nature portfolio

Peer Review File

An ubiquitous antibacterial toxin from human gut bacteria engenders neonatal colonization advantage

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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)
This paper by Valguarnera et al describes an impressive genomic and experimental characterization of a very large collection of Bacteroides fragilis isolates, with a focus on diversity in gene content in the GA3 subtype T6SS, a model interbacterial antagonism pathway. I have previously reviewed this work submitted to a different journal. While I felt before that the work was fairly strong although with some areas that could be strengthened, this revised paper is somewhat improved. As before, the writing is clear and well referenced. Highlights include the compilation and genomic analysis of a large isolate genome collection, the identification and characterization of genomic distribution of novel GA3 effector and immunity genes, importantly the finding that Enterobacteriaceae are targeted by the Bte3 effector, and the thorough and interesting in vivo mouse experimentation.
In general, I found the paper to be interesting and think it is a valuable addition to the growing Bacteroides T6SS literature. I have some comments outlined below but feel that these comments should not hinder the publication of this work but should be addressed in order for the findings to be robust and maximally interpretable.
Line 109, the authors comment on the division between B. fragilis genomospecies, as recently characterized in a recent publication by the Chu group in mSystems (Oles et al). This is notable because I do not see an analysis of the distribution of the genomes presented in this study into similar divisions. How did the authors assign division I versus division II without presenting a phylogenetic tree? This should be included here. Aside from classifying their isolates and allowing for comparison to published datasets like that of Oles et al, a phylogenetic tree would provide an opportunity to examine potential horizontal transfer of effectors across the phylogeny, as has been performed at smaller scale in other publications including Wexler et al 2016 PNAS.
Line 112-114, it is not clear what the authors mean by “no distribution bias” in their repository and how they assessed this without phylogenetic information. Again, phylogenetic information would be valuable to contextualize their comparison of effector and immunity distribution across strains.
A pre-secretion complex formed between Bte3, VgrG, ORF2, ORF3 is interesting but as the authors point out on line 232 there are some inconsistencies between the reciprocal pull downs. It would be strong to perform these pulldowns from the respective mutant backgrounds used to test competition function.
From the MS data, it appears that Hcp is pulled down by the FLAG tag-only control and this seems slightly concerning. Why does the ORF3-flag pulldown MS data not detect TssG and TssE?
Lines 284-287, the periplasmic localization of Bte3 in the tssC mutant is hard to reconcile. Why is there no Bte3 signal in the cell envelope CE lane of Extended Figure 6c if this includes periplasm? What possible mechanism could traffic Bte3 into the periplasm in the absence of a signal peptide or T6SS function? And how could Bte3 be exported out of the cell via the T6SS in a WT context if it is in the periplasm? Is periplasmic localization of Bte3 dependent on orf2 and orf3? To me, this finding of periplasmic localization of Bte3 seems artifactual even despite the presence of the rpoA and Fpn controls for cytoplasm and outer membrane.
Line 388, the claim that there are significant differences in effector genotypes between NTBF and ETBF strains may be confounded by phylogenetic relatedness. For example, if the ETBF strains all derive from several closely-related lineages while the NTBF strains are more diverse, the finding of effector difference may simply reflect differences in phylogenetic diversity rather than a biological effect or interaction between T6SS and BFT.
How do the authors interpret the strain-specific effect of Bte3 on E. coli strains, with HB101 and MG1655 showing a difference but Nissle only partially and the others do not? Perhaps it is a growth rate effect, since recovery of E. coli seems to vary about 3 logs across the different strains used and conditions. This should be investigated and/or discussed.
The in vivo finding that addition of “extra” effectors to B. fragilis strains increased competitive fitness against isogenic

competitors and different strains (Figure 4) is quite interesting. I wonder why the authors think that this is not observed to be common in genomes? The Bfe 1-5-6-7 combination seems to be the only observed 4-effector genotype – why? Perhaps an opportunity to discuss limitations on the effector payload.
I found the unlabeled figures to be quite challenging to go back and forth between. I recommended titling each figure for ease of review.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
Valguarnera et al. present a very thorough and compelling investigation of T6SS-mediated antagonism in Bacteroides fragilis. The work is highly significant and presents a major resource of a genome-sequenced clinical B. fragilis strain collection and an innovative mouse model of colonization in the developing microbiome. While there are many comments and questions below, most are minor with respect to the studies main findings, which are rigorously supported.

Major comments
1. Data and resource availability. The collection of >900 sequenced B. fragilis genomes a very valuable resource. The genome sequence data should be deposited in a publicly accessible database. This is basically required, because several supporting NIH awards are cited. There also appears to be no statement about how requests for plasmids and strains from this collection will be handled. Use of Addgene or other public resource for access to the pEV1 and pEV2 plasmids is encouraged.
2. It seems that all bte3 competitive experiments were done in the context of a bte1 + bte3 combination. Does Bte3 require a co-expressed Bte1 to mediate antagonism?

Minor comments
1. Results line 400, Figure 4g – In order to support conclusions about the effects of bte1 and bte3 in the attacker NTBF strain, the direct comparison needs to be made to the bfe1 + bfe8 only experiment. The statistical tests of CFU levels between attacker and defender are not very meaningful in isolation, because the strains are not isogenic and may have differences in colonization that are not T6SS-mediated. One approach would be to compare competitive indices between the right and left panels of Figure 4g.
2. Figure 4i – One representative colon from each group is not very informative. A plot of colon length measurements or other quantitative measure of “colonic architecture” with statistical test would support the conclusions better.
3. Figure 2b, results line 170. The conclusion about Bte3 requiring a specific VgrG would be better supported if a competitive advantage from VgrG alone could be more thoroughly excluded. A bte1 + vgrG complementation experiment would do it. Alternatively, protection of the defender by bti3 would support that this effect is Bte3-mediated.
4. Results line 135. Are the differences in AID detection rates by GA3 status statistically significant? It is not clear that this type of analysis would imply the presence or absence of selective pressures. GA3 status and AID acquisition may not be independent, e.g. GA3 strains may happen to be more susceptible to mobile elements, in which case it’s not necessarily expected that GA3+ and GA3- strains should have the same AID detection rates in the absence of selective pressures.
5. How is GA3 architecture related to strain phylogeny? Did the authors encounter genetically identical strains from separate patients and how was this handled in the comparative genomics?
6. Discussion – One of the more surprising findings was the dependency of competitive colonization phenotypes on the specific neonatal mouse age. No competitive advantage was observed in days 14 and 21 co-housing experiments. What do the authors think is happening between P10 and P14 to modulate this? In the results it’s suggested that this could be due to primary strain occupancy of the niche, but Figure 4d-e suggest that similar levels of colonization by the test strains are observed.
7. For the culture-based antagonism experiments, only CFUs of the defender are shown throughout, rather than both attacker and defender or a competitive index. I don’t think this needs to be changed, but the descriptions and conclusions in the results should not claim a competitive advantage of the attacker, but instead focus on disadvantage of the defender. Technically, the data don’t exclude the possibility that both attacker and defender CFUs are reduced in some of these experiments.
8. Results line 101. The statement about VgrG C-terminal variability suggesting effector-specific secretion mechanisms could be better supported by citations here or in the introduction. It’s also not clear if this really implies different mechanisms of secretion in terms of how effectors exit the cell, or more selective interactions between VgrGs and their effectors.
9. Extended data figure 3b – It’s unclear how to interpret the applied Dunnet’s test comparison to N1. The clinical isolates are not isogenic and may outcompete the N1 defender for reasons other than T6SS-mediated attack. A similar concern is for extended data Figure 4, where clinical isolates and N1 are directly compared.
10. Introduction line 51. The referenced study doesn’t necessarily show that there’s only one T6SS positive B. fragilis strain per adult microbiome. An alternative explanation is selection for multiple strains encoding similar effector/immunity pairs.
11. Extended data Fig 5d, results line 258 – The authors say there are striking differences between Pte3 and E. coli or Pte2 PAAR proteins, which is supported by a multiple sequence alignment. A structural comparison would be informative and illustrate the differences in VgrG-interacting beta-sheets.
12. All AlphaFold models require indicators of the confidence of prediction.
13. Have the prevalences of B. fragilis effector/immunity combinations changed over time in the isolate collection? It may support the general conclusion that these systems are important for strain transmission.
14. Figure 1b – It is difficult to distinguish the colors of GA2 and GA1-2 groups in the bar graph.

