

Transcriptional regulation of the pneumococcal capsule can dictate serotype-specific infection

Corresponding Author: Dr Andrew Ulijasz

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)

Marra and colleagues investigate the functional significance of promoter sequence regulatory elements upstream of the capsule (*cps*) operon of *Streptococcus pneumoniae*. Previous work, to which the authors contributed, had identified a 37 base-pair cis regulatory element (referred to throughout at 37-CE), which contains binding sites for the transcription factors SpxR and CpsR and which is an important determinant of infection outcomes. Here, they have expanded their studies of the 37-CE elements by investigating how serotype (i.e. capsule type) specific 37-CE elements modulate expression of the capsule and contribute to disease phenotypes.

I find the central hypotheses here compelling. That is, the idea that natural variation in regulatory regions of the *cps* promoter might contribute to serotype-specific attack rates or propensity to achieve vaccine escape. The authors perform a neat set of experiments to probe these ideas, using genetic and biochemical tools alongside phenotypic assays and infection models. They have produced a powerful toolkit with which to address the role of the 37-CE elements, including strains with *cps* genes from different serotypes with or without the accompanying regulatory regions. In this way, they are able to attempt to disentangle the contributions of capsule structure, and those of capsule expression, to disease phenotypes.

The manuscript is well presented. Figures are by and large clear and the text does an excellent job of guiding the reader through the series of investigations, with key comparisons identified throughout. It was an easy and enjoyable read.

Whilst the findings presented convince that 37-CE sequences influence capsule thickness and contribute to disease trajectories, some reported differences appear marginal and there are some important experiments and controls that could be included to substantially strengthen the manuscript.

Major comments

1. The central thesis here is that serotype-specific 37-CE sequences direct differential expression of capsule genes, via (at least in part) differential affinity for SpxR and CpsR binding and that this contributes to infection outcomes and/or vaccine evasion. The authors use capsule thickness as a readout to conclude that this is the case and those data are convincing but, as they acknowledge, capsule thickness is determined by multiple bacterial and environmental factors. Have the authors measured capsule gene expression by e.g. RT-qPCR in their various mutants, to confirm that different 37-CE sequences modulate capsule at the level of *cps* gene transcription? Can one of the 37-CE mutants be reconstructed in an SpxR null background to determine whether the effect on capsule expression is diminished? The process of making mutants in pneumococcus often inadvertently selects for reduced capsule production, so these additional experiments, or production of complemented strains, would be important to enable firm conclusions to be reached.

2. The authors make the important point that transcription factor activity, and therefore interactions with the 37-CE element are context dependent, with host or environmental factors playing a key role. These relationships could be explored more fully in the manuscript. For instance, in early parts of the manuscripts, the authors use C+Y media with shaking as 'airway-like conditions' and C+Y with FBS and without shaking as 'blood-like conditions'. I can see the value of comparing the influence of 37-CE elements in these two environments, but this focus is not carried through to later parts of the manuscript. For instance, the authors use a tail-vein infection model of bacteraemia but don't evaluate any of their strains in an airway infection model. They make the point that phagocytic clearance in spleen and liver is key to determining outcomes in bacteraemia, but they never directly assess phagocytosis of their various mutants. A phagocytic killing assay or efforts to

determine capsule thickness of strains recovered from different tissues (e.g. lungs, liver, spleen) would help to build the argument that transcription factor interaction with the 37-CE is both serotype-specific and environmentally regulated and that this influences disease phenotype.

