

Differential gut transmission of IncP plasmid clades involving hypervirulent *Klebsiella pneumoniae* reveals plasmid-specific ecological adaptation

Corresponding Author: Professor Yunn-Hwen Gan

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)

In this manuscript, Yong et al. investigate the conjugation dynamics of plasmid pKPC2, a carbapenem-resistance plasmid derived from *Klebsiella pneumoniae*, within a murine gut model. The study contrasts the conjugative behaviour of pKPC2 with that of RP4, a canonical member of the IncP plasmid family. The authors report three principal findings. The first one is the finding that pKPC2 demonstrates markedly higher conjugation efficiency in the gut than RP4. This disparity is attributed to the differing sensitivities of the plasmids to anaerobic conditions, suggesting that oxygen availability plays a critical role in modulating horizontal gene transfer (HGT) within the gut environment. Overall, I find this point well supported by the experimental results.

The second key idea is that pKPC2 appears to form a distinct clade within the IncP group which is significantly more prevalent in mammalian and human-associated microbiota. In contrast, RP4-like plasmids are underrepresented in these niches and are more commonly found in environmental contexts. These observations support the notion of ecological specialisation within the IncP group, with pKPC2 representing a lineage adapted to the anoxic conditions of the mammalian gut. As I explain later, I have some major concerns regarding this point.

Finally, the authors show that the capsule acts as a permeable barrier for horizontal gene transfer. Although the *Klebsiella* capsule is traditionally considered a barrier to conjugation, the authors provide evidence that, under in vivo gut conditions, it does not entirely inhibit plasmid transfer. Capsulated strains were shown to receive plasmids at frequencies approaching those of non-capsulated strains, indicating that the barrier is more porous than previously thought.

The manuscript is well written, and the results are clearly presented. However, the structure could benefit from a more unified conceptual thread. The current presentation at times lacks narrative coherence, making it challenging to discern the principal message. In my view, the most significant contribution of the work lies in its demonstration that microenvironmental factors (specifically oxygen availability) profoundly shape plasmid conjugation dynamics, with consequences observable at the level of plasmid biogeography. This central insight, however, is somewhat obscured by a few issues.

Major Comments

1.- A fundamental issue pertains to the degree of genetic similarity between the plasmids designated as "IncP." This is not a shortcoming of the study per se, but rather a consequence of the reliance on historical incompatibility groups, which conflate two different meanings: plasmids that are highly similar at the genetic level, and plasmids that merely share a conjugation system. In the case of IncP (like in the IncF) these labels often obscure considerable genomic heterogeneity. I am afraid the pKPC2 belongs to the second category, and although it contains an IncP conjugation system, it cannot be strictly considered a bona fide IncP group (in the strict meaning of the term). A quick estimation indicates that the average nucleotide identity (ANI) between pKPC2 and RP4 is well below 70%, indicating that they do not belong to the same plasmid taxonomic unit. Looking at the relaxase (a key phylogenetic marker for plasmid distance), they belong to the same MOB family but their identity is way lower than you'd expect if they were from the same group. This divergence likely accounts for the phenotypic differences observed, including the restricted host range of pKPC2, which appears unable to colonise *Pseudomonas*, RP4's native host. While the authors' phylogenetic bifurcation of IncP plasmids into Clade I and Clade II is a reasonable starting point, it remains ambiguous whether these groupings reflect meaningful taxonomic units in the absence of defined similarity

thresholds.

To address this, I would strongly encourage the authors to undertake a more rigorous classification strategy, including:

a) Delineation of a Novel PTU: pKPC2 appears to represent a distinct and highly prevalent plasmid lineage within the human gut. The authors possess a dataset of closely related plasmids—an analysis of sequence conservation within this set would be highly informative. Specifically, do these sequences meet the proposed PTU criterion (>70% nucleotide identity across >50% of the genome)? If so, this would suggest that a specific plasmid “species” underpins a major pathway of antibiotic resistance dissemination in the gut.

b) Extrapolation of Oxygen Sensitivity Across Clade Members: The authors should assess the degree to which the anaerobic resistance trait is shared across related plasmids (e.g., those tested in Figure 5a). What is the similitude between these plasmids? Are these plasmids members of the same PTU as pKPC2? Given the substantial divergence between Clades I and II, it is conceivable that they represent separate PTUs altogether, with distinct ecological and functional profiles.

The authors have already performed a significant part of this, as shown in Supplementary Figure 5a and b. In this figure the existence of at least three separate identity clusters, pointing out to three different plasmid taxonomic units. A thorough reanalysis along these lines would greatly enhance the clarity and impact of the study.

2.- The data addressing the role of transconjugants in sustaining conjugation in vivo is compelling but raises certain concerns. The authors demonstrate that the pKPC2ΔtraF + pACYC_TraF strain conjugates less efficiently than the wild-type in mice, which, in principle, supports the hypothesis that transconjugants are the primary vectors of plasmid spread. However, Supplementary Figures 2 and 3 suggest that traF complementation in *Klebsiella* is not fully working, and that the pACYC vector exhibits substantial instability in *E. coli*. These findings undermine the inference that transconjugants are the dominant plasmid propagators, specially when a decline in effective donors is accompanied by a parallel decline of transconjugants (Supplementary Figure 3, final days). To cast out these doubts I would recommend the authors to conduct an experiment in which recipient cells vastly outnumber donors (maybe 2-3 logs). Under such conditions, any sustained conjugation would necessarily rely on secondary transfer events. A comparison of pKPC2 and pKPC2ΔtraF + pACYC_TraF would thereby offer a more definitive test of the hypothesis.

Minor Comments

Line 28-29: “ongoing donor input was unnecessary after establishment”. Maybe I am missing something, but I could not find an experiment that supported this claim. Did the authors perform any experiment in which donors were specifically eliminated and the transconjugants kept increasing?

Line 43: The citations provided for early work on F and P plasmids (Refs 2 and 3) are somewhat anachronistic. For IncP plasmids, a more appropriate reference is the foundational work by Pühler (Gen Microbiol 1979, DOI: 10.1099/00221287-114-2-341). For the IncF group, the early taxonomic efforts of Naomi Datta are probably more historically accurate (DOI: 10.1017/s0016672300009538).

Line 93: Change “mammalian gut” to “murine gut” for precision

Paragraph starting in Line 161. The authors claim that the Bayesian analysis revealed the initial T population as the most significant contributor to the final conjugation frequency. However, conjugation frequency is just T/R and final T cells may descend from other T cells not just by conjugation but also by vegetative replication. How is this factor taken into account? If this is not factored in the analysis, I find it hardly surprising that the final T population depends mostly on the initial T population, given the average conjugation frequencies observed.

Lines 187–188: This paragraph is potentially misleading. It first states that traF complementation fully restores conjugation frequency in *E. coli*, and then adds that “similarly, with SGH10 as donors carrying pKPC2ΔtraF, no transfer occurred unless traF was complemented in trans.” This phrasing may lead readers to assume that complementation in *Klebsiella* is as effective as in *E. coli*. However, the data indicate otherwise: in *Klebsiella*, complementation results in conjugation frequencies that are, at first glance, at least an order of magnitude lower than those observed with the wild-type plasmid. This discrepancy should be explicitly acknowledged in the text.