15. Figure 4 – Please specify what the horizontal lines and error bars represent. It is recommended to show both top and bottom error bars, and to position these on top of the individual data points to help with visualization.
16. Extended data figure 6d – RMSD calculations are shown but need clarification on how multidomain proteins were handled. AlphaFold and other models typically have high confidence for each domain, but lower accuracy in modeling the domain relationships. Were comparisons made for individual domains, how were Calpha atoms trimmed, and how many atoms were included in each calculation?
17. Methods line 579 – Please specify the cell density (e.g. OD600) that was spotted on agar for antagonism experiments.
18. Figure 1g – In the orphan immunity analysis, bti3 is not shown. Please include it or specify in the Results that no examples of orphan bti3 were found.

(Remarks on code availability)
The code is sufficient.

Reviewer #3

(Remarks to the Author)
In this study, Valguarnera et al. identify and characterize Bte3, a prevalent Bacteroides fragilis T6SS effector exhibiting broad-spectrum antibacterial activity against both Bacteroides and Enterobacteriaceae—a target range previously unreported. Biochemical and genetic analyses demonstrate that Bte3 functions as a periplasmic toxin requiring novel adaptor proteins (Ate3 and Pte3) for secretion. In infant mouse models, Bte3-producing strains outcompete susceptible Bacteroides fragilis competitors, underscoring the critical role of T6SS effector-immunity pairs in early-life niche establishment. Furthermore, engineered non-toxigenic B. fragilis strains expressing Bte3 restrict colonization by enterotoxigenic strains and ameliorate gut pathology. These findings reveal how T6SS effector diversity—shaped by functional potency and temporal exposure—drives microbial competition during neonatal microbiota assembly, offering strategic insights for targeted interventions. The discovery of this broad-spectrum T6SS effector in B. fragilis represents a significant advancement in the field. However, certain technical aspects require further clarification (see specific comments) before publication in Nature Communications.

Q1. In Fig. 1b, why are blood samples associated with the highest isolation frequency of Bacteroides? Are certain GA3 T6SS gene clusters significantly positively correlated with specific diseases or sample types?

Q2: The nomenclature of the predicted effectors requires clarification. For instance, in Fig. 1d, the full names or functional meanings of the predicted effectors Ble and Cte1-3 should be provided. It is unclear whether these names are related to their predicted biological activities. Additionally, there is currently no explanation regarding the criteria or methodology used to predict that certain ORFs encode effector proteins and immunity proteins. Notably, Bfe6 appears to be an exceptionally small putative effector.

Q3: Line 191-193 (As a final validation of this.....dependent on T6SS function): Given the complex interbacterial interactions between attackers and defenders, as well as their diverse genetic backgrounds, the conclusion regarding T6SS-mediated killing of defenders require further investigation. For instance, even clinical isolates with identical effector combinations displayed distinct killing phenotypes (Extended Data Fig. 3a), suggesting that these phenotypes might also be influenced by additional bactericidal toxins beyond T6SS effectors. Although the authors validated the T6SS-dependence of killing phenotypes in the bte1_2 and bte1_3_4 isolates, the extent to which this conclusion applies to other isolates remains uncertain. Considering the manuscript's primary focus on Bte3 function, the authors may consider revising or removing this broader claim to better align with the central objectives of the study.

Q4: In Extended Data Fig. 2j, why does the pEV1/bte1_3 strain exhibit reduced Hcp secretion compared to the pEV1/bte1_3_4 strain? Does this suggest a role for Bte4 in effector delivery? Similarly, in Fig. 2c, the bactericidal activity of the Bte1_bte3 group is lower than that of the Bte1_3_4 group. Is this difference attributable to enhanced effector secretion by the Bte1_3_4 strain, or does it result from a combined effect introduced by the inclusion of Bte4?

Q5: In Extended Data Fig. 3a–b, the three bte1_3_4 isolates exhibit normal Hcp secretion, yet they display distinct killing phenotypes. Could this discrepancy be attributed to differences in Bte3 delivery efficiency or to the presence of other toxins produced by these strains? Could you detect the secretion of Bte3?

Q6: The competition experiments shown in Fig. 2f and 2g would be strengthened by the heterologous expression of Bti3 in the defender strain, similar to the setup in Fig. 2e, to more rigorously establish Bte3-dependent killing. Such an experimental design would directly determine whether Bte3 alone is sufficient to mediate the observed killing phenotype, independent of other effector proteins.

Q7: Given the distinct intestinal niches occupied by Bacteroides fragilis and Enterobacteriaceae, it remains unclear whether Bte3-mediated killing could occur via a contact-independent mechanism. To address this question, the authors could consider supplementary experiments: for instance, exogenously adding purified Bte3 protein to liquid cultures of Enterobacteriaceae, or employing a 0.22 μm filter membrane to physically separate attacker and defender strains while co-culturing.

Q8: Although co-immunoprecipitation and structural predictions imply the involvement of Ate3/Pte3 in Bte3 secretion, direct experimental evidence remains lacking. To substantiate the functional role of Ate3/Pte3 in this process, the authors are

encouraged to conduct additional experiments comparing Bte3 secretion or bactericidal activity between wild-type and ate3/pte3-deficient mutant strains. Such data would provide critical support for the proposed mechanism of Ate3/Pte3-dependent Bte3 secretion.

Q9: Although the detailed mechanism of Bte3-mediated broad killing may fall beyond the scope of this manuscript, it is nonetheless crucial to explore. A key question remains: what is the periplasmic target of Bte3 that enables its broad-spectrum antimicrobial activity?

