

Klebsiella pneumoniae employs a type VI secretion system to overcome microbiota-mediated colonization resistance

Corresponding Author: Dr M. Ammar Zafar

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 is an interesting study that identifies the type six secretion system (T6SS) as important in the ability of the opportunistic pathogen *Klebsiella pneumoniae* (Kp) to colonise the mammalian gut. The authors perform a transposon screen that leads to the identification of the T6SS and they follow this up by making T6SS mutants to confirm its importance for Kp colonisation. They also show that some features of the gut environment appear to lead to upregulation of the T6SS. Finally, they study the potential impacts of the T6SS on members of the microbiota using metagenomics. This finding suggests that some members of the Betaproteobacteria are suppressed by the T6SS and a model species from this group (*Burkholderia cepacia*) was shown to indeed be targeted by the T6SS of Kp, which is consistent with this effect.

Overall, this study contributes to the field by showing the importance of the T6SS for Kp colonisation. However, a potential issue for this contribution is that one of the studies (Merciecca et al 2022 - reference 40) cited at L133 and L257 already demonstrates the importance of the T6SS for *K. pneumoniae* strain CH1157 gut colonisation, albeit less thoroughly and only for longer time scales. Nevertheless, to justify publication in Nature Communications, it is arguable that there needs to be a clear and major advance from this previous study. Certainly, it seems that the authors should have discussed this previous work in more detail and how their work is distinct from it (at present it is only cited in passing). In terms of data, one route to make this distinction clearer would be to go after the interesting results on which members of the microbiota are targeted by the T6SS. The fact that the data appear to suggest that the key species being targeted by Kp are from a quite unrelated group of bacteria is surprising, as the typical expectation is that it would be more closely-related species. Moreover, at present, the data do not show a causal link between the suppression of members of Betaproteobacteria and the ability of Kp to colonise. Showing this link, and providing more evidence that the T6SS is directly targeting these strains, would bring additional depth to this study and mark it out as more distinct from the previous work.

Reviewer #2

(Remarks to the Author)

The paper entitled "Transposon mutagenesis screen reveals a critical role for the type VI secretion system of *Klebsiella pneumoniae* in overcoming microbiota mediated colonization resistance." describes very clearly the role of *K. pneumoniae* T6SSs in intestinal colonization by original approaches focusing both on i) the regulation of T6SSs gene expression and ii) the impact on resident bacteria of the intestinal tract.

I have few remarks concerning the manuscript:

1. A publication very close to the subject needs to be discussed (Calderon-Gonzalez R., et al., 2023, Modelling the Gastrointestinal Carriage of *Klebsiella pneumoniae* Infections. mBio 14:e0312122)
2. Please make a better description of the different potential effectors present in the genome of the KPPR1S strain.
3. Do the regulators, associated with the environmental cues, have a synergistic effect on T6SSs expression (Fig 3B) and antibacterial activity (Fig 6)?

Minor Comments:

1. Figure 1: Statistical analysis is missing in panel D. Could you shed some light on how such different conditions were

compared?

2. Figure 2B: The interesting results presented in this panel are not directly linked to the story of the intestinal colonization process.
3. Lines 194-203 and lines 216-219: There is a lot of discussion on these parts of the Results section. Please modify the text.
4. Are *astC*, *ssuE* and *fepD* genes significantly identified in the first screening (Fig 1A)?
5. Results Fig 5B and D seems redundant to Fig 5A and C. The "rate of decline" could be mentioned in the text.
6. Figure 6, please, at least mention the CFU count in the text.
7. Conclusion line 332-333 over-interpretation of the results.
8. Line 362-363 "Lastly, the role of trans-acting factors such as siderophores would be masked when random mutants are tested en masse", could you better explain?
9. In method, make a presentation in the same order as in the results section. How was the FNR protein obtained? Please clarify the oropharynx CFU count.
10. Will the INseq data be available online?

Reviewer #3

(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 #4

(Remarks to the Author)

The authors performed a transposon mutagenesis screen using a murine model of *K. pneumoniae* GI colonization with an intact microbiota and identified several factors that allow *K. pneumoniae* to overcome colonization resistance (CR). They identified the type VI secretion system (T6SS) is critical for *K. pneumoniae* gut colonization. Betaproteobacteria species in the GI track that were reduced by *K. pneumoniae* in a T6SS dependent manner. They also identified transcriptional regulators that control T6SS gene expression and found that expression is activated only under in vitro conditions that mimic the gut environment.