Reviewer #2

(Remarks to the Author)

Yong et al. investigate plasmid transfer dynamics in *Klebsiella pneumoniae*, which is particularly problematic in terms of AMR. The authors focus on hypervirulent *Klebsiella*, which produce a capsule that aids its virulence, but also is thought to slow plasmid spread based on in vitro studies. They study an IncP plasmid, pKPC2, which is prevalent in hospital settings and has previously been documented to spread with high efficiency in vitro (along with other plasmids).

The authors’ goal is to investigate plasmid transfer dynamics in vivo, which has so far not been well-studied with hypervirulent *Klebsiella*. Using a combination of mouse experiments and anaerobic culture, the authors state that clade II IncP plasmids (including pKPC2) transfer better in anaerobic conditions in the gut than clade I IncP plasmids, which may help explain their higher prevalence in hospital settings and in the mammalian gut. Lastly, the authors also show that the capsule of hypervirulent *Klebsiella* that has classically been thought to restrict plasmid spread for plasmids that use small pili to transfer plasmids like IncP plasmids actually does not pose a significant barrier under anaerobic conditions.

Overall, the authors study an interesting question of what drives plasmid spread in niches like the gut microbiome. The study

is nicely descriptive and well-controlled. I do not have many comments, aside from clarifying a bit better the limitations of the author's experimental system in the context of an intact microbiome. Please see below a list of comments:

Major comments:

1. In Fig. 2a, the difference between transfer of pKPC2 to *Klebsiella* with and without the capsule seems like it would be statistically different on Day 3 of the experiment (with secondary transfer). This seems to be a bit at odds with the results from Fig 1d. Indeed, in Table S1 it seems like there is a significant difference between transfer efficiencies in these two groups on D3 (p value= 0.017159). The authors should comment on this and adjust their language. Generally, are the early plasmid transfer dynamics not having differences a limitation of sample size? Perhaps the experimental system is not sensitive enough to see the effect of the capsule. Could it be that in the presence of an intact microbiota where the effect of transfer efficiency is likely more important, the role of the capsule would be amplified?
2. Related to the above comment, the authors should discuss more explicitly what the context of an intact microbiome would do to the transfer dynamics in their system. The authors state recipient amplification via transconjugants is the primary driver of plasmid spread, but what about cases where the niche for the recipient is much smaller than the donor? Presumably this would mean that in this case D to R transfer would outpace T to R transfer?

Minor comments:

1. Fig 1 labels – It would be helpful to label on the figure itself which strain is a donor and which is a recipient. With many combinations, it is much more readable to have all the information on the figure itself without having to rely on the figure legend.
2. Line 28 – “secondary transfer by transconjugants” instead of “transconjugant”
3. Fig 5b – it would be helpful to have an aerobic comparison side-by-side rather than referring back to Fig. 1. Perhaps one can plot fold-change resulting from aerobic -> anaerobic in both the capsule mutant and the wild-type to clearly show the effect.
4. Lines 178-179, the authors state that donor to recipient transfer is expected to be the primer driver plasmid dynamics and cite reference 31. In cases where recipient densities are exceedingly high, a rare donor has been shown to have dramatic consequences. E.g. <https://doi.org/10.1038/s41586-019-1521-8>. The authors may want to expand their discussion on this point slightly, as their finding is not entirely unexpected. Also see major point #2.

Reviewer #3

(Remarks to the Author)

The manuscript by Yong and colleagues characterizes the ability of IncP plasmids to conjugate to and from hypervirulent *K. pneumoniae* in vitro and in a gut colonization model in vivo. They find that Clade II IncP plasmids have high efficiency in the gut, and that secondary transfer of the plasmid from transconjugants to new recipients seems to drive high transconjugant levels. They also show that unlike in vitro, in vivo the capsule is not a barrier to conjugation, and find that anaerobic growth decreases mucoviscosity and increases conjugation efficiency. Overall, this is a valuable study demonstrating that conjugation efficiency depends on the environment, and some plasmids may be especially adept at transferring in the gut environment. This is a well-conceived study with appropriate experimental design, control, and analysis, and is clearly written. There are a few areas where methods and terms could be explained more clearly, particularly the Bayesian model and how it should be interpreted. Also, the measurement of conjugation efficiency over multiple days may not be an appropriate measurement of the transconjugant population. The data supporting a key role of secondary transfer does not support the strong statements in the abstract and discussion.

Major comments:

Lines 27-29,199-202: The authors seek to prove that secondary transfer is a key to sustaining transconjugants in the gut and that ongoing donor input is unnecessary, but the data are not definitive. The data with the transfer-deficient pKPC2 suggests this, but loss of pACYC_traF in the donor seems to confound the results, since the capacity to conjugate decreases over time. This caveat should be discussed, as this may over-estimate the importance of secondary transfer. The conclusions may need to be tempered or supported with additional experiments. For example, it seems that initial colonization could be done with recipients and transconjugants (as the donor, at different ratios) and compared to the standard donor and recipients, and primary-only donor and recipients, expecting higher early rates with the transconjugants as donors and proving that the donor is dispensable. In addition, recipients and transconjugants with the transfer deficient strain could be used for initial colonization, to test whether ongoing conjugation is needed to maintain the transconjugant population. It may be that this loss of primary donor capability over time could also be leveraged to prove the necessity of secondary transfer. A small amount of transconjugants could be mixed in with the primary transfer only donors and recipients, with the expectation that the population of transconjugants would rapidly expand. The authors may be able to devise other experiments to more definitively prove secondary transfer as a key process.

Figure 1D and 2B: It is not clear to this reviewer how conjugation efficiency was calculated over time, and how to interpret the results. For a single timepoint, the ratio of transconjugants to recipients provides some information about efficiency. But if done over time, is the efficiency calculated based on the recipient count that day, or at day 1? Some efficiencies go down over time, which could be changes in proportions of these strains in the gut and not necessarily changes in efficiency of transformation. In other words, are these really conjugation efficiencies or just a strain ratio at a point in time?

Lines 147-160 and Figure 2D: Similar to Supplemental Table 1, a statistical comparison across plasmid types is needed to support the statements provided here.

Lines 169-173: The Bayesian model will require more discussion to walk through possible outcomes of the model and what

a particular biological correlate would be. For example, it is not clear to this reviewer that a high early transconjugant count implies secondary transfer or clonal expansion, as opposed to a rapid transfer process that occurred within 24 hours and continued. Overall, it seems like the loss of part of the population through fecal shedding may need to be considered.

Minor comments:

Lines 53-55: It is not clear what multiplicity and plasticity mean in this context.

Lines 129-130: A brief description of the methods is needed here. Were the Ec and Kp donors cured of their endogenous plasmid before being used as donors? What are the endogenous plasmids and how might they interact with the plasmids being tested?

Lines 191-194: This summary does not seem appropriate here, which is describing control experiments.

Lines 205-206: How was vertical inheritance defined and measured?

Lines 316-317: Because oxic conditions are not shown, it would be helpful to state that the Clade 1 efficiency is reduced in anaerobic conditions, as opposed to Clade 2 efficiency improving.