Q10: However, as shown in Extended Data Fig. 4a, when strains such as E. coli Nissle or K. pneumoniae TOP52 are used as defenders, significant bacterial killing is only evident in the Bte1_3_4 isolate. Furthermore, when N1_bte1_3 is used as the attacker, the reduction in defender bacterial counts does not reach statistical significance. These findings raise the question: could Bte4 and other toxin components of these isolates also contribute to the observed killing of Enterobacteriaceae?

Minor point: Line 214: S. Typhimurium should be written in italics.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am satisfied with the author's thorough responses to my initial comments. I have no further comments or concerns and look forward to seeing this exciting manuscript published.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
My concerns are adequately addressed. I recommend publication.

(Remarks on code availability)
The code is sufficient.

Reviewer #3

(Remarks to the Author)
I fully acknowledge the authors for their comprehensive and rigorous response to the previous comments. All the raised points have been thoroughly addressed and validated through supplementary experiments. This study reveals the broad-spectrum antibacterial function of the T6SS effector Bte3 in Bacteroides fragilis, offering important new insights into the mechanisms underlying early-life gut microbiota colonization.

I only have one more minor suggestion.

Lines 523–533: The authors may consider briefly highlighting one or two key directions for future validation experiments in the section discussing the Bte3 target. For instance, transposon screening for resistant mutants in Enterobacteriaceae/Bacteroidales, or cross-linking co-immunoprecipitation for target protein identification. Providing such suggestions would offer clearer reference points for subsequent research.

(Remarks on code availability)

REVIEWER COMMENTS

We are highly appreciative of the reviewers for their thoughtful and constructive critique of our manuscript. We consider that the extensive and careful reviews that were prompted by your concerns have certainly improved the quality of our studies, and we sincerely hope that our rebuttal and revised manuscript version have taken your concerns into consideration.

Our revised manuscript now includes a phylogenetic analysis of our isolate collection that positions the T6SS GA3 as an ancestral acquired niche of the *B. fragilis* evolutionary history. In that context, we have found that GA3-positive clusters of genetic similarity are typically dominated by a single *locus I/II* combination. Our analysis also unveiled an early divergent GA3-low prevalence cluster of Division I, and confirmed the presence of clade-specific effectors for Division II isolates. Regarding the role of *bte3* and *bti3* in *in vitro* antagonism, we have included new data on *bti3*-dependent immunity of *V. cholerae* and *S*. Typhimurium, and included experiments that explain the differential magnitude of toxicity of *B. fragilis* laboratory and clinical strains against diverse *E. coli* strains. Additionally, we incorporate a genetic dissection of the *btei4* locus that is supportive of *bte4* and *bti4* as an effector-immunity pair that is not required for *bte3*-specific toxicity or T6SS function. We also conducted further studies on Bte3 function in the attacker strain, including evidence of Bte3 as essential for T6SS function. On the secretion of Bte3, we now provide evidence supportive of a protein complex that includes Bte3, Ate3 and Pte3. Finally, we have dissected the localization of both Bte3 and Bti3 using a more rigorous construct strategy that provides evidence to our hypothesis of Bte3 as a periplasm/outer membrane-targeting effector.

Reviewer #1 (Remarks to the Author):
This paper by Valguarnera et al describes an impressive genomic and experimental characterization of a very large collection of Bacteroides fragilis isolates, with a focus on diversity in gene content in the GA3 subtype T6SS, a model interbacterial antagonism pathway. I have previously reviewed this work submitted to a different journal. While I felt before that the work was fairly strong although with some areas that could be strengthened, this revised paper is somewhat improved. As before, the writing is clear and well referenced. Highlights include the compilation and genomic analysis of a large isolate genome collection, the identification and characterization of genomic distribution of novel GA3 effector and immunity genes, importantly the finding that Enterobacteriaceae are targeted by the Bte3 effector, and the thorough and interesting in vivo mouse experimentation.
In general, I found the paper to be interesting and think it is a valuable addition to the growing Bacteroides T6SS literature. I have some comments outlined below but feel that these comments should not hinder the publication of this work but should be addressed in order for the findings to be robust and maximally interpretable.

- Line 109, the authors comment on the division between B. fragilis genomospecies, as recently characterized in a recent publication by the Chu group in mSystems (Oles et al). This is notable because I do not see an analysis of the distribution of the genomes presented in this study into similar divisions. How did the authors assign division I versus division II without presenting a phylogenetic tree? This should be included here.

We have now included in the Results, Materials and Methods, and Supplementary Information sections a Prot-Spam based phylogenetic analyses of predicted proteomes for all isolates.

Phylogeny revealed two groups of similarity within *B. fragilis* isolates, corresponding with Division I or II predicted proteomes (Extended Data Fig. 1a). Division assignment by phylogenetic analysis fully matched the mutually-exclusive presence of the genes encoding the broad spectrum β-lactamase CepB (*cepB*, Division I) and the carbapenemase CfiA (*cfiA*, Division II), determined by BLAST using *cepB* and *cfiA* sequences as queries against the whole *B. fragilis* database.

- Aside from classifying their isolates and allowing for comparison to published datasets like that of Oles et al, a phylogenetic tree would provide an opportunity to examine potential horizontal transfer of effectors across the phylogeny, as has been performed at smaller scale in other publications including Wexler et al 2016 PNAS.

We fully agree with the comment on the advantages of performing a whole collection phylogenetic analysis, as it would allow us to generate hypotheses on horizontal transfer of T6SS genes within *B. fragilis*. While our study is limited in sequencing depth and coverage, we have now provided a phylogenetic tree that describes genetically similar isolates containing the same *ei* locus combinations. The magnitude of our collection allowed us to appreciate a higher resolution of evolutionary patterns than the Wexler et al study in PNAS, suggesting early independent acquisition events of GA3 (consistent with horizontal transfer) followed in some cases by GA3 inactivation or acquisition of a different combination. Additionally, phylogenetic analysis captured the presence of an early divergent Division I clade that likely went through an early GA3 loss event from a GA3-positive last common ancestor, given the low percentage of GA3 genes in this clade.

- Line 112-114, it is not clear what the authors mean by "no distribution bias" in their repository and how they assessed this without phylogenetic information. Again, phylogenetic information would be valuable to contextualize their comparison of effector and immunity distribution across strains.

We have corrected this statement, which was intended to refer to the fact that we did not observe significant association between carriage of GA1/GA2 genes and GA3 positivity, or association GA1/2 genes with a specific GA3 *locus I/II* combination. We have modified the text based on a re-examination of the associations between strains harboring GA3 and strains harboring GA1 and/or GA2, which showed a positive association between GA2 and carriage of GA3. The presence of GA1 positively associated with the GA3 combination *ble1_bfe2*, and GA2 with *bfe1_bte2* (Extended Data Fig. 1g, Supplementary Dataset 1). Our phylogenetic analysis for GA1 and GA2 is not graphed for the manuscript, but it can be recapitulated from the tables provided for genetically similar cluster associations. The overall generalized distribution of GA1 and GA2 is representative of active HGT dissemination in human microbiomes.

