Pneumococcal cell biology in a new light

Beilharz, Katrin

Chapter 3

Improved red fluorescent proteins for gene expression and protein localization studies in *Streptococcus pneumoniae*

Beilharz, Kjos and Veening, submitted

Abstract

During the last decades, a wide range of fluorescent proteins (FPs) have been developed and improved. This has had a great impact on the possibilities in biological imaging and the investigation of cellular processes at the single-cell level. Recently, we have benchmarked a set of green fluorescent proteins (GFPs) and generated a codon-optimized superfolder GFP for efficient use in the important human pathogen *Streptococcus pneumoniae* and other low-GC rich Gram-positive bacteria. In the present work we constructed and compared four red fluorescent proteins (RFPs) in *S. pneumoniae*. Two orange-red variants, mOrange2 and TagRFP, and two far-red FPs, mKate2 and mCherry, were codon-optimized and examined by fluorescence microscopy and plate reader assays. Our data show that mCherry is the fastest maturing RFP in *S. pneumoniae* and is best suited for studying gene expression while mKate2 and TagRFP are more stable and are the preferred choices for protein localization studies. The RFPs described here will be useful for cell biology studies that require multi-color labeling in *S. pneumoniae* and related organisms.

Introduction

Streptococcus pneumoniae is a human pathogen that annually kills about 1.5 million people worldwide[158]. It causes harmful infections such as pneumonia, meningitis, and sepsis, especially in elderly people, young children and immunosuppressed patients. The increase of antibiotic resistance and the lack of long-lasting vaccines against this organism make it crucial to better understand the cell biology of *S. pneumoniae* and gain insights into essential processes such as cell division, chromosome segregation and cell wall synthesis. Therefore, the development of tools for cell biological studies in this organism becomes important.

Studies of protein localization and gene expression at the single-cell level brought new insights into the cell biology of bacteria. However, most of our knowledge on bacterial cell biology is based on experiments performed in rod-shaped model organisms such as *Escherichia coli* and *Bacillus subtilis* and only recently have researchers been able to examine these processes in coccoid bacteria such as *Staphylococcus aureus* and *S. pneumoniae*[23].

S. pneumoniae is a microaerophilic organism that cannot tolerate high levels of oxygen. However, folding and maturation of fluorescent proteins often requires sufficient concentrations of oxygen, whereas microaerophilic conditions might have a negative effect on maturation[16]. Nevertheless, recent efforts have resulted in a set of usable molecular tools to study the cell biology of *S. pneumoniae*[21,22,159]. In particular, a green fluorescent protein variant was identified (a codon-optimized superfolder GFP) that yields adequate fluorescence levels in live *S. pneumoniae* cells[159].

The green fluorescent protein, GFP, from jellyfish *Aequorea victoria* was the first FP to be described[14] and cloned[15,160]. Other variants such as blue, cyan and yellow fluorescent proteins, have been engineered from the jellyfish-derived GFP, whereas orange, red and far-red variants were found in other sea animals[161–163]. Because RFPs are spectrally far away from GFP, they are ideal FPs to use in double-label experiments with GFP. Most RFPs naturally occur in tetrameric form and monomeric mutants often resulted in a great loss of photostability and fluorescent intensities. However, in recent years improved variants have been developed[162,164]. The four different RFPs that we benchmarked in this study were codon-optimized for low-GC Gram-positive bacteria. We chose for monomeric RFPs that can possibly be used in protein fusions. The orange-red fluorescent protein we tested, mOrange2, is a monomeric derivative of DsRed from coral *Dictiosoma sp.* with improved photostability[162]. TagRFP, a monomeric derivate of eqFP578 from sea anemone

Entacmaea quadricolor[164], is an orange-red FP for which high brightness but a low photostability has been reported[164]. For far-red variants we tested mCherry[161], another derivative of DsRed and one of the most widely used RFPs in bacterial research, and mKate2 derived from *E. quadricolor* with excellent pH resistance and photostability[163]. While the four selected RFPs share a high degree of sequence and structural similarity, mKate2 has the highest reported quantum yield when examined *in vitro*[163].