Lines 349-350: This paragraph could be moved to the discussion.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this revised version of the manuscript, the authors have effectively addressed all of my major comments. I now have only a minor suggestion.

The absence of a known PTU in COPLA that can be associated with these plasmids is, in my view, of significant importance. It suggests the existence of an entire class of plasmids; a class that is highly abundant in the human gut, and it is playing a key role in the dissemination of antibiotic resistance among hypervirulent *Klebsiella* strains, which is a major concern in the context of rising antimicrobial resistances. Given their clinical relevance and apparent capacity for transmission under anaerobic conditions, I expect these plasmids will attract significant attention in the near future. I would therefore encourage the authors to propose a designation for this PTU (PTU-hVK1 or something similar?) that clearly differentiate this novel family from "classical" PTU-P1 plasmids, which seem to behave very differently.

Reviewer #2

(Remarks to the Author)

Yong et al. have addressed my comments as well as the other reviewer's comments satisfactorily. I have no further comments.

Reviewer #3

(Remarks to the Author)

In this revised submission, the authors have provided convincing new evidence that secondary transmission of the pKPC2 plasmid drives the high ratio of transconjugants seen in the gut. They have also switched to presenting transconjugant/recipient ratios instead of conjugation efficiencies over time, which still allows these results to be discussed and interpreted for the role of secondary conjugation in the ratios observed. My suggestions for minor changes and clarifications were all adequately addressed. Despite the response to reviewers comments, I still find the Bayesian analysis a bit confusing. It's unclear what data was used as inputs for the modeling. It sounds like data from each day was used to model densities on the next day. If so, stating that would help to clarify the approach. If this was the approach, it's important to note that the model doesn't apply to day 1 (where there were no preceding transconjugants on Day 0). A few final edits in the section would be helpful. Overall, the manuscript is stronger for the changes that were made.

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Response to Reviewers' Comments

We sincerely thank the reviewers for their thoughtful and constructive comments. In response, we have carried out additional analyses including the experiments presented in Fig. 2c–d, in which we integrated the plasmid gene *traF* into the chromosomes of both *K. pneumoniae* SGH10 and *E. coli* Nissle. We believe these experiments provide a robust approach to address the reviewers' suggestions regarding the importance of a sustained donor role, and to clarify vertical versus horizontal transfer, thereby supporting our conclusion on the predominance of secondary transfer in our system. Additional analyses are included in new supplementary figures 4, 7, 9, and 2 more supplementary tables (2, 3) while significantly expanding supplementary table 1. Detailed responses are provided below.

Reviewer #1 (Remarks to the Author):

In this manuscript, Yong et al. investigate the conjugation dynamics of plasmid pKPC2, a carbapenem-resistance plasmid derived from *Klebsiella pneumoniae*, within a murine gut model. The study contrasts the conjugative behaviour of pKPC2 with that of RP4, a canonical member of the IncP plasmid family. The authors report three principal findings. The first one is the finding that pKPC2 demonstrates markedly higher conjugation efficiency in the gut than RP4. This disparity is attributed to the differing sensitivities of the plasmids to anaerobic conditions, suggesting that oxygen availability plays a critical role in modulating horizontal gene transfer (HGT) within the gut environment. Overall, I find this point well supported by the experimental results.

The second key idea is that pKPC2 appears to form a distinct clade within the IncP group which is significantly more prevalent in mammalian and human-associated microbiota. In contrast, RP4-like plasmids are underrepresented in these niches and are more commonly found in environmental contexts. These observations support the notion of ecological specialisation within the IncP group, with pKPC2 representing a lineage adapted to the anoxic conditions of the mammalian gut. As I explain later, I have some major concerns regarding this point.

Finally, the authors show that the capsule acts as a permeable barrier for horizontal gene transfer. Although the *Klebsiella* capsule is traditionally considered a barrier to conjugation, the authors provide evidence that, under in vivo gut conditions, it does not entirely inhibit plasmid transfer. Capsulated strains were shown to receive plasmids at frequencies approaching those of non-capsulated strains, indicating that the barrier is more porous than previously thought.

The manuscript is well written, and the results are clearly presented. However, the structure could benefit from a more unified conceptual thread. The current presentation at times lacks narrative coherence, making it challenging to discern the principal message. In my view, the most significant contribution of the work lies in its demonstration that microenvironmental

factors (specifically oxygen availability) profoundly shape plasmid conjugation dynamics, with consequences observable at the level of plasmid biogeography. This central insight, however, is somewhat obscured by a few issues.

Response: We thank the reviewer for the astute distilling of the paper and we have included the central insight in the abstract, and incorporate this into the introduction (lines 97-99) and discussion to emphasize the central insight.

Major Comments

1.- A fundamental issue pertains to the degree of genetic similarity between the plasmids designated as “IncP.” This is not a shortcoming of the study per se, but rather a consequence of the reliance on historical incompatibility groups, which conflate two different meanings: plasmids that are highly similar at the genetic level, and plasmids that merely share a conjugation system. In the case of IncP (like in the IncF) these labels often obscure considerable genomic heterogeneity. I am afraid the pKPC2 belongs to the second category, and although it contains an IncP conjugation system, it cannot be strictly considered a bona fide IncP group (in the strict meaning of the term). A quick estimation indicates that the average nucleotide identity (ANI) between pKPC2 and RP4 is well below 70% , indicating that they do not belong to the same plasmid taxonomic unit. Looking at the relaxase (a key phylogenetic marker for plasmid distance), they belong to the same MOB family but their identity is way lower what you’d expect if they were from the same group. This divergence likely accounts for the phenotypic differences observed, including the restricted host range of pKPC2, which appears unable to colonise *Pseudomonas*, RP4’s native host. While the authors’ phylogenetic bifurcation of IncP plasmids into Clade I and Clade II is a reasonable starting point, it remains ambiguous whether these groupings reflect meaningful taxonomic units in the absence of defined similarity thresholds.

To address this, I would strongly encourage the authors to undertake a more rigorous classification strategy, including:

a) Delineation of a Novel PTU: pKPC2 appears to represent a distinct and highly prevalent plasmid lineage within the human gut. The authors possess a dataset of closely related plasmids—an analysis of sequence conservation within this set would be highly informative. Specifically, do these sequences meet the proposed PTU criterion (>70% nucleotide identity across >50% of the genome)? If so, this would suggest that a specific plasmid “species” underpins a major pathway of antibiotic resistance dissemination in the gut.

b) Extrapolation of Oxygen Sensitivity Across Clade Members: The authors should assess the degree to which the anaerobic resistance trait is shared across related plasmids (e.g., those tested in Figure 5a). What is the similitude between these plasmids? Are these plasmids members of the same PTU as pKPC2? Given the substantial divergence between Clades I and

II, it is conceivable that they represent separate PTUs altogether, with distinct ecological and functional profiles. The authors have already performed a significant part of this, as shown in Supplementary Figure 5a and b. In this figure the existence of at least three separate identity clusters, pointing out to three different plasmid taxonomic units. A thorough reanalysis along these lines would greatly enhance the clarity and impact of the study.

Response: We thank the reviewer for insightful and constructive suggestions. We fully agree that the traditional classification of plasmids into incompatibility groups may have limitations. We also concur that the concept of plasmid taxonomic units (PTUs) provides a more rigorous and informative framework for plasmid classification. In response, we have undertaken additional analyses of our dataset following the reviewer's recommendations.

