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TITLE:

Constructing Mutants in Serotype 1 *Streptococcus pneumoniae* strain 519/43

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KEYWORDS:

Serotype 1, *S. pneumoniae*, Mutagenesis, natural competence, Meningitis belt

SUMMARY:

Here, we describe a *S. pneumoniae* serotype 1 strain 519/43 that can be genetically modified by using its ability to naturally acquire DNA and a suicide-plasmid. As proof of principle, an isogenic mutant in the pneumolysin (*ply*) gene was made.

ABSTRACT:

Streptococcus pneumoniae serotype 1 remains a huge problem in low-and-middle income countries, particularly in sub-Saharan Africa. Despite its importance, studies in this serotype have been hindered by the lack of genetic tools to modify it. In this study, we describe a method to genetically modify a serotype 1 clinical isolate (strain 519/43). Interestingly, this was achieved by exploiting the Pneumococcus' ability to naturally acquire DNA. However, unlike most pneumococci, the use of linear DNA was not successful; to mutate this important strain, a suicide plasmid had to be used. This methodology has provided the means for a deeper understanding of this elusive serotype, both in terms of its biology and pathogenicity. To validate the method, the major known pneumococcal toxin, pneumolysin, was mutated because it has a well-known and easy to follow phenotype. We showed that the mutant, as expected, lost its ability to lyse red blood cells. By being able to mutate an important gene in the serotype of interest, we were able to observe different phenotypes for loss of function mutants upon intraperitoneal and intranasal infections from the ones observed for other serotypes. In summary, this study proves that strain 519/43 (serotype 1) can be genetically modified.

INTRODUCTION:

Streptococcus pneumoniae (*S. pneumoniae*, the pneumococcus) is one of the principal causes of morbidity and mortality globally. Up until recently, close to 100 serotypes of *S. pneumoniae* have

been discovered[1–7]. Yearly, invasive pneumococcal disease (IPD) claims around 700,000 deaths, of children younger than 5 years old[8]. S. *pneumoniae* is the major cause of bacterial pneumonia, otitis media, meningitis and septicaemia worldwide[9].

In the African meningitis belt, serotype 1 is responsible for meningitis outbreaks, where sequence type (ST) ST217, an extremely virulent sequence type, is dominant[10–15]. Its importance in meningitis pathology has been likened to that of *Neisseria meningitidis* in the African meningitis belt[16]. Serotype 1 is often the main cause of IPD; however, it is very rarely found in carriage. In fact, in the Gambia, this serotype is accountable for 20% of all invasive disease, but it was only found in 0.5% of healthy carriers[14,17–19]. Genetic exchange and recombination in competent pneumococci occurs generally in carriage rather than in invasive disease[20]. Furthermore, serotype 1 has been shown to have one of the shortest carriage rates described amongst pneumococci (only 9 days). Therefore, it has been proposed that this serotype might have a much lower recombination rate than others[21].

In depth studies are necessary to understand the reason behind serotype 1 strains' low rate of carriage and its importance in invasive disease in sub-Saharan Africa.

Here we report a protocol that allows genome-wide mutagenesis of a particular serotype 1 strain, 519/43. This strain can easily acquire and recombine new DNA into its genome. This method is not yet inter-strain, but it is very efficient when done in 519/43 background (other targets have been mutated, manuscripts in preparation). By simply using 519/43 strain, and exploit its natural competence, as well as substituting the way that the exogenous DNA is provided, we were able to mutate the pneumolysin gene (*ply*) in this serotype 1 strain. This method represents an improvement on the one presented by Harvey et al.[22] as it is done in one-step without the need to passage the DNA through a different serotype. Nevertheless, and due to inter-strain variability, no method has been standardized to all strains. The ability to mutate specific genes and observe its effects will allow a profound understanding of serotype 1 *S. pneumoniae* strains and it will provide answers for the role of these strains in meningitis in sub-Saharan Africa.

PROTOCOL:

1. Generation of the mutating amplicon by SOE-PCR[23] and amplification of the spectinomycin cassette

1.1. Start by performing PCR for the amplification of the homology arms (ply 5' (488 bp) and ply3' (715 bp) respectively) of the flanking regions of the *ply* gene from strain 519/43. Use primers plyFw1_NOTI (TTT GCGGCCGCCAGTAAATGACTTTATACTAGCTATG), ply5'R1_BamHI (CGAAATATAGACCAAAGGACGC*GGATCC*AGAACCAAACTTGACCTTGA), ply3'F1_BamHI (TCAAGGTCAAGTTTGGTTCT*GGATCC*GCGTCCTTTGGTCTATATTTCG) and plyRv2_NotI (TTTGCGGCCGCCATTTTCTACCTTATCCTCTACC).

1.2. Use the following PCR conditions for ply5': denaturing at 94 °C for 60 s, step 2: denaturing at 94 °C for 30 s, step 3: annealing at 58 °C for 30 s, step 4: extension at 72 °C for 30 s, step 5: go back to step 2 and repeat for 35 cycles, step 6: final extension at 72°C for 30 s.

1.2.1. Use the same PCR conditions for ply3' with the exception of the extension time on step 4 and step 6 where it should be 60 s.

1.3. Analyze the PCR products by gel electrophoresis and excise the amplicon from the gel.

1.4. Purify the PCR amplicons following the protocol described in the manufacturer's instructions (**Table of Materials**).

1.5. Use equimolar amounts of both homology arms as templates in the SOE-PCR. Fuse the two amplicons using primers plyFw1_ NOTI (TTT GCGGCCGCCAGTAAATGACTTTATACTAGCTATG), and plyRv2_NotI (TTTGCGGCCGCCATTTTCTACCTTATCCTCTACC).

1.6. Use the following SOE-PCR conditions (step1: denaturing at 94 °C for 2 min, step 2: denaturing at 94 °C for 30 s, step 3: annealing 58 °C for 30 s, Step 4: extension at 68 °C for 60 s, step 5: go back to step 2 and repeat for 25 cycles, step 6: final extension at 68 °C for 90 s).