Minor Comments

1. Can the data presented in Figure 1C be statistically analysed? The differences between the various 37-CE sequences appear fairly marginal, with the exception of sequence 1, which clearly stands out as low affinity.
2. Do pneumococci grow comparably well in C+Y with and without FBS and aeration? Could differences in growth rate between the two conditions influence the capsule thickness differences reported?
3. Line 188: How do the authors reconcile the observations that D39+seq4 and D39+seq10 show unaltered capsule expression relative to WT under anaerobic conditions (i.e. blood-like conditions), and yet they are cleared more rapidly from the spleen in vivo? As a related point, in Fig 4, could the authors confirm that the D39+seq10 strain expresses less capsule in liver? E.g. by doing a 2 and 4 hour time point with the strain they used in Figure 3 and then assessing capsule thickness of recovered bacteria?
4. Is FITC-dextran exclusion a sufficiently sensitive measure for determination of capsule characteristics? Have the authors tried quantifying precursor sugars by NMR or performing more sensitive direct measurements by EM?
5. A few additional notes on the text and figures:
Line 333: Contrast, rather than contract
Line 359: Sepsis is a clinical definition for the response to an infection, rather than a term for the infection per se, so 'bacteraemia clearance' might be a more suitable phrase than 'sepsis clearance'.
Line 393: 'Achilles heel', not 'Achillies heal'
Line 399: The meaning of the phrase 'vaccine resistance persons' isn't clear to me
Fig 4B lacks a y-axis label
I can appreciate the difficulties in presenting the statistics in Figure 6, but it might be clearer to just highlight the key comparisons with asterisks and to omit the comparisons that aren't central to the conclusions reached in the text.

Reviewer #2

(Remarks to the Author)

This manuscript asks the question of why some serotypes of pneumococcus can switch their capsules by transformation and some can't. They have previously determined there is an upstream regulatory sequence that is instrumental in regulating the amount of capsule in response to various environmental signals. In this paper, they find that there are 13 varieties of the regulatory sequence and created swaps in the canonical pneumonia strain D39. They then swapped the capsule synthesis locus with different regulatory sequences. The results demonstrate that the sequence plays a significant role in the amount of certain capsules but not others.

Comments:

- 1) The validity of the study leans heavily on whether the multiple genetic manipulations really make the mutant they think they made. The Janus system is fairly accurate but experience strongly suggests that the mutants are validated with more than just one mutant/colony. Significant variations occur even within a successful swap, so it is recommended to test a panel of 3-5 to ensure the phenotype holds for a given mutation/genotype.
- 2) Each mutant should be whole genome sequenced (not just the region of interest) given the propensity for spurious mutations.
- 3) The original concept indicated that the capsule amount varied between the respiratory tract and the blood. That link should be tested in the in vivo assays as well to complete the circle of the story.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

My thanks to the authors for meaningful engagement with the comments provided and for performing the additional suggested experiments. Congratulations on completion of a very interesting study.

Reviewer #2

(Remarks to the Author)

The authors have addressed all concerns of reviewer 2. They fully sequenced all their mutants, found SNPs and addressed this issue adequately.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

We would like to thank this reviewer for their insightful comments which we believe has greatly improved our manuscript.

We have recruited Dr. Carlos Orihuela, Dr. Herve Tettelin and others to produce additional data requested for acceptance. We hope you find it satisfactory for publication.

New data include:

1. New in SpxR fluorescence polarization binding in a higher, more physiological salt concentration that better reflects *in vivo* conditions, is statistically significant, and parallels EMSA data (**new Fig. 1C**).
2. Phagocytosis assays with 37-CE variants (**new Fig. 3A**).
3. Pneumonia model of infection with 37-CE variants (**new Fig. 3B**).
4. Genome sequences of strains from Fig. 3 (37-CE variants) and Fig. 6 (capsule switch strains) (**new Fig. S3**; **new Table S2**; **new Extended Data 1**). These are available on the Figshare link provided in the text, and will be placed in NCBI before acceptance.
5. qRT-PCR of 37-CE variants to demonstrate RNA correlates with *cps* reporter and capsule production (**new Fig. S3B**).

From these data we have added descriptive text throughout.

