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Integrative bacterial artificial chromosomes for DNA integration into the *Bacillus subtilis* chromosome

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ABSTRACT

Bacillus subtilis is a well-characterized model bacterium frequently used for a number of biotechnology and synthetic biology applications. Novel strategies combining the advantages of *B. subtilis* with the DNA assembly and editing tools of *Escherichia coli* are crucial for *B. subtilis* engineering efforts. We combined Gibson Assembly and λ red recombineering in *E. coli* with RecA-mediated homologous recombination in *B. subtilis* for bacterial artificial chromosome-mediated DNA integration into the well-characterized *amyE* target locus of the *B. subtilis* chromosome. The engineered integrative bacterial artificial chromosome iBAC(cav) can accept any DNA fragment for integration into *B. subtilis* chromosome and allows rapid selection of transformants by *B. subtilis*-specific antibiotic resistance and the yellow fluorescent protein (*mVenus*) expression. We used the developed iBAC(cav)-mediated system to integrate 10 kb DNA fragment from *E. coli* K12 MG1655 into *B. subtilis* chromosome. iBAC(cav)-mediated chromosomal integration approach will facilitate rational design of synthetic biology applications in *B. subtilis*.

1. Introduction

The rod-shaped Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus subtilis* are well characterized model bacteria frequently used for metabolic engineering and a number of biotechnology and synthetic biology applications (Schallmey et al., 2004; Juhas et al., 2013a; Juhas, 2015b; Chen et al., 2013; Yim et al., 2011; Ajikumar et al., 2010). Although the vast majority of the good DNA assembly and editing tools are in *E. coli*, *B. subtilis* is a better host for certain applications. *B. subtilis* can secrete proteins into the medium and has been successfully used for the production of a number of industrially relevant bioproducts, such as riboflavin (Hao et al., 2013; Manabe et al., 2013; Zweers et al., 2008). Furthermore, *B. subtilis* forms dormant endospores that enable survival in the environments lacking nutrients (McKenney et al., 2013). *B. subtilis* is also considered to be a promising host for the construction of the minimal cell factories (Juhas et al., 2014b).

Novel tools combining the advantages of *B. subtilis* with the efficient and reliable DNA assembly and editing methods available in *E. coli* are crucial for *B. subtilis* engineering efforts.

Integration of genetic circuits into the bacterial chromosome has numerous advantages over their introduction into the genome on plasmids. Strategies for *E. coli* chromosomal integration are usually based on the λ bacteriophage red recombinase system-mediated homologous recombination (Cunningham et al., 2009; Marcellin et al., 2010; Das et al., 2010; Juhas et al., 2014a; Juhas and Ajioka, 2015a, 2015b). *B. subtilis* is a naturally competent bacterium that is readily transformable with extracellular DNA. DNA is integrated into the *B. subtilis* chromosome via RecA-mediated homologous recombination (Yadav et al., 2014; Shi et al., 2013). The natural competence permits use of the entire 4.2 Mb *B. subtilis* genome as a vector in the *B. subtilis* genome (BGM) vector system (Ogawa et al., 2015). BGM allows stable integration of large DNA fragments into the *B. subtilis* chromosome by homologous recombination (up to 3.5 Mb of *Synechocystis* PCC6803 DNA has been stably integrated into BGM) (Itaya et al., 2000, 2008; Yonemura et al., 2007; Kaneko et al., 2003; Iwata et al., 2013; Itaya et al., 2005). Horizontal gene transfer is mediated by mobile genetic elements, such as plasmids and genomic islands (Juhas, 2015a, 2015d; Juhas et al., 2007a, 2007b, 2008, 2009, 2013b; Robinson et al., 2010; Dobrindt et al., 2004, 2010). The integration of DNA fragments into the *B. subtilis* chromosome relies often on plasmids that have limited cloning capacity (Vojcic et al., 2012; Brigidi et al., 1990). Bacterial artificial chromosomes (BACs) based on the *E. coli* fertility factor (F-factor) are frequently used for engineering high molecular weight DNA fragments in *E. coli* (Hall et al., 2012; Shizuya et al., 1992). BACs are maintained in *E. coli* in the single copy extrachromosomal supercoiled form and support stable propagation of inserted DNA over multiple generations (O'Connor et al., 1989; Hall et al., 2012). DNA shuttling between BACs and BGM was reported previously (Kaneko et al., 2005, 2009). Integration into BGM requires two DNA sequences, called landing pad

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sequences to determine the integrated DNA fragment. Landing pad sequences, correctly aligned in an *E. coli* pBR322 plasmid are introduced into a pBR sequence locus of the *B. subtilis* chromosome prior to integration of DNA (Kaneko et al., 2003; Itaya et al., 2000).

Here we present an alternative approach for simple and reliable BAC-mediated DNA integration into the *B. subtilis* chromosome at well-characterized *amyE* locus using the engineered integrative iBAC(cav).