Table 1 List of red fluorescent proteins

Fluorescent protein	Max λ_{ex} (nm)	Max λ_{em} (nm)	Color	Brightness	Reference
mOrange2	549	565	orange	35	[162]
TagRFP	555	584	red	40	[164]
mCherry	587	610	far-red	16	[162]
mKate2	588	633	far-red	25	[165]

To test the performance of these four RFPs for *in vivo* single-cell gene expression studies in *S. pneumoniae*, we placed the respective genes under a Zn^{2+}-inducible promoter and integrated them stably as a single copy within the *S. pneumoniae* genome. These assays identified the far-red RFP mCherry as the brightest and the most rapidly maturing variant *in vivo*, while no signals were obtained with mOrange2. Speed of FP maturation is not always important, for instance when the question is simply where the protein localizes. In such cases brightness or functionality of the FP-fusion might be more crucial. Therefore, we tested the properties of TagRFP, mCherry and mKate2 when used as a C-terminal protein fusion to the histone-like protein HlpA. This showed that the most accurate localization pattern was obtained with mKate2 and TagRFP, whereas the mCherry fusion is more prone to degradation, leading to a more diffuse localization signal. Together, the RFPs presented in this study expand the cell-biological toolbox available for *S. pneumoniae* and related organisms.

Results and Discussion

Expression of codon-optimized RFPs in *S. pneumoniae*

New variants of monomeric red fluorescent protein have been discovered and developed in the recent years. They are important tools for creation of protein fusions to study protein localization and dynamics and for multi-color labeling, for instance in combination with GFP or cyan and yellow fluorescent proteins (CFP and YFP). In the past,

brightness of monomeric RFPs has been problematic, but more recently improved variants have been described. We selected four of these 'next generation' variants of red fluorescent proteins and codon-optimized these genes (Table 1) for low-GC Gram-positive bacteria, including mOrange2 and mCherry from the 'mFruits' palette[162], as well as TagRFP[164] and mKate2[163]. mCherry is one of the most commonly used RFPs with a reported short maturation time (varying between 15 min (11) and 40 min (13)), but a relatively poor photostability[162]. mOrange2 is an orange-red emitting FP variant and was reported to have a high photostability and high intrinsic brightness, but a relatively long maturation time (~4.5 hours) compared to the doubling time of *S. pneumoniae* (~30 min under our experimental conditions)[162]. TagRFP is a bright orange-red fluorescent protein with a reported maturation time of 100 min and high brightness (Table 1)[162,164]. mKate2 was also reported to have a short maturation time (< 20 min) and good photostability (Table 1)[163]. mKate2 and mCherry are far-red variants and therefore highly suitable for the use in combination with green and yellow fluorescent proteins. All variants are reported to be monomeric which makes them suitable for their use in protein fusions.

In order to directly compare the four RFPs (mOrange2, TagRFP, mCherry and mKate2), we used plasmid pJWV102[159], a pPP2 derivative[144] containing the Zn^{2+}-inducible promoter P_{Zn}[21], that integrates into the chromosome by double crossover at the non-essential *bgaA*-locus. To exclude translational differences, the same RBS was used for all RFPs. This allows for evaluation and unbiased comparison of the fluorescence intensities and folding times of the individual RFPs. A schematic presentation of *rfp*-carrying vectors is shown in Figure 1.

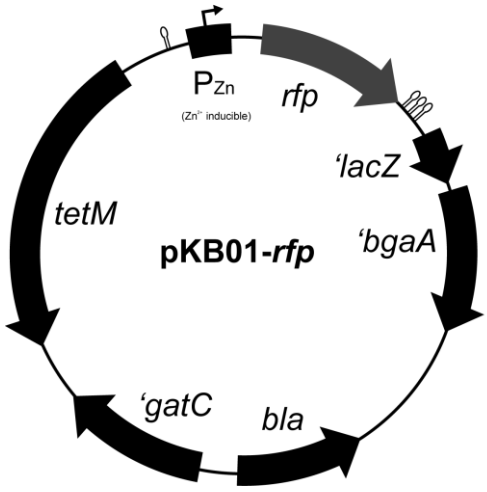

Fig. 1 Vector for inducible RFP expression in *Streptococcus pneumoniae*. The pKB01-derivate plasmid carries a copy of an *rfp* gene (*mCherry*, *mOrange2*, *TagRFP* or *mKate2*) and integrates in the *S. pneumoniae* chromosome at the non-essential *bgaA* locus by double crossover. The gene is under control of a Zn^{2+}-inducible promoter, P_{Zn}.