Marra and colleagues investigate the functional significance of promoter sequence regulatory elements upstream of the capsule (*cps*) operon of *Streptococcus pneumoniae*. Previous work, to which the authors contributed, had identified a 37 base-pair cis regulatory element (referred to throughout at 37-CE), which contains binding sites for the transcription factors SpxR and CpsR and which is an important determinant of infection outcomes. Here, they have expanded their studies of the 37-CE elements by investigating how serotype (i.e. capsule type) specific 37-CE elements modulate expression of the capsule and contribute to disease phenotypes.

I find the central hypotheses here compelling. That is, the idea that natural variation in regulatory regions of the *cps* promoter might contribute to serotype-specific attack rates or propensity to achieve vaccine escape. The authors perform a neat set of experiments to probe these ideas, using genetic and biochemical tools alongside phenotypic assays and infection models. They have produced a powerful toolkit with which to address the role of the 37-CE elements, including strains with *cps* genes from different serotypes with or without the accompanying regulatory regions. In this way, they are able to attempt to disentangle the contributions of capsule structure, and those of capsule expression, to disease phenotypes.

The manuscript is well presented. Figures are by and large clear and the text does an excellent job of guiding the reader through the series of investigations, with key comparisons identified throughout. It was an easy and enjoyable read.

Whilst the findings presented convince that 37-CE sequences influence capsule thickness and contribute to disease trajectories, some reported differences appear marginal

We have addressed the marginal numbers in the bacterial titers shown in Fig. 3 and Fig. 6 by also including **how many** mice still contained bacteria in the liver and spleen (above the limit of detection, LOD). We did this because infections often give bimodal data and therefore 'regular' statistical analyses (e.g. ANOVA) might not be the best way to present and/or emphasize the real differences. Nevertheless, we have also applied more stringent (and correct) statistical analyses, showing the **median** value instead of the mean, and using parametric and nonparametric analyses when appropriate. Finally, to make the data easier to interpret in Fig. 6, we split the graphs into wild-type strains (A-C) and switch strains (D-F) for the blood, spleen and liver.

Taking this additional information into account, *i.e.* the ability of the animal to clear the infection or not, in some cases gave stark 'all or nothing' differences that paralleled CFU counts (see Fig. 3 and 6 numbers below the graphs and descriptions in the text). An example of how changing the 37-CE can result in large differences in infected organs is highlighted in a green box in Fig. 6E and 6F, showing that seq-10 (19C serotype) 37-CE produces almost complete organ colonization in the spleen, but very little in the liver (where likely the antigenicity of the 19C capsule plays a more important role in this organ).

Overall, having a few nucleotide changes in the 37-CE in the same genetic background can make the difference in clearing an infection or not. We hope that we have now strengthened this argument with these thoughts and new data.

Finally, we have addressed the marginal binding affinities of our FP data in Fig. 1 (see below).

and there are some important experiments and controls that could be included to substantially strengthen the manuscript.

We have added the above-described new experiments on your suggestions and the other reviewer, which has indeed improved the rigor of the manuscript.

Major comments

1. The central thesis here is that serotype-specific 37-CE sequences direct differential expression of capsule genes, via (at least in part) differential affinity for SpxR and CpsR binding and that this contributes to infection outcomes and/or vaccine evasion. The authors use capsule thickness as a readout to conclude that this is the case and those data are convincing but, as they acknowledge, capsule thickness is determined by multiple bacterial and environmental factors.

Have the authors measured capsule gene expression by e.g. RT-qPCR in their various mutants, to confirm that different 37-CE sequences modulate capsule at the level of *cps* gene transcription?

We have now done RT-qPCR on aerated cultures for WT D39, D39 + Seq. #13, D39 + Seq. #4 and D39 + Seq. #10 strains and see the expected reduction in variant RNA levels compared to WT cells (which parallels reduction in capsule content in Fig. 2B). These new data are shown in Fig. S3A, and are statistically significant.

Can one of the 37-CE mutants be reconstructed in an SpxR null background to determine whether the effect on capsule expression is diminished?