With respect to point (a), we performed pairwise comparisons of the pKPC2-like and RP4-like sequences from the ENA database to determine their average nucleotide identity (ANI). We subsequently carried out clustering using the threshold of $\geq 70\%$ nucleotide identity across $\geq 50\%$ of the genome. The results, now presented in Supplementary Fig. 7 and elaborated in lines 315-339, show that pKPC2-like and RP4-like sequences form clearly distinct clusters, without interconnection, consistent with the reviewer's suggestion that they likely represent separate PTUs. Within these clusters, the RP4-like sequences form a single group, whereas pKPC2-like sequences segregate into two closely related subclusters. These subclusters remain interconnected, suggesting they satisfy the ANI criteria. However, there may be core differences specific to each subcluster, such that plasmids within the same subcluster are more similar to one another than to those in the other subcluster. Notably, the majority of pKPC2-like sequences sharing $\geq 95\%$ identity and coverage of the T4SS and replicon sequences (as defined in our original analyses) fall within the same subcluster. Overall, the pKPC2-like sequences likely form distinct PTU from the RP4-like sequences.

Regarding point (b), we re-analysed the plasmids (originally grouped into Clade I and Clade II) using COPLA to assess PTU assignment. However, COPLA was unable to assign PTUs to a number of plasmids owing to an sHSBM score of < 4 . We then constructed a clustering graph using the ANI threshold of $\geq 70\%$ identity across $\geq 50\%$ of the genome, while simultaneously annotating each plasmid with its COPLA-derived PTU classification. The results are shown in Supplementary Fig. 9 and elaborated in lines 352-370. This analysis revealed that Clade I and Clade II plasmids indeed segregate into two distinct clusters, with the exception of a single Clade I plasmid that did not cluster with the others. Of note, RP4 could not be assigned to a PTU by COPLA (sHSBM cluster size = 3), consistent with a recently published review co-authored by the COPLA developers (Table 1 of <https://doi.org/10.1093/nar/gkaf652>), which similarly did not assign RP4 to a PTU. Nevertheless, in our clustering analysis, RP4 grouped with plasmids assigned to PTU-P1. By contrast, pKPC2 was assigned to PTU-? (i.e., a novel, as yet unnamed PTU) by COPLA, and many other Clade II plasmids that clustered with pKPC2 were also designated PTU-?.

While we would ideally have preferred to apply the PTU naming to our plasmids, particularly pKPC2 and RP4, this was unfortunately not possible owing to the absence of established PTU categorisation within the current COPLA pipeline, most likely due to the scope of COPLA's plasmid database curation. Nevertheless, these analyses indicate that the IncP plasmids we defined as Clade I and Clade II exhibit substantial plasmid genomic similarity within each clade, extending beyond the shared transfer machinery, as they form distinct clusters using the ANI-based thresholds. Consequently, our observation that oxygen sensitivity appears to differ between the Clade I and Clade II plasmids that we tested may also extend to other members within each clade, given their broadly similar genomic relatedness. This, in turn, lends support to our central conclusion regarding the ecological differentiation of these plasmids.

2.- The data addressing the role of transconjugants in sustaining conjugation *in vivo* is compelling but raises certain concerns. The authors demonstrate that the pKPC2 Δ traF + pACYC_TraF strain conjugates less efficiently than the wild-type in mice, which, in principle, supports the hypothesis that transconjugants are the primary vectors of plasmid spread. However, Supplementary Figures 2 and 3 suggest that traF complementation in *Klebsiella* is not fully working, and that the pACYC vector exhibits substantial instability in *E. coli*. These findings undermine the inference that transconjugants are the dominant plasmid propagators, especially when a decline in effective donors is accompanied by a parallel decline of transconjugants (Supplementary Figure 3, final days). To cast out these doubts I would recommend the authors to conduct an experiment in which recipient cells vastly outnumber donors (maybe 2-3 logs). Under such conditions, any sustained conjugation would necessarily rely on secondary transfer events. A comparison of pKPC2 and pKPC2 Δ traF + pACYC_TraF would thereby offer a more definitive test of the hypothesis.

Response: We are grateful for this valuable suggestion, which was also raised by other reviewers. We have carefully considered the proposed experiment to modulate the donor-to-recipient ratio in order to achieve a substantially higher recipient density in the gut. However, based on our previous experience, we believe this would be technically challenging, as gavaging EcN or SGH10 at different concentrations typically results in an eventual equilibrium of approximately 10^9 – 10^{10} CFU per gram of stool at day 1 after gavage in our mouse model. This makes it difficult to achieve a differential donor–recipient ratio *in vivo*.

To overcome the instability associated with pACYC_traF, we have performed new experiments where we integrated traF into the chromosomes of both SGH10 and EcN, thereby ensuring stable expression of the transfer machinery. In the new experiment (Fig. 2c-d), we compared the transfer efficiency of pKPC2 Δ traF from EcN with chromosomally integrated traF (EcN::traF pKPC2 Δ traF) into SGH10 and into SGH10::traF. In the former recipient (SGH10), secondary transfer is not possible because once pKPC2 Δ traF is acquired, the plasmid cannot be further transferred from this recipient. In contrast, the SGH10::traF recipient is capable of secondary

transfer, as chromosomally encoded *traF* restores the complete transfer machinery of pKPC2Δ*traF*. We observed that pKPC2Δ*traF* transferred at higher frequencies into SGH10::*traF* compared with SGH10, with transfer rates comparable to those of the original pKPC2.

These results provide additional support for our conclusion that transconjugants play an important role as effective propagators of the plasmid.

Minor Comments

Line 28-29: “ongoing donor input was unnecessary after establishment”. Maybe I am missing something, but I could not find an experiment that supported this claim. Did the authors perform any experiment in which donors were specifically eliminated and the transconjugants kept increasing?

Response: Our original wording may not have been sufficiently clear. While we did not perform an experiment in which the donor population was deliberately eliminated, the statement was made based on observations from previous experiments in Fig. 2a-b. Specifically, we found that transfer of pKPC2Δ*traF* from EcN to SGH10 was lower than that of the original pKPC2 on day 1, when EcN still largely retained the pACYC_*traF* plasmid. By days 2 and 3, however, pACYC_*traF* was progressively lost from EcN, substantially reducing the proportion of effective donors. Despite this decline, transconjugant numbers remained relatively stable, suggesting that sustained donor input was not critical to maintain the transconjugant population. This interpretation is further supported by our new data (Fig. 2c-d). When comparing transfer from EcN carrying pACYC_*traF* versus EcN with a chromosomal *traF* (EcN::*traF*) insertion into SGH10 with chromosomal *traF* (SGH10::*traF*), the transconjugant-to-recipient ratios (T/R) over three days showed no statistical difference (Supplementary Table 3) even though pACYC_*traF* was progressively lost in the former. Thus, the persistence of high T/R is better explained by secondary transfer rather than continued donor contribution.