1.7. Analyze the SOE-PCR product by gel electrophoresis. Excise it from the gel using a gel extraction kit and follow the instructions provided.

1.8. Amplify the spectinomycin cassette from plasmid pR412 using the following PCR conditions: step 1: denaturing at 94 °C for 60 s, step 2: denaturing at 94 °C for 30 s, step 3: annealing at 55 °C for 30 s, step 4: extension at 68 °C for 60 s, step 5: go back to step 2 and repeat for 25 cycles, step 6: final extension at 68 °C for 60 s.

1.8.1. Use primers BamHI_SP2F2 (GGATCC CTA GAA CTA GTG GAT CCC CC) and BamHI_SP2R2 (GGATCC AAT TCT GCA GAT TTT AC ATG ATC). Plasmid pR412 was acquired from Dr Marc PrudHomme (CNRS-Universite Paul Sabatier Toulouse France).

1.9. Analyze the PCR amplicons by gel electrophoresis. Excise and purify the resulting PCR amplicon as described above.

2. Generation of plasmid pSD1 and Chemical transformation of *E. coli* Dh5α

2.1. Perform a ligation following the pGEMT-easy system I manufacturer instructions (**Table of Materials**). In a microcentrifuge tube, add 5 µL of 2x ligation buffer, 1 µL of pGEMTeasy, 2 µL of the ply_SOE product, 1 µL of T4 DNA ligase and water to a 20 µL total volume. Incubate overnight at 4 °C. This generates plasmid pSD1.

2.2. Transform chemically competent *E. coli* Dh5α with pSD1. Start by incubating 50 µL of chemically competent *E. coli* Dh5α with 3 µL of pSD1 ligation reaction for 15 min on ice. Then continue by exposing the cells to thermic shock (42 °C, 30 s). Place the cells on ice for 2 min.

2.3. Remove the cells from ice and add 350 µL of S.O.C media. Incubate the culture for 2 h at 37 °C, 120 rpm.

2.4. Plate the transformation on Luria Bertani Agar (LBA) supplemented with 0.4 mM IPTG, 0.24 mg/mL X-Gal for blue/white selection and 100 µg/mL ampicillin to ensure all colonies growing in the plate have the plasmid backbone. White colonies contain pSD1.

2.5. Pick three white colonies and set up overnight growths in 10 mL of LB, supplemented with 100 µg/mL ampicillin. Incubate the cultures overnight at 37 ^{o}C with shaking.

2.6. The next day centrifuge the cultures at 3,082 x *g* and use the pellet for plasmid extraction.

3. Plasmid DNA extraction, restriction digestion of pSD1 and spectinomycin gene and assembly of pSD2

3.1. Extract the plasmid DNA following the instructions provided with the commercial kit (**Table of Materials**).

3.2. Set up a BamHI-restriction digestion for both pSD1 plasmid and the spectinomycin cassette previously amplified and purified. Use the following conditions and quantities described in **Table 1**.

3.3. Incubate the restriction digestion reactions and controls at 37 °C for 3 h.

3.4. Analyze the restriction digest by electrophoresis, excise the band and purify following the instructions provided with the commercial kit (**Table of Materials**).

3.5. Next, prepare a ligation reaction following the manufacturer instructions (**Table of Materials**) using the BamHI- digested pSD1 and spectinomycin (from step 3.2). In a microcentrifuge tube, add the following reaction components: 5 µL of 2x ligation buffer, 2 µL pSD1, 2 µL of spectinomycin cassette, 1 µL of T4 DNA ligase and incubate overnight at 4 °C. This generates plasmid pSD2.

3.6. Transform plasmid pSD2 into chemically competent *E. coli* Dh5α as described in step 2.2.

3.7. Select the transformants carrying plasmid pSD2 based on their ability to grow in LBA supplemented with 100 µg/mL of spectinomycin and ampicillin.

3.8. Perform a plasmid DNA extraction (pSD2) as described above and following the manufacturer's instructions (µL).

4. Transformation of *S. pneumoniae* strain 519/43

4.1. Prepare an overnight culture of *S. pneumoniae* 519/43 in BHI and allow it to grow statically at 37 °C, 5% CO_2.

4.2. The following day dilute the cultures 1:50 and 1:100 in 10 mL of fresh BHI broth. Incubate the cultures statically at 37 °C until the OD_{595nm} is between 0.05 and 0.1 (optimal acquisition of DNA closer to 0.1 OD).

4.3. Once an OD of 0.1 is reached, take 860 µL and transfer into a microcentrifuge tube. In this same microcentrifuge tube add: 100 µL of 100 mM NaOH, 10 µL of 20% (w/v) BSA, 10 µL of 100 mM $CaCl_2$, 2 µL of 50 ng/mL $CSP1^{24}$ and 500 ng of pSD2.

4.4. Incubate the reaction statically at 37 °C for 3 h.

4.5. Plate 330 µ µL onto 5% blood agar plates (BA) supplemented with 100 µg/mL spectinomycin, every hour over the 3 incubation hours.

4.6. Incubate plates overnight at 37 °C, 5% CO_2. Patch spectinomycin resistant colonies onto another BA plate supplemented with 100 µg/mL spectinomycin as well as onto BA plates supplemented with 100 µg/mL of ampicillin. Incubate both sets of plates overnight under the conditions stated above. The ampicillin plates are to test for the presence of the plasmid backbone.

4.7. Confirm the presence of the spectinomycin cassette by PCR using primers plyFw1_ NOTI (TTT GCGGCCGCCAGTAAATGACTTTATACTAGCTATG) and SPEC_REV (TAATTCCTCTAAGTCATAATTTCCG). Confirm the mutation by PCR using primers plySCN1 (CCAATGGAAATCGCTAGGCAAGAGATAA) and plySCN2 (ATTACTTAGTCCAACCACGGCTGAT) which attach outside of the mutated region.