- A pre-secretion complex formed between Bte3, VgrG, ORF2, ORF3 is interesting but as the authors point out on line 232 there are some inconsistencies between the reciprocal pull downs. It would be strong to perform these pulldowns from the respective mutant backgrounds used to test competition function.

We appreciate the comment, and have generated additional data and modifications of the manuscript to address these concerns. C-terminal tagging of VgrG does not interfere with T6SS function and secretion of Hcp, Bte3 and VgrG itself, yet appears as artifactual in the context of

pulldowns, showing a different pattern of interacting partners than eluates from Orf2 and Orf3-tagged constructs when focusing on Orf2 and Orf3 tagged constructs. Thus, we now provide new experiments in Fig. 3g including a non-tagged version of Bte3-specific VgrG and further dissection of the interactions between Bte3, Orf2 (Ate3) and Orf3 (Pte3). We have incorporated pulldowns in which we alternatively tagged Ate3 or Pte3 with a Flag tag in the presence or absence of Pte3 or Ate3, respectively. Our data revealed that the absence of any of the accessory genes fully disrupts T6 function, evidenced by loss of secretion (Hcp and Bte3-HA) and competitive phenotypes (Extended Data 6d and 6e). Furthermore, while the lack of Pte3 showed a partial reduction in the interaction between Bte3 and Ate3, lack of Ate3 fully disabled Bte3-Pte3 interactions. Thus, we have now provided more rigorous support that Ate3 and Pte3 as essential components of the Bte3-specific spike complex.

- From the MS data, it appears that Hcp is pulled down by the FLAG tag-only control and this seems slightly concerning. Why does the ORF3-flag pulldown MS data not detect TssG and TssE?

Our rationale for the presence of Hcp1 in the FLAG tag-only negative control is that Hcp1 is one the most abundant proteins in the *B. fragilis* proteome. In these studies, we did not utilize detergent during the anti-FLAG and anti-HA pulldowns to maintain native complexes; this facet of our experimentation likely explains the residual amounts of Hcp in the negative control. Despite the presence of Hcp1 in the negative control eluate, peptide intensity values shown in Supplementary Dataset 3 were considerably lower than eluates for *vgrG*-FLAG (15588.2 x higher than FLAG control), *orf2*-FLAG (67.6 x higher than FLAG control), and *orf3*-FLAG (22.6 x higher than FLAG control). Additionally, there are over 100 other proteins that appear more abundant in the negative control eluates than Hcp1, which probably underscores the limitations of detergent-free pulldown as it contributes to increased background in the negative control eluate. For the revised version, we have incorporated $\log_2$ fold change ($\log_2$FC) and Z-score values for each protein in every pairwise comparison (lines 757-763).

We speculate that the lack of signal of TssE/G and lower signal of TssF in the ORF3-FLAG pulldown in comparison to other constructs could be that while VgrG has been documented to interact with the baseplate formed by TssE/F/G/K, this is not the case for PAAR proteins [1]. Cryo-EM-based models have predicted baseplate-VgrG interactions to occur closer to the N-terminus (base) of the VgrG protein, thus it is likely that the distance between ORF3/PAAR and the baseplate would prevent the latter interactions from occurring.

- Lines 284-287, the periplasmic localization of Bte3 in the tssC mutant is hard to reconcile.

We agree with the concern on this unexpected phenotype, as the possibility of fractionation artifacts could not be discarded. We decided to address this matter based on the hypothesis that if Bte3 is normally leaking into the periplasm in the absence of a signal peptide and other accessory proteins, it would be lethal in attacker cells lacking Bti3. Thus, in Fig. 3h we introduced only Bte3 or Bti3 alone, or Bte3 together with Bti3 in *wild type* N1 strain. The *bte3*-harboring strain displayed normal viability and cytosolic localization of Bte3; *bti3*-strain showed periplasmic and outer membrane localization for Bti3. Notably, the strain harboring both *bte3* and *bti3* showed partial periplasmic localization of Bte3, and periplasmic/OM localization of Bti3, as previously depicted in the original submission (Now in Extended Data Fig. 7a and 7d).

Fractionation controls did not indicate contamination artifacts, yet we cannot discard that Bti3-dependent Bte3 localization phenotype is at least partially due to non-cognate expression levels of our genomic insertion constructs. Revised version includes the statement “We cannot discard that partial periplasmic localization of Bte3 was due to expression of *bte3* from a non-GA3 genomic locus lacking cognate expression regulation.” (Lines 371-372). Future studies that exceed the scope of this manuscript are poised to genetically and biochemically dissect such surprising phenotype.

- Why is there no Bte3 signal in the cell envelope CE lane of Extended Figure 6c if this includes periplasm?

We apologize for the lack of clarity on this specific matter. The absence of signal in the CE lane can be explained because this fraction was not concentrated by TCA precipitation before SDS-PAGE. This fraction was used as the input for ultracentrifugation that rendered an outer membrane pellet (OM) and a soluble periplasmic fraction (PE), the latter concentrated by TCA precipitation. Differences in total protein amounts between the fractions can be appreciated in the Coomassie Blue stain panel in Extended Data Fig. 6c.

- What possible mechanism could traffic Bte3 into the periplasm in the absence of a signal peptide or T6SS function? And how could Bte3 be exported out of the cell via the T6SS in a WT context if it is in the periplasm?

We are unaware of a specific mechanism that could lead to trafficking a portion of the Bte3 pool into the periplasm in an apparent Bti3-dependent manner, and we cannot exclude the possibility of over-expression artifacts. We hope that the new data provided in Figure 3h provides more rigor to the valid concerns raised.

- Is periplasmic localization of Bte3 dependent on orf2 and orf3?

As noted above, the constructs used in the new version of the manuscript in Fig. 3h show that Bti3 appears to be sufficient for periplasmic localization of Bte3.

- To me, this finding of periplasmic localization of Bte3 seems artifactual even despite the presence of the rpoA and Fpn controls for cytoplasm and outer membrane.

We hope that the concerns of fraction contamination are at least partially addressed with the new data provided in Fig. 3h.

An important note is that the vast majority of Bte3 is cytosolic, both as a 75 kDa band and high molecular weight aggregate. Periplasmic fractions analyzed have been concentrated by TCA, leading to an increase in the signal and more periplasmic equivalents relative to the corresponding protoplast (cytoplasm) fraction analyzed.

- Line 388, the claim that there are significant differences in effector genotypes between NTBF and ETBF strains may be confounded by phylogenetic relatedness. For example, if the ETBF strains all derive from several closely-related lineages while the NTBF strains are more diverse, the finding of effector difference may simply reflect differences in phylogenetic diversity rather than a biological effect or interaction between T6SS and BFT.