Comments:

The results are generally supported by the data. However, quantification of Betaproteobacteria species or F/B ratio is suggested.

Minor comments:

1. Line 31: delete "(*K. pneumoniae*)"
2. Line 131: cite a reference for NTUH-K2044
3. Line 176: change "KPPR1" to "KPPR1S"
4. Line 218: change "V" to "Vibrio"
5. Line 302: ddPCR may include *Duncaniella freteri* for reconfirmation
6. Line 539: OD600 = 4.0, how about the CFU numbers?
7. Line 543: wild type color should also be indicated.
8. Line 661: change "KPPR1" as point 3

Reviewer #5

(Remarks to the Author)

The study of Bray et al takes a very broad approach to study the factors of *Klebsiella pneumoniae* that allow it to overcome colonization resistance. The authors begin with a transposon bank to identify colonization fitness factors and then focused on the T6SS which they identified in the screen. Subsequent experiments were directed at understanding the contribution to colonization of the two distinct T6SS loci, how these loci are regulated, and what organisms they may antagonize in the gut to overcome the colonization resistance. This study provides some additional data that were not previously reported; however, this work does not represent a great leap in knowledge of the T6SS and colonization resistance (CR) in *K. pneumoniae*.

The work with the transposon bank is particularly troublesome if the goal is to determine in vivo fitness data of *K. pneumoniae* gene. For such studies, a much more robust transposon bank is necessary. There are no data showing in vitro essential genes, the number of tn hits per gene in their library, hits across the genome, etc. Figure 1 should be a much better description of the bank itself. It is also not adequate to use a bank of 5000 mutants for Inseq fitness data. A mutant bank of only 5000 mutants would not be expected to even have 1 insertion in every gene as there are 5159 genes, albeit, some will be essential. This is too low and if only 5000 mutants can be used due to bottle neck effects, numerous pools of the library must be analyzed in different groups of mice. It appears that only two mice were used for these analyses which is not adequate.

What is the basis for the following statement "Thus, an inoculum of 10⁹ CFU containing ~5000 mutants was considered appropriate for transposon-insertion sequencing (INSeq) studies"? The sparsity of the mutant bank is exemplified by the fact

that the authors conclude that the T6SS is important for CR yet only a minority of the T6SS core genes were detected in their screen.

The supplemental table 1 is poorly described and does not include enough data. In the input library (5000 mutants), how many times are each of the 5130 genes have a tn insertion? There needs to be a column for this information.

Table S1 is not marked as such and why are there "sheet 2 and sheet 3" but only sheet 2 has information and there is no sheet 1.

There are no table legends to allow the reader to understand this table. What exactly is being shown?

Please state what each column lists. What is column G "candidate". This can be removed as all values are 1.

Adding the numbers of column B "NumInser" for which there is no description, there are 10,841. What is this number? Is column B the number of hits in these gene in the input library and column C the number in the output library? This seems very unlikely if there are only 5000 mutants used. If a gene is conditionally essential in the murine gut, the number of insertions tolerated should be close to 0. There are 470 genes listed on this table that the authors state significantly contributing to the ability of *K. pneumoniae* to colonize the mouse gut. Most still have a large number of hits however, and most of these genes, like the T6SSs, do not seem critical.

The title should be changed as I do not think it helps to mention the transposon mutagenesis screen. Also the word "critical" should be changed. The data show it has an auxiliary role, but it is certainly not critical, or perhaps "contributes to robust colonization". Along the same lines, Fig 1E shows that the *clpV* mutant has shedding defect compared to WT, but it is still able to colonize the mouse gut. It is expected that low level colonization with *K. pneumoniae* rapidly expands when treated with antibiotics.

In the methods, the RNA was not isolated from GI tissues, but rather from the cecal contents.

Please indicate on Figure 1C what regions were deleted in the T6SS-1 (T6-/+), T6SS-2 (T6+/-), or both T6SS-1 and T6SS-2 (T6-/-) mutants. This is very important to judge the significance of the data. This is especially true as you show that the WT strain was unable to rescue the colonization defect of the single and double T6SS locus mutants as expected by "cheating". Were the effector and immunity genes part of the deletion? If so, the WT strain would antagonize these mutants in the mouse gut and on plates.

In the section of novel regulators, please give more description of the functions of the deleted genes.

The manuscript by Storey et al. published in PLoS Pathogens in 2020 performed some overlapping regulation studies. These authors showed that pH, iron, oxygen tension and osmolarity control the expression of *K. pneumoniae* T6SS (Storey et al, 202 PLoS Path). Some of this is repeated here. They also showed that PmrAB, Hfq, Fur, RpoS and RpoN positively regulate the T6SS.

