Construction of fluorescent pneumococci for *in vivo* imaging and labelling of the chromosome

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Running Head: Fluorescent pneumococci

Abstract

Advances in fluorescence imaging techniques and development and optimization of fluorescent proteins recent years have made major impacts on different fields of pneumococcal research. This chapter provides methodology for construction of fluorescent pneumococcal strains using fusions to DNA-binding proteins. By expressing fluorescent proteins fused to HlpA, a pneumococcal nucleoid binding protein, brightly fluorescent pneumococci are generated. HlpA fusions may be used both for *in vivo* imaging of pneumococci as well as for marking the nucleoid in cell biology studies. Furthermore, it will also be explained how to construct strains for imaging of specific chromosomal loci in pneumococci, using a heterologous ParBS system.

Key Words

GFP, mKate2, HlpA, fluorescent fusions, ParB

1. Introduction

Fluorescence microscopy imaging of live cells is instrumental for different fields of pneumococcal research, from mechanistic studies of proteins during various cellular processes to *in vivo* imaging of bacteria during infection. These techniques rely on the availability of bright fluorescent proteins (FP) and construction of functional protein-FP fusions. Various optimized FPs as well as vectors and methods to construct protein-FP fusions in *Streptococcus pneumoniae* have been described recent years [1-6]. These include FPs of various colors, from green (GFP [4, 6]), red (RFP [1]), yellow (YFP [5]) and cyan (CFP [5]) to FPs optimized for super-resolution techniques [7].

In vivo imaging of live pneumococci in infection settings has long been limited by the lack of sufficiently bright FP-expressing cells. In a study from 2015, it was found that FP fusions to the nucleoid binding protein HlpA (SPV_0997, spr1020, histone-like protein A, also referred to as protein HU or Hup) generated bright fluorescent cells suitable for *in vivo* imaging [8]. Stains expressing HlpA-FP fusions have later been used to image pneumococci during biofilm formation, adherence to epithelial cells, phagocytosis, growth in macrophages and during infection in a zebrafish meningitis model [8-11]. HlpA-FP fusion have also been utilized as markers for the nucleoid in pneumococcal cell biology studies [1, 3, 6, 12, 13].

While HlpA-FP label the entire nucleoid, other methods are needed to label specific chromosomal loci. One recently developed chromosomal labelling system is based on expression of a heterologous ParB protein ($ParB_p$) derived from the lactococcal plasmid pLP712 (NC_019377). $ParB_p$ binds specifically to a 18-bp palindromic $parS_p$ site (GGGGCTAAATTTAGCCCC) [6]. Thus, by simply integrating this 18 bp sequence into the chromosome of strains expressing a $ParB_p$-FP fusion, specific loci of the pneumococcal

chromosome can be labelled and visualized by fluorescence microscopy [6]. The pneumococcal chromosome also encodes a ParB-*parS* system, which is involved in chromosome segregation [14]. However, the plasmid-derived $ParS_p$-$parS_p$ system used for chromosome labelling here does not interfere with the function of the native, pneumococcal ParB-*parS* [6]. In addition to the $ParB_p$-$parS_p$ system, other heterologous chromosomal labelling systems have also recently been published for pneumococci, allowing for visualization of multiple loci simultaneously. These include a TetR/*tetO* repressor-operator system [6] as well as the ParB-parS system from *Enterococcus faecalis* [13].

In this chapter, a protocol for construction of strains expressing HlpA-mKate2 is described, in which a *hlpA-mKate2* fusion gene (mKate2 is a far-red RFP) is integrated in tandem with the native pneumococcal *hlpA* [8]. Specific notes on how to generally generate efficient and functional fluorescent fusions in pneumococci are also provided. Secondly, construction of strains with the $ParB_p$-$parS_p$ chromosome labelling system will be described.