Comparison of RFP intensities at the population and single-cell level

The intensities of the different RFPs were analyzed at the population level using a plate reader and at the single-cell level using epifluorescence microscopy. First, to check whether the expression levels were comparable and whether all RFPs were expressed under our experimental conditions, we performed immunodetection on whole-cell lysates using an anti-RFP polyclonal antibody (Molecular Probes®, Life Technologies). Due to the structural homology between the different RFPs, this polyclonal antibody recognizes all tested variants (Molecular Probes®, Life Technologies). Pneumococcal cells were grown in C+Y liquid medium at 37°C to OD_{600} 0.1, diluted 1:100 in C+Y containing 0.15 mM $ZnCl_2$ for induction and harvested at OD_{600} 0.15 (mid-exponential growth). All fluorescent proteins were efficiently produced at comparable levels in *S. pneumoniae* and only mCherry showed signs of protein degradation (Figure 2A).

Prior to fluorescence measurement using a plate reader, induced cells were harvested at OD_{600} 0.15 and were washed and resuspended in PBS. The plate reader we used contains a standard RFP filter set (excitation: λ_{ex} 590/20 nm, emission: λ_{em} 635/10 nm; 50% dichroic mirror), which is best suitable for the far-red FPs. Not surprisingly, the signals for cells expressing TagRFP and mOrange2 hardly appeared above background. Interestingly, mCherry had the highest signal to noise level with approximately 6-fold higher fluorescence compared to mKate2 (Figure 2B), while mKate2 was reported to be brighter *in vitro*[163].

For fluorescence microscopy, cells were grown to OD_{600} 0.05 before expression of RFPs was induced with 0.15 mM $ZnCl_2$. Cells were then harvested for microscopy at different time points. We used filter sets that are standard in most fluorescence microscopes; mCherry filter set (λ_{ex} 575/25 nm and λ_{em} 632/60 nm, QUAD 2 polychroic (580-630 nm)) and TRITC filter set (λ_{ex} 542/27 nm and λ_{em} 594/45 nm, QUAD 1 polychroic (550-630 nm)). The same light output and exposure times were used for all samples. Using the mCherry filter set, signals from mCherry and mKate2 were already visible after 30 min of induction (Figure 2C). After 120 min also TagRFP showed good fluorescence, similar in strength to mKate2, but still approximately 6 times less bright than mCherry (Figure 2C). With these imaging conditions, signals for mOrange2 could not be detected. When the TRITC filter set was applied, which should be better suited for mOrange2 and TagRFP than for the far-red RFPs, mCherry still outperformed the other RFPs (Figure 2D). With this filter set, TagRFP gave a good signal above background with approximately half of that of

mCherry for the different time-points, while the signal from mKate2 was consistently very low and mOrange2 still gave no signals above background levels after 3.5 hrs. It should be noted that the TRITC filter set and the QUAD1 polychroic mirror might block a large amount of the emitted photons from mOrange2 (Table 1). Also, the slow fluorophore maturation time of mOrange2 at 37°C[162] might be a possible explanation for the absence of signal and thus makes this FP unsuitable for imaging in *S. pneumoniae*. TagRFP gives appreciable signals with a standard TRITC filter set and is a good orange-red RFP protein for use in *S. pneumoniae* (Figure 2), but might be less suitable in multi-color experiments that include yellow fluorescent proteins due to possible spectral overlap. In total, these results show that mCherry is the fastest maturing RFP in *S. pneumoniae* with the highest fluorescence signal when used in promoter fusions. Importantly, this is not simply because of more efficient protein production since all four RFPs were produced to approximately similar levels (Figure 2A).