We are asked this question a lot. The answer is essentially 'yes', but would not tell us much as deletion of SpxR has profound effects on central metabolism and pleiotropic effects (it regulates more than just the capsule which we intend to describe in an upcoming publication). Thus, we prefer to focus on mutations of a **single** transcription factor binding site controlling a **single** promoter so we can better claim its effects on the **specific gene(s)/operon** in question (in this case the *cps* locus).

The process of making mutants in pneumococcus often inadvertently selects for reduced capsule production, so these additional experiments, or production of complemented strains, would be important to enable firm conclusions to be reached.

We have teamed up with Dr. Herve Tettelin to analyze the genomes of the 37-CE mutants seen in Fig. 3, as well as the capsule switch strains in Fig. 6. Data showed there are 5 SNPs in other parts of the genome that are shared between all 37-CE variants from Fig. 3, but are not present in the WT D39 strain. Thus these 5 SNPs must have been introduced in the parent construct used to make the 37-CE variants. Also, we found two additional SNPs in the Seq-13 variant, and a single unique SNP in Seq-1 and Seq-8 37-CE switches. As expected, we found many SNPs in our capsule switch strains that were introduced with the new capsule, but were, again, >99% shared among all switch strains.

After examining the SNPs the nature of these changes is suggestive of them not affecting capsule phenotypic outcomes. Moreover, comparing all the 37-CE variants to each other (who again all share most SNPs) still gives stark infection variation, which must be due to the 37-CE because that is essentially all that is different between them.

Genome sequences have been deposited by Herve and Adonis at this link if you want to have a look:

<https://figshare.com/s/06b23f97b527c2c48e8c>

If the paper is accepted, we intend to move these to NCBI.

Table 2 contains 37-CE SNP descriptions. Figure S3B shows that the *cps* region was correct in the switch strains. Fig. S3C displays a Venn diagram of mutations between 19C capsule switch strains. Data used to generate the Venn diagram is given in Extended Data 1.

2. The authors make the important point that transcription factor activity, and therefore interactions with the 37-CE element are context dependent, with host or environmental factors playing a key role. These relationships could be explored more fully in the manuscript. For instance, in early parts of the manuscripts, the authors use C+Y media with shaking as 'airway-like conditions' and C+Y with FBS and without shaking as 'blood-like conditions'. I can see the value of comparing the influence of 37-CE elements in these two environments, but this focus is not carried through to later parts of the manuscript.

For instance, the authors use a tail-vein infection model of bacteremia but don't evaluate any of their strains in an airway infection model.

We have now evaluated the strains in an airway infection model (teaming up with Dr. Carlos Orihuela here at UAB). The airway model did not result in the difference we saw with our sepsis model (no statistical difference between the strains by one-way ANOVA – Fig. 3E). This was expected as the capsule is not that critical for lung infection (due to capsule shedding and a need for adherence¹⁻⁴), but would be expected to be more so during bacteremia / blood infection due to its role in avoiding phagocytosis. Our new phagocytosis assays further exemplify this point in that during sepsis, when bacteria are presumably most susceptible to phagocytic interactions, we see the biggest 37-CE-driven differences in clearance from reticuloendothelial (blood-rich) organs that contain their own resident phagocytes (liver and spleen) (**Fig. 3**).

It is important to point out that it is difficult to extrapolate *in vitro* data to *in vivo* data. These discrepancies are due to the culture conditions not entirely reflecting *in vivo* conditions (*e.g. in vitro* there is not an immune system, or complete immune system in the case of our phagocytosis assays, and nutrient sources differ greatly *etc.*). Thus, we can simply say that such 37-CE changes result in 'differences' *in vitro*, and that differences are manifested in some way during infection.

Overall, these *in vivo* versus *in vitro* discrepancies point to the 37-CE sequence being important for regulation of the capsule *in vitro*, but the consequences of these differences can only truly be realized during an actual infection situation. We have added some Discussion to better highlight these discrepancies (starting with Line 469 in the manuscript).