2. **Materials**

- Genomic DNA and genome sequence of *S. pneumoniae*
- Plasmids
    - pMK11-01 or pMK11-02; for amplification of *mKate2* (Addgene #99605 or #99606)
    - pPEP1 (Addgene #61046)
    - pMK17-02 encoding $parB_p^{mut}$-*gfp* (Addgene #99604) (*see* **Note 1**).
    - pAE03 (Addgene #61044)
- Reagents for PCR, including Phusion polymerase, buffer, dNTPs and primers (**Table 1**)
- Equipment for agarose gel electrophoresis.
- PCR purification kit
- High-fidelity restriction enzymes and buffers: SphI, BamHI, EcoRI, NotI

- T4 DNA ligase and buffer.
- Pneumococcal strain and transformation protocol
- Todd Hewitt agar plates (Todd Hewitt broth supplemented with 1.5 % agar) with 2 µg/ml chloramphenicol
- C+Y-medium [15, 16] or other suitable liquid medium.
    - C+Y medium contains (total 110 mL): 100 mL PreC, 2.5 mL Adams III, 2.5 mL 10 % yeast extract, 1 mL 8 % BSA, 1.5 mL 2 % sodium pyruvate, 1 mL 20 % glucose, 0.5 mL 2 mg/mL uridine, 0.5 mL 2 mg/mL adenosine, 0.1 mL 0.4 mM $MnCl_2$, 0.073 mL 3 % glutamine, 0.327 mL 0.3 M sucrose. pH can be adjusted with HCl.
    - PreC contains 8.5 g/L K_2HPO_4, 5 g/L casein hydrolysate, 2 g/L sodium acetate, 11.25 mg/L cysteine, 6 mg/mL tryptophane.
    - Adams III contains 24 mg/L biotin, 24 mg/L nicotinic acid, 28 mg/L pyridoxine HCl, 96 mg/L calcium pantothenate, 26 mg/L thiamine HCl, 11 mg/L riboflavin, 20 mg/L $FeSO_4 \cdot 7H_2O$, 20 mg/L $CuSO_4 \cdot 5H_2O$, 20 mg/L $ZnSO_4 \cdot 7H_2O$, 8 mg/L $MnCl_2 \cdot 4H_2O$, 20 g/L $MgCl_2 \cdot 6H_2O$, 1,75 g/L L-asparagine, 200 mg/L choline, 0.5 g/L $CaCl_2$.
- Induction agents ($ZnCl_2$ and $MnCl_2$)
- Fluorescence microscope

3. Methods

3.1 Construction of *hlpA-mKate2* strain

The nucleoid binding protein *hlpA* will be fused to *mKate2*, encoding a monomeric red fluorescent protein [1] (*see* **Note 2**). The fusion construct will integrate immediately downstream of the native *hlpA* gene (*see* **Note 3**) and transcription will thus be driven by the highly active *hlpA* promoter [17] (*see* **Note 4**). The construct is designed to encode a domain-

breaking linker (*see* **Note 5**), separating *hlpA* and *mKate2* (RGSGSGGEAAAKGTS). A chloramphenicol resistance gene is placed immediately downstream of *hlpA-mKate2* for selection (*see* **Note 6**). A schematic overview of the construct is shown in **Fig. 1**, including an outline of how the construct is assembled using conventional restriction and ligation (*see* **Note 7**).

1. Design/order primers corresponding to **Fig. 1** and **Table 1**.
2. Amplify the five DNA fragments using the primer combinations and template DNA indicated in Fig. 1. Standard PCR reactions and PCR cycling conditions are used:

	Volume (µl)
Phusion polymerase	0.5
HF buffer (10x)	10
dNTPs (2.5 mM each)	1
Forward primer (100 µM)	0.5
Reverse primer (100 µM)	0.5
Template DNA (50-100 ng/µl)	1
dH_2O	36.5
Total	*50*

Temperature (°C)	*Time*	
98	5 min	Initial denaturation
98	20 sec	30 cycles
60	30 sec	
72	30 sec/Kbp	
72	10 min	Final elongation

3. Cast a 1 % agarose gel and check that the products are amplified correctly.
4. Purify all PCR fragments and elute in 16 µl elution buffer from the PCR purification kit.
5. Digest fragment 2, 3 and 4 using restriction enzymes BamHI, BamHI/EcoRI and EcoRI, respectively, using standard reactions.

