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METHODS AND PROTOCOLS

A method to generate recombinant *Salmonella typhi* Ty21a strains expressing multiple heterologous genes using an improved recombineering strategy

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Abstract Live attenuated *Salmonella enterica* serovar Typhi Ty21a (Ty21a) is an important vaccine strain used in clinical studies for typhoid fever and as a vaccine vector for the expression of heterologous antigens. To facilitate the use of Ty21a in such studies, it is desirable to develop improved strategies that enable the stable chromosomal integration and expression of multiple heterologous antigens. The phage λ Red homologous recombination system has previously been used in various gram-negative bacteria species to mediate the accurate replacement of regions of chromosomal DNA with PCR-generated 'targeting cassettes' that contain flanking regions of shared homologous DNA sequence. However, the efficiency of λ Red-mediated recombineering in Ty21a is far lower than in *Escherichia coli* and other *Salmonella typhimurium* strains. Here, we describe an improved strategy for recombineering-based methods in Ty21a. Our reliable and efficient method involves the use of linear DNA-targeting cassettes that contain relatively long flanking 'arms' of sequence (ca. 1,000 bp) homologous to the chromosomal target. This enables multiple gene-targeting procedures to be performed on a single Ty21a chromosome in a straightforward, sequential manner. Using this strategy, we inserted three different influenza antigen expression cassettes as well as a green fluorescent protein gene reporter into four different loci on the Ty21a chromosome, with high efficiency and accuracy. Fluorescent microscopy and Western blotting analysis confirmed that strong inducible expression of all four heterologous genes could be achieved. In summary, we have developed an efficient, robust, and versatile method that may be used to construct recombinant Ty21a antigen-expressing strains.

Keywords Recombineering *Salmonella* vaccine

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Introduction

Salmonellae are a group of rod-shaped gram-negative facultative anaerobic bacteria of the Enterobacteriaceae family. *Salmonella enterica* serovar Typhi is a notable human pathogen and is the causative agent of typhoid fever (Crump et al. 2004; Parry et al. 2002). In 1975, Germanier and Furer used chemical mutagenesis to generate multiple mutations in the *Salmonella typhi* Ty2 strain, resulting in the creation of a live attenuated vaccine strain, *S. typhi* Ty21a (ATCC 33459) (Germanier and Fuer 1975). The identified mutations resulted in galactose sensitivity (*galE* mutation), isoleucine, and valine auxotrophy (*ilvD* mutation), Vi

polysaccharide capsule deficiency (*viaB* mutation), and a reduction in stress resistance (*rpoS* mutation) (Kopecko et al. 2009). Ty21a is not only a unique live oral vaccine against typhoid fever, but is also an effective carrier for the delivery of heterologous antigens. For example, Bumann and Metzger successfully expressed the *Helicobacter pylori* antigens urease A and B subunits (encoded on plasmid pDB1) within Ty21a (Bumann et al. 2001) (Metzger et al. 2004). Christian et al. (Hotz et al. 2009) notably used Ty21a to deliver antigens using plasmid-based *Escherichia coli* hemolysin (HlyA) type I secretion system.

Although high copy plasmid-based antigen expression systems may be easily constructed and often achieve high antigen expression levels resulting in strong humoral and cellular immune responses, they have several critical defects, as outlined below. Plasmids are generally not well-retained within the cell without selective pressures favoring their retention, e.g., plasmid-encoded antibiotic resistance genes combined with the corresponding antibiotic in their environment. When live bacteria-carrying plasmids are orally administered, the bacteria will face stresses from the host immune system as well as poor growth conditions. The high-level expression of heterologous antigens, whether within the bacterial cytoplasm or secreted from the cell, places a high metabolic burden on the carrier, further enhancing plasmid loss (Galen and Levine 2001). Furthermore, the delivery of antibiotic resistance genes on mobile genetic elements (such as plasmids) into animal bodies is not desirable, as there may be horizontal transfer of these genetic genes, e.g., into clinically important strains. Also, because of incompatibilities between various different plasmid types, it is difficult to introduce and maintain multiple antigens within a single bacterial cell.

Conversely, chromosome-based antigen-expressing system does not have these problems (Husseiny and Hensel 2006). Procedures involving homologous recombination are often used to introduce genes encoding heterologous antigens into the chromosome of the bacterial carrier. In such procedures, segments within two DNA molecules that share high levels of sequence homology are exchanged, resulting in genetic 'crossover'. In the vast majority of bacteria species, such as *E. coli*, genetic recombination is primarily catalyzed by DNA recombinase enzymes (e.g., RecA), and usually only occurs when there are common regions of DNA sequence homology longer than ca. 100 bp; in general, the longer the regions of sequence homology, the higher the recombination efficiency (Shen and Huang 1986; Watt et al. 1985).

Over recent years, a powerful chromosome-engineering technique has been developed and utilized within gram-negative bacteria, which has been termed recombineering technology (recombination-mediated genetic engineering). This employs the homologous recombination-promoting proteins from phage λ encoded by the *exo*, *bet*, and *gam* genes), which are collectively referred to as λ-Red. In such procedures, polymerase chain reaction (PCR)-generated double-stranded DNA molecules known as targeting cassettes, which encode the desired genetic alteration as well as an antibiotic resistance gene for selection, are introduced into cells expressing the Red proteins. In some strains of *E. coli*, only ca. 40–50 bases of flanking DNA sequence homology are required for efficient genetic recombination between the targeting cassette and the chromosomal or episomal target locus. These short regions of sequence homology may be included in the oligonucleotides used to synthesize the targeting cassettes (Datsenko and Wanner 2000; Yu et al. 2000).

λ Red-mediated recombineering approaches have previously been used to create both chromosome-and episome-based gene replacements in *E. coli*, *Salmonella typhimurium*, and *Shigella* spp. (Datta et al. 2006; Ranollo et al. 2006; Karlinsey 2007). In this study, we explored the use of λ Red-mediated recombineering technology within *S. typhi* strain Ty21a. We found that the efficiency of λ Red recombineering in Ty21a was ca. 100-fold lower than reported previously in *E. coli* (10^2 per 10^8 viable cells versus 10^4 per 10^8 viable cells, respectively) (Sawitzke et al. 2007; Datta et al. 2006). We therefore set about developing an optimized recombineering procedure for the efficient modification of the Ty21a chromosome, which could be used in an iterative manner for the integration of multiple antigen genes.