We appreciate this observation and have thus introduced changes in the manuscript that reflect findings from our phylogenetic analyses. Effectively, there are likely non-T6SS mechanisms that

can confound competitive phenotypes. Our initial criteria (pre-phylogeny) to only include NTBF and ETBF isolates that were matched not only in GA3 combination but also in their genetic repertoire of GA1/GA2, AID, and non-T6SS secreted antibacterial toxins (BSAPs, Ubb) resulted in NTBF/ETBF pairs that were mostly restricted to the same clusters of genetic similarity. Every pair tested in *in vitro* competitions in Extended Data Fig. 10c clustered together except for isolates 13051^{NTBF} (cluster 19.9.10) and 13360^{ETBF} (cluster 19.9.8).

- How do the authors interpret the strain-specific effect of Bte3 on E. coli strains, with HB101 and MG1655 showing a difference but Nissle only partially and the others do not? Perhaps it is a growth rate effect, since recovery of E. coli seems to vary about 3 logs across the different strains used and conditions. This should be investigated and/or discussed.

We have set up growth curves of both the *B. fragilis* (*bte1_3_4* isolate and N1 variants) (Extended Data Fig. 3e) and *E. coli* strains (Extended Data Fig. 4c) that reflect the hypothesis by the reviewer that these findings relate to growth rate. Additionally, we tested anaerobic growth in solid media followed by plating in LB in aerobiosis for *E. coli* strains (Extended Data Fig. 4b), showing strong inhibition in the case of S17 that now explains the high sensitivity of this strain. Our current hypothesis with the new data included is that varying degree of survival of defender strains is dependent upon the growth rate and T6SS payload for each attacker, and defender growth rate in anaerobiosis.

The in vivo finding that addition of "extra" effectors to B. fragilis strains increased competitive fitness against isogenic competitors and different strains (Figure 4) is quite interesting. I wonder why the authors think that this is not observed to be common in genomes? The Bfe 1-5-6-7 combination seems to be the only observed 4-effector genotype – why? Perhaps an opportunity to discuss limitations on the effector payload.

We are unaware of biologic limitations in incorporation of more effectors into the T6SS repertoire in a given strain. To address this, however, we have added additional discussion on effector payload natural limitation based. We speculate, based on our phylogeny, that diversity and number of GA3 effector and combination repertoires expanded early during branching of Division I Major clade. *Effector-immunity* pairs are always restricted to *locus I* and *locus II*; in the discussion we now hypothesize that unknown HGT and effector-immunity "shuffling" mechanisms that have driven payload landscape are either presently absent or constitute rare events.

I found the unlabeled figures tbe quite challenging to go back and forth between. I recommended titling each figure for ease of review.

Thank you for pointing out this difficulty. This is now modified in the revised version of the figure files.

Reviewer #2 (Remarks to the Author):
Valguarnera et al. present a very thorough and compelling investigation of T6SS-mediated antagonism in Bacteroides fragilis. The work is highly significant and presents a major resource

of a genome-sequenced clinical B. fragilis strain collection and an innovative mouse model of colonization in the developing microbiome. While there are many comments and questions below, most are minor with respect to the studies main findings, which are rigorously supported.

We thank the reviewer for their favorable evaluation of our studies and significance to the field. We have addressed each of the relevant comments below.

Major comments
- 1. Data and resource availability. The collection of >900 sequenced B. fragilis genomes a very valuable resource. The genome sequence data should be deposited in a publicly accessible database. This is basically required, because several supporting NIH awards are cited.

We apologize for the lack of availability of the genomes during the review process, the final 882 *B. fragilis* annotated genomes (*.gff) are available within a Harvard Dataverse dataset which can be accessed using the following link:

https://dataverse.harvard.edu/previewurl.xhtml?token=f8e89886-925e-4a31-abc7-8b1dd3fa04c8

Genomes will effectively be uploaded to the genome repository at NCBI for public access upon publication acceptance. We have incorporated a Data and Resource Availability Statement in the manuscript to include the NCBI BioProject number that will contain all submitted annotated genomic sequences.

- There also appears to be no statement about how requests for plasmids and strains from this collection will be handled. Use of Addgene or other public resource for access to the pEV1 and pEV2 plasmids is encouraged.

We appreciate the comment and we commit to transfer vectors pEV1, pEV2, and the newly introduced pK-*bes*, an allelic exchange vector with sucrose-based negative selection via Addgene. We will readily begin the process and make the vectors available upon publication acceptance. For the rest of the T6SS-specific constructs, readers are encouraged to contact us for acquisition and we will provide those promptly. We have incorporated a Data and Resource Availability Statement in the manuscript to include the Addgene catalog numbers for each vector (upon manuscript acceptance) and to enable researchers to contact us for sharing T6SS-specific constructs.

2. It seems that all bte3 competitive experiments were done in the context of a bte1 + bte3 combination. Does Bte3 require a co-expressed Bte1 to mediate antagonism?

We have always expressed Bte3 in N1 strain derivatives in the context of *locus I/II* combination *bte1_3*, however we do note that co-expression of Bte1 is not necessary for Bte3-based intoxication. Fig. 3a includes new data showing deletion of *bte1* and *bte4* in isolate 13051 as non-essential for *bte3*-specific *in vitro* competitive advantage.

Minor comments
1. Results line 400, Figure 4g – In order to support conclusions about the effects of bte1 and bte3 in the attacker NTBF strain, the direct comparison needs to be made to the bfe1 + bfe8 only experiment. The statistical tests of CFU levels between attacker and defender are not very

meaningful in isolation, because the strains are not isogenic and may have differences in colonization that are not T6SS-mediated. One approach would be to compare competitive indices between the right and left panels of Figure 4g.

We have now reanalyzed the data for Figure 4b and 4g to segregate the analysis into fecal and cecum samples. Each panel (left, feces; right, cecum) was analyzed and means of each group were tested all vs all using 2-way ANOVA and Tukey multiple comparison test.

2. Figure 4i – One representative colon from each group is not very informative. A plot of colon length measurements or other quantitative measure of "colonic architecture" with statistical test would support the conclusions better.

We have now included a plot with all the individual data for colon length measurements in Fig. 4i. The representative image panel is now in Fig. 4j.

3. Figure 2b, results line 170. The conclusion about Bte3 requiring a specific VgrG would be better supported if a competitive advantage from VgrG alone could be more thoroughly excluded. A bte1 + vgrG complementation experiment would do it. Alternatively, protection of the defender by bti3 would support that this effect is Bte3-mediated.

We have now incorporated a *bti3*-containing prey and repeated experiments for Fig. 2b, showing specific intoxication by Bte3 and not by VgrG. Additionally, we included an attacker strain that contains *bte1* and *bte3*-specific accesory ORFs *orf1/2/3*, which showed only Bte1-based toxicity.

- 4. Results line 135. Are the differences in AID detection rates by GA3 status statistically significant?

We have now incorporated in the manuscript and in the cluster associations table within Supplementary Dataset 1 the degree of association between AID carriage and specific clones within the newly generated *B. fragilis* phylogenetic analysis.

- It is not clear that this type of analysis would imply the presence or absence of selective pressures. GA3 status and AID acquisition may not be independent, e.g. GA3 strains may happen to be more susceptible to mobile elements, in which case it's not necessarily expected that GA3+ and GA3- strains should have the same AID detection rates in the absence of selective pressures.