Component	*Volume (µl)*
Restriction enzyme	1[a]
Buffer (10x)	2
DNA	16
dH_2O	[a]
Total	*20*

[a] 1 µl of each restriction enzymes should be used. dH_2O should be added to a total volume of 20 µl.

Incubate the reaction at 37°C for 30 min.

6. Cast a 1 % agarose gel and run the digested fragments on the gel.
7. Purify the digested fragments from gel using a PCR purification kit. Elute in 15 µl from the purification kit.
8. Ligate the fragments. The reaction should be set up with equimolar ratios of the three fragments (1:1:1 molar ratio of each fragment, 1 µl T4 Quick ligase and 2 µl 10x reaction buffer) at room temperature for 2 hours or overnight at 16°C.
9. Use the ligation mix as template DNA in a PCR reaction (same conditions as above) to amplify the 2+3+4 fragment using primers C and H.
10. Purify the 2+3+4 fragment using PCR purification kit.
11. Assemble fragment 1 and 5 to the 2+3+4 fragment by repeating the procedure in steps 5-11; digest fragment 1 with SphI, fragment 2+3+4 with SphI and NotI and fragment 5 with NotI. Purify the digested fragments and ligate. Finally amplify the full fragment 1+2+3+4+5 using primers A and J.
12. Transform the full linear fragment (**Fig. 1**) into the *S. pneumoniae* strain. Transformats are selected on TH agar plates containing 2 µg/ml chloramphenicol (*see* **Note 8**).
13. Pick and grow colonies in C+Y medium containing 2 µg/ml chloramphenicol (*see* **Note 9**).
14. Verify transformants by colony PCR using primer pairs K + F and G + L.
15. The resulting strain should display bright fluorescence from the constitutively expressed nucleoid localized fusion protein (**Fig. 2**).

3.2 Construction of strain for localization of chromosomal loci using a Zn^{2+}-inducible *$parB_p$-gfp* fusion.

First, a construct for expression of a Zn^{2+}-inducible *$parB_p^{mut}$-gfp* fusion is introduced into the pneumococcal strain.

1. Plasmids encoding fusions of fluorescent proteins to $parB_p^{mut}$ is available via Addgene (*see* **Note 10**). Transform pMK17-02, encoding $parB_p^{mut}$-*gfp* (*see* **Note 11**) into *S. pneumoniae*. The construct will integrate by double crossover in the *bgaA*-locus of *S. pneumoniae* (*see* **Note 12**). Select transformants on TH agar containing 1 µg/ml tetracycline.
2. Pick colonies and grow in C+Y medium containing 1 µg/ml tetracycline (*see* **Note 9**). Verify integration by colony PCRs using primers M + N and O + P, which will produce a 1 kb product only upon correct integration by double crossover (Fig. 2A).

Next, a chromosomal locus of choice is tagged by inserting the 18 bp palindromic $parS_p$ (GGGGCTAAATTTAGCCCC) site into the chromosome. As an example, insertion of $parS_p$ into the terminus region of the chromosome is explained. The sequence is integrated together with an erythromycin resistance cassette (for selection of transformants) between *rbgA* and *iga* as depicted in **Fig. 2**.

3. Amplify the three DNA fragments using the primer combinations and template DNA indicated in Fig. 2. Standard PCR reactions and PCR cycling conditions (*see* **Section 3.1**).
4. Purify all PCR fragments.
5. Digest fragment 1, 2 and 3 using restriction enzymes BamHI, BamHI/NotI and NotI, respectively, using standard reactions (*see* **Section 3.1**).
6. Cast a 1% agarose gel and purify the digested fragments from gel using a PCR purification kit
7. Ligate the fragments in equimolar ratios (1:1:1 molar ratio of each fragment, 1 µl T4 Quick ligase, 2 µl 10x reaction buffer) at room temperature for 2 hours or overnight at 16°C.
8. Transform the ligation product directly into the pneumococcal strain made in step 2. Select transformants on TH plates containing 0.25 µg/ml erythromycin.