Fig 3 - As the authors use DPI-ELISA instead of EMSA for protein-DNA binding assays, the control with competing unlabeled DNA is necessary to show specificity.

You have incorrectly stated that *Oscillibacter* is a Gram negative bacterium, it is a Clostridia. In addition, a previous analysis of *K. pneumoniae* competition in the gut also revealed that it likely competes with *Oscillibacteraceae* (Merciecca, T. et al. Sci Rep 2022)

Strain level variation in the microbiome are frequent and may occur normally without *K. pneumoniae* colonization. Were there any reductions in strains when colonized with the T6SS mutant compared to WT? Fig 4D – It is unclear if this metagenomic analysis was performed with both the WT and the T6S mutant.

The T6SS-mediated antagonism shown in Figure 6 are very minimal. Antagonism is usually at least half a log and for more robust killing T6SS mediated killing is on the order of 3 log. This study reports 2-fold killing under the best conditions. Although the data are reproducible.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for the comments. However, the case for publication appears no stronger than at the last round of review, because the initial review has not been sufficiently addressed. For instance, the authors state that "*K. pneumoniae* reduces *Parasutterella* in a T6SS dependent manner", but it is still unclear whether the reduction of *Parasutterella* is due to direct killing of *Parasutterella* by the T6SS or if it is due to the increased abundance of *K. pneumoniae* in the intestines afforded by its T6SS. Moreover, it is not clear if this effect on *Parasutterella* impacts *Klebsiella* colonisation. Given this, the paper unfortunately still comes across as too similar to earlier work (e.g. Merciecca et al 2022, as mentioned in the previous round of review) to justify publication in a top journal.

Reviewer #2

(Remarks to the Author)

The manuscript entitled "Transposon mutagenesis screen reveals a role for the type VI secretion system of *Klebsiella pneumoniae* in overcoming microbiota mediated colonization resistance" by Bray et al. was revised following comments from the reviewer. A point-by-point response has been provided which addresses and clarifies the main criticisms and suggestions. I propose that this manuscript is now acceptable for publication.

Reviewer #3

(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 #5

(Remarks to the Author)

The authors have included the new information requested and provided clarification of methods and results that make this manuscript much easier to understand and the resulting data easier to interpret. The new information and data help validate their conclusions.

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Reviewer #1 (Remarks to the Author):

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Overall, this study contributes to the field by showing the importance of the T6SS for Kp colonisation. However, a potential issue for this contribution is that one of the studies (Merciecca et al 2022 - reference 40) cited at L133 and L257 already demonstrates the importance of the T6SS for *K. pneumoniae* strain CH1157 gut colonisation, albeit less thoroughly and only for longer time scales. Nevertheless, to justify publication in Nature Communications, it is arguable that there needs to be a clear and major advance from this previous study. Certainly, it seems that the authors should have discussed this previous work in more detail and how their work is distinct from it (at present it is only cited in passing).

We appreciate the reviewer for highlighting the contribution of our study to *K. pneumoniae* pathogenesis. We have provided further context to our data, discussed the previous study by Merciecca et al, and how our results build upon their initial causal study by providing evidence that *K. pneumoniae* reduces specific members of the gut microbiota through the use of its T6SS. These changes have been highlighted in yellow throughout the manuscript.

In terms of data, one route to make this distinction clearer would be to go after the interesting results on which members of the microbiota are targeted by the T6SS. The fact that the data appear to suggest that the key species being targeted by Kp are from a quite unrelated group of bacteria is surprising, as the typical expectation is that it would be more closely-related species. Moreover, at present, the data do not show a causal link between the suppression of members of Betaproteobacteria and the ability of Kp to colonise. Showing this link, and providing more evidence that the T6SS is directly targeting these strains, would bring additional depth to this study and mark it out as more distinct from the previous work.

We agree with the reviewer that rather than provide a causal link that other studies have provided, we need to establish that *K. pneumoniae* reduces these novel betaproteobacteria strains in a T6SS dependent manner. We direct the reviewer to our results where we performed droplet digital PCR (ddPCR) which allows for absolute quantification of specific nucleotide sequences, in this case that show that *Parasutterella* is targeted in a T6SS manner. These results are included in the section "*K. pneumoniae* reduces *Parasutterella* in a T6SS dependent manner". We would like to highlight here that this is the first instance where a gut commensal that naturally occupies the GI tract (human and mice), has been shown to be reduced in a T6SS dependent manner. Further novelty is added, as commensal betaproteobacteria have not been shown to be targeted by the T6SS.