Linear DNA molecules containing relatively long flanking regions of sequence homologous to the target loci (i.e., targeting cassettes with 'arms' of ca. 1,000 bp in length) were used to increase the efficiency of λ Red-mediated recombination with the chromosome. These were PCR-amplified from a set of vectors incorporating a loxP-flanked (floxed) chloramphenicol (Cm) resistance gene, to be used as a selectable marker. To validate the utility of our 'long homology arm' strategy, we used it to sequentially insert three different heterologous antigen genes and a *gfp* reporter gene at four distinct loci on the Ty21a chromosome (*htrA*, *hsdR*, *msbB*, and *asd*, respectively). After each gene-targeting step, the floxed Cm genes were successfully removed using Cre/loxp site specific recombination without interference to adjacent genes. Our results clearly demonstrate that up to four separate expression cassettes may be precisely integrated into the *S. typhi* Ty21a chromosome using our 'long homology-arm' strategy. This enables the simultaneous expression of multiple antigen and/or reporter genes from a single Ty21a cell.

Materials and methods

Bacterial strains, media, chemicals, enzymes, and plasmids

E. coli strain DH10B (Invitrogen) was used for all plasmid construction experiments. *S. typhi* strain Ty21a was

purchased from the ATCC. Bacterial strains were routinely cultured at 37°C with 230 rpm shaking in Luria–Bertani (LB) broth. Minimal medium based on the N-salts recipe (Hensel 2000) was used for protein induction. The Hela cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplied with 10% FBS (GIBCO) with 37°C and 5% CO_2. When required, antibiotics and chemicals (Sigma) were used at the following concentrations: ampicillin (100 μg/ml), kanamycin (Km, 50 μg/ml), and Cm (25 μg/ml) for both chromosomal selection and plasmid maintenance and diaminopimelic acid (DAP) (50 μg/ml) for the *asd* mutant strain. Restriction endonuclease and ligase enzymes were purchased from New England BioLabs (NEB), and *Taq* polymerase was from Takara. Plasmid *p*Sim6 was a gift from Dr. D.L. Court (Datta et al. 2006). DNA encoding the HA, NA, and NP antigens was supplied by the Department of Microbiology at the University of Hong Kong and were cloned from influenza A viruses H5N1/Vietnam/1194/2004; the remainder of the plasmids were from laboratory stocks (Table 1).

Plasmid construction

*p*Bluescript II SK (pBSK) was used as the backbone for all constructs. The *prosseA* promoter sequence was cloned from the promoter region of *sseA* gene in the pathogenicity island 2 (SPI2) of Ty21a by PCR reaction with the NotI-prosseA-F and HindIII-prosseA-R primers (Table 2),

Table 1 Strains and plasmids used in this work

Strain	Relevant genotype or characteristics	Ref. or source
S. typhi		
Ty21a	*galE ilvD viaB RpoS*	ATCC 33459
YBS001	Ty21a; *hsdR<>prosseA-HA*	This study
YBS002	Ty21a; *hsdR<>prosseA-HA*; *htrA<>prosseA-NP*	This study
YBS003	Ty21a; *hsdR<>prosseA-HA*; *htrA<>prosseA-NP*; *msbB<>prosseA-NA*	This study
YBS004	Ty21a; *hsdR<>prosseA-HA*; *htrA<>prosseA-NP*; *msbB<>prosseA-NA*; *asd<>prosseA-gfp*	This study
YBS005	Ty21a; Cm^R; *asd<>prosseA-gfp-cm*	This study
YBS006	Ty21a; Cm^R; *hsdR<>prosseA-HA*; *asd<>prosseA-gfp-cm*	This study
YBS007	Ty21a; Cm^R; *hsdR<>prosseA-HA*; *htrA<>prosseA-NP*; *asd<>prosseA-gfp-cm*	This study
YBS008	Ty21a; Cm^R; *hsdR<>prosseA-gfp-cm*	This study
YBS009	Ty21a; Cm^R; *htrA<>prosseA-gfp-cm*	This study
E. coli		
DH10B	endA1 recA1 galE15 galK16 nupG rpsL ΔlacX4 Φ Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-	Lab stock
Plasmid		
*p*Bluescript II SK	Ap^R; cloning vector	Stratagene
*p*Sim6	Ap^R, lambda-red recombinase plasmid	(Datta et al. 2006)
*p*loxp-cm-loxp	Ap^R, Cm^R; pBSK derivative containing EGFP and loxp-cm-loxp fragment	(Jin et al. 2009)
*p*705Cre-Km	Cre-recombinase expressing plasmid	(Jin et al. 2009)
*p*YBS-prosseA	Ap^R; pBSK derivative containing prosseA promoter	This study
*p*YBS-prosseA-cm	Ap^R, Cm^R; pBSK derivative containing prosseA promoter, and loxp-cm-loxp fragment	This study
*p*YBS-pro-HA-cm	Ap^R, Cm^R; pBSK derivative containing prosseA promoter, HA gene, and loxp-cm-loxp fragment	This study
*p*YBS-pro-NP-cm	Ap^R, Cm^R; pBSK derivative containing prosseA promoter, NP gene, and loxp-cm-loxp fragment	This study
*p*YBS-pro-NA-cm	Ap^R, Cm^R; pBSK derivative containing prosseA promoter, NA gene, and loxp-cm-loxp fragment	This study
*p*YBS-pro-GFP-cm	Ap^R, Cm^R; pBSK derivative containing prosseA promoter, GFP gene, and loxp-cm-loxp fragment	This study
*p*YBS-hsdR	Ap^R; pBSK derivative with long homology arms of *hsdR* sites;	This study
*p*YBS-htrA	Ap^R; pBSK derivative with long homology arms of *htrA* sites;	This study
*p*YBS-msbB	Ap^R; pBSK derivative with long homology arms of *msbB* sites;	This study
*p*YBS-asd	Ap^R; pBSK derivative with long homology arms of *asd* sites;	This study
*p*YBS-hsdR-HA	Ap^R, Cm^R; pBSK derivative with long homology arms of*hsdR* sites; *prosseA-HA-cm*	This study
*p*YBS-htrA-NP	Ap^R, Cm^R; pBSK derivative with long homology arms of *htrA* sites; *prosseA-NP-cm*	This study
*p*YBS-msbB-NA	Ap^R, Cm^R; pBSK derivative with long homology arms of *msbB* sites; *prosseA-NA-cm*	This study
*p*YBS-asd-GFP	Ap^R, Cm^R; pBSK derivative with long homology arms of *asd* sites; *prosseA-gfp-cm*	This study