9. Pick and grow transformants in C+Y medium containing 0.25 µg/ml erythromycin (Note 9). Verify transformants by PCR using outer primers W+X, and sequence the $parS_p$-site using primer T (**Fig. 2**).
10. When the strain is verified, it can be used for fluorescence microscopy. To obtain optimal signal strength from the $ParB_p^{mut}$-GFP fusion, first grow the strain in C+Y without antibiotics until OD_{600} = 0.4. Then, dilute the culture 1/100 in C+Y medium. When OD_{600} reach 0.05, add 0.1 mM $ZnCl_2$ and 0.01 mM $MnCl_2$ for induction of $parB_p^{mut}$-*gfp* expression from the Zn^{2+}-promoter. Incubate further until OD_{600} = 0.1-0.2 before performing fluorescence microscopy (*see* Note 13).

4. Notes

1. The original *$parB_p$*-sequence from plasmid pLP712 contains an internal *$parS_p$* sequence [6]. This sequence has been mutagenized in *$parB_p^{mut}$*, which is the version utilized in this protocol.
2. A large number of different fluorescent proteins of various colors have been utilized in protein fusions in pneumococci. Studies have also been performed to compare the performance of different GFP variants [4] and RFP variants [1]. Based on these studies, the GFP of choice is sfGFP(Bs) or it's monomeric counterpart m(sf)GFP (Addgene #96603 or #96604), and the RFP of choice is mKate2 (Addgene #96605) for protein-FP fusions. Note, however, that the optimal fluorescent protein will depend on the setup of your fluorescence microscope or fluorescence detection unit, and for super-resolution microscopy techniques, fluorescent proteins with specific features are often required.
3. The *hlpA-mKate2* fusion gene is integrated in tandem with the native *hlpA* gene, because introduction of the *hlpA-mKate2* fusion without the presence of a native *hlpA* has not been successful [8]. On the other hand, construction of strains expressing *hlpA* fused to superfolder *gfp* can be made in the absence of a native *hlpA* [8].
4. Expression of *hlpA*-fusions has been shown to be highly efficient, producing strains with bright fluorescence [8]. In cases where expression levels of fluorescent fusions is too low, several adjustments can be made for optimization; these include ectopic expression with an inducible promoter (see 3.2) and to add N-terminal tags of 5-10 amino acids to increase translation efficiency [2, 5]. The latter is particularly usable for fusions where the fluorescent protein is fused to the N-terminus of the target protein.

5. To provide structural flexibility between the fluorescent protein and the target proteins, a linker sequence should be added. Different linkers have been utilized in pneumococci [2, 5, 18], including the domain-breaking linker [19] in the *hlpA-mKate2* construct.
6. Other antibiotic resistance genes could be used instead. In this case, the resistance gene is driven by the *hlpA*-promoter. In other cases, it may be necessary to include full resistance cassette (i.e., promoter – resistance gene – terminator) to obtain sufficiently high expression.
7. Any cloning strategy for fusing multiple fragments can in principle be used, including overlap-extension PCR [20], infusion cloning [21, 22] or Gibson (isothermal) assembly [1, 23].
8. Chloramphencol concentrations ranging from 2 – 4.5 µg/ml can be used for selection, depending on the pneumococcal strain.
9. Instead of picking and growing the colonies in liquid medium containing antibiotics, the colonies can also be re-plated on antibiotic plates and incubated over-night. Re-plated colonies can then be picked and grown in liquid medium without antibiotics.
10. In addition to pMK17-02, which is used here, other versions of similar plasmids are available, including pMK17-01 encoding *$parB_p$-gfp* (Addgene #99603), pMK11-01 encoding *$parB_p$-mKate2* (Addgene #99605) pMK11-02 encoding *$parB_p^{mut}$-mKate2* (Addgene #99606).
11. The *gfp* version in these plasmid is monomeric superfolder *gfp, m(sf)gfp* [6]. Utilizing monomeric versions is an advantage in many applications to avoid artefacts due to multimerization of the fluorescent proteins.
12. The homology regions to *bgaA* in the plasmid are from the strain D39. Utilization of this plasmid for integration into other pneumococcal strains will depend on the degree of similarity to the sequence of D39, and this should be checked prior to starting the experiment.