Line 43: The citations provided for early work on F and P plasmids (Refs 2 and 3) are somewhat anachronistic. For IncP plasmids, a more appropriate reference is the foundational work by Pühler (Gen Microbiol 1979, DOI: 10.1099/00221287-114-2-341). For the IncF group, the early taxonomic efforts of Naomi Datta are probably more historically accurate (DOI: 10.1017/s0016672300009538).

Response: We thank the reviewer for bringing this to our attention and have updated the citations accordingly.

Line 93: Change “mammalian gut” to “murine gut” for precision

Response: We have revised the text accordingly, changing “mammalian gut” to “murine gut” for precision.

Paragraph starting in Line 161. The authors claim that the Bayesian analysis revealed the initial T population as the most significant contributor to the final conjugation frequency. However, conjugation frequency is just T/ R and final T cells may descend from other T cells not just by conjugation but also by vegetative replication. How is this factor taken into account? If this is not factored in the analysis, I find it hardly surprising that the final T population depends mostly on the initial T population, given the average conjugation frequencies observed.

Response: We thank the reviewer for highlighting this point. A similar comment was also raised by Reviewer 3 under the major comments, and we have addressed both points together in our combined response in that section.

Lines 187–188: This paragraph is potentially misleading. It first states that *traF* complementation fully restores conjugation frequency in *E. coli*, and then adds that "similarly, with SGH10 as donors carrying pKPC2Δ*traF*, no transfer occurred unless *traF* was complemented in trans." This phrasing may lead readers to assume that complementation in *Klebsiella* is as effective as in *E. coli*. However, the data indicate otherwise: in *Klebsiella*, complementation results in conjugation frequencies that are, at first glance, at least an order of magnitude lower than those observed with the wild-type plasmid. This discrepancy should be explicitly acknowledged in the text.

Response: We have revised the paragraph to clarify the distinction between the effects of *traF* complementation in EcN versus SGH10. Specifically, we now explicitly acknowledge that (lines 206-212), while complementation fully restores conjugation frequency from EcN to SGH10, conjugation from SGH10 to EcN was not restored fully to wildtype-levels (approximately at least an order of magnitude lower) despite *traF* complementation. This effect may be attributable to the capsule under *in vitro* conditions, which likely hinders the primary transfer event from SGH10 to EcN, thereby making secondary transfer the more dominant pathway. Indeed, when pKPC2Δ*traF* is transferred from the SGH10Δ*wcaJ* background, the efficiency approaches that of the wild-type plasmid, although a modest difference remains, suggesting that EcN-to-EcN transfer continues to play an important role for this pairing under *in vitro* conditions.

Reviewer #2 (Remarks to the Author):

Yong et al. investigate plasmid transfer dynamics in *Klebsiella pneumoniae*, which is particularly problematic in terms of AMR. The authors focus on hypervirulent *Klebsiella*, which produce a capsule that aids its virulence, but also is thought to slow plasmid spread based on in vitro studies. They study an IncP plasmid, pKPC2, which is prevalent in hospital settings and has previously been documented to spread with high efficiency in vitro (along with other plasmids).

The authors' goal is to investigate plasmid transfer dynamics in vivo, which has so far not been well-studied with hypervirulent *Klebsiella*. Using a combination of mouse experiments and anaerobic culture, the authors state that clade II IncP plasmids (including pKPC2) transfer better in anaerobic conditions in the gut than clade I IncP plasmids, which may help explain their higher prevalence in hospital settings and in the mammalian gut. Lastly, the authors also show that the capsule of hypervirulent *Klebsiella* that has classically been thought to restrict plasmid spread for plasmids that use small pili to transfer plasmids like IncP plasmids actually does not pose a significant barrier under anaerobic conditions.

Overall, the authors study an interesting question of what drives plasmid spread in niches like the gut microbiome. The study is nicely descriptive and well-controlled. I do not have many comments, aside from clarifying a bit better the limitations of the author's experimental system in the context of an intact microbiome. Please see below a list of comments:

Major comments:

1. In Fig. 2a, the difference between transfer of pKPC2 to *Klebsiella* with and without the capsule seems like it would be statistically different on Day 3 of the experiment (with secondary transfer). This seems to be a bit at odds with the results from Fig 1d. Indeed, in Table S1 it seems like there is a significant difference between transfer efficiencies in these two groups on D3 (p value= 0.017159). The authors should comment on this and adjust their language. Generally, are the early plasmid transfer dynamics not having differences a limitation of sample size? Perhaps the experimental system is not sensitive enough to see the effect of the capsule. Could it be that in the presence of an intact microbiota where the effect of transfer efficiency is likely more important, the role of the capsule would be amplified?

Response: Thank you for the comment. In our study, Fig. 2a depicts the donor, recipient, and transconjugant populations for the pKPC2 Δ *traF* experiment, while Fig. 2b shows the comparison of transconjugant-to-recipient (T/R) ratios under conditions of primary transfer alone versus combined primary and secondary transfer. We did not, however, directly compare the T/R ratios between capsulated and non-capsulated recipients in this experiment. That said, we agree with the reviewer's observation that at least for pKPC2 in Fig. 1d, the

capsule effect is statistically significant on day 3 ($p = 0.017159$, with a difference of -1.324 , indicating a lower T/R ratio for the capsulated compared with the non-capsulated recipient). We have now also performed statistical analyses for the pKPC2 $\Delta traF$ experiment in Fig. 2a–b, comparing capsulated and non-capsulated recipients (as shown below). While the differences on days 1 and 2 were not statistically significant, they did reach significance on day 3 ($p = 0.034790$, with a difference of -1.018).

	p value	Mean_1	Mean_2	Difference	SE of difference
EcN pACYC_ <i>traF</i> pKPC2 $\Delta traF$ to SGH10 vs EcN pACYC_ <i>traF</i> pKPC2 $\Delta traF$ to SGH10 $\Delta wcaJ$					
D1	0.142278	-4.621	-3.773	-0.8489	0.4740
D2	0.071840	-5.153	-4.185	-0.9676	0.4460
D3	0.034790	-5.298	-4.280	-1.018	0.3831

We interpret these findings as suggesting that capsule-mediated impediment in the gut may be less pronounced, possibly owing to capsule regulation *in vivo* differing from that observed under aerobic *in vitro* conditions, in a manner that is context-specific. At the same time, we recognise that our ability to detect differences may be limited by the relatively small sample size, even though the observed effect sizes in the gut appear smaller than expected.

Accordingly, we have revised the manuscript to more carefully reflect this. Specifically, in lines 162–166, we now write:

*“The non-capsulated SGH10 $\Delta wcaJ$ accepted pKPC2 ~10-fold more efficiently than wild-type SGH10, although this difference was only statistically significant on day 3 (Supplementary Table 2). Thus, the hvKp capsule may have influenced plasmid transfer in the gut, although the effect appears significantly less pronounced than that observed under *in vitro* conditions.”*

We have also adjusted phrasing elsewhere for greater accuracy and balance: in line 33, “largely vanished” has been revised to “markedly reduced”, and in line 583, “essentially nullified” has been revised to “less pronounced”.