Reviewer #2 (Remarks to the Author):

The paper entitled “Transposon mutagenesis screen reveals a critical role for the type VI secretion system of *Klebsiella pneumoniae* in overcoming microbiota mediated colonization resistance.” describes very clearly the role of *K. pneumoniae* T6SSs in intestinal colonization by original approaches focusing both on i) the regulation of T6SSs gene expression and ii) the impact on resident bacteria of the intestinal tract.

I have few remarks concerning the manuscript:

1. A publication very close to the subject needs to be discussed (Calderon-Gonzalez R., et al., 2023, Modelling the Gastrointestinal Carriage of *Klebsiella pneumoniae* Infections. mBio 14:e0312122)

We thank the reviewer for bringing to our attention the recent study. While we had cited that work, we have now incorporated their results into our section describing the Δ clpV mutant. We build on their work and provide a more comprehensive understanding of *K. pneumoniae* T6SS with regards mucosal colonization.

2. Please make a better description of the different potential effectors present in the genome of the KPPR1S strain.

We have added further details about the putative effector proteins from either locus. This information is now included in the section titled “**The T6SS of *K. pneumoniae* is a colonization determinant**”. To further aid the detail, we have included a **Supplemental fig. 3**.

3. Do the regulators, associated with the environmental cues, have a synergistic effect on T6SSs expression (Fig 3B) and antibacterial activity (Fig 6)?

We tested anaerobic growth with arginine as the sole nitrogen source, which both individually lead to upregulation of the T6SS and killing of the prey strain. However, when combined (anaerobiosis + arginine), we did not observe any increased killing, so it does not appear that these conditions are synergistic. These new results are described in the “T6SS dependent interbacterial competition” section.

Minor Comments:

1. Figure 1: Statistical analysis is missing in panel D. Could you shed some light on how such different conditions were compared?

Statistical analysis has now been added to Fig. 1D.

RNA was isolated from cecal contents, and minimal media (M63) grown culture (mid-log phase). We used primers specific to *K. pneumoniae* and 16s served as normalization control. Fold-change was calculated using the delta-delta Ct method. These details are provided now in the Methods section and in figure legend.

2. Figure 2B: The interesting results presented in this panel are not directly linked to the story of the intestinal colonization process.

The focus of our current study is on factors identified through InSeq and their importance in gut colonization, which included the T6SS. However, we wanted to provide a comprehensive understanding of *K. pneumoniae* T6SS loci in their contribution in both gut and lung colonization. For context in a previous transposon mutagenesis screen the T6SS was identified to be important for lung colonization (Lawlor et al 2005) and a recent study showed that the *K. pneumoniae* T6SS was upregulated in the lungs (Wong Fok Lung et al 2022). However, neither of these studies showed whether the T6SS of *K. pneumoniae* is important for lung colonization. Thus, we believe that our results provide important context for the importance of *K. pneumoniae* T6SS as it relates to mucosal colonization of the GI tract and the lung.

3. Lines 194-203 and lines 216-219: There is a lot of discussion on these parts of the Results section. Please modify the text.

Based on the reviewer feedback we have streamlined those sections.

4. Are *astC*, *ssuE* and *fepD* genes significantly identified in the first screening (Fig 1A)?

astD, *astE*, *ssuE* and *fepD* were identified in our INSeq approach to be important for gut colonization. We have added this detail to the result section.

5. Results Fig 5B and D seems redundant to Fig 5A and C. The “rate of decline” could be mentioned in the text.

While we agree with the reviewer that the rate of decline panel in **Fig. 5** is redundant, we would like to emphasize that providing a visualization of the slopes in combination with the ddPCR results furnishes a strong argument for our ddPCR results which show that *K. pneumoniae* reduces *Parasutterella* in a T6SS dependent manner. Therefore, we prefer to keep both the ddPCR and the corresponding rate of decline slopes as part of **Fig. 5**.

6. Figure 6, please, at least mention the CFU count in the text.

CFU count has been added to the figure legend of Figure 6.

7. Conclusion line 332-333 over-interpretation of the results.

We have modified the sentence to make it specific to the conditions and the strain tested.

8. Line 362-363 “Lastly, the role of trans-acting factors such as siderophores would be masked when random mutants are tested en masse”, could you better explain?

We have added another line to provide better context.