4.8. Confirm the integration in the correct location of the genome by sequencing using primers plySCN1 (CCAATGGAAATCGCTAGGCAAGAGATAA), plySCN2 (ATTACTTAGTCCAACCACGGCTGAT), as well as primers with their binding site in the spectinomycin cassette sqr1 (CCTGATCCAAACATGTAAGTACC) sqf2 (CGTAGTTATCTTGGAGAGAATA) spec_sqf1 (GGTACTTACATGTTTGGATCAGG) and spec_sqr2 TATTCTCTCCAAGATAACTACG.

REPRESENTATIVE RESULTS:

The protocol described here starts by using PCR to amplify the left and right homology arms, whilst simultaneously deleting 191 bp from the middle region of the *ply* gene. While performing the PCR a BamHI site is introduced at the 3' of the left homology arm and at the 5' end of the

right homology arm (**Figure 1A**). This is followed by PCR-SOE where left and right homology arms are fused into one amplicon (**Figure 1B**). This SOE-PCR amplicon is then cloned into pGEMTeasy using TA cloning to generate plasmid pSD1 (**Figure 1C**). Successful transformation will yield white colonies that are resistant to ampicillin. Any blue colonies will be transformants containing an empty pGEMTeasy plasmid. pSD1 will then be digested at the BamHI site that was introduced at the time of the SOE-PCR (**Figure 1D**) and ligated to a spectinomycin cassette (also BamHI digested for compatible ends). The new plasmid is termed pSD2 (**Figure 1E**). Correct assembly of pSD2 is confirmed by restriction digestion (**Figure 2A**). pSD2, that works as a suicide plasmid since it does not contain a Gram-positive compatible origin of replication, was used to transform 519/43WT (previously prepared to be competent). Positive transformants are colonies growing on 100 µg/mL spectinomycin after an overnight incubation. All colonies are patched onto newly fresh blood agar base plates supplemented with 100 µg/mL spectinomycin as well as blood agar base supplemented with 100 µg/mL of ampicillin. Any colonies that grow on the second overnight plates supplemented with spectinomycin are possible positives. Any colonies that grow on ampicillin plates will denote integration of the full plasmid or recombination of the ampicillin cassette elsewhere in the genome. So far and using strain 519/43 no colonies have grown on the plates supplemented with ampicillin. All colonies positive by PCR must be confirmed by sequencing (**Figure 2C**) to assess and confirm the location of the insertion. To this end, primers must have their binding site outside of the homology region (**Figure 2C**). The chosen target has a very marked phenotype (hemolysis of red blood cells (RBC)), and therefore the mutation can also be confirmed phenotypically (**Figure 2B**). The mutant lost its ability to lyse RBC's.

FIGURE AND TABLE LEGENDS:

Table 1: **Restriction digestion reaction components for pSD1 and spectinomycin cassette.**

Figure 1: Overview of the mutagenesis strategy A) Amplification of homology arms (Ply3' and Ply5' (lanes 2 until 5); and Splicing by Overlapping Extension PCR (Lanes 6 and 7). L- hyperladder I (Bioline), lane 1- negative control for the reaction, lane 2 and 3- ply5' (488 bp) and ply3' (715 bp) homology arms amplified from D39 gDNA, lanes 4 and 5- ply5' (488 bp) and ply3' (715 bp) homology arms amplified from 519/43 gDNA. Right hand side lane 6- D39 SOE PCR product, lane 7- 519/43 SOE PCR product (1235 bp). **B**) Schematic depicting the final SOE-PCR construct obtained (ply_SOE); Indicated by arrows are the primers used to obtain the homology region between both homology arms as well as the restriction digestion chosen. **C**) Plasmid pSD1, depicting the cloning of ply_SOE; **D**) Restriction digestion of pSD1 and spectinomycin cassette. **E**) Final construct pSD2 that is then used as a suicide plasmid to transform *S. pneumoniae*.

Figure 2: A) Confirmation of the presence of the spectinomycin cassette in pSD2. L- hyperladder 1kb (Bioline); 1- pSD1 digested with BamHI; 2-pSD2 digested with BamHI; L hyperladder 1 kb (Bioline); 3- Spectinomycin cassette amplified from pR412, 4- Spectinomycin cassette digested with BamHI*;* **B**) Phenotypic confirmation of the pneumolysin mutation by determination of haemolytic activity for D39, 519/43WT and mutant 519/43Δply. This was compared to haemolysis of red blood cells by 0.5% saponin. Saponin-derived haemolysis is considered 100% and the rates for 519/43Wt and 519/43Δply were calculated against it. Each data point is the

mean of 5 technical and 3 biological replicates. **C**) Sequencing data mapped to the mutant genome region where pneumolysin was interrupted. Primers are indicated as arrows and by name. PLYSCN1 and PLYSCN2 bind outside of the homology arms. Sequencing obtained from primers PLYSCN1 and 2 showed that there was uninterrupted sequence from the neighboring regions outside of the homology area until the spectinomycin cassette, demonstrating the insertion in the genome.

DISCUSSION:

Streptococcus pneumoniae, in particular serotype 1, continues to be a global threat causing invasive pneumococcal disease and meningitis. Despite the introduction of various vaccines that should be protective against serotype 1, in Africa, this serotype is still capable of causing outbreaks that lead to high morbidity and mortality[13] .The ability to genetically manipulate this serotype is of critical importance because of its clinical relevance. The method described in this study allows the genetic manipulation of a representative strain within this serotype. An invasive strain 519/43 (ST5316), a clinical isolate from a meningitis patient in Denmark[25].

The methodology presented here, was successful mostly due to the chosen strain as it can acquire exogenous DNA, but also due to changes made to the traditional protocols used for *S. pneumoniae* transformation, a typical success rate to our transformation protocol is of about 70%.

With this methodology, it is paramount to use a suicide plasmid instead of the usual linear DNA. Conventionally, linear DNA[26–28] would have been used; however all attempts to use the exogenous DNA in this form were unsuccessful. Furthermore, attempts to exploit the natural competence of *S. pneumoniae* 519/43 at lower absorbance were not successful. Troubleshooting demonstrated that natural competence for strain 519/43 was higher when OD_{595} was 0.1, which is different from the data observed for other serotypes of *S. pneumoniae* where highest natural competence was observed at very low OD[24].