Table 2 Primers used in this work

Primer name	Primer sequence (5′ to 3′)
NotI-prosseA-F	ATTTGCGGCCGCAGAAGAGAACAACGGCAAGTTAC
HindIII-prosseA-R	CCCAAGCTTACGATAGATAATTAACGTGC
XhoI-floxed-F	CCGCTCGAGCCGATCATATTCAATAACCCT
XhoI-floxed-R	CCGCTCGAGGACTAGTGAACCTCTTCGAGGG
SacI-hsdR-up-F	TCCGAGCTCGTCGACCTCAAAGGCGTCAACGACTTCA
SacII-hsdR-up-R	TCCCCGCGGTCTTGCTGGCCGTTAACGATAGCC
XhoI-hsdR-down-F	CCGCTCGAGCTACGATACGCCGCAGGACTTT
kpnI-hsdR-down-R	CGGGGTACCGTCGACGATTTGGTTTCGTTAGCGTTCTTCTG
SacI-htrA-up-F	TCCGAGCTCGTCGACGCCTACGTGGAAGTCGTCAGTA
SacII-htrA-up-R	TCCCCGCGGCGTCGGTCTGAATAAAGTTCTCGTAA
XhoI-htrA-down-F	CCGCTCGAGGGATGTCATTACCTCGCTGAACGG
kpnI-htrA-down-R	CGGGGTACCGTCGACTCCCTAAACGCTGTGCCCATTC
SacI-msbB-up-F	TCCGAGCTCGTCGACGTTCCGCATCTCCTCCAACTTCAA
SacII-msbB-up-R	TCCCCGCGGCGATAATCTCCAGACCTTCCCAGT
XhoI-msbB-down-F	CCGCTCGAGCTATATCTGGAATACCGTGCGTCG
kpnI-msbB-down-R	CGGGGTACCGTCGACGCATGAAGCTGTTAATCTGCTGCG
SacI-asd-up-F	TCCGAGCTCGTCGACATGATGGAAACTATCCTCGGCACG
SacII-asd-up-R	TCCCCGCGGCGACATCAACATCAGGCTAACGGT
XhoI-asd-down-F	CCGCTCGAGCGGAAACCAACAAGATCCTCAATA
kpnI-asd-down-R	CGGGGTACCGTCGACGACACTTCTTTGACCTGAACGGCG
HindIII-HA-F	CCCAAGCTTAAGAAGGAGATATACATATGGACCAAATCTGCATTGGT
XhoI-Ter-HA-R	CCGCTCGAGCGGCCGCAAAAAACCCCTCAAGACCCGTTTAGAGG
	CCCCAAGGGGTTATGCTAGCTAAATGCAAATTCTGCATTGTAAC
HindIII-NP-F	CCCAAGCTTAAGAAGGAGATATACATATGGCGTCTCAAGGCACCAAAC
XhoI-Ter-NP-R	CCGCTCGAGCGGCCGCAAAAAACCCCTCAAGACCCGTTTAGAGG
	CCCCAAGGGGTTATGCTAGTCAATTGTCATATTCCTCTGCATTG
HindIII-NA-F	CCCAAGCTTAAGAAGGAGATATACATATGAATCCAAATCAGAAGATAATAAC
XhoI-Ter-NA-R	CCGCTCGAGCGGCCGCAAAAAACCCCTCAAGACCCGTTTAGAGG
	CCCCAAGGGGTTATGCTAGCTACTTGTCAATGGTGAATGGCAAC
HindIII-GFP-F	CCCAAGCTTAAGAAGGAGATATACATATGGTGAGCAAGGGCGAGGAGC
XhoI-Ter-GFP-R	CCGCTCGAGCGGCCGCAAAAAACCCCTCAAGACCCGTTTAGAGG
	CCCCAAGGGGTTATGCTAGTTACTTGTACAGCTCGTCCATGCC
Long-arm-hsdR-F	GTCGACCTCAAAGGCGTCAACGACTTCA
Long-arm-hsdR-R	GTCGACGATTTGGTTTCGTTAGCGTTCTTCTG
Long-arm-htrA-F	GTCGACGCCTACGTGGAAGTCGTCAGTA
Long-arm-htrA-R	GTCGACTCCCTAAACGCTGTGCCCATTC
Long-arm-msbB-F	GTCGACGTTCCGCATCTCCTCCAACTTCAA
Long-arm-msbB-R	GTCGACGCATGAAGCTGTTAATCTGCTGCG
Long-arm-asd-F	GTCGACATGATGGAAACTATCCTCGGCACG
Long-arm-asd-R	GTCGACGACACTTCTTTGACCTGAACGGCG
Asd-arm-800-F	TACCGTAAGCCGGGTAAGGCG
Asd-arm-800-R	TGCATCCTATCTGCGTCGTC
Asd-arm600-F	CACTGGACTTTCTGCTTGCG
Asd-arm600-R	CCGTGGGATTAAGCTACCCT
Asd-arm400-F	AATGGTCGGCTCTGTTCTCA
Asd-arm400-R	ACCACACGCAGGCCCGATAA
Asd-arm200-F	ATACCAACGAAATTTATCCAAAGCTG
Asd-arm200-R	CATAGTGATATCACGATCGT
Asd-arm100-F	TCTCGACCCGGTCAACCAGG

Table 2 (continued)

Primer name	Primer sequence (5′ to 3′)
Asd-arm100-R	ATGGTGAACGCCTGGCTGTG
Short-arm-asd-F	TGAAGACCTTTGTGGGCGGT
Short-arm-asd-R	ACCATCAACCGGGATCACAG
primer-1 (hsdR)	TAATAACGTCTTCCCGCTCAG
primer-4 (hsdR)	CCAGATCGTTGGTCTGCGACTTCA
primer-3 (hsdR)	CTAAATGCAAATTCTGCATTGTAAC
primer-1 (htrA)	TAGAACAGCTTGAACTACAGGGAT
primer-4 (htrA)	TTGAGCACCACCATCTCGGTAAGG
primer-3 (htrA)	TCAATTGTCATATTCCTCTGCATTG
primer-1 (msbB)	TGGATGGCAAGCGTGAACAGAGTC
primer-4 (msbB)	CGCTAATGACTTCCCGTATCAGCT
primer-3 (msbB)	CTACTTGTCAATGGTGAATGGCAAC
primer-1 (asd)	CTCATGTTAACGACGCTGGCTTCT
primer-4 (asd)	CGATACGACTGGACATGGTTTGTT
primer-3 (asd)	TTACTTGTACAGCTCGTCCATGCC
primer-2	AGAAGAGAACAACGGCAAGTTAC

digested with NotI and HindIII, and ligated into pBSK, to generate plasmid pYBS-prosseA. An XhoI-loxp-Cm-loxp-XhoI fragment was amplified from plasmid ploxp-Cm-loxp using primers XhoI-floxed-F and XhoI-floxed-R (Jin et al. 2009), and ligated into pYBS-prosseA at the XhoI site, to create plasmid pYBS-prosseA-cm. The *HA*, *NP*, *NA*, and *gfp* genes were PCR-amplified using: HindIII-HA-F and XhoI-Ter-HA-R; HindIII-NP-F and XhoI-Ter-NP-R; HindIII-NA-F and XhoI-Ter-NA-R; and HindIII-GFP-F and XhoI-Ter-GFP-R pairs of primers, respectively. PCR products were digested with HindIII and XhoI then ligated into pYBS-prosseA-cm, to generate plamids pYBS-pro-HA-Cm, pYBS-pro-NP-Cm, pYBS-pro-NA-Cm, and pYBS-pro-GFP-Cm, respectively.

