0259-1) demonstrated that the Mpa daptides are not antimicrobial but exhibit hemolytic activity. The authors suggested that the combined internal hydrophobicity and the charge modification of the terminus may indicate a shared membrane-targeting mode of action among daptides. To address this, we revised the text (Line 85, 497) to clarify that the proposed mode of action pertains to hominicin.

4) Although the authors showcase the in vivo activity of hominicin (mouse skin infection model), I think the therapeutic applicability remains doubtful unless additional experiments such as cytotoxicity and hemolytic activity are done. Do the authors have data on this?

Response: We thank the reviewer for these suggestions and have now included hemolysis data using whole human blood as well as cytotoxicity data using immortalized N/TERT keratinocytes (Supplementary Fig. 13). We did not observed differences in hemolysis of the AH5011 extract when compared to treatment with the Δp1Δp2 extract. This finding is supported by data using live bacteria. While the *S. aureus* strains exhibited pronounced hemolysis and cytotoxicity, *S. hominis* AH5011 is comparable to the control treatment (PBS). Additionally, we've included the hominicin treatment alone without *S. aureus* infection on mouse skin and observed minimal skin damage (Fig. 7 and Supplementary Fig. 16).

5) Hominicin does not seem to inhibit S. capitis, any plausible explanation?

Response: We observed varying levels of susceptibility to AH5011's conditioned media (CM) in *S. capitis*, and one plausible explanation is that the concentration of hominicin in the CM is insufficient to elicit observable sensitivity in *S. capitis.* While this work primarily focuses on the

mechanism of producer immunity–specifically the role of HomI–we cannot rule out other potential mechanism(s). It is also possible that *S. capitis* may encode homologs of HomI or canonical transporter systems for bacteriocins (e.g., ATP-binding cassette transporters).

6) I have not been able to find any information on the S. aureus strain AH6350, which was used in the work to show bioactivity in vitro and in vivo (mouse skin infection model). The authors should add an appropriate reference(s) on this pathogenic strain, particularly those related to antimicrobial resistance mechanisms and/or membrane-level characterization.
Response: The *S. aureus* strain AH6350 has not been previously published. Characterization of this strain was primarily done through this work, which included genomic information, antimicrobial susceptibility test, and hemolysis and cytotoxicity data. We rationalized that AH6350 is a suitable organism for *in vivo* studies due to its ability to cause cutaneous damage on mouse skin. To support this, we now include hemolysis and cytotoxicity data for AH6350 (Supplementary Fig. 13), comparing it to the well-known USA300 lineage strain AH1263 and the Δ*agr* mutant to highlight its pathogenic potential. While we cannot provide references to cite this specific clinical strain, we have added additional work citing the epidemiology, antimicrobial resistance, virulence, and characterization of the sequence type 15 (ST15).

C. Finally, there are other very minor comments:

Line 139: This part of the sentence is a bit confusing; the reader could understand that there are some structural differences between hominicin and the compound identified in this work (which is actually the same). Please rewrite it
Response: We believe the confusion arises from a textual error. The difference is at positions 10 and 17 of the core peptide, not the "precursor peptide" as originally stated. We apologize for this error and have revised the text accordingly.

"We detected differences at the 10^{th} and 17^{th} residues between the core peptide of the AH5011 bacteriocin and the final structure of hominicin (Supplementary Fig. 14d). Although the genetic and biosynthetic information of the hominicin-producing strain MBBL 2-9 are not publicly available, we infer that the AH5011 bacteriocin is likely hominicin."

Line 168: I would not say that NMR was just an orthogonal approach to confirm the structure, but that it was applied to unequivocally confirm the structure.
Response: We thank Reviewer 3 for the observation and agree with the comment. We have addressed the suggestion and rephrased the sentence accordingly. "1D and 2D NMR experiments were carried out to confirm the proposed structure of AH5011 hominicin."

Line 179: I miss the data of the amount of compound isolated, and I could not find this data in the supporting information. Please include this data.
Response: We have now included the amount of compound isolated (approximately 6.4 mg) in the main text.

Line 207: The reading of abbreviation for the mutant HomACIM1PlanMD was a bit confusing to me. I would change it to HomACIM1PLanMD in line 207 and the successive times it appears (at least in the manuscript text).
Response: We thank the reviewer for their thoughtful suggestion and have revised the text accordingly.

Line 216: Actually, the first ion of the MS cluster should be the previous m/z (1026.0642, calculated with Bruker Isotope Pattern tool). Please reflect this also in Figure S9C.

Response: We believe this confusion may arise from the way the mass spectrum was labeled in Figure 9a. According to ChemDraw's isotope distribution calculator, the most abundant isotope has an *m/z* of 1026.5659, which aligns with our experimental data (*m/z* 1026.5684). The *m/z* 1026.0642 corresponds to the second most abundant isotope. We have revised Figure S9a, which is now Supplementary Fig 10a, to clarify this.

Line 339: I would write "understudied" instead of "unstudied".
Response: We thank the reviewer for the comment and the typo has been fixed.