In order to validate the method, a pneumolysin mutant was constructed because it exhibits an easy to follow phenotype; however, to prove that the method can be applied to any gene within this strain, other genes have been successfully targeted (manuscripts in preparation). Such a method, using a suicide vector that has no Gram-positive compatible origin, could also be used for chromosomal complementation, overexpression of genes of interest, as well as introduction of reporter systems, all by using the neighbouring genes as homology regions.

The expansion of genetic tools to *S. pneumoniae* serotype 1, strain 519/43 is important because, we can now genetically manipulate representative strains directly. Strain 519/43 is of interest as it is genetically pliable, is pathogenic as it was isolated from a meningitis patient, and its manipulation will provide clues to better understand the development and establishment of meningitis. Previously, understanding certain determinants within the species was done by inserting the gene in question in one of the very well characterized strains of *S. pneumoniae*, such as D39 (serotype 2). Such approach was used by Paton et al., due to difficulties with the mutagenesis on serotype 1[29]. The results reported by them on D39 carrying a less haemolytic

allele of serotype 1 *ply* in comparison to 519/43 Δ*ply* differ from the ones presented by us[30] highlighting the importance of being able to mutate a gene within the original strain background. Later on, the same group was able to mutate a non-lineage A serotype 1 strain[22]. Interestingly, their protocol is quite distinct from ours, as it is a two-step approach that requires the mutation to be done first in serotype 2 strains and this is then used as a template to be transformed in their serotype 1 strain.

Currently, there is one limitation in the method presented. For now, this method works only for the 519/43 representative strain. The same exact protocol was tried in other strains, namely clinical isolates from ST3081 and ST303 and it was not successful. Furthermore, electroporation as a method of delivery of exogenous DNA to the cell was also attempted on all three sequence types, with positive results observed only for 519/43. Expanding and standardizing the methodology to all serotype 1 strains is of paramount importance as there is enormous variability throughout the group. Studies are undergoing presently to expand the applicability of the method to all strains within serotype 1.

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DISCLOSURES:
The authors have nothing to disclose.

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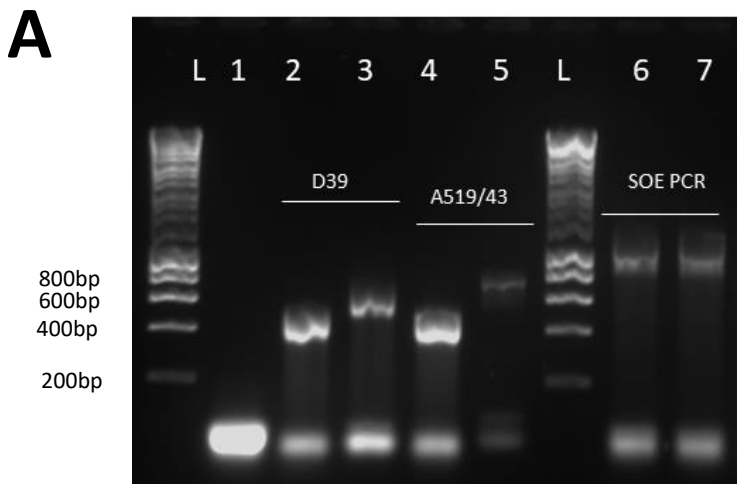
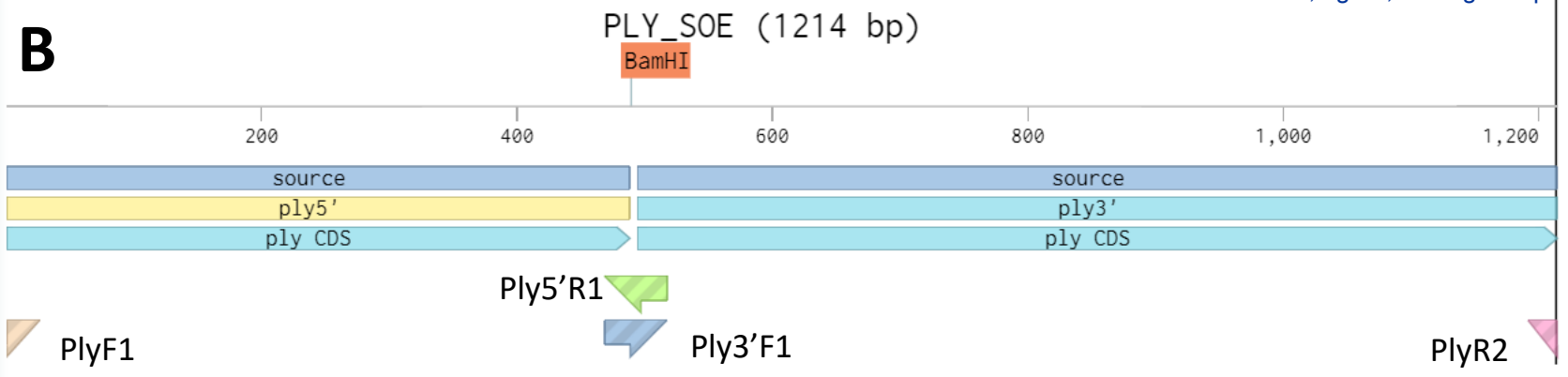
Figure 1