With regard to the role of the microbiome, our mouse model involved microbiota perturbation by ampicillin, which drastically reduced community composition, though we expect recovery to occur over time (e.g., on day 3 or beyond). It is therefore possible that in the context of an intact microbiota, different outcomes might be observed, including potentially greater differences between capsulated and non-capsulated strains. As this aspect was beyond the scope of our study, we have added a statement to the Discussion (lines 532–534):

“This study did not address the influence of an unperturbed microbiota on plasmid transfer, nor its potential impact on transfer into capsulated versus non-capsulated strains.”

2. Related to the above comment, the authors should discuss more explicitly what the context of an intact microbiome would do to the transfer dynamics in they system. The authors state recipient amplification via transconjugants is the primary driver of plasmid spread, but what

about cases where the niche for the recipient is much smaller than the donor? Presumably this would mean that in this case D to R transfer would outpace T to R transfer?

Response: Thank you for the insightful and extremely valid point. The microbiome is indeed an interesting aspect, but as noted in our previous response, it was not examined in this study and our mouse model involves a dysbiotic microbiota. Therefore, we are unable to provide direct insights on this aspect. We acknowledge this as a limitation of our work. Nevertheless, plasmid conjugation requires physical contact between bacterial cells, so the proximity and density of donors and recipients are important determinants of transfer dynamics. In scenarios where donors vastly outnumber recipients, the likelihood of donor-to-recipient transfer may be higher than that of transconjugant-to-recipient transfer, which would mean that primary transfer could remain the dominant mode under such conditions.

To reflect this consideration, we have added the following passage to the Discussion (lines 534-552):

“Second, in our model, the donor-to-recipient ratio does not differ substantially. A previous study examining plasmid transfer of Salmonella in the mouse gut demonstrated a positive correlation between donor density and transconjugant yield⁶². The relative contribution of secondary transfer in the gut may differ under conditions where recipients are rare and donors are abundant. Because plasmid transfer requires physical contact, a high donor density could facilitate primary transfer events, unless donors and recipients occupy distinct ecological niches within the gut, in which case the dynamics may favour secondary transfer. The balance between primary and secondary transfer is therefore likely context-dependent and may also be influenced by microbiome composition. In an intact microbiome, competition for ecological niches could substantially alter transfer dynamics. While our model, with roughly comparable donor and recipient abundances, demonstrated transconjugant-driven spread, other scenarios may produce different outcomes. For example, when the ecological niche available to recipients is much smaller than that available to donors, such as in competitive microbiome environments where donors have colonization advantages, primary donor-to-recipient transfer may outpace transconjugant-to-recipient transfer. This consideration is particularly relevant in natural gut microbiomes, where intense niche competition may constrain the ability of transconjugants to establish themselves. Future studies investigating the conjugation dynamics of hvKp in more complex microbial communities will be essential to determine how these principles operate across diverse ecological contexts.”

Minor comments:

1. Fig 1 labels – It would be helpful to label on the figure itself which strain is a donor and which is a recipient. With many combinations, it is much more readable to have all the information on the figure itself without having to rely on the figure legend.

Response: We have revised the figure to improve clarity by adding the label “(donor)” beside the donor name and including an arrow to indicate the direction of transfer.

2. Line 28 – “secondary transfer by transconjugants” instead of “transconjugant”

Response: Thank you for the suggestion. We have updated the text in line 28 to read “secondary transfer by transconjugants.”

3. Fig 5b – it would be helpful to have an aerobic comparison side-by-side rather than referring back to Fig. 1. Perhaps one can plot fold-change resulting from aerobic -> anaerobic in both the capsule mutant and the wild-type to clearly show the effect.

Response: We appreciate the helpful suggestion. As the data points for the aerobic and anaerobic conditions are not paired, constructing a fold-change graph may not be appropriate. As an alternative, we overlaid the aerobic data in the graphs using lighter shading and added insets showing the direct fold-change between aerobic and anaerobic conditions, with details provided in the figure caption for clarity.

4. Lines 178-179, the authors state that donor to recipient transfer is expected to be the primer driver plasmid dynamics and cite reference 31. In cases where recipient densities are exceedingly high, a rare donor has been shown to have dramatic consequences. E.g. <https://doi.org/10.1038/s41586-019-1521-8>. The authors may want to expand their discussion on this point slightly, as their finding is not entirely unexpected. Also see major point #2.

Response: We thank the reviewer for these insightful suggestions and for highlighting this oversight. We have revised this section in lines 190-199 as follows:

“Conjugative transfer in a mixed population can occur via primary transfer (directly from an original donor) and secondary transfer (from an initial transconjugant to other plasmid-free recipients). A study using Salmonella in a murine gut model demonstrated that rare donors can achieve dramatic transfer efficiency when recipient densities are high, with up to 99% transconjugants observed within 2-3 days where donor re-seeding is the rate-limiting step. It is possible that this involves initial transfer from rare donors to available recipients, followed by rapid spread through secondary transfer among the recipient population to achieve such high transconjugant frequencies. This phenomenon, where secondary transfer may play an important role in plasmid spread, could occur in our model with comparable size of the donor and recipient populations. This was predicted by our mathematical modelling (Fig. 1e).”

In consideration of both this point and Major Point #2, we have also expanded the Discussion (lines 534-552) to address donor–recipient densities and the potential influence of the microbiome on plasmid transfer.

Reviewer #3 (Remarks to the Author):

The manuscript by Yong and colleagues characterizes the ability of IncP plasmids to conjugate to and from hypervirulent *K. pneumoniae* in vitro and in a gut colonization model in vivo. They find that Clade II IncP plasmids have high efficiency in the gut, and that secondary transfer of the plasmid from transconjugants to new recipients seems to drive high transconjugant levels. They also show that unlike in vitro, in vivo the capsule is not a barrier to conjugation, and find that anaerobic growth decreases mucoviscosity and increases conjugation efficiency. Overall, this is a valuable study demonstrating that conjugation efficiency depends on the environment, and some plasmids may be especially adept at transferring in the gut environment. This is a well-conceived study with appropriate experimental design, control, and analysis, and is clearly written. There are a few areas where methods and terms could be explained more clearly, particularly the Bayesian model and how it should be interpreted. Also, the measurement of conjugation efficiency over multiple days may not be an appropriate measurement of the transconjugant population. The data supporting a key role of secondary transfer does not support the strong statements in the abstract and discussion.

Major comments:

Lines 27-29,199-202: The authors seek to prove that secondary transfer is a key to sustaining transconjugants in the gut and that ongoing donor input is unnecessary, but the data are not definitive. The data with the transfer-deficient pKPC2 suggests this, but loss of pACYC_traF in the donor seems to confound the results, since the capacity to conjugate decreases over time. This caveat should be discussed, as this may over-estimate the importance of secondary transfer. The conclusions may need to be tempered or supported with additional experiments. For example, it seems that initial colonization could be done with recipients and transconjugants (as the donor, at different ratios) and compared to the standard donor and recipients, and primary-only donor and recipients, expecting higher early rates with the transconjugants as donors and proving that the donor is dispensable. In addition, recipients and transconjugants with the transfer deficient strain could be used for initial colonization, to test whether ongoing conjugation is needed to maintain the transconjugant population. It may be that this loss of primary donor capability over time could also be leveraged to prove the necessity of secondary transfer. A small amount of transconjugants could be mixed in with the primary transfer only donors and recipients, with the expectation that the population of transconjugants would rapidly expand. The authors may be able to devise other experiments to more definitively prove secondary transfer as a key process.