We agree that our analysis is not sufficient to conclude that selective pressures drive AID diversity in *B. fragilis* populations. We have thus modified this section altogether in the revised manuscript. Phylogenetic analysis now presented in Fig. 1 is suggestive of cluster-specific AID incorporation events that likely occurred after divergence between Division I Minor and Major clades, followed by clonal expansion. While AIDs appear to have traits of current mobility within Division I Major clade, and this phenotype has been experimentally shown by Ross et al. [2], harboring of AIDs by specific clusters as we now observe has given us a new perspective on the evolutionary history of orphan immunity repertoires.

- 5. How is GA3 architecture related to strain phylogeny? Did the authors encounter genetically identical strains from separate patients and how was this handled in the comparative genomics?

As mentioned, our phylogenetic analysis now shows clear associations between genetically similar clusters and dominance by a specific locus I/II combinations. Our samples lacked patient identifiers, thus we were not able to deduplicate or link genetically similar/identical strains to individual patients.

6. Discussion – One of the more surprising findings was the dependency of competitive colonization phenotypes on the specific neonatal mouse age. No competitive advantage was observed in days 14 and 21 co-housing experiments. What do the authors think is happening between P10 and P14 to modulate this? In the results it's suggested that this could be due to primary strain occupancy of the niche, but Figure 4d-e suggest that similar levels of colonization by the test strains are observed.

We hypothesize that changes in the development of the colonic epithelium allow for strain engraftment during the P10-P14 time window, thus determining the higher burden of the primary strain. We speculate that by P14, a single effector-immunity-based competition phenotype is not enough to enable disruption of primary niche occupancy. While aggregated data in figures 4d and 4e show similar colonization levels for the *bti3*-containing condition, pEV2-containing condition when comparing Fig 4d and e, together with disaggregated data in Extended Data Figure 9, do not allow us to reject the hypothesis of primary strain engraftment effect as a limiting factor for T6-based competitive advantage.

7. For the culture-based antagonism experiments, only CFUs of the defender are shown throughout, rather than both attacker and defender or a competitive index. I don't think this needs to be changed, but the descriptions and conclusions in the results should not claim a competitive advantage of the attacker, but instead focus on disadvantage of the defender. Technically, the data don't exclude the possibility that both attacker and defender CFUs are reduced in some of these experiments.

We agree with this observation and have thus modified sentences in which we stated competitive advantage when utilizing non-isogenic strains. In the case of intra-N1 competitions, despite not showing attacker CFUs for *in vitro* competitions, there is no basis for antagonism of defenders against attackers in the exact same genetic background.

8. Results line 101. The statement about VgrG C-terminal variability suggesting effector-specific secretion mechanisms could be better supported by citations here or in the introduction. It's also not clear if this really implies different mechanisms of secretion in terms of how effectors exit the cell, or more selective interactions between VgrGs and their effectors.

We appreciate this comment and have introduced references[3-8] and modifications accordingly. We were referring to highly selective interactions between specific VgrGs and their corresponding *locus II* VgrG-associated effector. Key studies referenced belong to the groups of Sarah Coulthurst (Cianfanelli et al., PLOS Pathogens, 2016), Tao Dong (Liang et al., PLOS

Pathogens, 2021), Allan Filloux (Wettstadt et al., Frontiers in Microbiology, 2019; Howard et al., mBio, 2021), and Erh-Min Lai (Bondage et al., PNAS, 2016; Wu et al., EMBO Reports, 2019).

9. Extended data figure 3b – It's unclear how to interpret the applied Dunnet's test comparison to N1. The clinical isolates are not isogenic and may outcompete the N1 defender for reasons other than T6SS-mediated attack. A similar concern is for extended data Figure 4, where clinical isolates and N1 are directly compared.

In both figures we compared the means of all defender CFU/ml versus the mean CFU/ml of pEV2 defender versus pEV1 attacker condition. This is the condition where we see the lowest competitive disadvantage for our N1 and *E. coli* S-17 defenders. In that sense, no statistical analysis would provide evidence in favor or against non-T6-based competitive features by non-isogenic strains to our N1 model strain. Thus, we generated Δ*tssC* mutant strains for representative clinical isolates of *bte1_2* and *bte1_3_4* combinations, allowing us to propose T6-based targeting of *Bacteroidales* and non-*Bacteroidales* by *bte3* only for the genetically dissected strains (Fig. 2d and Extended Data Fig. 3c). A paragraph addressing non-T6 confounding factors in *in vitro* competition phenotypes is now included Results section, lines 231-236.

10. Introduction line 51. The referenced study doesn't necessarily show that there's only one T6SS positive B. fragilis strain per adult microbiome. An alternative explanation is selection for multiple strains encoding similar effector/immunity pairs.

Thank you for raising this point. We have now included a reference from the group of Eric Alm that performed longitudinal sampling of *B. fragilis* strains, showing clonality and evolution within individual hosts [9], further supporting the hypothesis of single strain colonization in adults.

11. Extended data Fig 5d, results line 258 – The authors say there are striking differences between Pte3 and E. coli or Pte2 PAAR proteins, which is supported by a multiple sequence alignment. A structural comparison would be informative and illustrate the differences in VgrG-interacting beta-sheets.

We apologize for the lack of description of how we performed comparisons between PAAR proteins, which is now detailed in Extended Data Figure 6f. Comparative analysis of PAAR and PAAR-like proteins was carried out using PROMALS3D, a software for structure-sequence-based alignments [10]. Our inputs for the analysis were the experimentally-determined and Alphafold2 modelled PDB files corresponding to PAAR and PAAR-like proteins. We have now included the proper reference and details in the revised manuscript.

12. All AlphaFold models require indicators of the confidence of prediction.

We have now included AlphaFold confidence values (average pLDDT) in Supplementary Data Table 1 (Alphafold2_structures_metadata tab), as determined in Uniprot or by ColabFold in the case of in-house generated models.

13. Have the prevalences of B. fragilis effector/immunity combinations changed over time in the

isolate collection? It may support the general conclusion that these systems are important for strain transmission.

We have tested this hypothesis with our dataset; we found that the few significant associations of specific clusters with a Year or period of years were not informative of those combinations as drivers of transmission/dissemination at the human population level. This analysis is now present in Supplementary Dataset 1, Temporal associations tab.

14. Figure 1b – It is difficult to distinguish the colors of GA2 and GA1-2 groups in the bar graph.

We have modified the figure accordingly, now Extended Data Fig.1b.

15. Figure 4 – Please specify what the horizontal lines and error bars represent. It is recommended to show both top and bottom error bars, and to position these on top of the individual data points to help with visualization.

Horizontal lines represent comparisons between means (2-way ANOVA). Error bars represent SD. Because of the magnitude of CFU counts, error bars mainly extend upwards; bars are still set to represent both directions and now they are placed on top of the individual points.