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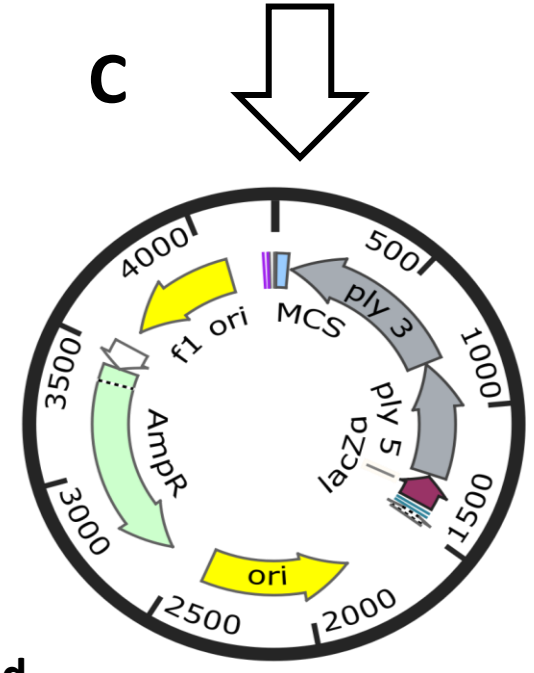
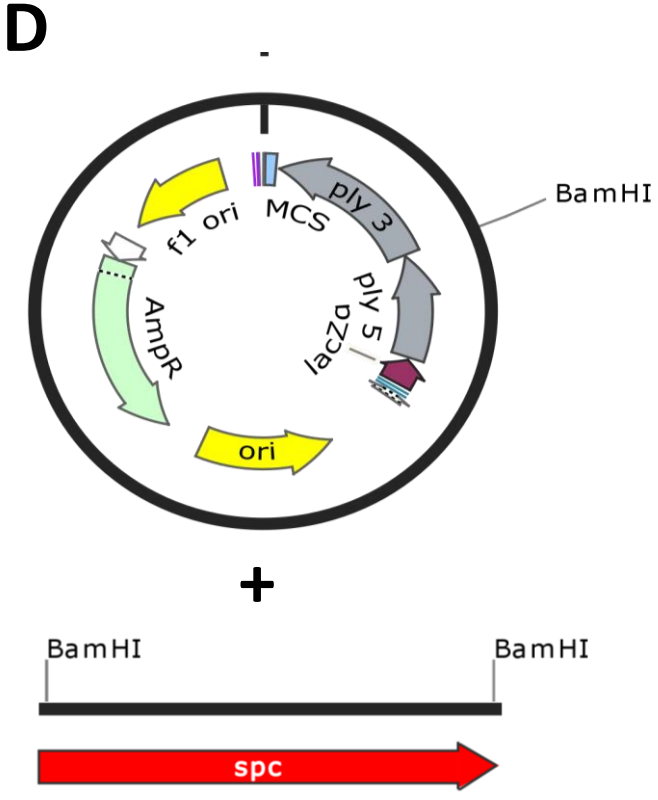
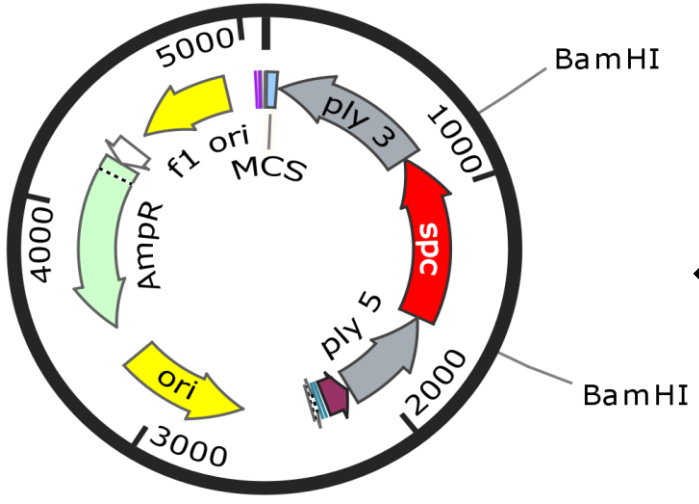