9. In method, make a presentation in the same order as in the results section. How was the FNR protein obtained? Please clarify the oropharynx CFU count.

We have further streamlined and rearranged the method section.

The FNR gene was cloned into the pET expression vector and then the active site was mutated (D154A), using site-directed mutagenesis. The mutant protein was shown to be active under aerobic conditions (PMID 8406003; 7837268). These references have now been included in the protein purification section.

The oropharyngeal lavage was performed by passing PBS buffer using a needle through the esophagus and collecting through mouth. Buffer (200 ul) was collected, serially diluted and plated on selective media plates to enumerate *K. pneumoniae*. Further details are provided in our *K. pneumoniae* murine model study (Taylor et al 2020).

10. Will the INseq data be available online?

We have added the INSeq results in the excel file included (**Supplementary Table I**).

Reviewer #3 (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 #4 (Remarks to the Author):

The authors performed a transposon mutagenesis screen using a murine model of *K. pneumoniae* GI colonization with an intact microbiota and identified several factors that allow *K. pneumoniae* to overcome colonization resistance (CR). They identified the type VI secretion system (T6SS) is critical for *K. pneumoniae* gut colonization. Betaproteobacteria species in the GI track that were reduced by *K. pneumoniae* in a T6SS dependent manner. They also identified transcriptional regulators that control T6SS gene expression and found that expression is activated only under in vitro conditions that mimic the gut environment.

Comments:

The results are generally supported by the data. However, quantification of Betaproteobacteria species or F/B ratio is suggested.

To quantify betaproteobacteria species, we performed droplet digital PCR (ddPCR) which allows for absolute quantification of specific nucleotide sequence, in this case that targets specifically *Parasutterella* in a T6SS manner. Our results conclusively show that that *Parasutterella* is reduced in a T6SS dependent manner. These results are described in the section titled "*K. pneumoniae* reduces *parasutterella* in a T6SS-dependent manner."

Per reviewer suggestion, we performed the F/B ratio analysis to determine if there was a microbial imbalance. While there was slight difference when comparing day 0 to day 2 post infection, no other days were significantly different when compared to pre-inoculation (day 0). Furthermore, when comparing day 0 to all the days pooled together, no differences were observed, suggesting that *K. pneumoniae* colonization of the gut does not cause major microbial disruption. These results have been added to the supplemental figure 6.

Minor comments:

1. Line 31: delete "(*K. pneumoniae*)" **Fixed**
2. Line 131: cite a reference for NTUH-K2044 **Added**
3. Line 176: change "KPPR1" to "KPPR1S" **The strain used in that previous screen (Lawlor et al 2005) was KPPR1 and not KPPR1S, so this was not a typo.**
4. Line 218: change "V" to "Vibrio" **Fixed**
5. Line 302: ddPCR may include *Duncaniella freteri* for reconfirmation **Added**
6. Line 539: OD₆₀₀ = 4.0, how about the CFU numbers? **Predator strains were grown overnight and adjusted to OD₆₀₀ 4.0, and then subcultured again to 1:100 into fresh media. We did not do CFU count when adjusting to 4.0, as we wanted a qualitative adjustment to have all strains at equal OD₆₀₀ before subculture.**
7. Line 543: wild type color should also be indicated. **Fixed**
8. Line 661: change "KPPR1" as point 3 **Fixed**

Reviewer #5 (Remarks to the Author):

The study of Bray et al takes a very broad approach to study the factors of *Klebsiella pneumoniae* that allow it to overcome colonization resistance. The authors begin with a transposon bank to identify colonization fitness factors and then focused on the T6SS which they identified in the screen. Subsequent experiments were directed at understanding the contribution to colonization of the two distinct T6SS loci, how these loci are regulated, and what organisms they may antagonize in the gut to overcome the colonization resistance. This study provides some additional data that were not previously reported; however, this work does not represent a great leap in knowledge of the T6SS and colonization resistance (CR) in *K. pneumoniae*.

The work with the transposon bank is particularly troublesome if the goal is to determine in vivo fitness data of *K. pneumo* gene. For such studies, a much more robust transposon bank is necessary. There are no data showing in vitro essential genes, the number of tn hits per gene in their library, hits across the genome, etc. Figure 1 should be a much better description of the bank itself. It is also not adequate to use a bank of 5000 mutants for Inseq fitness data. A mutant bank of only 5000 mutants would not be expected to even have 1 insertion in every gene as there are 5159 genes, albeit, some will be essential. This is too low and if only 5000 mutants can be used due to bottle neck effects, numerous pools of the library must be analyzed in different groups of mice. It appears that only two mice were used for these analyses which is not adequate.