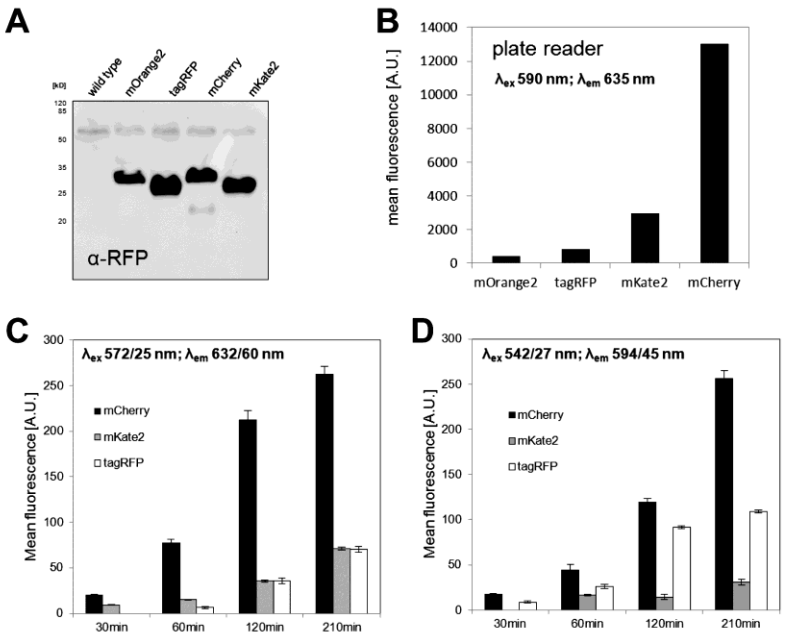

Fig. 2 Fluorescence intensities of the different red fluorescent proteins. (**A**) Western blot analysis of expression levels of RFPs in whole-cell extract detected using anti-RFP antibody. (**B**) Mean fluorescence on the population level using a plate reader equipped with a standard RFP filter set (excitation: λ_{ex} 590/20 nm, emission: λ_{em} 635/10 nm). See Materials and Methods for details of the filters used. (**C**) Average fluorescence intensities on the single-cell level measured by fluorescence microscopy using an mCherry filter set (excitation: λ_{ex} 572/25 nm, emission: λ_{em} 632/60 nm), polychroic mirror QUAD2 or (**D**) a TRITC filter set (excitation: λ_{ex} 542/27 nm, emission: λ_{em} 542/45 nm); polychroic mirror QUAD1. Values are in arbitrary units [A.U.]. Single-cell fluorescence levels were determined using MicrobeTracker[148]. Error bars depict the standard deviation. mOrange2 values are not shown in the plots since signals above background level could not be detected.

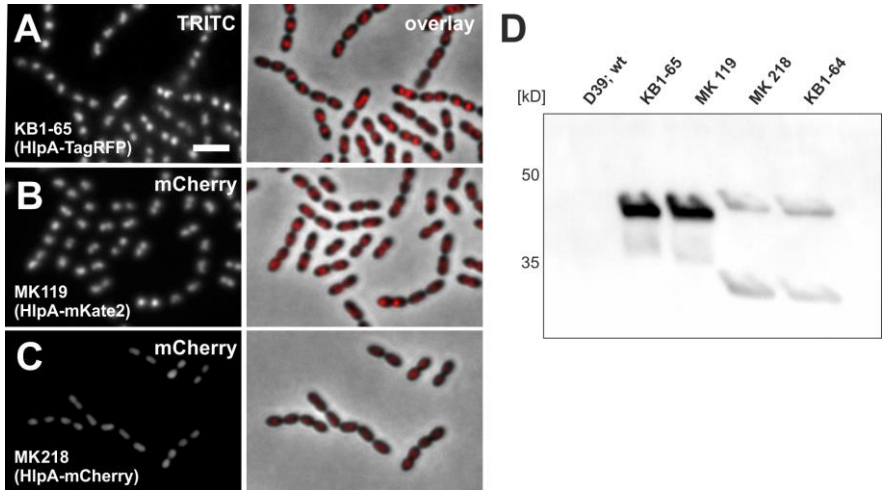

Fig. 3 Comparison of TagRFP, mCherry and mKate2 in a C-terminal protein fusion to the histone-like protein HlpA. Non-deconvolved micrographs of merodiploid D39 strains expressing (**A**) HlpA-TagRFP (KB1-65), (**B**) HlpA-mKate2 (MK119) and (**C**) HlpA-mCherry (MK218). The scale bar equals 2 µm. The filter sets used (TRITC or mCherry) are indicated in the figures. Note that the contrast for the MK218 cells was increased to better visualize the localization of HlpA-mCherry. (**D**) Immunodetection of fusion proteins in whole-cell extracts of strains KB1-65 (*hlpA, hlpA-TagRFP*), MK119 (*hlpA, hlpA-mKate2*), MK218 (*hlpA, hlpA-mCherry*) and KB1-64 (*hlpA-mCherry*) using anti-RFP antibodies.

Characterization of RFPs in a fusion to HlpA

Far-red fluorescent proteins are particularly suitable for double-labeling experiments in combination with GFP or YFP due to non-overlapping excitation and emission spectra and thus limited chance of crosstalk. mCherry has been successfully used to tag *S. pneumoniae* FtsZ[54] and the capsule protein Wze (aka CpsD)[22] and mKate2 has also been successfully used to tag FtsZ[166] and additionally to tag the eukaryotic-type serine/threonine kinase StkP[43]. However, a systematic comparison has not been made. In order to compare the performance of different RFPs in protein fusions, the far-red and fast folding mCherry and mKate2, as well as the red-orange TagRFP, which has been suggested as a suitable FP for protein fusions, but not yet been used for this purpose in *S. pneumoniae*, were all fused to the C-terminus of HlpA (Hup, SPD_0997). HlpA is the only known histone-like protein encoded in the *S. pneumoniae* D39 genome[167] and binds aspecifically to DNA and thus localizes to the nucleoid in a similar fashion as the intercalating DNA dye DAPI[168]. For all three FP-encoding genes, we attempted to construct C-terminal fusions to HlpA by integrating the fusion gene at the native locus. Interestingly, we obtained the hlpA-mCherry fusion strain but we were unable to make the strains expressing the HlpA-mKate2 and HlpA-TagRFP fusions as sole copies of HlpA. In our attempts to make these strains, we always observed mutations in the linker sequence