pSD1

BamHI digestion and T4 DNA ligation





pSD2

Figure 2

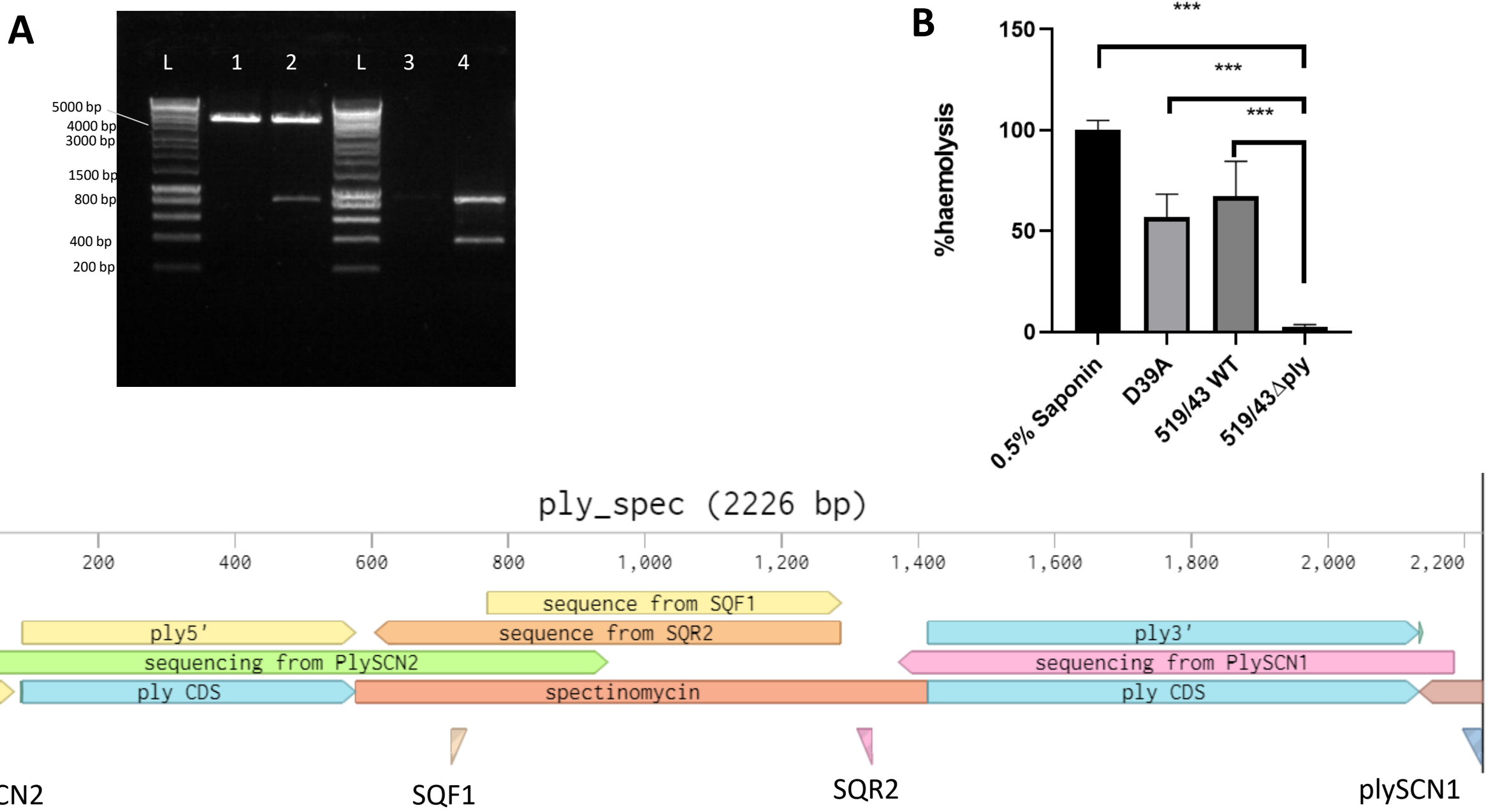


Table 1

Components	Reaction (µL)	Control (µL)	Reaction (µL)	Control (µL)
Buffer	2	2	2	2
pSD1	4	4		-
Spectinomycin cassette	-	-	6	6
BamHI-HF	1	-	1	-
Water	13	14	11	12
Total Volume	20	20	20	20

Table of Materials

Click here to access/download;Table of Materials;Copy of JoVE_Materials_table.xlsx

Name of Material/ Equipment	Company	Catalog Number
AccuPrime *Pfx* DNA polymerase	Invitrogen	12344024
Ampicillin sodium salt	Sigma Aldrich	A9518
Blood Agar Base	Oxoid	CM0055
Bovine Serum Albumine	sigma	55470
Brain Heart Infusion	Oxoid	CM1135
Calcium Chloride $Cacl_2$	Sigma	449709
Competence stimulating peptide 1	AnaSpec	AS-63779
Luria Broth Agar	Gibco	22700025
Luria Broth Base (Miller's formulation)	Gibco	12795027
Monarch Gel Extraction Kit	NEB	T1020S
Monarch Plasmid Miniprep Kit	NEB	T1010S
pGEM T-easy	Promega	A1360
S.O.C.	Invitrogen	15544034
Sodium Hydroxide (NaOH)	Sigma	S0899
Spectinomycin Hydrochloride	SigmaAldrich	PHR1426
Subcloning Efficiency DH5α Competent Cells	Invitrogen	18265017

Comments/Description
Used for amplification of the fragments
Used for bacterial selection on stage 1(pSD1)
Used to plate *S. pneumoniae* transformants
used for *S. pneumoniae* Transformation
used to grow *S. pneumoniae* cells
used for *S. pneumoniae* Transformation
used for *S. pneumoniae* Transformation
used for plating and selection of pSD1 and pSD2
used for plating and selection of pSD1 and pSD2
Used to extract the bands from the DNA gel
Used to extract plasmid from the cells
used as suicide plasmid
used for recovery of cells after transformation
used for *S.pneumoniae* Transformation
Used for bacterial selection
used for the creation of pSD1 and pSD2

Dear Editor,

Please accept this rebuttal letter with all points mentioned by the reviewers answered, as well as all editorial comments.
We believe we have now answered all points and the manuscript is much clearer.

Best wishes,

Dr Vanessa Terra
(on behalf of all authors)

Dear Dr. Terra,

Your manuscript, JoVE61594R1 "Construction of a Pneumolysin deficient mutant in Streptococcus pneumoniae serotype 1 strain 519/43," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Jul 01, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888
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About JoVE

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer

comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 63-68, 108-111, 129-141, 144-146,to avoid this overlap.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

• **Protocol Numbering:** all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• **Figures:** Mark weights on Fig 3

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response to the Editor:
Textual overlap:
Lines 63-68:
62 causes of morbidity and mortality worldwide. Up until now, close to 100 serotypes of S. pneumoniae have been discovered 1–7. Invasive pneumococcal disease (IPD) is responsible for around 700,000 deaths yearly, in children younger than 5 years old 8 with S. pneumoniae being the principal cause of bacterial pneumonia, otitis media, meningitis and septicaemia globally 9. Serotype 1 is one of the main causes of meningitis outbreaks particularly in the African meningitis belt, where sequence type (ST) ST217, a highly virulent sequence type, is predominant 10–15

Was modified to:

Up until recently, close to 100 serotypes of *S. pneumoniae* have been discovered [1–7]. Yearly, invasive pneumococcal disease (IPD) claims around 700,000 deaths, of children younger than 5 years old [8]. S. *pneumoniae* is the major cause of bacterial pneumonia, otitis media, meningitis and septicaemia worldwide [9].

In the African meningitis belt, serotype 1 is responsible for meningitis outbreaks, where sequence type (ST) ST217, an extremely virulent sequence type is dominant[10–15].