They make the point that phagocytic clearance in spleen and liver is key to determining outcomes in bacteraemia, but they never directly assess phagocytosis of their various mutants.

A phagocytic killing assay or efforts to determine capsule thickness of strains recovered from different tissues (e.g. lungs, liver, spleen) would help to build the argument that transcription factor interaction with the 37-CE is both serotype-specific and environmentally regulated and that this influences disease phenotype.

We have now directly assessed phagocytosis of our mutants (see new **Figure 3A**). There are indeed differences, and once again the seq-10 37-CE is more easily phagocytosed compared to all other sequences tested. Most of these results, but not all, were consistent with our sepsis model of infection. These data also reflect the Pcps::luminescence reporter data in Fig. 4 which shows **no detectable signal** in the liver for the 37-CE sequence 10 (37-CE sequence taken from the 19C serotype). We think this was a good suggestion and has added a substantial amount of rigor to our findings.

Minor Comments

1. Can the data presented in Figure 1C be statistically analysed? The differences between the various 37-CE sequences appear fairly marginal, with the exception of sequence 1, which clearly stands out as low affinity.

These binding studies were done in triplicate and indeed have very different Kds, perhaps not as evident by looking at the FP graphs. On the other hand, CpsR binding was not shown using FP as its size does not allow this assay to be used (due to the protein size versus DNA - we tried extensively). It is important to note that **small changes in observed *in vitro* affinities can result in large regulatory changes *in vivo*** (see references ⁵ and ⁶). We have added some references and a line of text in the main manuscript in the appropriate results section. Moreover, CpsR and SpxR changes in binding affinity can both be seen in the gel shift (EMSA) data in Figure 1 (see also **Table S1**), which show dramatic binding differences.

Another thing to keep in mind is we do not know the ligands for these two transcription factors yet, which would likely dramatically affect the differences in affinity here since the factors cannot practice their ligand bound affinities (whether the ligands binding would increase or decrease affinities is not known – keeping in mind that SpxR likely has several ligands to add to this complexity...). Therefore, with the binding data we can simply say with the two versions of the assay we did (EMSAs and FP) ‘they are different affinities which would be expected to translate to some kind of altered state of gene expression during infection’.

Finally, since the paper resubmission we have realized that the conditions used in the FP were not stringent enough (i.e. not enough salt), likely resulting in many of these binding curves not being properly compared. *i.e* low salt = more DNA binding / less stringency and would theoretically bring the S-curves ‘closer together’. Also, lower salt can give more non-specific binding. We therefore used the more physiological salt concentration of 150 μ M, which resulted in more dramatic (statistically-significant) differences that paralleled EMSA results. The new figure to supplant the old has been added (**Fig. 1C**).

2. Do pneumococci grow comparably well in C+Y with and without FBS and aeration? Could differences in growth rate between the two conditions influence the capsule thickness differences reported?

Aerated pneumococci grow more slowly and anaerobically faster. FBS is known to assist in capsule growth (PMID: [22412375](#)) and aeration to hinder it (e.g. PMID: [11500414](#)). So yes, the different growth mediums and conditions can alter capsule thickness. However, since all the

strains are grown under the same conditions, we can compare differences within the same condition, or in this case between WT and the 37-CE variants.

3. Line 188: How do the authors reconcile the observations that D39+seq4 and D39+seq10 show unaltered capsule expression relative to WT under anaerobic conditions (i.e. blood-like conditions), and yet they are cleared more rapidly from the spleen *in vivo*?

The answer here would be the same as above, where it is difficult to extrapolate between *in vitro* results to *in vivo* outcomes. We have added the words 'attempted to mimic' when describing these conditions that are essentially differing only by anaerobic versus aerobic states – which should not be taken to mean they are the same as the 'real thing' – i.e. *in vivo* conditions during infection. The discrepancies between *in vitro* versus *in vivo* data the reviewer points out exemplify this discrepancy, and is why we should only note that the 37-CE provides "differences" in capsule phenotypes *in vitro*. More than likely, *in vivo* there are additional signaling inputs feeding into SpxR/CpsR etc. that account for *in vivo* versus *in vitro* differences. A section in the Discussion has been added on this subject.