13. The best signal is observed when cells are in early and mid-exponential growth phase.

References

1. Beilharz, K., van Raaphorst, R., Kjos, M., and Veening, J.W. (2015). Red fluorescent proteins for gene expression and protein localization studies in *Streptococcus pneumoniae* and efficient transformation with DNA assembled via the Gibson assembly method. Appl Environ Microbiol 81, 7244-7252.
2. Eberhardt, A., Wu, L.J., Errington, J., Vollmer, W., and Veening, J.W. (2009). Cellular localization of choline-utilization proteins in *Streptococcus pneumoniae* using novel fluorescent reporter systems. Mol Microbiol 74, 395-408.
3. Kjos, M., and Veening, J.W. (2014). Tracking of chromosome dynamics in live *Streptococcus pneumoniae* reveals that transcription promotes chromosome segregation. Mol Microbiol 91, 1088-1105.
4. Overkamp, W., Beilharz, K., Detert Oude Weme, R., Solopova, A., Karsens, H., Kovacs, A., Kok, J., Kuipers, O.P., and Veening, J.W. (2013). Benchmarking various green fluorescent protein variants in *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Lactococcus lactis* for live cell imaging. Appl Environ Microbiol 79, 6481-6490.
5. Henriques, M.X., Catalao, M.J., Figueiredo, J., Gomes, J.P., and Filipe, S.R. (2013). Construction of improved tools for protein localization studies in *Streptococcus pneumoniae*. PLoS One 8, e55049.
6. van Raaphorst, R., Kjos, M., and Veening, J.W. (2017). Chromosome segregation drives division site selection in *Streptococcus pneumoniae*. Proc Natl Acad Sci U S A 114, E5959-E5968.

7. Jacq, M., Adam, V., Bourgeois, D., Moriscot, C., Di Guilmi, A.M., Vernet, T., and Morlot, C. (2015). Remodeling of the Z-ring nanostructure during the *Streptococcus pneumoniae* cell cycle revealed by photoactivated localization microscopy. MBio 6, pii: e01108-01115.
8. Kjos, M., Aprianto, R., Fernandes, V.E., Andrew, P.W., van Strijp, J.A., Nijland, R., and Veening, J.W. (2015). Bright fluorescent *Streptococcus pneumoniae* for live-cell imaging of host-pathogen interactions. J Bacteriol 197, 807-818.
9. Ercoli, G., Fernandes, V.E., Chung, W.Y., Wanford, J.J., Thomson, S., Bayliss, C.D., Straatman, K., Crocker, P.R., Dennison, A., Martinez-Pomares, L., et al. (2018). Intracellular replication of *Streptococcus pneumoniae* inside splenic macrophages serves as a reservoir for septicaemia. Nat Microbiol 3, 600-610.
10. Jim, K.K., Engelen-Lee, J., van der Sar, A.M., Bitter, W., Brouwer, M.C., van der Ende, A., Veening, J.W., van de Beek, D., and Vandenbroucke-Grauls, C.M. (2016). Infection of zebrafish embryos with live fluorescent *Streptococcus pneumoniae* as a real-time pneumococcal meningitis model. Journal of neuroinflammation 13, 188.
11. Reddinger, R.M., Luke-Marshall, N.R., Sauberan, S.L., Håkansson, A.P., and Campagnari, A.A. (2018). *Streptococcus pneumoniae* modulates *Staphylococcus aureus* biofilm dispersion and the transition from colonization to invasive disease. MBio 9, pii: e02089-02017.
12. Nourikyan, J., Kjos, M., Mercy, C., Cluzel, C., Morlot, C., Noirot-Gros, M.F., Guiral, S., Lavergne, J.P., Veening, J.W., and Grangeasse, C. (2015). Autophosphorylation of the bacterial tyrosine-kinase CpsD connects capsule synthesis with the cell cycle in *Streptococcus pneumoniae*. PLoS Genet 11, e1005518.
13. Mercy, C., Lavergne, J.-P., Slager, J., Ducret, A., Garcia, P.S., Noirot-Gros, M.-F., Dubarry, N., Nourikyan, J., Veening, J.-W., and Grangeasse, C. (2018). RocS drives chromosome segregation and nucleoid occlusion in *Streptococcus pneumoniae*. bioRxiv.