generating premature stop codons or deletion of (parts of) the *rfp* genes. Therefore, we instead constructed merodiploid strains where the hlpA fusions are present as an extra copy immediately downstream of the native *hlpA* gene and thus also under the control of the endogenous *hlpA* promoter. These strains could be readily made resulting in a total of four benchmarked strains: KB1-64 (*hlpA-mCherry*), MK218 (*hlpA*, *hlpA-mCherry*), KB1-65 (*hlpA*, *hlpA-TagRFP*) and MK119 (*hlpA*, *hlpA-mKate2*). Fluorescence microscopy on exponentially growing cells showed that the total amount of fluorescence of the HlpA-TagRFP strain was comparable to the HlpA-mKate2 strain, whereas HlpA-mCherry signals were lower (data not shown). Importantly, a clear difference in the localization pattern was observed: signals for the merodiploid HlpA-mKate2 and HlpA-TagRFP strain appeared as sharp defined nucleoid-localized spots in the cytoplasm (Fig. 3A and B), whereas both HlpA-mCherry strains frequently demonstrated a more diffuse and heterogeneous signal (Figure 3C). Western blot analysis showed the presence of degradation products in both HlpA-mCherry strains whereas no such degradation was observed for the HlpA-mKate2 and HlpA-TagRFP strains (Fig. 3C). Degradation of mCherry might lead to a pool of untagged HlpA, which could be the reason why the single copy HlpA-mCherry could be made, while this was not possible with mKate2 and TagRFP. All in all, these results show that mCherry, originating from our codon-optimized gene, is more readily degraded than mKate2 and TagRFP in *S. pneumoniae* (Figs. 2D and 3C) making the latter RFPs more suitable for protein fusions. Note that protein functionality always needs to be tested since FP fusions may not always be fully functional, as probably is the case for the HlpA-mKate2 and HlpA-TagRFP fusions.

Concluding remarks

Here we benchmarked four commonly used RFPs (mOrange2, TagRFP, mCherry and mKate2) in the important human pathogen *S. pneumoniae.* The genes encoding these RFPs were codon-optimized and Western blotting showed that all proteins are efficiently produced in *S. pneumoniae.* Under our experimental conditions no fluorescent signals were detected with mOrange2, likely because of its long maturation time. The three other RFPs were all reported to be fast-folding. However, mCherry clearly was the fastest folding variant in our assays and generated the highest fluorescence signals and is thus the best RFP to use in promoter fusions. On the other hand, our version of mCherry is also partially degraded making it less suitable in protein fusions. Therefore, we recommend the use of mKate2 or TagRFP as a red fluorescent protein localization marker in *S. pneumoniae*.

Materials and Methods

Bacterial strains, plasmids, media and growth conditions. *Streptococcus pneumoniae* was grown in liquid C+Y medium at 37°C[156]. Blood agar plates were prepared from Columbia agar with addition of 3% defibrinated sheep blood (Johnny Rottier, Kloosterzade, The Netherlands). The P_{Zn} promoter was induced with addition of $ZnCl_2$ to a final concentration of 0.1 mM or 0.15 mM. *Escherichia coli* DH5α was used for cloning and grown in LB medium at 37°C with shaking or on LB medium solidified with 1.5% (wt/vol) agar. When required, 100 μg ml^{-1} ampicillin (Amp) for *E. coli* or either 1 μg ml^{-1} tetracycline (Tet) or 4.5 μg ml^{-1} chloramphenicol (Cm) for *S. pneumoniae* were used for selection. Strains are listed in Table 2.

Recombinant DNA techniques and oligonucleotides. Procedures for DNA isolation, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were performed as described by Sambrook and Russel[155]. Plasmid DNA and PCR products were isolated and purified using the High Pure Plasmid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Enzymes were purchased from Fermentas (Vilnius, Lithuania) and used as described by the manufacturer. For PCR amplification, Phusion- and Taq DNA polymerase (Fermentas) were used. *S. pneumoniae* was transformed as described by Martin et al.[156]. Oligonucleotides used in this study are listed in Table 3 and were purchased from Biolegio (Nijmegen, the Netherlands). Constructs were sequence-verified.