Line 394: I agree that the differences between Mpa daptides and hominicin regarding their bioactivity profiles are probaly due to the presence of dehydroamino acids in the latter, but the authors should not forget that the other key difference is the N-terminal dimethylation. This kind of modification has been proved essential for the bioactivity of cypemycin and other linaridins, as commented above (comment 1).
Response: We have revised the text to mention that the Mpa daptides lack a dimethylated N-terminus.

Line 553: The NMR spectra were recorded in methanol-d3 (CD3OH). Were the experiments carried out under a solvent-presaturation scheme? If so, please indicate it in the experimental section.
Response: Following the reviewer's suggestion, we have included the pulse sequence used for the NMR data collection, and the corresponding section in the method has been rephrased as follows: NMR experiments were performed on an Agilent 700 MHz NMR spectrometer equipped with a cryoprobe, operating at 700 MHz for ^{1}H and 175 MHz for ^{13}C. Spectra were collected using a water suppression enhanced through T1 effects (WET) pulse sequence. The sample was dissolved in methanol-d_3, and the residual solvent signals (δ_H = 3.31 ppm and δ_C = 49.0 ppm) were used as reference peaks.

Figure 7C: Images are not clear. They appear blurry or pixelated. I think they should be improved.
Response: The blurry images of mouse skin have been replaced with the highest resolution available.

Supporting Information, Figure S3: I´m a bit surprised by the impressive accuracy of the LC-MS1 data shown across different samples. The retention time and the m/z are completely identical for both hominicin AH5011(wt) and homABCDIJM1M2PlanMD mutant. Perhaps the authors have inadvertently duplicated the same figure.
Response: We thank the reviewer for catching this error. Upon review, we found that panels Supplementary Fig. 3a and 3c were inadvertently duplicated during figure assembly. Panels 3b and 3d are correct as presented. We have now replaced Supplementary Fig. 3a with the correct data and updated the Supporting Information accordingly.

Supporting Information, Figure S9C:

--At entry 1 of the table, ion y9 is not correct. It should be m/z 868.xxx instead 852.4864. In the corresponding peptide sequence, there is another typo: it reads Glu-Ile-Dhb-Dha-Ala-Val-Ile-Ala-Thr, but it should be Glu-Ile-Dhb-Ala-Dha-Val-Ile-Ala-Thr.

--From entry 7 (ion b4) to entry 12 (ion b11) there are some erroneous annotations of m/z values and/or aa sequence. Please check it before publishing.

Response: We thank the reviewer for their careful examination of the table. Upon review, we confirmed that ion y9 was not observed in the fragmentation spectrum and have removed this entry from the table. Additionally, we have corrected the erroneous entries noted from entry 7 -12. The updated table now accurately reflects the correct *m/z* values and fragment assignments. These revisions have been incorporated into the Supporting Information.

Supporting Information, Table S4: The coupling constant (J) value for γ´-CH3 signal of Val-18 is not feasible. Please revise this probable typo.
Response: We apologize for this typo and thank Reviewer 3 for pointing it out. The J value for γ´-CH3 signal of Val-18 was corrected. The new J value is 6.6.

Reviewer #4

This manuscript is an activity-based natural product isolation story focused on a molecule made by members of the skin microbiome. The molecule in question is a RiPP (ribosomal natural product) called a daptide. These RiPPs are distinguished by the presence of methylated amine groups found at both the N- and C-termini. Previous work on daptides clearly laid out the biosynthesis of these molecules but showed only minimal characterization of bioactivity. The current manuscript convincingly shows that daptides originating from Staph. are membrane active and depolarize the membrane. In this current work the authors also discover an immunity factor for daptides, a YidC homolog. Finally, the authors also show that the daptide, named hominicin, is effective at combatting Staph aureus skin infections in mice. If there is any substantial criticism of the work it is lack of novelty of the hominicin compound, but in my opinion this weakness is overcome by the new insights into the structure-activity relationship, the discovery of a highly unusual immunity factor, the clear experiments into the mechanism of action, and the demonstration of efficacy in a mouse skin infection model. I am highly supportive of publication pending some clarifications/edits delineated below.

1) Line 60, make sure to include the AD abbreviation when atopic dermatitis is first mentioned.
Response: We appreciate the reviewer's comment and have included the abbreviation for atopic dermatitis (AD).

2) Line 139, this sentence should be altered a bit to make it clear that the disagreement is at positions 10 and 17 of the core peptide, not the precursor. This is only clear when looking at Figure 2. Moreover, it is still unclear to me after reading the paper if there is any difference between the hominicin in ref 17 and what is reported here. Finally, and related to this, can the authors include a comparison or sequence alignment of hominicin(s) (if they are different) and the daptide from ref 12?
Response: We thank the reviewer for catching this error and have revised the text to clarify that the differences are located in the core peptide. "We detected differences at the 10^{th} and 17^{th} residues between the core peptide of the AH5011 bacteriocin and the final structure of hominicin (Supplementary Fig. 14d). Although the genetic and biosynthetic information of the hominicin-producing strain MBBL 2-9 are not publicly available, we infer that the AH5011 bacteriocin is likely hominicin."