16. Extended data figure 6d – RMSD calculations are shown but need clarification on how multidomain proteins were handled. AlphaFold and other models typically have high confidence for each domain, but lower accuracy in modeling the domain relationships. Were comparisons made for individual domains, how were Calpha atoms trimmed, and how many atoms were included in each calculation?

We appreciate this concern as it became a key consideration in our revision, mainly for Bte3 structural comparisons.

Initially for Bte3 we performed whole protein structure comparisons instead of domain-specific comparisons, and employed the cealign algorithm for protein superimposition in PyMOL. While this method is the most rigorous for proteins with low sequence similarity, it primarily aligned only the C-term of Bte3 orthologs, with the following RMSD and atom values: Bfe3: 4.310 (192 atoms); *Pv*Bte3: 5.702 (160 atoms); *Fs*Bte3: 5.360 (304 atoms); *Ec*Bte3: 6.725 (176 atoms).

For the revised version, we employed a less stringent superimposition method for all alignments (super, with 5 cycles and a cutoff=2 Å for outlier rejection)(included in Materials and Methods); for Bte3 we split each model into separate domains before comparison (β1-β2, β3, C-term open β-barrel). New RMSD values are incorporated in the figures and Supplementary Dataset 1. In the case of Bti3 ortholog comparison, we trimmed the predicted signal peptides prior to comparison. Supplementary Dataset 1 has been modified to display the metrics for alignments, including the RMSD values, range of residues used for comparison and the amount of atoms included per calculation.

17. Methods line 579 – Please specify the cell density (e.g. OD600) that was spotted on agar for antagonism experiments.

We appreciate this comment to improve the presentation of our data. We have modified the manuscript accordingly to reflect specific OD600 values used to normalize attackers and defenders.

18. Figure 1g – In the orphan immunity analysis, bti3 is not shown. Please include it or specify in the Results that no examples of orphan bti3 were found.

We have now modified the results section of the manuscript, indicating that we only found 3 genomes with orphan *bti3* orthologs in them.

Reviewer #2 (Remarks on code availability):

The code is sufficient.

Reviewer #3 (Remarks to the Author):
In this study, Valguarnera et al. identify and characterize Bte3, a prevalent Bacteroides fragilis T6SS effector exhibiting broad-spectrum antibacterial activity against both Bacteroides and Enterobacteriaceae—a target range previously unreported. Biochemical and genetic analyses demonstrate that Bte3 functions as a periplasmic toxin requiring novel adaptor proteins (Ate3 and Pte3) for secretion. In infant mouse models, Bte3-producing strains outcompete susceptible Bacteroides fragilis competitors, underscoring the critical role of T6SS effector-immunity pairs in early-life niche establishment. Furthermore, engineered non-toxigenic B. fragilis strains expressing Bte3 restrict colonization by enterotoxigenic strains and ameliorate gut pathology. These findings reveal how T6SS effector diversity—shaped by functional potency and temporal exposure—drives microbial competition during neonatal microbiota assembly, offering strategic insights for targeted interventions. The discovery of this broad-spectrum T6SS effector in B. fragilis represents a significant advancement in the field. However, certain technical aspects require further clarification (see specific comments) before publication in Nature Communications.

Q1. In Fig. 1b, why are blood samples associated with the highest isolation frequency of Bacteroides?

While blood isolates are one of the most common in our collection (ranked 4th, n=176), we purposefully enriched our subset of isolates in different isolation sites (including blood) to potentially uncover a broader genetic diversity. We have now added a sentence in the Materials and Methods section. The revised manuscript includes a table within Supplementary Data 1 that shows the total numbers for each isolation site versus the subset that we sequenced.

Are certain GA3 T6SS gene clusters significantly positively correlated with specific diseases or sample types?

We have not seen positive or negative correlations of any GA3 T6SS *ei* combination with different isolation sites.

Q2: The nomenclature of the predicted effectors requires clarification. For instance, in Fig. 1d,

the full names or functional meanings of the predicted effectors Ble and Cte1-3 should be provided. It is unclear whether these names are related to their predicted biological activities.

Predicted effector nomenclature is not based on putative biological activity. Instead, we incorporated previous nomenclature from the Comstock and Goodman groups used for *bfe1/bfe2* and *bte1/bte2* (lines 89-92 and 94-95) and established a nomenclature based on genetic linkage between different *locus I* and *locus II* ei pairs. Predicted protein sequence alignment of *locus I* effectors allowed for segregation into 4 separate groups: *bfe1/bfe1b*, *bte1/bte1b/*, *ble1*, *cte1/cte1b*. We apologize for the lack of definition for *ble1*: *Bacteroides locus I* effector 1, and *cte1/2/3*: *B. fragilis* clade 2 effector. These changes have been incorporated to the Results section and Figure 1C legend.

Additionally, there is currently no explanation regarding the criteria or methodology used to predict that certain ORFs encode effector proteins and immunity proteins. Notably, Bfe6 appears to be an exceptionally small putative effector.

We have now included the reference for the study by the Mougous group (Verster, Ross, et al., CHM, 2017) that compiled multiple predicted effector-immunity coding genes [11]. We employed the effector sequences provided in such study to search against our local BLAST database. Detailed comparison of our nomenclature and the numeric nomenclature used in the effector database we employed are provided in Supplementary Data 1, Locus_I_II_nomenclature tab.

Q3: Line 191-193 (As a final validation of this.....dependent on T6SS function): Given the complex interbacterial interactions between attackers and defenders, as well as their diverse genetic backgrounds, the conclusion regarding T6SS-mediated killing of defenders require further investigation. For instance, even clinical isolates with identical effector combinations displayed distinct killing phenotypes (Extended Data Fig. 3a), suggesting that these phenotypes might also be influenced by additional bactericidal toxins beyond T6SS effectors. Although the authors validated the T6SS-dependence of killing phenotypes in the bte1_2 and bte1_3_4 isolates, the extent to which this conclusion applies to other isolates remains uncertain. Considering the manuscript's primary focus on Bte3 function, the authors may consider revising or removing this broader claim to better align with the central objectives of the study.

We agree with this and we have modified the paragraph accordingly to narrow the claim to the T6-mutant tested isolates.

Q4: In Extended Data Fig. 2j, why does the pEV1/bte1_3 strain exhibit reduced Hcp secretion compared to the pEV1/bte1_3_4 strain? Does this suggest a role for Bte4 in effector delivery? Similarly, in Fig. 2c, the bactericidal activity of the Bte1_bte3 group is lower than that of the Bte1_3_4 group. Is this difference attributable to enhanced effector secretion by the Bte1_3_4 strain, or does it result from a combined effect introduced by the inclusion of Bte4?