As a related point, in Fig 4, could the authors confirm that the D39+seq10 strain expresses less capsule in liver? E.g. by doing a 2 and 4 hour time point with the strain they used in Figure 3 and then assessing capsule thickness of recovered bacteria?

The main reason we avoided this experiment was that few bacteria would be present (they are all cleared at 8 hours) ruling out direct visualization using TEM. Another reason we avoided this experiment stemmed from our own research experiences, where we are unable to account for capsule that is shed by bacteria at other sites but captured by the liver – giving false results. Fluorescence microscopy has also been used to visualize the capsule from an excised organ. However, with this method it is impossible to tell if the capsule is free capsule from lysed bacteria, from the blood, or actually attached to the pathogen. The best way to measure capsule size in an organ would be to use cryo-EM, as the capsule size would remain intact. Unfortunately, this has never been accomplished by any lab (to the best of our knowledge). Sven Hammersmith did this in a paper of his, but the organ was infected post excision (PMID: 16040978).

4. Is FITC-dextran exclusion a sufficiently sensitive measure for determination of capsule characteristics? Have the authors tried quantifying precursor sugars by NMR or performing more sensitive direct measurements by EM?

FITC-dextran is sensitive enough and can be quantitated (see references ^{1,7,8}). Several other labs have asked us how we do this as it is considered a gold standard for capsule thickness measurement. We also have deposited a new script for ImageJ we wrote to specifically account for pneumococcal chains to better and more accurately capture the volume. EM can be tricky as the treatments are harsh and you can lose volume easily, making it hard to quantitate. NMR is a great method, but we did not feel the need to do this in addition to the new phagocytosis assay which is directly tied to capsule expression (see WT versus Δ cps control strain in Figure 3A).

5. A few additional notes on the text and figures:

Line 333: Contrast, rather than contract

Fixed.

Line 359: Sepsis is a clinical definition for the response to an infection, rather than a term for the infection per se, so 'bacteraemia clearance' might be a more suitable phrase than 'sepsis clearance'.

Changed. Thank you.

Line 393: 'Achilles heel', not 'Achillies heal'

Fixed.

Line 399: The meaning of the phrase 'vaccine resistance persons' isn't clear to me

We changed to this for clarity to say that we are referring specifically to people who have received the vaccine, yet still became infected with a serotype contained within the vaccine.

Fig 4B lacks a y-axis label

We have added the y-axis label. Thank you.

I can appreciate the difficulties in presenting the statistics in Figure 6, but it might be clearer to just highlight the key comparisons with asterisks and to omit the comparisons that aren't central to the conclusions reached in the text.

We have re-done these statistics and separated the graphs into basically WT strains and the fixed/37-CE switches. Comparisons now focus on statistics for these two groups. Importantly, after discussions with Carlos and Hasan, we now include discussion of **how many mice had detectable bacteria over the Limit of Detection (LOD)** (or not), rather than relying simply on the variable CFU counts. When we observe the data through this lens the data can sometimes be stark, and can be found below the graphs. We applied a Fisher's test for statistics and found significance in Fig. 3F (spleen) with seq-4 and seq-10.

Reviewer #2 (Remarks to the Author):

This manuscript asks the question of why some serotypes of pneumococcus can switch their capsules by transformation and some can't. They have previously determined there is an upstream regulatory sequence that is instrumental in regulating the amount of capsule in response to various environmental signals. In this paper, they find that there are 13 varieties of the regulatory sequence and created swaps in the canonical pneumonia strain D39. They then swapped the capsule synthesis locus with different regulatory sequences. The results demonstrate that the sequence plays a significant role in the amount of certain capsules but not others.