To clarify, our mutant library included 56,757 unique insertions in the open reading frame of the genome of strain KPPR1S, which provides a 10-fold coverage of the genome, and not only 5000 mutants. The genome hits are now included in **Supplemental figure 1** which shows all the inserts in the KPPR1S genome. We did not identify any insert (68 genes) or only 1 insert (120 genes) in KPPR1S genome, suggesting these genes to be essential for growth under *in vitro* conditions. This information now has been added in to the results section.

Furthermore, a total of 9 independent pools of mutants, with each pool containing at least ~5000 mutants was tested at a high dose 10^9 CFU to overcome potential GI bottlenecks (Shown in Supplemental figure 2, and supplemental table 1). Bottleneck analysis, or stochastic loss of mutants, was used to determine the number of mutants that would eventually occupy the GI tract (Supplemental Figure 2). For this purpose, we performed inoculation studies with two distinct antibiotic resistant KPPR1 strains (Apramycin and Streptomycin) and determined that we could test 10^3 mutants (Supplemental Figure 2). By inoculating mice at 10^9 CFU infectious dose each mutant is present in high enough number to potentially overcome the intrinsic GI bottleneck. Therefore, a reduction in a specific mutant (output compared to input) is because of an intrinsic defect and not due to bottleneck.

Each pool was inoculated to two mice; thus, we would have eighteen mice and not two mice tested for our screen. In total we had 9 input pools and 18 output pools.

What is the basis for the following statement “Thus, an inoculum of 10^9 CFU containing ~5000 mutants was considered appropriate for transposon-insertion sequencing (INSeq) studies”? The sparsity of the mutant bank is exemplified by the fact that the authors conclude that the T6SS is important for CR yet only a minority of the T6SS core genes were detected in their screen. **Please see the response above**

The supplemental table 1 is poorly described and does not include enough data. In the input library (5000 mutants), how many times are each of the 5130 genes have a tn insertion? There needs to be a column for this information.

Table S1 is not marked as such and why are there “sheet 2 and sheet 3” but only sheet 2 has information and there is no sheet 1.

There are no table legends to allow the reader to understand this table. What exactly is being shown? Please state what each column lists. What is column G “candidate”. This can be removed as all values are 1.

Adding the numbers of column B “NumInser” for which there is no description, there are 10,841. What is this number? Is column B the number of hits in these gene in the input library and column C the number in the output library? This seems very unlikely if there are only 5000 mutants used. If a gene is conditionally essential in the murine gut, the number of insertions tolerated should be close to 0. There are 470 genes listed on this table that the authors state significantly contributing to the ability of *K. pneumoniae* to colonize the mouse gut. Most still have a large number of hits however, and most of these genes, like the T6SSs, do not seem critical.

We have provided further details in the INSeq supplemental table, including a key to help guide the user. The genes if they are listed as 1, are those that meet the criteria of being important for gut colonization. Many of these would likely not be important for growth under *in vitro* conditions, hence not critical for growth. However, they provide an advantage to *K. pneumoniae* in the unique environment of the GI tract, which is considerably different from *in vitro* broth growth condition.

The title should be changed as I do not think it helps to mention the transposon mutagenesis screen. Also the word “critical” should be changed. The data show it has an auxiliary role, but it is certainly not critical, or perhaps “contributes to robust colonization”. Along the same lines, Fig 1E shows that the *clpV* mutant has shedding defect compared to WT, but it is still able to colonize the mouse gut. It is expected that low level colonization with *K. pneumoniae* rapidly expands when treated with antibiotics.

Per the suggestion of the reviewer, we have removed the word critical. However, our transposon mutagenesis screen was performed with an intact gut microbiota and as there is no previous instance of such a screen being performed without antibiotics, we consider it to be novel and for that purpose we would like to highlight that in our title.

In the methods, the RNA was not isolated from GI tissues, but rather from the cecal contents.

We have corrected that to include cecal contents

Please indicate on Figure 1C what regions were deleted in the T6SS-1 (T6-/+), T6SS-2 (T6+/-), or both T6SS-1 and T6SS-2 (T6-/-) mutants. This is very important to judge the significance of the data. This is especially true as you show that the WT strain was unable to rescue the colonization defect of the single and double T6SS locus mutants as expected by “cheating”. Were the effector and immunity genes part of the deletion? If so, the WT strain would antagonize these mutants in the mouse gut and on plates.