Based on the MS and NMR analysis, we confirmed the AH5011 daptide is structurally identical to the hominicin reported by Kim *et al.* (DOI: 10.1016/j.bbrc.2010.07.024). This information is stated in the Result and Discussion sections. Importantly, with the genetic data, we resolved the biosynthetic gap between the precursor peptide, which to our knowledge has not been reported, and the final structure of hominicin.

Additionally, we have included a comparison of the precursor sequences between hominicin from *S. hominis* and MpaA1, MpaA2, and MpaA3 from *M. paraoxydans* in Supplementary Fig. 14.

3) Line 147, related to the above point, how does the hominicin BGC compare to the daptide BGC in ref 12?
Response: We appreciate the suggestion by the reviewer and have included a visual comparison of the hominicin BGC from *S. hominis* AH5011 and the Mpa BGC from *M. paraoxydans* DSM 15019 in Supplementary Fig. 14. The presence of *homBC* (decarboxylase), *homD* (aminotransferase), and *homM* (methyltransferase) is shared between the two gene clusters. However, key differences include the presence of predicted ATP-binding cassette transporters (MpaT) in DSM 15019, which are absent in AH5011, and the presence of lanthipeptide dehydratase (LanM) and oxidoreductase (HomJ) in AH5011, which are absent in DSM 15019.

4) Line 172, is the RN4220 strain resistant because it is a hominicin producer? Or does it just harbor the gene encoding HomI?
Response: We have confirmed that *S. aureus* strain RN4220 (GenBank Accession CP076105) does not encode either a biosynthetic gene cluster for hominicin or harbor the *homI* gene. Furthermore, we did not observe antimicrobial activity in either the live bacteria or its conditioned media (Fig. 4b and 4c), indicating that that it is not a hominicin producer. While RN4220 does not harbor *homI,* it does encode two putative *yidC_1* and *yidC_2* genes (Supplementary Fig. 15), which share structural similarity with HomI and may contribute to the observed resistance phenotype.

5) Line 179, can the authors provide the peptide yield after purification to homogeneity?
Response: We have now included the peptide yield in the manuscript. Specifically, we isolated ~6.4 mg of purified peptide from approximately 802 mg of crude chloroform extract, corresponding to a yield of approximately 0.8%. This information has been added to the main text.

6) Line 183: the reference to Figure S5 is incorrect, this is a standard curve.
Response: We thank the reviewer's comment. We have made changes to the main text, figure, and legend to reflect that the graph is a standard curve.

7) Line 203: too many significant figures on the report zone sizes.
Response: The reported diameters of the zones have been adjusted to reflect the appropriate significant numbers.

8) Line 207-217: this section was hard for me to follow because it is difficult to keep track of what all of the genes do. This may be improved when the Figure 4 is put near this text, but I might still suggest that the authors explain briefly what each variant is omitting.
Response: We thank the reviewer for their suggestion and have added a brief statement clarifying the omitted components of each variant in this section.

9) Line 222: ref 12 did show that the original daptides had hemolysis activity. I agree that this isn't the same as forming pores, but the hemolysis activity should probably be mentioned here.
Response: We thank the reviewer for their suggestion and have added a brief statement about the hemolytic activity of Mpa daptides from *M. paraoxydans* to the main text.

10) Line 283: this section discusses the presence of homI in the Staph BGCs, but what about the set of ~500 daptides predicted in ref 12? Is the presence of homI more general or is it Staph-specific?

Response: Previous work by Ren *et al.* (DOI: 10.1007/s12223-013-0259-1) reported that the global co-occurrence percentage of HomI and YidC (PF02096, TIGR03592) with daptide gene clusters is 0.8%, which corresponded to 4 out of 483 gene clusters. All four entries originated from genomes of *Staphylococcus pseudintermedius*. This information has been added to the main text (Line 326).

11) Line 286: when I see the phrase "homI mutant" I think of a deletion mutant, but this is an overexpression mutant. I suggest the authors make this clear and mention whether the homI expression plasmid is multicopy.
Response: We agree with the reviewer and have rephrased the sentence to read, "we cloned *homI* on a multicopy plasmid and observed no growth defects between the wild-type and *homI*-expressing mutant in TSB."

12) Line 326: express should be expresses.
Response: We thank the reviewer's comment and fixed the typo.

13) Line 328: I find the statement that "infection with S. aureus was augmented by the topical addition…" confusing. The word augment could suggest that hominicin was helping colonization of S. aureus.
Response: We agree with the comment from the reviewer and rephased the sentence to read, "infection with *S. aureus* was attenuated by the topical addition…," to emphasize that the addition of hominicin reduced *S. aureus* infection on mouse skin.

14) Line 339: I think the authors mean understudied.
Response: We thank the reviewer's comment and fixed the typo.

15) Figure 1: there is an extra letter D floating in panel F.
Response: We thank the reviewer for the comment and removed the misplaced label.

16) Figure 1B caption: Table S1 in the SI does not seem to include information about all of the strains used for susceptibility testing. This information is important to include if others want to follow up on the work.
Response: We appreciate the reviewer for their comment. The strains tested for susceptibility in the conditioned media of AH5011, as represented in Fig. 1b, are listed in Supplementary Table 6.