Codon optimization. *mKate2*, *TagRFP* and *mOrange2* were codon-optimized for *S. pneumoniae* using OptimumGene™ (Genscript Inc., Piscataway, NJ, USA), resulting in genes *mKate2(Sp), TagRFP(Sp)* and *mOrange2(Sp)*, while the sequence for a codon-harmonized variant of *mCherry* (based on the *B. subtilis* codon usage table) was obtained from DSM Biotechnology Center (Delft, The Netherlands), resulting in gene *mCherry(Bs)*. Genes were synthesized by Genscript (*mKate2(Sp), TagRFP(Sp) and mOrange2(Sp)*) or by GeneArt (Regensburg, Germany) (*mCherry(Bs)*). Note that throughout the text we refer to these codon-optimized genes as *mKate2*, *TagRFP, mOrange2* and *mCherry*. The sequences of *mKate2(Sp)*, *TagRFP(Sp), mCherry(Bs)* and *mOrange2(Sp)* have been submitted to Genbank (accession number pending).

Construction of plasmids. *mKate*2 and *TagRFP* were amplified from the synthetic genes using primer pairs mKate-F+XbaI/mKate-R-SpeI and TagRFP-F+XbaI/TagRFP-R-SpeI, respectively, and cut using XbaI and SpeI. *mCherry* was cut from the GeneArt plasmid pMA-*mCherry* using XbaI and SpeI and purified. Digested *mKate2*, *TagRFP* and *mCherry* were ligated in XbaI and SpeI cut plasmid pJWV102[159], resulting in plasmids pKB01-*mKate2*, pKB01-*TagRFP* and pKB01-*mCherry* respectively. *mOrange2* was amplified using mOrange-F+NotI/mOrange-R+SpeI, cut using NotI and SpeI and ligated in similarly digested pJWV102. Fragments were ligated accordingly resulting in plasmid pKB01-*mOrange2*.

Construction of strains.

S. pneumoniae strains KB1-69, KB1-70, KB1-71 and KB1-72, expressing different *rfp* variants under the Zn^{2+}-inducible promoter (P_{Zn}), were obtained by transformation of strain D39 with pKB01 derivatives as described previously[156]. Correct integration by double crossover in the *bgaA* locus was tested by colony PCR using primer pairs integration 1/integration 2 and integration 5/integration 6.

The construction of strain MK119 (*hlpA, hlpA-mKate2-cm*^{R}), containing a *hlpA-mKate2* fusion (in which *hlpA* and *mKate2* are separated by a flexible linker) and a chloramphenicol resistance gene on the same transcriptional unit as *hlpA*, is described elsewhere[168].

To construct strain KB1-64 (D39; *hlpA-mCherry-cm*^{R}), the *hlpA*-up fragment (containing the *hlpA* gene and its promoter) was amplified from D39 chromosomal DNA using hlpA-up-F/hlpA-up-R+BamHI and the *hlpA*-down fragment (containing ~1000 bps immediately downstream of the *hlpA* stop codon including a chloramphenicol resistance gene) was amplified from strain MK119 (D39; *hlpA, hlpA-mKate2*; *cm*^{R}) using primer pair camR-up-F+EcoRI/*hlpA*-down-R. *mCherry* including a flexible linker was amplified from pMA-*mCherry* using linker-mCherry-F+BamHI/mCherry-R+EcoRI. Fragments were purified and cut using BamHI (fragment *hlpA*-up), BamHI and EcoRI (linker-*mCherry*) or EcoRI (*hlpA*-down), respectively. Cut and purified fragments were ligated. *S. pneumoniae* D39 was transformed with

the ligation mix and transformants were selected on plates containing chloramphenicol. A single transformant was sequence verified and this strain was named KB1-64.

To construct strain MK218 (D39; *hlpA, hlpA-mCherry-cm^R)*, an *hlpA*-up fragment (containing *hlpA* and the upstream sequence) was amplified from genomic DNA of D39 using primers *hlpA*-up-F and *hlpA*-R-SphI. The *hlpA-mCherry-cm^R* fragment (containing the *hlpA-mCherry* fusion with a downstream *cm^R* resistance gene and the *hlpA* downstream sequence) was amplified from strain KB1-64 using primers *hlpA*-F-rbs-SphI and *hlpA*-down-R. The two fragments were digested with SphI and ligated. The ligation product was transformed into D39 and transformants were selected on plates containing chloramphenicol. Correct transformants were verified by PCR and sequencing.