The deletion strains constructed were either missing T6SS locus 1 (structural genes and effector immunity pairs), locus 2 (structural genes and effector immunity pairs) or both (completely missing all structural genes and effector immunity pairs). We have added further details for clarification in the results section about these mutants, and their construction is detailed in the methods section. The reviewer is correct that the WT would outcompete the mutant as they are missing effector immunity pairs. However, our data show that both loci contribute to colonization with a greater defect with the double mutant, suggesting the importance of having both T6SS loci. Furthermore, we also show that the *clpV* mutant is defective in gut colonization when inoculated alone, but in coinfection studies it behaves as WT acting as a social cheater. Collectively these studies are important in our understanding of the contribution of each locus in gut colonization in the presence of an intact microbiota.

In the section of novel regulators, please give more description of the functions of the deleted genes.

We have added further details about *astC*, *ssuE* and *fepD*. These genes were not deleted, instead we tested their expression in the GI tract, to provide evidence that regulators ArgR, FNR and Fur are active in the GI tract. *astC* is regulated by ArgR, *ssuE* through FNR, and *fepD* through Fur.

The manuscript by Storey et al. published in PLoS Pathogens in 2020 performed some overlapping regulation studies. These authors showed that pH, iron, oxygen tension and osmolarity control the expression of *K. pneumoniae* T6SS (Storey et al, 202 PLoS Path). Some of this is repeated here. They also showed that PmrAB, Hfq, Fur, RpoS and RpoN positively regulate the T6SS.

The role of Fur and iron levels has been explored by Storey et al in modulating *K. pneumoniae* T6SS, and by other groups for other gram-negative bacteria. However, roles of arginine and the anaerobic environment, which are likely encountered by *K. pneumoniae* in the GI tract has not been examined in

previous studies. Together with the two regulators ArgR and FNR, our results identify novel conditions and regulators involved in modulating the activity of the T6SS. We have further incorporated the results from Storey et al in the discussion section with regards to iron, oxygen tension, and the role of Fur and RpoN. We thank the reviewer for highlighting this study as we believe these additions to the text add further strength to our manuscript.

Fig 3 – As the authors use DPI-ELISA instead of EMSA for protein-DNA binding assays, the control with competing unlabeled DNA is necessary to show specificity.

The control for these assays as described by the authors (PMID: 21108821) is considered binding to non-specific DNA. However, we have addressed the reviewer's concerns and performed competition assays using unlabeled oligomers (non-biotinylated) containing the putative Fur binding site or a scrambled control sequence as competitor to show as an example that Fur binding to the *t6ss* promoter region is specific. These results are part of **Supplemental figure 5H-I**.

You have incorrectly stated that *Oscillibacter* is a Gram negative bacterium, it is a Clostridia. In addition, a previous analysis of *K. pneumoniae* competition in the gut also revealed that it likely competes with Oscillibacteraceae (Merciecca, T. et al. Sci Rep 2022).

Oscillibacter sp. 1-3 is an unclassified taxon, so its precise gram-stain characteristic remains unknown but all other classified species from genus *Oscillibacter* have been found to be gram negative (PMID: 32712374; 24501034). Furthermore, while it is generally believed that all Clostridia are gram positive, there are several genera within Clostridia that are gram negative (Medical Microbiology: Clostridia: Spore forming anaerobic Bacilli; PMID: 23834245). For these reasons, we focused on *Parasutterella* as it been characterized in greater detail compared to *Oscillibacter* as a gram negative gut commensal. As suggested by the reviewer, we have also further highlighted the Merciecca et al. study in our manuscript.

Strain level variation in the microbiome are frequent and may occur normally without *K. pneumoniae* colonization. Were there any reductions in strains when colonized with the T6SS mutant compared to WT? Fig 4D – It is unclear if this metagenomic analysis was performed with both the WT and the T6S mutant.

Our metagenomics screen was performed on mice inoculation with the WT strain (KPPR1S) and samples were collected at either day 0 pre-inoculation and at day 2, 4 and 7 post inoculation. Post inoculation samples (WT infection) were compared to their corresponding non infection control at day 0.

As the T6SS mutant colonizes at lower density, any microbiota changes could be due to either lack of the T6SS or reduced colonization. Our metagenomics screen with the WT inoculated mice identified gram-negative bacterial species that might serve as *K. pneumoniae* T6SS prey. These members were then validated to serve as T6SS dependent prey strain using our ddPCR assay.