Comments:

1) The validity of the study leans heavily on whether the multiple genetic manipulations really make the mutant they think they made. The Janus system is fairly accurate but experience strongly suggests that the mutants are validated with more than just one mutant/colony. Significant variations occur even within a successful swap, so it is recommended to test a panel of 3-5 to ensure the phenotype holds for a given mutation/genotype.

We completely agree, we test multiple clones and that was also the case with this work. For our luciferase reporter strains we checked 8 clones per strain and assessed reporter activity consistency before moving forward. A final clone was picked that reflected the majority of clones tested (*i.e.* we did not pursue outliers). For other constructions we picked 8 clones and tested them for growth phenotype abnormalities to each other and to wild-type (D39) parent strains. Finally, we sequenced the capsule loci and compared to the parent strain. In our lab we take these variations very seriously to enhance the rigor of the lab's data and convince ourselves that the results are real.

Importantly, we have now teamed up with Dr. Herve Tettelin (U Maryland), whose lab is known for pneumococcal genome sequencing and analysis (Herve sequenced and annotated the first

pneumococcal genome), to analyze all the genomes presented here for any SNPs, deletions, etc. within or outside the cps locus.

We sequenced and analyzed all genomes in Fig. 3, and all genomes in Fig. 6, including the WT parent strains from serotypes 2, 6A, 6B, and 19C. Fig. 3 genomes were compared to WT D39 and for Fig. 6 we compared sequences to the WT strains as well, including the donor strains for the switches in D39 (i.e. 19C, 6A, 6B). All these have been deposited in Figshare and will later be available in NCBI.

Figshare genomes:

<https://figshare.com/s/06b23f97b527c2c48e8c>

Because our answer is essentially the same as above (for Reviewer 1) I will re-paste below:

What we found:

Data showed there are 5 SNPs in other parts of the genome that are shared between all 37-CE variants from Fig. 3, but are not present in the WT D39 strain. Thus these 5 SNPs must have been introduced in the parent construct used to make the 37-CE variants. Also, we found two additional SNPs in the Seq-13 variant, and a single unique SNP in Seq-1 and Seq-8 37-CE switches. Conversely and as expected for any capsule switch, we found many SNPs in our capsule switch strains that were introduced with the new capsule, but were >99% shared among all switch strains (see new **Fig. S3C**).

All the data, *in vitro* and *in vivo*, suggests these SNPs do not affect the phenotypic outcomes. Importantly we checked each one, especially the few non-synonymous ones - even checking available atomic structures - and were convinced they would not affect protein (and capsule) function/expression. Moreover, comparing the 37-CE variants (who again all share most SNPs) still gives stark infection variation, which must be due to the 37-CE because that is essentially all that is different between them. These sequences have been deposited by Drs. Tettelin and D'Mello at this link:

<https://figshare.com/s/06b23f97b527c2c48e8c>

If the paper is accepted, we intend to move these to NCBI.

Sequence data can be found at:

Table 2 contains 37-CE SNP descriptions and Figure S3 shows that the cps region was correct in the switch strains (S3A) and S3B shows a Venn diagram of mutations between 19C capsule switch strains. Data used to generate the Venn diagram is given in Extended Data 1.

2) Each mutant should be whole genome sequenced (not just the region of interest) given the propensity for spurious mutations.

As above we have now done this. Genomes can be accessed on Figshare:

<https://figshare.com/s/06b23f97b527c2c48e8c>

3) The original concept indicated that the capsule amount varied between the respiratory tract and the blood. That link should be tested in the in vivo assays as well to complete the circle of the story.

Teaming up with Carlos Orihuela's lab we have now included a lung infection model for the mutants. We observed no significant differences in the lung, which makes sense as the capsule is not known to play a major role in this organ during infection, due in part to shedding capsule for adhesion purposes (see references ^{1,7,8}). This work further solidifies that the capsule plays a more significant role during sepsis, especially regarding SpxR/CpsR regulation through the 37-CE.

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