14. Attaiech, L., Minnen, A., Kjos, M., Gruber, S., and Veening, J.W. (2015). The ParB-*parS* chromosome segregation system modulates competence development in *Streptococcus pneumoniae*. MBio 6, e00662.
15. Lacks, S., and Hotchkiss, R.D. (1960). A study of the genetic material determining an enzyme in Pneumococcus. Biochimica et biophysica acta 39, 508-518.
16. Martin, B., Garcia, P., Castanie, M.P., and Claverys, J.P. (1995). The *recA* gene of *Streptococcus pneumoniae* is part of a competence-induced operon and controls lysogenic induction. Mol Microbiol 15, 367-379.
17. Aprianto, R., Slager, J., Holsappel, S., and Veening, J.-W. (2018). High-resolution analysis of the pneumococcal transcriptome under a wide range of infection-relevant conditions. bioRxiv.
18. Straume, D., Stamsås, G.A., Berg, K.H., Salehian, Z., and Håvarstein, L.S. (2017). Identification of pneumococcal proteins that are functionally linked to penicillin-binding protein 2b (PBP2b). Mol Microbiol 103, 99-116.
19. Arai, R., Ueda, H., Kitayama, A., Kamiya, N., and Nagamune, T. (2001). Design of the linkers which effectively separate domains of a bifunctional fusion protein. Protein engineering 14, 529-532.
20. Higuchi, R., Krummel, B., and Saiki, R.K. (1988). A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic acids research 16, 7351-7367.
21. Irwin, C.R., Farmer, A., Willer, D.O., and Evans, D.H. (2012). In-fusion(R) cloning with vaccinia virus DNA polymerase. Methods Mol Biol 890, 23-35.
22. Liu, X., Gallay, C., Kjos, M., Domenech, A., Slager, J., van Kessel, S.P., Knoops, K., Sorg, R.A., Zhang, J.R., and Veening, J.W. (2017). High-throughput CRISPRi phenotyping

identifies new essential genes in *Streptococcus pneumoniae*. Molecular systems biology 13, 931.

23. Gibson, D.G. (2011). Enzymatic assembly of overlapping DNA fragments. Methods in enzymology 498, 349-361.
24. Slager, J., Kjos, M., Attaiech, L., and Veening, J.W. (2014). Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell 157, 395-406.