The T6SS-mediated antagonism shown in Figure 6 are very minimal. Antagonism is usually at least half a log and for more robust killing T6SS mediated killing is on the order of 3 log. This study reports 2-fold killing under the best conditions. Although the data are reproducible.

We agree with the reviewer that antagonism studies with T6SS generally tend to show a much greater impact. However, those studies are usually performed for a much longer duration (6 hours or more). Because of faster growth kinetics of *K. pneumoniae* compared to the prey strain, our *in vitro* antagonism

studies were carried out for only two hours. Furthermore, our results consistently show a T6SS dependent effect under the three conditions tested, with two different *K. pneumoniae* isolates, which highlights the robustness of our assay and results.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you for the comments. However, the case for publication appears no stronger than at the last round of review, because the initial review has not been sufficiently addressed. For instance, the authors state that “*K. pneumoniae* reduces *Parasutterella* in a T6SS dependent manner”, but it is still unclear whether the reduction of *Parasutterella* is due to direct killing of *Parasutterella* by the T6SS or if it is due to the increased abundance of *K. pneumoniae* in the intestines afforded by its T6SS. Moreover, it is not clear if this effect on *Parasutterella* impacts *Klebsiella* colonisation. Given this, the paper unfortunately still comes across as too similar to earlier work (e.g. Merciecca et al 2022, as mentioned in the previous round of review) to justify publication in a top journal.

We appreciate the reviewer's concerns. However, our study is different from the Merciecca et al. paper in that 1) we performed a transposon mutagenesis screen with an intact gut microbiome, 2) we performed a metagenomic analysis that identified the strain level changes that occur due to *K. pneumoniae* gut colonization, and 3) we identified three regulators of t6ss gene expression (Fur, Fnr, and ArgR) and showed that expression is induced under in vitro conditions that are consistent with the known functions of these regulators and these conditions mimic those likely found in the GI tract. These results provide a much more thorough understanding of *K. pneumoniae* gut colonization than the Merciecca study, which focused solely on the T6SS.

Regarding the reduction of *Parasutterella* by *K. pneumoniae*, we direct the reviewer to Figure 5, where we demonstrate through ddPCR that colonization by *K. pneumoniae* leads to a reduction in *Parasutterella* levels. To account for the differences in colonization density between the WT and the full T6SS mutant (T6-/-) we normalized *Parasutterella* DNA levels in the fecal samples to *K. pneumoniae* fecal CFU. This data strongly suggests that *K. pneumoniae* reduces *Parasutterella* in a T6SS-dependent manner. It is difficult to tell if Kp is killing *Parasutterella* or just interfering with its replication, so we limit the language to ‘reduction’. To further strengthen our ddPCR results, we show that *Duncaniella*, a strain that appeared unaffected by *K. pneumoniae* in our metagenomics screen, is also not affected in the ddPCR results. Thus, our ddPCR results provide strong evidence for a novel relationship between *K. pneumoniae* and *Parasutterella* in the murine GI tract.

Reviewer #2 (Remarks to the Author):

The manuscript entitled ‘Transposon mutagenesis screen reveals a role for the type VI secretion system of *Klebsiella pneumoniae* in overcoming microbiota mediated colonization resistance’ by Bray et al. was revised following comments from the reviewer. A point-by-point response has been provided which addresses and clarifies the main criticisms and suggestions. I propose that this manuscript is now acceptable for publication.

We thank the reviewer for providing us with critical feedback that allowed us to further improve our studies.

Reviewer #3 (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 #5 (Remarks to the Author):

The authors have included the new information requested and provided clarification of methods and results that make this manuscript much easier to understand and the resulting data easier to interpret. The new information and data help validate their conclusions.

We appreciate the reviewers feedback that helped us make this a more convincing study.

Guidance from independent reviewer:

Reviewers 1 and 4 are true. Indeed, the direct action of T6SS of Kp on *Parasutterella* is not demonstrated but the use of proxy species allows to validate T6SS activity under the conditions of the intestinal tract.

Authors should be careful, as the wording “demonstrates a link between T6SS *K. pneumoniae* function and *Parasutterella* depletion” is correct. The section titled “*K. pneumoniae* reduces *parasutterella* in a T6SS-dependent manner.” could be modified as “In the gut, the T6SS *K. pneumoniae* reduces *parasutterella* directly or indirectly.

Based on the reviewers comment we have tampered down the language. We have changed the title of section to “*K. pneumoniae* colonization reduces *Parasutterella* in the gastrointestinal tract.”