The upstream and downstream long homology arms for the *hsdR*-targeting cassettes were amplified from Ty21a genomic DNA using the SacI-hsdR-up-F and SacII-hsdR-up-R, and the XhoI-hsdR-down-F and KpnI-hsdR-down-R for hsdR pairs of primers, respectively. The 'upstream arm' PCR product was digested with SacI and SacII, and the 'downstream arm' PCR product was digested with XhoI and KpnI, enabling a two-step ligation into a suitably digested pBSK plasmid, to create plasmid pYBS-hsdR. The 'long homology arms' for the *htrA*-, *msbB*-, and *asd*-targeting cassettes were similarly generated by PCR and cloned into pBSK to generate plasmids pYBS-htrA, pYBS-msbB, and pYBS-asd, respectively. The *pro-HA-Cm*, *pro-NP-Cm*, *pro-NA-Cm*, and *pro-GFP-Cm* NotI/XhoI fragments from plasmids pYBS-pro-HA-Cm, pYBS-pro-NP-Cm, pYBS-pro-NA-Cm, and pYBS-pro-GFP-Cm, respectively, were ligated into plasmids pYBS-hsdR, pYBS-htrA, pYBS-msbB, and pYBS-asd, respectively, to create plasmids pYBS-hsdR-HA, pYBS-htrA-NP, pYBS-msbB-NA, and pYBS-asd-GFP, respectively. Maps for the 'long homology arm' vectors are shown in Fig. S1.

λ Red-recombineering procedures

Linear dsDNA was generated by PCR using the long homology arm vectors as template, with the length of homology arms varied by using the appropriate primer pairs. PCR products were gel purified (Qiaquick gel extraction kit, Qiagen), digested with DpnI (NEB) for 2 h, purified (Qiaquick PCR purification kit, Qiagen), and its concentration was measured by UV spectroscopy.

Plasmid *p*Sim6 was transformed into the appropriate *Salmonella* strain by electroporation. After plating onto LB agar-containing ampicillin, single colonies were inoculated into fresh LB medium (5 ml) supplemented with ampicilin, then incubated at 32°C for 12 h. The bacterial cultures were diluted 1:50 into fresh LB medium (100 ml) then incubated at 32°C until the optical density (OD_{600}) reached 0.3 AU (ca. 2 h). Culture flasks were incubated for 15 min at 42°C (with swirling, using a water bath) to induce λ Red expression, then chilled in ice water (without swirling) for 20 min. Cultures were centrifuged (4,000×*g*, 5 min), supernatant was discarded, and cells were gently resuspended in the same volume ice-cold autoclaved ddH_2O. The washing step was repeated twice, all residual water was removed, then the cell pellet was resuspended in fresh ice-cold ddH_2O (50 μl) in a 1.5-ml eppendorf tube (ca. 50 ml cell culture corresponding to one tube). The 'recombination-competent' cells were mixed with linear-targeting cassette

DNA (150 ng), transferred to 0.1-cm electroporation cuvettes, and electroporated (Bio-Rad electoporator, 1.8 kV). LB without antibiotics (1 ml) was added to the cuvette and the transformed cell mixture was transferred to a sterile culture tube. After 2 h recovery at 32°C, bacterial culture (200 μl) was plated evenly onto LB agar containing the Cm antibiotics, after further incubation at 32°C for 18–24 h, colonies were enumerated and 50–100 individual clones were selected for screening.

Confirmation of accurate chromosomal integration of DNA-targeting cassettes

Colony PCR was used to screen the (Cm resistant) *htrA*, *hsdR*, and *msbB* recombinant clones obtained, using two different primer sets that corresponded to chromosomal positions located inside the heterologous genes, and chromosomal positions inside, as well as outside the regions of sequence homology with the targeting cassettes (Fig. 2a). For the *asd*-targeting procedure, single colonies were also streaked onto LB plates with and without DAP, as strains lacking *asd* will not grow in the absence of DAP (Kong et al. 2008).

Removal of chloramphenicol selectable markers using the Cre recombinase

Plasmid *p*705Cre-Km was transformed (by electroporation) into correctly targeted recombinant strains; the transformed cell mixtures were plated onto LB agar + kanamycin and incubated at 32°C overnight. Single colonies were inoculated into fresh LB medium without antibiotics (5 ml), incubated for 16 h at 37°C, then diluted 1:1,000 with fresh LB medium, and 20 μl was plated onto an LB agar. After incubation for a further 16 h, single colonies were picked and streaked onto LB agar plates containing no antibiotics, as well as ones containing ampicillin, kanamycin, or chloramphenicol. Colonies that could only grow on LB plates (i.e., had lost all antibiotic markers) were deemed to be correctly targeted.

Analysis of in vitro and in vivo heterologous gene expression in YBS004

To analyze in vitro induction, a single colony of YBS004 was inoculated into LB medium (5 ml) containing DAP and incubated for 12 h at 37°C. Cells were collected by centrifugation, washed twice with N-salts media, inoculated into fresh N-salts media (5 ml) containing DAP, then incubated at 37°C for a further 12 h. The expression of green fluorescent protein (GFP) was observed using fluorescence microscopy (OLYMPUS BX51 with SPOT SOFTWARE 4.6, and GFP excitation and emission wavelengths are 488 and 518 nm). The expression of the three chromosomally integrated heterologous influenza antigens was analyzed by Western blotting using monoclonal antibodies specifically targeting the HA (ab8262, Abcam), NP (ab20711, Abcam), and NA (ab39804, Abcam) proteins.

For in vivo induction of antigen expression, Hela cells were cultured on coverslips in six-well plates to ca. 90% confluence. 10^7 Salmonella YBS004 cells (freshly grown in LB medium overnight, then washed three times with PBS buffer) were then directly added to 10^5 Hela cells in DMEM-containing DAP (50 μg/ml). After bacteria and Hela cells had been co-incubated for 1 h at 37°C, the medium was aspirated and replaced with fresh DMEM (without DAP), and cells were further incubated for 12 h. After washing three times with PBS buffer, the coverslip was placed into 4% PBS-buffered paraformaldehyde containing 10 ug/ml DAPI (Sigma) for 30 min, and then washed three times with PBS. Fluorescence microscopy was used to monitor GFP expression.

Results

Construction of inducible heterologous protein expression cassettes and long homology arm recombination vectors

Three major antigen proteins from the influenza A virus (H5N1/Vietnam/1194/2004): haemagglutinin (HA) (Wei et al. 2008), internal nucleoprotein (NP) (Guo et al. 2010), and neuraminidase (NA) (Rawangkhan et al. 2010) were selected for expression in *S. typhi* Ty21a. We used a strategy whereby the expression of these three heterologous antigen genes was placed under the control of the intracellularly activated *prosseA* promoter (Hensel 2000), which is present in the Ty21a SPI2. A green fluorescent protein *gfp* gene reporter was also selected for targeting, so that the expression levels from the *prosseA* promoter could be determined using fluorescence microscopy. The *hsdR*, *htrA*, *msbB*, and *asd* genes were selected as the sites for the chromosomal insertion of the HA, NP, NA, and *gfp* genes, respectively.