Lines 108-111

From:

PCR amplify the spectinomycin cassette from plasmid pR412 (Dr Marc PrudHomme

108 CNRS-Universite Paul Sabatier Toulouse France) using primers BamHI_SP2F2 (GGATCC

109 CTA GAA CTA GTG GAT CCC CC) and BamHI_SP2R2 (GGATCC AAT TCT GCA GAT TTT AC

110 ATG ATC).

TO:
On lines 126 onwards
Amplify the spectinomycin cassette from plasmid pR412 using the following PCR conditions: step 1: denaturing at 94 ^{o}C for 60 sec, step 2: denaturing at 94 ^{o}C for 30 sec, step 3: annealing at 55 ^{o}C for 30 sec, step 4: extension at 68 ^{o}C for 60 sec, step 5: go back to step 2 and repeat for 25 cycles, step 6: final extension at 68 ^{o}C for 60 sec. Use primers BamHI_SP2F2 (GGATCC CTA GAA CTA GTG GAT CCC CC) and BamHI_SP2R2 (GGATCC AAT TCT GCA GAT TTT AC ATG ATC). Plasmid pR412 was (acquired from Dr Marc PrudHomme at CNRS-Universite Paul Sabatier Toulouse France).

Lines 129-141:
Extract the plasmid DNA following the instructions provided with the Kit (NEB, Monarch, UK).
Set up a BamHI-restriction digestion for both pSD1 plasmid and the spectinomycin cassette previously amplified and purified. Use the following conditions and quantities.

Components	Reaction (µl)	Control (µl)	Reaction (µl)	Control (µl)
Buffer CutSmart (NEB)	2	2	2	2
pSD1	4	4	-	-
Spectinomycin cassette	-	-	6	6
BamHI-HF	1	-	1	-
Water	13	14	11	12
Total volume	20	20	20	20

Incubate the restriction digestion reactions and controls at 37 °C for 3 hr.

Analyse the restriction digest by electrophoresis, excise the band and purify following the instructions provided with the Kit (Monarch, UK)**.**

Next, prepare a ligation reaction following the pGEMT-easy system I manufacturer instructions (Promega, UK) using the BamHI- digested pSD1 and spectinomycin (from 3.2). In a microcentrifuge tube, add the following reaction components: 5 µl 2X ligation buffer, 2 µl pSD1, 2 µl of spectinomycin cassette, 1 µl T4 DNA ligase and incubate overnight at 4 °C. This generates plasmid pSD2.

Transform plasmid pSD2 into chemically competent *E. coli* Dh5α as described in point 2.2.

Select the transformants carrying plasmid pSD2 based on their ability to grow in LBA supplemented with 100 µg/ml of spectinomycin and ampicillin.

Perform a plasmid DNA extraction (pSD2) as described above and following the manufacturer's instructions (Monarch, NEB, UK).

Lines: 144-146
Text was changed from :
Start by growing *S. pneumoniae* 519/43 statically, overnight in BHI at 37 ºC, 5% CO_2**.**
The next morning dilute the cultures 1:50 and 1:100 in 10 ml of fresh BHI broth (Oxoid, UK).
Incubate the bacterial suspension statically, at 37 ºC until the OD_{595nm} is between 0.05 to 0.1 (optimal acquisition of DNA closer to 0.1 OD).

To:

1.1. Prepare an overnight culture of *S. pneumoniae* 519/43 in BHI and allow it to grow statically, at 37 °C, 5% CO_2**.**

1.2. The following day dilute the cultures 1:50 and 1:100 in 10 ml of fresh BHI broth (Oxoid, UK). Incubate the cultures statically, at 37 °C until the OD_{595nm} is between 0.05 and 0.1 (optimal acquisition of DNA closer to 0.1 OD).

Protocol detail: As the Editor my notice, I have added a lot more detail to the protocol as a whole, particularly PCR conditions were noted and a table for restriction digested was added (see table 1 on the Figure file attached).

Protocol numbering: The protocol numbering was corrected, and the indentations removed.

Discussion: We believe that all the points raised by the Editor have been covered, we have highlighted the modifications and troubleshooting as well as limitations to the technique and its significance when compared to existing methods (one only). We have covered future applications and highlighted all critical steps within the protocol.

Figures: We have modified the figures so that they are unique to this publication, please see the figure file attached.

Comments from Peer-Reviewers:

Reviewer #1:

Comments

My major concern with this is the extremely limited scope of this study. The authors utilize a suicide vector to generate a gene deletion in *S. pneumoniae*, which is pretty routine across the board. The aspect that is interesting, is that they were able to genetically manipulate a strain that has not yet been genetically tractable using a modified transformation protocol. This is particularly important for not only serotype 1 strains but also other isolates that have extremely poor transformation efficiencies.

Major Concerns

1. The reference to the lack of genetic manipulation in serotype 1 strains points to study that just utilized two strains. Strains of S. pneumoniae vary considerably in their level of transformability, often by multiple orders of magnitude. Can the authors provide additional studies demonstrating lack of genetic tools for serotype 1 strains that use a considerably larger collection of strains? I understand this may be the situation of negative data not seeing publication, but if the authors have attempted multiple serotype 1 isolates by this methodology these negative results (along with the one positive) would be extremely useful

for inclusion in this manuscript. This also needs to be shown to demonstrate the efficacy of the protocol.
Reply: We have included the other two sequence types I have tried it on. And the information that it wasn't successful (lines 318-320). We argue that given the importance of the serotype 1 lineage that although the study is limited, its importance in resolving a previously genetically intractable lineage is a breakthrough that will enable the full genetic characterisation of this key serotype

2. This is also evident by their findings that just a single serotype 1 strain can be genetically manipulated by this technique. This calls into question the applicability of these findings- is this one strain the rule or the exception? Could transformation efficiencies with a panel of serotype 1 strains be provided? This would be extremely helpful for individuals attempting to replicate these genetic manipulations. This also needs to be shown to demonstrate the efficacy of the protocol.

Reply: We do not have an efficiency panel, as it was a case of colonies on strain 519/43 versus no colonies at all in the other two sequence types tried. However, for every 10 colonies that appeared on the first overnight plate, 7 regrew on the second overnight plate and went on to be confirmed by PCR as positive for integration and negative for growth in Ampicillin, which leads to believe that the antibiotic resistance marker was lost and did not recombine elsewhere. A typical success rate on strain 519/43 is of 70%. This information was added to the text (line 288-290). Furthermore, however this method is for now only for strain 519/43, mutations in this background will be very informative on how meningitis progresses and establishes itself, what genes are implicated and it could indeed provide valuable information. Furthermore, we continue our efforts to extend the method to other sequence types.