<p>Response: We thank the reviewer for highlighting the importance of carefully interpreting the role of secondary transfer in sustaining transconjugants <i>in vivo</i>. We agree that loss of donor</p>
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capability due to pACYC_ *traF* instability could confound interpretation and may overestimate the contribution of secondary transfer.

We have addressed this concern through stable expression of *traF* by integrating it into the chromosome of EcN as well as SGH10. In the new experiments (Fig. 2c-d), we compared the transfer efficiency of pKPC2Δ*traF* from EcN::*traF* into SGH10 and SGH10::*traF*. In SGH10 recipients, secondary transfer is not possible, whereas in SGH10::*traF* recipients, secondary transfer is enabled by the chromosomally encoded *traF*. Consistent with the hypothesis, we observed substantially higher transfer frequencies into SGH10::*traF* than SGH10, with rates comparable to wildtype pKPC2.

In a parallel experiment, we also leveraged the gradual loss of pACYC_ *traF* in the donor, which reduces the effective donor population over time, and included both SGH10 and SGH10::*traF* as recipients. We observed that transfer to SGH10::*traF* increased and was sustained over a three-day period, comparable to the scenario with a stable donor population (EcN::*traF* pKPC2Δ*traF*). These results indicate that even when donor numbers decline, secondary transfer from transconjugants is sufficient to maintain plasmid spread, demonstrating that transconjugants play an important role as effective propagators *in vivo*.

Together, these findings strengthen our conclusion that transconjugants are key drivers of pKPC2 propagation in the gut, while mitigating confounding effects from donor instability. We sincerely appreciate the reviewer's suggestion, which guided us to refine our experimental design and more rigorously address this question.

Figure 1D and 2B: It is not clear to this reviewer how conjugation efficiency was calculated over time, and how to interpret the results. For a single timepoint, the ratio of transconjugants to recipients provides some information about efficiency. But if done over time, is the efficiency calculated based on the recipient count that day, or at day 1? Some efficiencies go down over time, which could be changes in proportions of these strains in the gut and not necessarily changes in efficiency of transformation. In other words, are these really conjugation efficiencies or just a strain ratio at a point in time?

Response: The conjugation efficiencies were calculated as the number of transconjugants divided by the number of recipients at each timepoint. We agree that, when measured over time such as in our *in vivo* experiments, this metric may not fully reflect true conjugation efficiency. Accordingly, we have revised the y-axis in the *in vivo* data figures to show T/R ratios and we now use T/R throughout the text when discussing *in vivo* data, providing a more accurate representation of the data under these conditions.

Lines 147-160 and Figure 2D: Similar to Supplemental Table 1, a statistical comparison across plasmid types is needed to support the statements provided here.

Response: We have performed the statistical analysis and have included the results in the updated Supplementary Table 1.

Lines 169-173: The Bayesian model will require more discussion to walk through possible outcomes of the model and what a particular biological correlate would be. For example, it is not clear to this reviewer that a high early transconjugant count implies secondary transfer or clonal expansion, as opposed to a rapid transfer process that occurred within 24 hours and continued. Overall, it seems like the loss of part of the population through fecal shedding may need to be considered.

Combined Response to Reviewer 3 and 1: We appreciate the comments from both reviewers 3 and 1, which highlight important points about the interpretation of our Bayesian analysis, the distinction between clonal expansion and secondary transfer, and the potential impact of fecal shedding on our results. These concerns are closely related, so we address them together here.

1. Bayesian Model Interpretation and Biological Mechanisms

The Bayesian regression model was designed to assess the relative contributions of different bacterial subpopulations (original donors, plasmid-free recipients, and existing transconjugants) to the observed increase in transconjugant numbers over time. The model predicts the change in transconjugant counts as a function of the densities of each subpopulation, rather than simply correlating static endpoint measurements.

Both reviewers rightly point out that the final transconjugant population reflects both new conjugation events and the clonal expansion of existing transconjugants. Our model does not directly disentangle these processes, but it does reveal that the existing transconjugant population is consistently the strongest predictor of further increases in transconjugant numbers. This suggests that, once established, the transconjugant population itself, rather than the original donor pool, drives ongoing plasmid dissemination.

However, as Reviewer #1 notes, this could theoretically result from either secondary transfer (transconjugants acting as new donors) or enhanced clonal expansion. To resolve this ambiguity, we performed experiments with a transfer-deficient pKPC2 Δ traF mutant plasmid. In this setup, transconjugants can still replicate vegetatively but cannot act as donors. The dramatic reduction in transconjugant numbers with pKPC2 Δ traF, compared to wildtype pKPC2, provides direct evidence that secondary transfer (rather than clonal expansion) is critical for sustained plasmid spread.

2. Addressing Fecal Shedding and Gut Dynamics

Reviewer #3 raises an important consideration: the continuous-flow nature of the gut environment and the potential loss of bacteria through fecal shedding. Our model does not explicitly include a shedding term, but it does capture the net change in population sizes as measured in stool samples. This net change inherently reflects the combined effects of replication, conjugation, and loss.

While this is a simplification, we believe the model's conclusions remain valid for two reasons. First, all co-colonizing populations (donors, recipients, and transconjugants) are subject to similar shedding rates, so this factor should not bias the relative comparison of their contributions. Second, our experimental design controls for shedding effects: the pKPC2ΔtraF experiments were conducted under identical housing and sampling conditions as the wildtype pKPC2, ensuring that any shedding would impact both scenarios equally. The observed differences between wildtype and mutant conditions thus reflect differences in conjugation dynamics, not differential shedding.

3. Clarifying the Model's Role and Experimental Validation

The Bayesian model served as a hypothesis-generating tool, identifying the transconjugant population as the primary driver of plasmid spread. The pKPC2ΔtraF experiments were then designed to test this hypothesis directly. The convergence of statistical predictions with experimental validation strengthens our conclusion that secondary transfer is an important mechanism of sustained plasmid dissemination in the gut.

Minor comments:

Lines 53-55: It is not clear what multiplicity and plasticity mean in this context.

Response: We have revised the text to present the original statement more clearly. Rather than using the terms “multiplicity” and “plasticity,” the revised text now conveys that the presence of multiple blaKPC gene variants on individual pKpQIL plasmids, together with the structural flexibility of these plasmids through recombination, enables *K. pneumoniae* ST307 to develop simultaneous resistance to both carbapenems and newer antibiotics such as ceftazidime-avibactam, while generating heterogeneous resistance profiles that facilitate adaptation under diverse selective pressures.

Lines 129-130: A brief description of the methods is needed here. Were the Ec and Kp donors cured of their endogenous plasmid before being used as donors? What are the endogenous plasmids and how might they interact with the plasmids being tested?