We first constructed a pBSK-derived plasmid (pYBS-prosseA-Cm), which contained a PCR-amplified *prosseA* promoter fragment (from the Ty21a SPI2) and a Cm resistance gene flanked by loxp sites (i.e., a 'floxed Cm gene; see Figs. 1a and S1). The HA, NP, NA, and *gfp* genes were then individually cloned into pYBS-prosseA-Cm, placing their expression under the control of the *prosseA* promoter. Then, ca. 1,000 bp sections of DNA corresponding to the sequence of the chromosomal regions immediately upstream and downstream of the respective gene being targeted (*hsdR*, *htrA*, *msbB*, or *asd*) were then

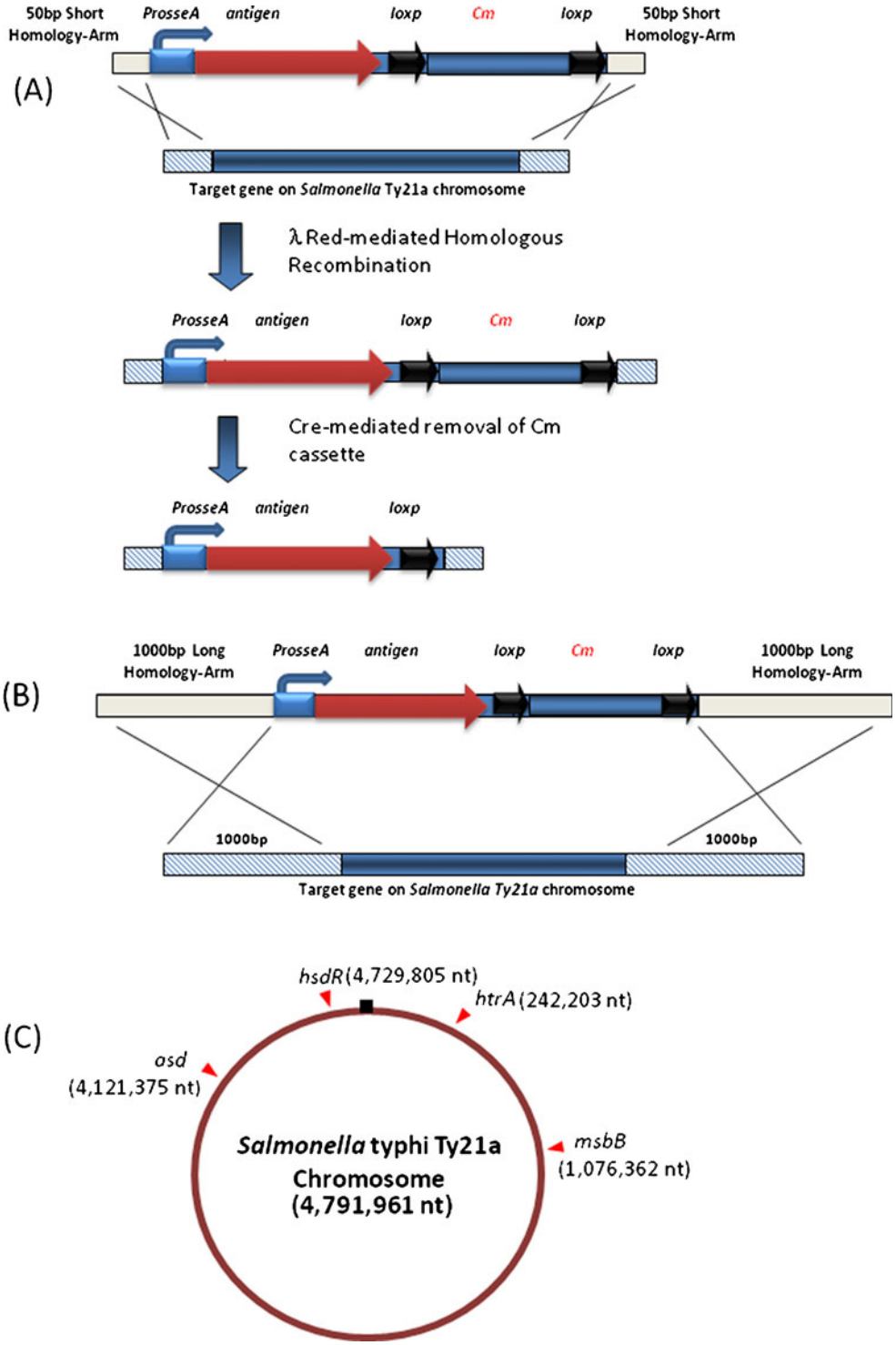

Fig. 1 Overview of recombineering strategy used. **a** In a 'traditional' recombineering approach, the PCR-generated targeting cassette contains short-flanking regions (ca. 50 bp in length) homologous to the target loci on the chromosome, as well as an antibiotic resistance gene (e.g., chloramphenicol acetyltransferase, *Cm*) as a selectable marker. If flanked by *loxP* sites, the selectable marker can be removed in a subsequent step by site-specific recombination, via transient expression of the Cre recombinase. The targeting cassette is electroporated into competent cells induced for the expression of λ Red proteins (recombination-competent cells), and clones are selected by plating onto selective media (containing chloramphenicol). **b** Composition of the 1,000 bp long homology arm vector containing the *prosseA*-controlled antigen expression cassette and loxP-Cm-loxP selective marker. **c** Positions of the four loci on theTy21a chromosome that were selected for the insertion of heterologous genes

inserted into the appropriate plasmid so as to flank the *prosseA-antigen/gfp gene*-loxP-Cm-loxP fragment. The four resultant plasmids constructed: pYBS-hsdR-HA, pYBS-htrA-NP, pYBS-msbB-NA, and pYBS-asd-GFP acted as templates for the PCR amplification of linear DNA-targeting cassettes for the *hsdR*, *htrA*, *msbB*, and *asd* genetic loci, respectively.

Recombination of four heterologous genes into a single bacterial chromosome

Recombination-competent *Salmonella* Ty21a cells harboring the *p*Sim6 (heat shock-inducible) λ Red expression plasmid (Datta et al. 2006) were separately transformed with 'long arm' and 'short arm' versions (Fig. 1) of the

prosseA-HA-loxp-Cm-loxp-targeting cassette. These targeted the *hsdR* gene for replacement with an inducible influenza haemagglutinin gene expression cassette. Colonies that grew on an LB agar + Cm plates were screened by colony PCR, and the ratio of 'successful recombinants' (i.e., ones where the targeting cassette was deemed to have been correctly chromosomally inserted) versus non-specific integrants was determined (Fig. 2).