Newly incorporated experiments in Fig. 3a-b and Extended Data Fig. 5a-c show that *bte4* is an effector gene that contributes to toxicity phenotypes; toxicity can be prevented by the *bti4* cognate immunity gene. This was shown through deletion of *btei4* in isolate 13051 (Fig. 3a, 3b and Extended Data Fig. 5b), as well as new studies using the N1 strain platform, now incorporating *bti4* into immunity constructs (Extended Data Fig. 5a). Differences in Hcp secretion of *bte1_bte3* and *bte1_bte3_bte4* constructs appear to be clone I-specific (Extended

Data Fig. 5c), consistent with invariant Hcp expression and secretion of *btei4* deletion mutant in comparison to *wt* 13051 isolate.

Q5: In Extended Data Fig. 3a–b, the three bte1_3_4 isolates exhibit normal Hcp secretion, yet they display distinct killing phenotypes. Could this discrepancy be attributed to differences in Bte3 delivery efficiency or to the presence of other toxins produced by these strains? Could you detect the secretion of Bte3?

We cannot rule out intoxication by other non-T6SS toxins or differences in Bte3 delivery. The only means to assess Bte3 secretion in our model is by using a HA-tagged Bte3 copy in the context of employed genomic insertion constructs. Thus, we cannot rigorously answer this concern without raising specific antibodies against Bte3 or tagging the *bte3* locus in *cis* to examine expression and secretion driven by cognate regulation. We have included a statement on the limitations of using a genomic tagged copy as we have used to study Bte3 expression and secretion (lines 743-747).

Q6: The competition experiments shown in Fig. 2f and 2g would be strengthened by the heterologous expression of Bti3 in the defender strain, similar to the setup in Fig. 2e, to more rigorously establish Bte3-dependent killing.

We have now incorporated experiments in Fig. 2f and 2g using pEXT20 containing *bti3*, which provided *V. cholerae* and *S.* Typhimurium with Bte3-specific immunity.

Q7: Given the distinct intestinal niches occupied by Bacteroides fragilis and Enterobacteriaceae, it remains unclear whether Bte3-mediated killing could occur via a contact-independent mechanism. To address this question, the authors could consider supplementary experiments: for instance, exogenously adding purified Bte3 protein to liquid cultures of Enterobacteriaceae, or employing a 0.22 μm filter membrane to physically separate attacker and defender strains while co-culturing.

We have not been able to express Bte3 as a recombinant protein in *E. coli*, thus the suggested recombinant protein addition experiment cannot be performed. We have now incorporated experiments in Extended Data Fig. 4d that show that wt and Δ*tssC* isolate 13051 are not toxic towards *E. coli* S17-1 defenders in liquid media competitions, consistent with Bte3 as a contact-dependent T6SS effector.

Q8: Although co-immunoprecipitation and structural predictions imply the involvement of Ate3/Pte3 in Bte3 secretion, direct experimental evidence remains lacking. To substantiate the functional role of Ate3/Pte3 in this process, the authors are encouraged to conduct additional experiments comparing Bte3 secretion or bactericidal activity between wild-type and ate3/pte3-deficient mutant strains. Such data would provide critical support for the proposed mechanism of Ate3/Pte3-dependent Bte3 secretion.

We have now included an experiment in the revised version of the manuscript showing Ate3 and Pte3 as both necessary for Bte3 and Hcp secretion, using HA-tagged Bte3 and FLAG-tagged Ate3 or Pte3. This was carried out by complementation of the N1 Δ*t6p* strain with different combinatorial constructs (Figure 3g and Extended Data Fig. 6e).

Q9: Although the detailed mechanism of Bte3-mediated broad killing may fall beyond the scope of this manuscript, it is nonetheless crucial to explore. A key question remains: what is the periplasmic target of Bte3 that enables its broad-spectrum antimicrobial activity?

We are currently exploring the mechanism of intoxication of Bte3, which remains elusive. We speculate that such target of intoxication is a shared outer membrane moiety between *Bacteroidales* and non-*Bacteroidales*, given our overall data. We have now included statements in the results and discussion sections that mentions our working hypothesis.

Q10: However, as shown in Extended Data Fig. 4a, when strains such as E. coli Nissle or K. pneumoniae TOP52 are used as defenders, significant bacterial killing is only evident in the Bte1_3_4 isolate. Furthermore, when N1_bte1_3 is used as the attacker, the reduction in defender bacterial counts does not reach statistical significance. These findings raise the question: could Bte4 and other toxin components of these isolates also contribute to the observed killing of Enterobacteriaceae?

We appreciate the comment, and have now addressed the genetic interaction between *bte3* and *bte4* in clinical isolate 13051. As shown in Fig. 3a and Extended Data Fig. 5, *bte4* has been shown to encode a T6SS effector that is not required for overall T6SS function or Bte3-based toxicity. We also incorporated experiments in which we show identical *E. coli* CFU loss by wt and ∆*bte4* 13051 isolate, further supporting Bte3 as the main driver of broad species toxicity. Additionally, we have added growth curves for *B. fragilis* and *Enterobacteriaceae* defenders, as well as anaerobic solid media growth for defender strains (Extended Data Fig. 3e, 4b and 4c). Thus, our newly added data led us to hypothesize that differences in toxicity phenotypes between *B. fragilis* strains and *Enterobacteriaceae* are the resultant of attacker T6SS payload and growth rate versus the defender anaerobic tolerance and growth rate. Clonal-specific variations in Hcp secretion in the N1 ∆*t6p* strain complemented with pEV1/*bte1_bte3* (Extended Data Fig. 5d) could partially account for lower overall T6SS effector delivery and toxicity against *Enterobacteriaceae* than the 13051 isolate.

Minor point: Line 214: S. Typhimurium should be written in italics.

We consider this to be a stylistic preference even within the field of *Salmonella enterica*. Because Typhimurium is a serovar and not a species *per se*, we prefer to retain the nomenclature used by the groups of Jorge Galán, Wolf-Dietrich Hardt and Andreas Bäumler.

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Dear Reviewers,

We sincerely thank you for recommending publication of our manuscript. We have included a sentence in lines 533-536 that addresses a thoughtful suggestion by Reviewer #3 on directions for identification of Bte3 targets in susceptible bacteria.

Best regards,

Juliane Bubeck-Wardenburg

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):
I am satisfied with the author's thorough responses to my initial comments. I have no further comments or concerns and look forward to seeing this exciting manuscript published.

Reviewer #2 (Remarks to the Author):
My concerns are adequately addressed. I recommend publication.

Reviewer #2 (Remarks on code availability):
The code is sufficient.

Reviewer #3 (Remarks to the Author):
I fully acknowledge the authors for their comprehensive and rigorous response to the previous comments. All the raised points have been thoroughly addressed and validated through supplementary experiments. This study reveals the broad-spectrum antibacterial function of the T6SS effector Bte3 in Bacteroides fragilis, offering important new insights into the mechanisms underlying early-life gut microbiota colonization.

I only have one more minor suggestion.
Lines 523–533: The authors may consider briefly highlighting one or two key directions for future validation experiments in the section discussing the Bte3 target. For instance, transposon screening for resistant mutants in Enterobacteriaceae/Bacteroidales, or cross-linking co-immunoprecipitation for target protein identification. Providing such suggestions would offer clearer reference points for subsequent research.