3. Are the serotype 1 strains not amenable to electroporation? While not frequently undertaken, electroporation methods are fairly efficient at manipulating pneumococcal strains recalcitrant to genetic manipulation.
Reply: Electroporation was tried and proved unsuccessful for other 2 sequence types tested. This has been the case for other investigators for this genetically intractable lineage. It was successful for the strain in question albeit less efficient (lines 320-321).

4. Rather than gels showing the various PCRs, cloning, etc., it would have been more informative to actually confirm the correct integration event on the chromosome- was this a single crossover event? While the presence of the Spec cassette on the chromosome confirms plasmid integration, further confirmation of the specific cross-overs would be useful.
Reply: We have never looked for the single crossover, however if there was one it might have been a fairly quick event as the colonies were never ampicillin resistant. Primers were designed outside of the homology region and used for confirmation of correct integration, furthermore the phenotype was assessed and the loss of haemolytic activity was confirmed (Figure 2B).

Reviewer #2:

Manuscript Summary:

The article "Construction of Pneumolysin deficient mutant in Streptococcus pneumoniae serotype 1 strain 519/43" presents a molecular method used to construct deletion mutants in serotype 1 Pneumococcus using as a proof of concept the knock-out of the pneumolysin gene. The article is clear and easy to read, the different steps and the procedure are "easy to follow". The issue dealing with the lack of genetic tools for such an important pathogen is clearly stated. Of note, the mention that maximum competence is reached at OD=0.1 is a nice addition to the field and may give clues to improve genetic manipulations of the various serotypes present among this challenging bacterium. Nonetheless a couple of minor modifications may be needed in order to help the reader

Minor Concerns:

#1. A mention or quick discussion about how this procedure may also be used besides mutant construction could be added. The same protocol (SOE+suicide vector) may be used to perform genes overexpression, introduce reporter systems or in general manipulate Spn bacterial genome for example.

Reply: Indeed, this is a very good point and it has been amended on the text to include all possible future applications (lines 300-303)

#2. Figure 3 and 4, reading seems unusual, the authors may flip the figures in order to allow top to bottom reading of the vector construction.

Reply: Figures were re-done and we believe are now clear. Figure 1A-E depict all the mutagenesis strategy and Figure 2 contains confirmation of mutants by restriction digestion, phenotypically as well as mapping of sequencing reads to the chromosomal region.

#3. The use of a suicide vector is critical in this protocol, as stated by the authors (L211-212), however it is barely mentioned. The authors should add emphasis on this key element depicting the reasons (loss of vector after introduction of the construct) and the features (absence of gram-positive origin?) explaining this importance.

Reply: The plasmid was chosen because it does have a Gram positive compatible origin of replication. And this was added to the text on lines 237 and 301.

#4. Ply KO being the proof of concept here, an illustration of the mutant phenotype (haemolysis on blood agar, haemolysis assay using red blood cells) could be a great visual addition to the paper.

Reply: We have included the figure (Figure 2B) as we felt this comment made sense. The target was chosen because it had a clear phenotypic output, however we did not include the method as the paper is about making the mutants.

#5. Mapping of the primers used in the study should be added to the figures.

Reply: All primers used for the mutagenesis and for confirmation are indicated in Figure 1B and Figure 2C.

Reviewer #3:

Manuscript Summary:

This manuscript presents a protocol for transformation of virulent strains of S. pneumoniae type 1 (recently published in Microbial Pathogenesis).

Major Concerns:

The only significant concern is the authors' claim to be the first to transform virulent type 1 pneumococci. A previous study (see Infect Immun 2016; 84:822-832. doi: 10.1128/IAI.01454-15) used natural (CSP-1-dependent) transformation, using linear rather than plasmid DNA as donor, to successfully delete two distinct gene clusters from a highly virulent type 1 strain belonging to ST3018 (CC615; type 1 lineage C). The Introduction and Discussion sections need to be reworded to correct this oversight.

Reply: This was indeed an oversight- this has been fixed on the text to highlight that it was in this strain that it was new and that this technique is genome-wide in this particular strain. We have interrogated other genes and have at least two manuscripts in preparation. Furthermore, we have included their method in the discussion as a comparison to the one described here.

Minor Concerns:

There are a few typographical/grammatical errors which need to be tidied up.

Reply: These have been corrected.

Reviewer #4:

Manuscript Summary:

This manuscript describes a method to transform a capsule type 1 pneumococcus strain using a plasmid vector. Protocols which describe pneumococcal transformation are always of interest, particularly with difficult strains like capsule type 1. This method uses a commercially available plasmid vector which is commonly used in many labs to clone and express recombinant DNA.

Major Concerns:

The plasmid vector contains a beta-lactamase gene, which is traditionally never introduced into pneumococci. How did the authors assure that this gene did not recombine somewhere at a distant location in the genome? Although this is unlikely, the implications are very significant. I think whole-genome sequencing should be reported or, alternatively, phenotypic comparison of parent and mutant with ampicillin MIC for a functional beta-lactamase.

Reply: Whole genome sequencing was not performed, however checking for ampicillin resistant was. Throughout the protocol the obtained colonies were always plated in both spectinomycin as well as spectinomycin/ ampicillin and ampicillin alone. All mutants that were positive for spectinomycin from different transformation events were tested for ampicillin resistance. We have never observed any growth in the plates that had ampicillin added. This suggests the loss of the plasmid and that the ampicillin cassette did not recombine elsewhere in the genome.

However, for future studies we intend to modify the suicide plasmid as different strains have different resistances profiles.

Minor Concerns:
Sequencing is mentioned as method to demonstrate the replacement of the pneumolysin gene. At least some cursory description or reference to a sequencing platform and methodology should be given.
Reply: Sequencing was outsourced to Eurofins genomics; it was not done in house and therefore further details weren't included. We have modified the figures (figure 2C) to include all primers used, exactly where the primers bind (outside of the homology region) and the reads obtained and how they covered the insertion area.