Several positive colonies were individually transformed with the *p*705Cre-Km plasmid (Buchholz et al. 1996) to remove the chloramphenicol resistance selectable marker via Cre/loxP site-specific recombination. Again, colony PCR was used to identify accurate Cm gene removal, which left one residual loxP site after the HA gene. The resultant strain was named YBS001, which was used for the next round of recombineering.

Three rounds of analogous experiments targeting the *htrA*, *msbB*, and *asd* genetic loci were performed in an iterative manner. These generated the YBS002, YBS003, and YBS004 recombinant *Salmonella* Ty21a strains, which incrementally harbored *prosseA-NP*, *prosseA-NA*, and *prosseA-gfp* at the *htrA*, *msbB*, and *asd* loci, respectively. Three separate sets of PCR reactions (named 'F', 'R', and 'W') were performed after each gene-targeting experiment or Cre-mediated Cm gene removal step. The 'F' PCR reactions (using primers 1 and 3) amplified the chromosomal regions that included the sites of integration (genetic crossover) at the 5′-end of the appropriate targeting cassette. The respective forward primers (primer 1) annealed to the chromosomal regions just upstream of the integration site, and the reverse primers (primer 3) annealed to a region in the antigen (or *gfp*) gene. Correspondingly, the 'R' PCR reactions (using primers 2 and 4) examined the chromosomal regions that included the sites of integration at the 3′-end of the targeting cassette. The 'W' PCR reactions (primers 1 and 4) amplified the entire inserted heterologous antigen cassette regions (Fig. 2a). Thus, the genetic composition at the targeted loci was thoroughly investigated, ensuring accurate cassette integration has occurred. After the construction of YBS004, each genetic insertion loci was again verified by colony PCR (Fig. 2b).

Comparison of recombineering frequencies using targeting cassettes containing 'long arms' and 'short arms' of sequence homology

We focused on the *asd* gene locus to directly compare the λ Red-mediated recombineering frequency in *S. typhi* strains using two different primer sets, two different targeting cassettes were synthesized for each genetic loci: one that contained 'long arms' of flanking sequence homology (ca. 1,000 bp), and another that contained 'short arms' of only ca. 50 bp flanking sequence homology (Table 3).

The original Ty21a strain, as well as the derived YBS001 and YBS002 and YBS003 strains, were analogously transformed with targeting cassettes containing both

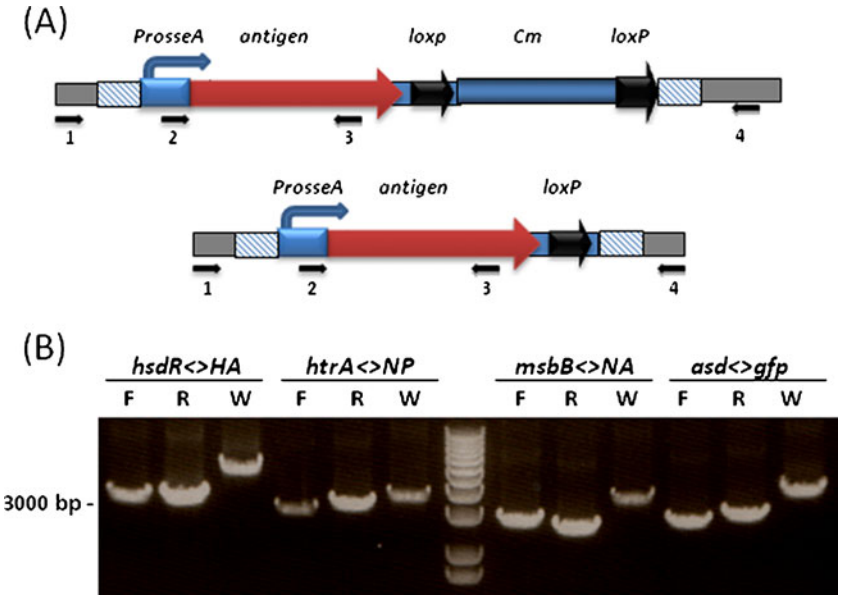

Fig. 2 Verification of chromosome-based multiple antigen insertion and expression by colony PCR. **a** The relative locations of primers 1 to 4 on the chromosome of the recombinant strain. Primers 1 and 4 are positioned outside the 1,000-bp region of homology shared with the targeting cassettes. Primers 2 and 3 are positioned within the heterologous regions. **b** Colony PCR verification of the accurate insertion the four antigen genes at their respective loci. *F*: PCR reaction to amplify the chromosomal region containing the 5′-junction formed after integration; *R*: PCR reaction to amplify the chromosomal region containing the nascent 3′-junction; *W*: PCR reaction to amplify the entire integrated targeting cassette region

Table 3 Summary of recombineering efficiency for chromosomal insertion of heterologous genes from targeting cassettes containing 1,000 or 50 bp of flanking homology

Strains	Length of homology arms on targeting cassette (bp)	No. of correct recombinant clones per 10^8 viable cells[a]	Average targeting success rate (ATSR)[b], (%)	Efficiency ratio (long arm to short arm homology)[c]
Ty21a	50	0	0	–
	1,000	0	0	
TY21a (pSim6)	50	1.55×10^2	51	17:1
	1,000	2.68×10^3	82	
YBS001 (pSim6)	50	1.45×10^1	4.7	115:1
	1,000	1.66×10^3	41	
YBS002 (pSim6)	50	2.81×10^1	4.1	96:1
	1,000	2.70×10^3	53	
YBS003 (pSim6)	50	1.62×10^1	4.2	33:1
	1,000	5.40×10^2	46	

[a] Values indicated the average of six experiments

[b] Targeting success rate is the number of correct recombinant clones divided by the number of resistant colonies ($n>100$) examined in one single experiment, and the ATSR is the average of TSR in total experiments

[c] Efficiency ratio is the comparison of correct recombinant clones per 10^8 viable cells between the long arm and short arm

long (1,000 bp) and short (50 bp) flanking regions of sequence homologous to the *asd* locus. Thus, it would also be possible to determine whether or not the accuracy or efficiency of the recombination deteriorated in strains containing one or more previously integrated antigen expression cassettes. The recombination efficiency was determined by dividing the number of recombinant clones obtained that were chloramphenicol resistant and did not contain the *asd* gene, by the total number of viable cells that survived electroporation (evaluated by plating the transformed cell mixture onto LB agar containing no antibiotics). The targeting 'success rate' was determined by dividing the number of Cm-resistant colonies that exhibited the correct pattern after colony–PCR screening, by the number of Cm-resistant colonies that contained a *ProsseA*-gfp-loxP-Cm-loxP fragment that had been inserted in an incorrect manner.