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *B. subtilis* strains were routinely grown in Luria-Bertani broth (LB). For generating competent *B. subtilis*, cells were cultivated in the starvation medium as described below. When needed, growth media were supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) for cultivating *E. coli*, or kanamycin (5 μg/ml) and chloramphenicol (5 μg/ml) for growing *B. subtilis*. Liquid cultures of *E. coli* and *B. subtilis* were grown in LB (supplemented with the antibiotics when needed) and incubated at 37 °C with shaking at 200 rpm on a rotatory shaker, depending on the requirements. All *E. coli* and *B. subtilis* plate cultures were grown for about 24 h at 37 °C.

2.2. DNA amplification and modification procedures

PCR amplification of DNA sequences was performed in 25 or 50 μl reaction volumes using Dream Taq master mix kit (Thermo Scientific) or Phusion DNA polymerase (Thermo Scientific) according to the supplier's specifications. PCR primers were synthesized by Sigma-Aldrich. A modified Gibson Assembly procedure (Gibson et al., 2009; Merryman and Gibson, 2012) in a 5.2 μl final reaction volume was employed to assemble DNA fragments (Juhas et al., 2014a). The successful assemblies were validated by PCR amplification and sequencing. Standardized kits were used for extracting DNA fragments from the agarose gel (Qiaquick gel extraction kit, Qiagen) and for plasmid isolation (Qiaprep Spin Miniprep kit, Qiagen), according to the supplier's instructions. Bacterial artificial chromosomes were isolated using Qiaquick gel extraction kit (Qiagen) or PhasePrep BAC DNA kit (Sigma-Aldrich). *B. subtilis* genomic DNA was isolated with GeneJET genomic DNA purification kit (Thermo Scientific) according to manufacturer's instructions.

Table 1
Bacterial strains, plasmids and BACs used in this study.

	Characteristics	Reference
Strains		
K12 MG1655	*E. coli* wild type	Hayashi et al. (2006)
Bs	*B. subtilis* Marburg wild type 168, *trpC2*	Burkholder and Giles (1947)
Bs(cavQS)	*B. subtilis* 168, 10 kb DNA integrated in *amyE* locus	This study
Plasmids		
pSB1K3	BioBrick assembly plasmid, kanamycin resistance	parts.igem.org/Part:pSB1K3
pKM208	IPTG-inducible Red recombinase system	Murphy and Campellone (2003)
pCP20	Flippase (FLP) recombinase	Datsenko and Wanner (2000)
pSB1K3(FRTK)	pSB1K3 with kanamycin resistance cassette	Juhas et al. (2014a)
pJScav	pJS209 plasmid, *amyE* integr sites, *ven/cat* circuits	Lab collection
pSB1K3(FRTKpJScav)	pSB1K3(FRTK), *amyE* integr sites, *ven/cat* circuits	This study
BACs		
pBeloBAC11	BAC with chloramphenicol resistance	Lab collection
iBAC(cav)g	pBeloBAC11, *amyE* sites, *ven/cat*, Gibson Assem.	This study
iBAC(cav)r	pBeloBAC11, *amyE* sites, *ven/cat*, λ red assembled	This study
iBAC(cavQS)	iBAC(cav) with 10 kb *E. coli* DNA fragment	This study

2.3. λ red system-mediated DNA integration into BACs

The electro-competent and chemically competent *E. coli* cells were produced employing the modified Miller and Nickoloff (Miller and Nickoloff, 1995) and Hannah (Hanahan et al., 1991) methods, respectively. Integration into BACs was performed using the streamlined λ red system-mediated method (Juhas et al., 2014a; Juhas and Ajioka, 2015a, 2015b). Briefly, *E. coli* harbouring BAC was transformed with plasmid pKM208 encoding the IPTG-inducible λ red system and incubated on ampicillin plates at 30 °C. Overnight culture of the pKM208-harbouring *E. coli* K12 MG1655 was inoculated into LB with ampicillin (1:100 dilution) and grown at 30 °C. After reaching OD_{600} 0.2, IPTG (1 mM) has been added and the bacterial culture was grown to the final OD_{600} 0.5. Cells were washed twice and resuspended in 100 μl of 10% glycerol per 100 ml of starting culture volume. 100 μl of the gel purified DNA fragment flanked by sequences of the integration target on BAC was electroporated into pKM208-harbouring electrocompetent cells. Transformants were selected on LB plates with kanamycin (50 μg/ml) and grown at 37 °C overnight and then incubated at 42 °C overnight to cure out pKM208 plasmid. Integrations were confirmed by diagnostic PCR using flanking primers and sequencing.