Tables

Table 1. Oligo list

Primer	Alternative name	Sequence (5'- 3'); restriction site (underlined); reference
A	hlpA-up-F	AACAAGTCAGCCACCTGTAG; [3]
B	hlpA-R-SphI	CGCGCATGCAGACTGATTATTTAACAGCGTC; SphI; [3]
C	hlpA-F-rbs-SphI	CGTGCATGCTGGAGGAATCATTAACATGGCA; SphI; [3]
D	hlpA-up-R-BamHI	CTGCGGATCCTTTAACAGCGTCTTTAAGAGCTTTACCAGC; BamHI; [3]
E	mKate2-link-BamHI	CGATGGATCCGGATCTGGTGGAGAAGCTGCAGCTAAAG GATCAGAACTTATCAAGGAAAATATGCACATG; BamHI; [3]
F	mKate2-R-EcoRI	GCATGAATTCTTATTAACGGTGTCCCAATTTACTAG; EcoRI; [3]
G	cam-up-F-EcoRI	ACTCGGAATTCGATAAAAATTTAGGAGGCATATC; EcoRI
H	camR-NotI	ACGTGCGGCCGCTTATAAAAGCCAGTCATTAG; NotI; [3]
I	hlpA-down-F-NotI	AGCGCGGCCGCTTAAAAAGCCTATTGTATCAAGCT; NotI; [3]
J	hlpA-down-R	CGTGGCTGACGATAATGAGG; [3]
K	hlpA-up-check	GATTGTAACCGATTCATCTG; [3]
L	hlpA-down-check	GGAATGCTTGGTCAAATCTA; [3]
M	integration 1	CTTGATGAAACCTACATTTG; [24]
N	integration 2	GCTTCCATTAAGGATAGTTC; [24]
O	integration 3	CCGGTCGCTACCATTACCAG; [24]
P	integration 4	TGGTCTTTAATGATAAAGAA; [24]
Q	rbg-up-F	CAGATCTTCAGAACTATGTCCA; [6]
R	rbg-up-R-BamHI	CCCGGGATCCAGCCTATCTTTTACCCTATATAGA; BamHI; [6]
S	insert-ter-1parSp-BamHI	ATGGATCCGGGGCTAAATTTAGCCCCCAACAGCAAA GAATGGCGGA; BamHI; [6]
T	ery-R-NotI	GTCAGCGGCCGCGTAGGCGCTAGGGACCTC; NotI; [6]
U	rbg-down-F-NotI	GTCAGCGGCCGCAAAAGATAGGGTAAAAGGCTATC; NotI; [6]
V	rbg-down-R	GACCACGACCAACCTCATCA; [6]
W	rbg-check-up	ATCAGATAGTACAGAGGGATC; [6]
X	rbg-check-down	GGCTTGGTCTTGAACGGCT; [6]

Figure legends

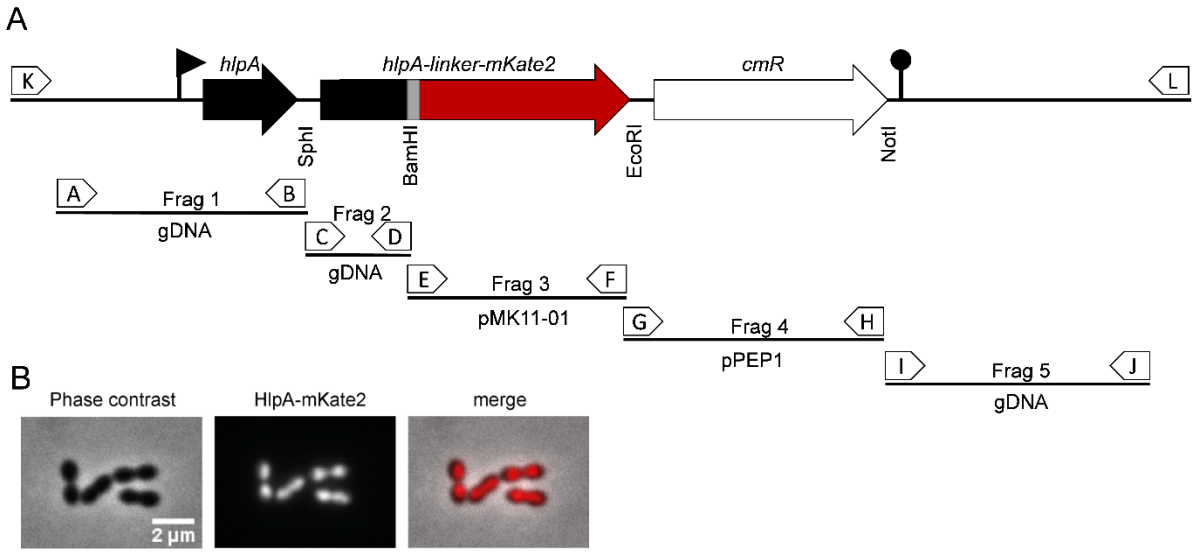

Figure 1. Construct for expression of HlpA-mKate2.