Results indicated that when the λ Red system was not present (Ty21a), neither the targeting cassette could be recombined with the chromosome to generate the correct recombinant strain. Similarly, when the Red proteins were present in Ty21a cells but no DNA-targeting cassette was introduced, no successful recombinants could be detected. In Ty21a cells where the λ Red proteins had been induced prior to transformation with targeting cassette [Ty21a (pSim6)], the efficiency of long homology arm recombineering could reach a frequency of 10^3 per 10^8 viable cells. This was ten times higher than the recombination frequency obtained using the short homology arm-targeting cassette (10^2 per 10^8 viable cells), with a correspondingly lower success rate of 51% versus 82% for the long homology arm cassette.

When recombineering experiments were performed in cells that contained one to three heterologous antigen gene cassettes that had previously integrated at distant loci (YBS001, YBS002, and YBS003), the efficiency of the long homology arm strategy remained relatively constant at about 10^2–10^3 per 10^8 viable cells. The levels of accurate DNA cassette recombination remained similarly constant, with ca. 41–53% of recombinant clones exhibiting the correct patterns after PCR analysis. Contrastingly, the efficiency of the short homology arm strategy fell to levels of ca. 10^1 per 10^8 viable cells, with far lower levels of insertion accuracy of ca. 4%. This made it difficult and tedious to correctly identify a clone containing the desired genetic alteration. Although the long homology arm-recombineering efficiency in the strain containing three pre-integrated targeting cassettes (YBS003) dropped to ca. 5×10^2 per 10^8 viable cells, the insertion accuracy (success rate) remained relatively high at 46% (Table 3).

Analysis of the effect of homology arm length on recombination efficiency

We used PCR to generate different size of homology arms (1,000, 800, 600, 400, 200, 100, and 50 bp) to test the recombination efficiency in YBS003 at *asd* gene locus. We found that when homology arm size was longer than 800 bp, the success rate was still around 46–54%; when the homology arm was adjusted to 400–600 bp, the success rate dropped into 20–28%; only 11% for 100-bp arm and 4.2% for 50-bp arm. The numbers of correct recombinant clones also decreased from 10^2 for long arms (>800 bp) to 10^1 for short arms (<800 bp). This result indicated that long arms offer both high efficiency and accuracy (Table 4).

Table 4 Recombineering efficiency analysis for different length of flanking homology in strain YBS003

Length of homology arm (bp)	No. of correct colonies per 10^8 viable cells	Average targeting success rate (ATSR)[a], (%)
1,000	5.40×10^2	46
800	8.40×10^2	54
600	2.31×10^1	28
400	3.17×10^1	20
200	6.23×10^1	13
100	4.17×10^1	11
50	1.62×10^1	4.2

[a] Targeting success rate (TSR) is the number of correct recombinant clones divided by the number of resistant colonies tested (n>100) in one single experiment, and the ATSR is the average of TSR in total experiments

Detection of heterologous protein expression

S. typhi strain YBS004 was generated after the fourth round of recombineering. The *prosseA-gfp* cassette was integrated into the *asd* locus, resulting in the creation of a DAP auxotrophic strain. When YBS004 was cultured in LB-containing DAP, GFP was not expressed. When late-log phase YBS004 cells grown in an LB media were washed and then cultured in N-salts minimal media for 24 h to induce expression from the *ProsseA* promoter, a strong GFP expression could be observed by fluorescence microscopy (Fig. 3a). The in vivo expression of GFP was determined by co-culturing YBS004 with Hela cells in DMEM media containing DAP for 1 h, and then in DMEM media lacking DAP for a further 12 h before analyzing cellular fluorescence. It was found that GFP expression was strongly induced in YBS004 cells that had invaded the mammalian cells (Fig. 3b). This result indicated that although four identical *prosseA* promoters had been integrated into the Ty21a chromosome at various loci, this did not significantly compromise the ability of this strain to invade epithelial cells, and the expression levels from this promoter did not appear to be significantly affected. Western blotting analysis clearly indicated that there was an expression of all three influenza virus antigens (HA, NP, NA) in the YBS004 strain. This confirmed that the expression of multiple heterologous genes positioned at discrete loci on the Ty21a chromosome, which were all under the control of the *ProsseA* promoter, were under relatively tight control and could be strongly induced under the appropriate conditions (Fig. 3c).

Discussion

Recombineering technology enables researchers to modify essentially any nucleotide sequence on the chromosome of certain gram-negative bacteria species within days, not weeks or months. This approach is simple, rapid, and precise. Chromosome-based protein expression systems avoid problems associated with plasmid instability or loss and provide a convenient platform for the insertion and expression of multiple heterologous genes. It has previously been reported that the genes within the SPI2 locus were specifically and strongly 'switched on' when *Salmonella* had invaded host cells (Hensel 2000; Cirillo et al. 1998; Hansen-Wester et al. 2002). Husseiny et al. isolated an intracellularly activated promoter, *prosseA*, derived from SPI2 (Husseiny and Hensel 2005). This promoter was controlled by a two-component regulatory system within SPI2 and was only activated after the invasion of host cells or by culturing in a minimal medium to simulate the environment inside host cells (Hensel 2000). In our study, we employed the *prosseA* promoter from *S. typhi* to achieve high-level inducible in vitro and in vivo expression of antigen genes from the influenza virus (Fig. 2).

We selected four genetic loci (*htrA*, *hsdR*, *msbB*, and *asd*) on the TY21a chromosome as the respective sites for the insertion of the four heterologous genes. The major reason for choosing these specific genes was that they could further attenuate Ty21a in various ways. HtrA is a stress-related protein (Lewis et al. 2009). HsdR acts as a chaperone associated with HsdS in a bacterial host restriction system (Rudd 2000). The MsbB enzyme plays an important role in lipid A biosynthesis, deleting the *msbB* gene will attenuate virulence without generating growth defects (Murray et al. 2001). Asd is involved in the biosynthesis of DAP, which is necessary for cell wall synthesis; *asd* mutants will not survival in medium without supplementation with DAP (Kong et al. 2008). A further reason was that the *htrA* (242,203 nt), *hsdR* (4,729,805 nt), *msbB* (1,076,362 nt) and *asd* (4,121,375 nt) genetic loci are each separated by at least 600,000 nt (the complete genome size of *S. typhi* Ty2 is 4,791,961 nt).

After completion of the final round of recombineering, the chromosome of the resultant YBS004 strain contains four isolated *loxP* sites, generated by four individual Cre recombinase-mediated excisions of the Cm resistance selectable markers. This indicated that the Cre recombinase did not mediate the excision of large sections of the Ty21a chromosome, probably due to physical factors as well as the presence of multiple genes encoding essential functions within these ca. 600,000 nt regions.