Response: Both SGH10 and EcN harbour endogenous plasmids, and we have now added this information to the text at the end of the relevant paragraph in lines 139-148. Specifically, SGH10 carries a large non-self-transmissible virulence plasmid of 231 kb with IncHIB and IncFIB(K) replicons, while EcN carries two small cryptic plasmids, pMUT1 (3.2 kb) and pMUT2 (5.5 kb), of unknown function with ColE1- and ColE2-like replicons. These endogenous plasmids were not cured, as we consider them intrinsic to the identity of the respective strains, particularly the SGH10 virulence plasmid, which underpins its hypervirulent phenotype. Importantly, most of the plasmids we tested possess replicons distinct from those of the resident plasmids, and thus no incompatibility issues were expected or observed. The only exception was pOX38 (IncFI), which proved unstable in SGH10, likely owing to incompatibility with the resident virulence plasmid carrying the IncFIB(K) replicon.

Lines 191-194: This summary does not seem appropriate here, which is describing control experiments.

Response: Thanks for pointing it out. We agree that the remark regarding secondary transfer was not appropriate in this section describing the control experiments, and we have removed it from the text.

Lines 205-206: How was vertical inheritance defined and measured?

Response: We intended to convey that the absence of *traF* on pKPC2 prevents its transfer from transconjugants lacking the *traF* gene, but does not impair the ability of these transconjugants to propagate vertically. As shown in Supplementary Fig. 2c, SGH10 carrying pKPC2Δ*traF* exhibited no growth difference compared to those with the original pKPC2. The text in lines 243-246 has been revised accordingly for clarity as follows:

“It is worth noting that in pairs 2 and 4, SGH10 recipients that acquire pKPC2ΔtraF become conjugatively deficient and cannot participate in subsequent horizontal transfer events, but they retain the ability to propagate the plasmid vertically to their progeny through cell division.”

Lines 316-317: Because oxic conditions are not shown, it would be helpful to state that the Clade 1 efficiency is reduced in anaerobic conditions, as opposed to Clade 2 efficiency improving.

Response: Thank you for pointing this out. We have revised the sentence (now in lines 406–408) to clarify that Clade I exhibits lower efficiency than Clade II under anaerobic conditions, as follows:

“... clade I plasmids (RP4-11, pMNCN064, pYKBP039) transferred at significantly lower frequencies than the the clade II plasmids (pKPC2, pVS0309, pVS1035) ...”

Lines 349-350: This paragraph could be moved to the discussion.

Response: We respectfully suggest retaining the sentence in its current position, as doing so allows readers to interpret the data immediately in the context in which it is presented. Relocating it to the Discussion section could separate it from the corresponding results, potentially making the connection less direct. The Discussion already addresses the broader interpretation of how anaerobic conditions influence hypermucoviscosity, along with relevant limitations, providing a more comprehensive context for readers.

Response to Reviewers' Comments

Reviewer #1 (Remarks to the Author):

In this revised version of the manuscript, the authors have effectively addressed all of my major comments. I now have only a minor suggestion.

The absence of a known PTU in COPLA that can be associated with these plasmids is, in my view, of significant importance. It suggests the existence of an entire class of plasmids; a class that is highly abundant in the human gut, and it is playing a key role in the dissemination of antibiotic resistance among hypervirulent *Klebsiella* strains, which is a major concern in the context of rising antimicrobial resistances. Given their clinical relevance and apparent capacity for transmission under anaerobic conditions, I expect these plasmids will attract significant attention in the near future. I would therefore encourage the authors to propose a designation for this PTU (PTU-hVK1 or something similar?) that clearly differentiate this novel family from "classical" PTU-P1 plasmids, which seem to behave very differently.

Response: We thank the Reviewer for this thoughtful suggestion. We agree that these plasmids likely represent an important and clinically relevant group that is associated with the human gut and mechanistically distinct from classical PTU-P1 plasmids. To avoid implying host specificity toward hypervirulent *K. pneumoniae*, we propose the designation "PTU-P2", which reflects their relatedness to canonical IncP plasmids while recognizing them as a distinct taxonomic unit. Accordingly, we have replaced "IncP clade II" with PTU-P2 and refer to canonical IncP plasmids as PTU-P1 throughout the manuscript. Specifically, in lines 373-378, we added the following sentences:

"We propose the designation "PTU-P2" for the clade II plasmids to reflect their evolutionary relatedness to the IncP family and to acknowledge that they constitute a distinct taxonomic unit from clade I, of which most members belong to PTU-P1. In this study, we subsequently refer to IncP clade I plasmids that cluster with PTU-P1 as PTU-P1, and to clade II plasmids that cluster with pKPC2 as PTU-P2."

From that point onward, we consistently use the PTU-P1 and PTU-P2 designations. In addition, we have revised the abstract and updated the figure legends for Fig. 4, Fig. 5, Supplementary Fig. 10, and Supplementary Fig. 11 accordingly. We appreciate the Reviewer's valuable suggestion, which we believe will benefit future comparative and surveillance studies.

Reviewer #2 (Remarks to the Author):

Yong et al. have addressed my comments as well as the other reviewer's comments satisfactorily. I have no further comments.

We thank the Reviewer for the positive feedback and for taking the time to evaluate our revised manuscript. We are grateful for the constructive input, which has helped improve the clarity and quality of our work.

Reviewer #3 (Remarks to the Author):

In this revised submission, the authors have provided convincing new evidence that secondary transmission of the pKPC2 plasmid drives the high ratio of transconjugants seen in the gut. They have also switched to presenting transconjugant/recipient ratios instead of conjugation efficiencies over time, which still allows these results to be discussed and interpreted for the role of secondary conjugation in the ratios observed. My suggestions for minor changes and clarifications were all adequately addressed. Despite the response to reviewers comments, I still find the Bayesian analysis a bit confusing. It's unclear what data was used as inputs for the modeling. It sounds like data from each day was used to model densities on the next day. If so, stating that would help to clarify the approach. If this was the approach, it's important to note that the model doesn't apply to day 1 (where there were no preceding transconjugants on Day 0). A few final edits in the section would be helpful. Overall, the manuscript is stronger for the changes that were made.

Response: We appreciate the Reviewer's careful reading and the opportunity to further clarify the inputs of our Bayesian analysis. The hierarchical Bayesian regression model quantified day-to-day changes in transconjugant counts as the outcome variable. We modeled the transitions D1→D2 and D2→D3, using donor, recipient, and transconjugant densities measured on the preceding day as predictors of the change observed on the following day. Because there were no measurements prior to Day 1, the D0→D1 transition was not included as an outcome. The model therefore starts from the first measurable populations and examines how their abundance influences subsequent transconjugant count, thereby capturing the contribution of secondary transfer to ongoing spread. Although Day 0 data were unavailable, we show experimentally (using the *traF*-deficient plasmid) that secondary transfer is also critical during the initial D0→D1 phase, supporting the biological interpretation drawn from the model. We found the Reviewer's suggestion helpful and have further clarified those points in the manuscript. Specifically, in lines 177-178, we revised the sentence from

"the size of the initial transconjugant population emerged as the strongest predictor of final conjugation frequency ..."

to

"the size of the initial transconjugant population emerged as the strongest predictor of the next-day increase in transconjugants ..."

And in lines 761-764, we added:

“We modelled per-mouse day-to-day changes in transconjugant counts as the outcome. For each subsequent day ($D1 \rightarrow D2$ and $D2 \rightarrow D3$), predictors were the donor, recipient and transconjugant counts measured on the preceding day. Because no $D0$ measurements were available, the model did not include $D0 \rightarrow D1$ ”