A. Top: Schematic overview of the construction. Flag and lollipop indicate promoter and transcriptional terminator, respectively, while the genes are shown as arrows. Below: Outline of the construction based on amplification of five fragments (Frag 1-5). Restriction sites are indicated and primers are shown as boxed arrows (primers A-L, see Table 1). The DNA templates used for amplification are also given. **B**. Images of *S. pneumoniae* D39 expressing HlpA-mKate2 from the construct above, indicating the nucleoid localized signal. Phase contrast and fluorescence signals are shown individually and merged.

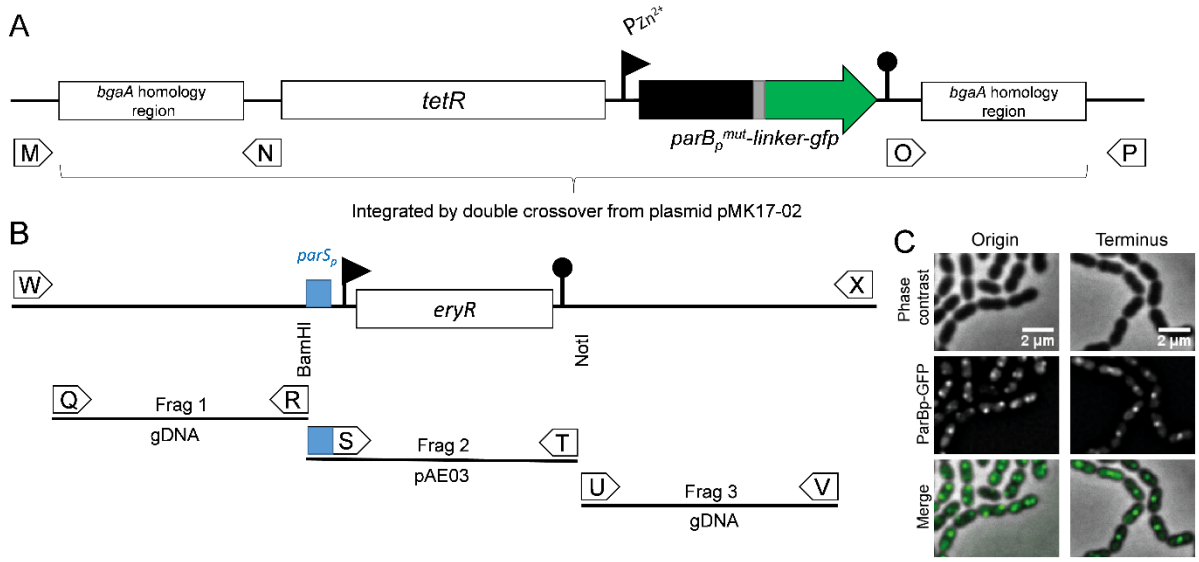

Figure 2. ParB$_p$-*parS$_p$* chromosome labelling system.

A. Schematic overview of the *parB$_p$-gfp* contruct integrated in the *bgaA*-locus. **B**. Schematic overview of the *parS$_p$* integration construct integrated between *rbgA* and *iga* in the terminus region. Promoters are indicated by flags and transcriptional terminators by lollipops. Primers (M-X, see Table 1) are shown as boxed arrows and restriction sites are indicated. The 18 bp *parS$_p$* site (GGGGCTAAATTTAGCCCC) is included as overhang in primer S and located upstream of the erythromycin resistance cassette. **C**. Microscopy images (phase contrast, GFP and merged images) of pneumococcal strain expressing ParB$_p$-GFP with a *parS$_p$* site introduced close to origin of replication (left panel) or close to the terminus region of the chromosome (right panel).