In the stepwise construction of the YBS001, YBS002, YBS003, and finally the YBS004 strains, starting from the Ty21a strain, each round of recombination introduced two additional regions of homologous DNA sequence into the chromosome (after Cre-mediated Cm gene excision) namely the *prosseA* promoter region (ca. 300 bp), and a region containing a transcriptional terminator and a single *loxp* site

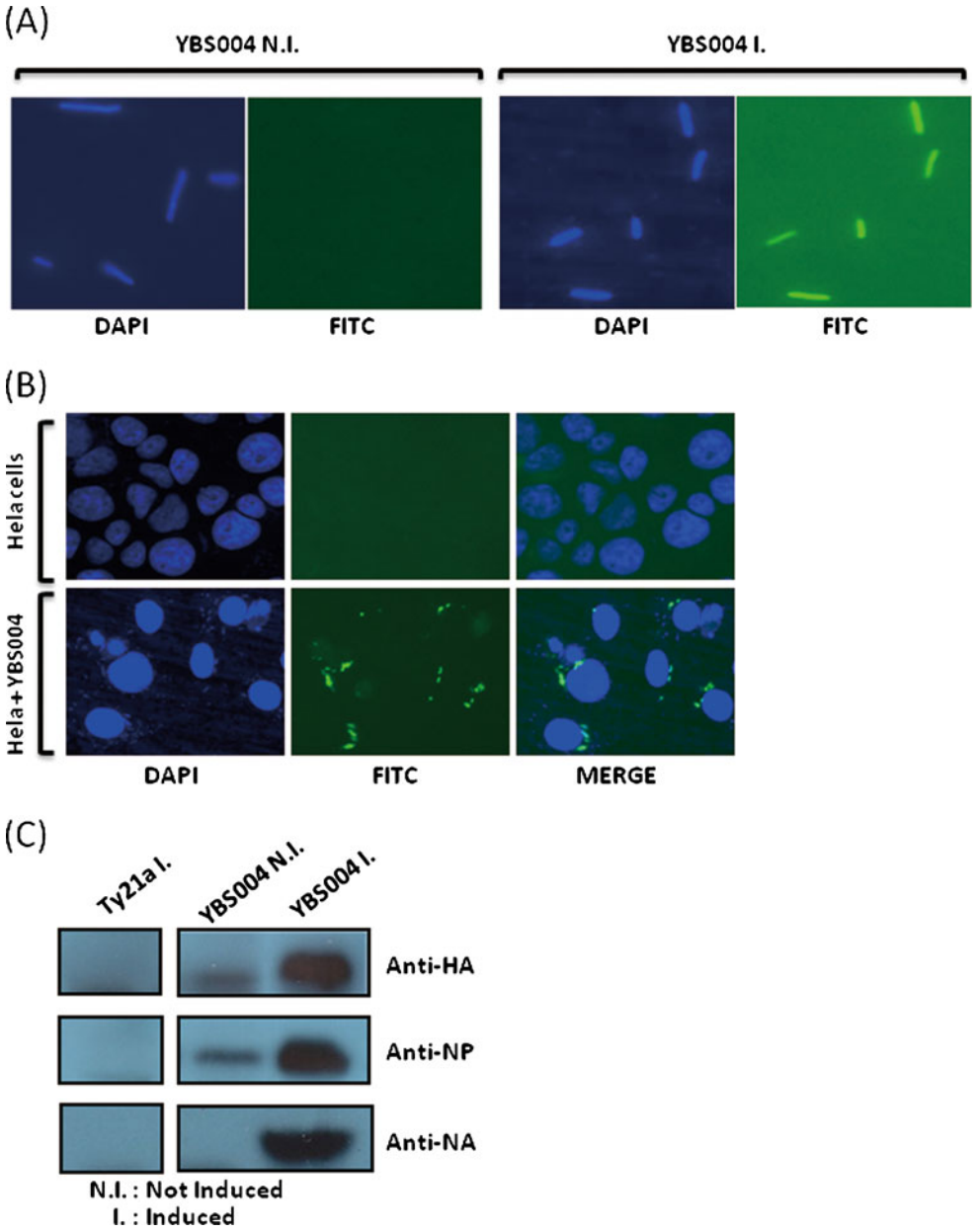

Fig. 3 In vivo expression of chromosome-based heterologous genes from the ProsseA inducible promoter in strain YBS004. **a** To test the in vitro induction of expression form the *prosseA* promoter in strain YBS004, cells cultured in LB media (*YBS004 N.I.*) or N-salts minimal media YBS004 (*YBS004 I.*) were analyzed by fluorescent microscopy, under conditions for DAPI visualization of DNA (excitation at 345 nm, emission at 458 nm) and green fluorescence protein visualization (FITC; excitation at 488 nm, emission at 518 nm). **b** To test the in vivo induction of expression form the *prosseA* promoter, YBS004 cells were co-cultured with Hela cells, and the expression of GFP was analyzed by fluorescence microscopy (*lower panel*). The *upper panel* shows fluorescence from Hela cells only. **c** Western blotting to detect expression of the three heterologous influenza antigen proteins in YBS004 cells cultured in LB media (*YBS004 N.I.*), and in N-salts induction medium (*YBS004 I.*). *S.* Typhi Ty21a cells cultured in LB medium was used as negative control (*Ty21a I*)

(ca. 100 bp). Consequently, during the construction of the YBS002, YBS003, and YBS004 strains, these homologous regions of DNA sequence would potentially be capable of acting as sites of genetic recombination (crossover). These sites may be expected to interfere with the desired genetic recombination process between the transformed targeting cassette and the respective *hsdR*, *msbB*, or *asd* loci. Our data (Table 3) are consistent with this hypothesis, in that the success rate dropped-off during the three rounds of recombineering. However, the results also demonstrated that using the 'long homology arm' strategy dramatically increased both the efficiency and the accuracy of the recombineering process. When the length of homology arms decreases, the efficiency also dramatically decreases (Table 4). Therefore, the λ Red-mediated 'long homology arm' recombineering strategy is more suitable for the construction of multiple chromosome insertions than the more common 'short homology arm' approach.

Since the precise genomic sequences of related bacterial subspecies and strains are not identical, another advantage of the 'long homology arm' strategy is that it can overcome sequence variation between these different strains. For instance, there is no published Ty21a genome sequence; only the *S. typhi* Ty2 sequence is available. Therefore, we do not know the precise locations of the thousands of estimated single or multiple nucleotide polymorphisms present on the chromosome. Specifically, we do not know if any of these nucleotide variations are crucially located

within the regions of homology included in the targeting cassettes. Using this approach, the predicted multiple mismatches should not influence the overall recombination frequency to a large extent, resulting in reliable gene targeting.

In summary, we have developed a robust and efficient method for generating genetically modified Ty21a strains using λ Red-mediated recombineering, combined with Cre/loxp technology. Four different heterologous genes were precisely inserted into the chromosome of a single bacterium. This reliable methodology may be of great utility in the vaccine development field, e.g., to generate carrier strains expressing multiple heterologous antigens. In the future, our approach may be further adapted for the construction of vaccine strains in other bacterial species.

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