To construct strain KB1-65 (D39; *hlpA, hlpA-TagRFP-cm^R*), the *hlpA*-linker-up fragment, containing an extra copy of *hlpA* including a flexible linker downstream of *hlpA*, was amplified from MK119 using primer pair *hlpA*-up-F/*hlpA*-linker-R+BglII and *hlpA*-down fragment was amplified as described for KB1-64. *TagRFP* was amplified from pUC57-*TagRFP* using primers mKate2-TagRFP-F+BamHI/TagRFP-R+EcoRI. Fragments were purified and cut with BglII (*hlpA*-linker-up), EcoRI (*hlpA*-down) and with BamHI and EcoRI (*TagRFP*), respectively. The fragments were ligated and directly transformed to *S. pneumoniae* D39. Positive transformants were selected on plates containing chloramphenicol. A single transformant was sequence-verified and the strain was named KB1-65.

Fluorescence measurements using plate reader. *S. pneumoniae rfp*-expressing strains and D39 wild-type strain were grown at 37°C in liquid C+Y medium to an OD_{600} of 0.1, diluted 1:100 with fresh C+Y containing 0.15 mM $ZnCl_2$ for induction of P_{Zn}. Cells were then further grown and harvested at OD_{600} of 0.15 (mid-exponential growth). For plate reader assays, cells were first collected by centrifugation and washed with PBS. Fluorescence was measured with the following equipment and settings: Infinite 200 Pro plate reader (Tecan Group Ltd.) with I-control™ 1.7.1.12 software (Tecan Group Ltd.), RFP filter set (excitation at 590/20 nm, emission at 635/10 nm with a 50% dichroic mirror); RFP signals were collected as top readings with a gain setting of 100. RFP fluorescence values were corrected for background fluorescence, OD_{600} and negative controls (values of the wild-type strains). The OD_{600} levels used were corrected for the background value of the corresponding buffer.

Fluorescence microscopy. Cells of *S. pneumoniae* were grown at 37°C in liquid C+Y medium to an OD_{600} of 0.1, diluted 1:100 with fresh C+Y and further grown until an OD_{600} of 0.05 before 0.15 mM $ZnCl_2$ was added to the cultures for induction of P_{Zn}. Cells were then further grown and collected at different time points. For microscopy, 0.4 µl of the cell suspension was directly spotted onto a microscope slide carrying a thin layer of 1.2% agarose in PBS and then covered by a coverslip. Images were taken with an Olympus IX71 Microscope (Personal DV, Applied Precision; assembled by Imsol (Preston, UK) and Chromaphor (Münster, Germany) using a CoolSNAP HQ2 camera (Princeton Instruments, Trenton, USA) with a 100× phase-contrast objective and trulight^{Tm} (Deltavision, GE Healthcare USA) illumination. The following standard fluorescence filter sets from Chroma Technology Corporation (Bellows Falls, USA) were used (mCherry: excitation, 575/25 nm; emission, 632/60 nm, polychroic mirror QUAD2 (580 – 630 nm); TRITC: excitation, 542/27 nm; emission, 594/45 nm, polychroic QUAD1 (550 – 630 nm)) to visualize RFPs. Note that the second number behind the slash symbol (/) indicates the bandwidth around the wavelength, i.e. 575/25 indicates excitation with light from 562.5 till 587.5 nm. For the Zn^{2+}-inducible constructs, the exposure time was 1.4 s with 100% excitation for all RFPs. To visualize the RFP fusions to HlpA, an exposure time of 0.9 s was used. Softworx 3.6.0 (Applied Precision, Washington, USA) software was used for image capturing. Phase contrast images were segmented automatically and fluorescence signal intensities were extracted using MicrobeTracker[148]. Fluorescence levels were corrected for background fluorescence of the medium.

Western blot analysis and immunodetection. Cells were grown in C+Y medium with or without addition of $ZnCl_2$ to a final concentration of 0.15 mM. Cells were harvested by centrifugation at 8000 rpm for 10 minutes. For lysis, the pellet was resuspended in 100 µl of SEDS lysis buffer and was incubated for 5 min at 37°C. Lysates were diluted in 100 µl 2x SDS-loading buffer and boiled for 5 minutes. After separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by Western blotting. Red fluorescent proteins were detected with polyclonal anti-RFP antibody (Molecular probes®) and anti-IgG-rabbit-HRP

antibody (GE Healthcare) according to the manufacturer's instructions. Note that the polyclonal anti-RFP antibodies recognize all four RFPs due to the high structural homology.

Acknowledgements

We thank Amaya Aramendía de Goñi for initial characterization of FPs in *S. pneumoniae*. We thank Hans Roubos (DSM) for kindly providing the *mCherry(Bs)* sequence. Work in the lab of J.-W.V. is supported by the EMBO Young Investigator Programme, a VIDI fellowship (864.12.001) from the Netherlands Organisation for Scientific Research, Earth and Life Sciences (NWO-ALW) and ERC starting grant 337399-PneumoCell.

Table 2. Bacterial strains and plasmids

Strains and plasmids	Relevant properties	Source or reference
E. coli DH5α	F-, *araD*139, Δ(*ara-leu*)7696, Δ(*lac*)X74, *galU*, *galK*, *hsdR2*, *mcrA*, *mcrB1*, *rspL*	Laboratory stock
S. pneumoniae		
D39	encapsulated strain	[1]
KB1-69	D39, tet, *bga*::P_{Zn}-*mOrange2*	This study
KB1-70	D39, tet, *bga*::P_{Zn}-*TagRFP*	This study
KB1-71	D39, tet, *bga*::P_{Zn}-*mCherry*	This study
KB1-72	D39, tet, *bga*::P_{Zn}-*mKate2*	This study
MK119	D39, cm, *hlpA hlpA-mKate2*	[168]
KB1-64	D39, cm, *hlpA-mCherry*	This study
MK218	D39, cm, *hlpA, hlpA-mCherry*	This study
KB1-65	D39, cm, *hlpA, hlpA-TagRFP*	This study
Plasmids		
pKB01_sfgfp(Sp)	*bla, tet, bgaA,* P_{Zn}-sf*gfp(Sp)*	[159]
pKB01_mOrange2	*bla, tet, bgaA,* P_{Zn}-*mOrange2(Sp)*	This study
pKB01_TagRFP	*bla, tet, bgaA,* P_{Zn}-*TagRFP(Sp)*	This study
pKB01_mCherry	*bla, tet, bgaA,* P_{Zn}-*mCherry(Bs)*	This study
pKB01_mKate2	*bla, tet, bgaA,* P_{Zn}-*mKate2(Sp)*	This study

Table 3. Oligonucleotides used in this study[a]

Oligo	Sequence (5' - 3')
mOrange-F+NotI	GCAGGCGGCCGCGGAGGAAAATTAATGTCTAAGGGAG
mOrange-R+SpeI	GCAGACTAGTCTCATGAATTCTTTCTCGAGTTAGG
mKate2-F+XbaI	GCAGTCTAGAGGAGGAAAATTAATGTCAGAACTTATCAAGG
mKate2-R+Spe1	GCAGACTAGTGCGAGATCTCATGAATTCTTTCTCG
TagRFP-F+XbaI	GCAGTCTAGAGGAGGAAAATTAATGTCAGAAC
TagRFP-R+SpeI	GCAGACTAGTCATGAATTCTTTCTCGAGTTAGGATCC
hlpA-up-F	AACAAGTCAGCCACCTGTAG
hlpA-up-R+BamHI	CTGCGGATCCTTTAACAGCGTCTTTAAGAGCTTTACCAGC

camR-up-F+EcoRI	ACTCGGAATTCTAATGAGCACTAGTAGGAGGCATATC
hlpA-down-R	CGTGGCTGACGATAATGAGG
Linker-mCherry-	CGATGGATCCGGATCTGGTGGAGAAGCTGCAGCTAAAGGAAGCAAAGGAGAAGAAGATAACATG
F+BamHI	GCAATCATC
mCherry-R+EcoRI	GCGCGAATTCTTATTATTTGTAAAGCTCATCCATTCCGCC
hlpA-F-rbs-SphI	CGTGCATGCTGGAGGAATCATTAACATGGCA
hlpA-R-SphI	CGCGCATGCAGACTGATTATTTAACAGCGTC
hlpA-linker-up-R+BglII	GCGCAGATCTTCCTTTAGCTGCAGCTTCTCC
mKate2-TagRFP-	
F+BamHI	CGCTGGATCCTCAGAACTTATCAAGG
TagRFP-R+EcoRI	GCATGAATTCTTATTAACGGTGTCCCAATTTACTAG
Integration 1	CTTGATGAAACCTACATTTG
Integration 2	GCTTCCATTAAGGATAGTTC
Integration 5	GCTATCGCTGAGCGCCGG
Integration 6	AGCTAGAGTTCCGCAATTGG

[a]Underlined sequences show the restriction sites.