2.4. Pulsed field gel electrophoresis analysis (PFGE) of BACs

Samples were loaded into the wells of a 1% pulsed field certified agarose gel in TBE buffer (BioRad Laboratories). The gel was then placed into the electrophoresis chamber of a CHEF DR II (BioRad Laboratories) PFGE apparatus filled with 0.5 × TBE buffer. The PFGE was run with the following electrophoretic conditions: run time of 11 h, switch time of 0.5–5 s, voltage gradient of 6 V/cm. The running temperature of 14 °C was provided by using the water pump and the cooling module (BioRad Laboratories). 8–48 kb CHEF DNA size Ladder (BioRad Laboratories) has been used as the molecular weight marker for PFGE. Following electrophoresis, the gel was stained with the SYBR®Safe DNA gel dye (Invitrogen) and DNA fragments were visualized with Safe Imager blue-light transilluminator.

2.5. Generation and transformation of competent B. subtilis cells

To generate competent *B. subtilis* cells, single colony was first inoculated into 10 ml minimal medium composed of 2 ml of 5× minimal salts solution (2 g ammonium sulphate, 14.8 g potassium hydrogen phosphate, 5.4 g potassium dihydrogen phosphate, 1.9 g sodium citrate, 0.2 g magnesium sulphate heptahydrate dissolved in 200 ml of deionised water), 0.1 ml glucose (50% w/v), 0.1 ml casamino acids (2% w/v), 0.02 ml tryptophan (10 mg/ml), 0.01 ml iron ammonium citrate (2.2 mg/ml) in a deionised water. Inoculated cells were grown at 200rpm on a rotatory shaker at 37 °C for 18 h. Then, 1.4 ml of the *B. subtilis* culture was inoculated into 10 ml of the fresh minimal medium and grown for another 3 h. Subsequently, 11 ml of the starvation medium composed of 2.2 ml of 5× minimal salt solution and 0.11 ml glucose (50% w/v) in a deionised water was added to the *B. subtilis* culture and cells were grown for additional 2 h and 45 min. 0.3 ml aliquots were transferred into 15 ml polypropylene tubes and transformed with 15 μl of BAC DNA or plasmid DNA. Transformed *B. subtilis* cells were incubated at 37 °C with shaking at 200 rpm on a rotatory shaker for 1 h prior to the addition of 700 μl LB. Cells were then continued to grow for 1.5–2 h. 20–200 μl of this culture was plated onto selection plates

and grown at 37 °C for 18–24 h. Integrations into *B. subtilis* chromosome were confirmed by diagnostic PCR and sequencing.

2.6. Fluorescence measurement with the microplate reader

Fluorescence was quantified in the flat-bottomed black 96 well plates (Greiner BioOne, UK). *B. subtilis* cultures were grown overnight and then normalized to OD_{600} of 0.05. 200 μl of these diluted cultures was aliquoted into the microplate wells and incubated in the microplate reader (Fluostar Omega, BMG Labtech, UK) at 37 °C for 10 h. *mVenus* fluorescence was measured with the automatically repeated protocol (excitation filter 485–12, emission filter 530–10, gain 1200, double orbital shaking at 200 rpm, and cycle time 60 min). The mean and standard errors were calculated from three biological replicates.

2.7. Microplate reader measurement of the absorbance

Absorbance was measured in the flat-bottomed clear 96 well plates (Sterilin Sero-Well, UK). The overnight *B. subtilis* cultures were diluted to OD_{600} of 0.05 and 200 μl of these normalized cultures per microplate well was incubated in the microplate reader (Fluostar Omega, BMG Labtech, UK) at 37 °C for 24 h. Absorbance was measured with the automatically repeated protocol with the following parameters (cycle time 60 min, double orbital shaking at 500 rpm, 600 nm absorbance filter). The experiment was carried out in triplicate.

2.8. Databases and sequence analyses

The National Center for Biotechnology Information (NCBI) website (http://ncbi.nlm.nih.gov) was exploited to compare DNA sequences using the BLASTN (Altschul et al., 1990), TBLASTX and position-specific iterated BLAST (PSI-BLAST) algorithms (Altschul et al., 1997). The sequences of the pJS209 and pSB1K3 plasmids and BAC pBeloBAC11 were obtained from the websites of the Registry of Standard Biological Parts (http://parts.igem.org/Main_Page) and the Addgene nonprofit plasmid repository (http://www.addgene.org/), respectively. The Registry of Standard Biological Parts is a repository of genetic parts that can be mixed and matched to build systems for synthetic biology applications. The *E. coli* genome sequence was obtained from the *E. coli* K-12 project website (http://www.xbase.ac.uk/genome/escherichia-coli-str-k-12-substr-mg1655). The *B. subtilis* genome sequence was obtained from the BioCyc (http://bsubcyc.org/) and SubtiWiki (http://subtiwiki.uni-goettingen.de/) databases. Sequencing of DNA constructs was performed by Source Bioscience.

3. Results and discussion

3.1. B. subtilis integrative bacterial artificial chromosome iBAC(cav) construction

To integrate synthetic DNA into *B. subtilis*, we engineered the well-characterized BAC pBeloBAC11 by inserting sequences homologous to *amyE* target locus on the *B. subtilis* chromosome from the plasmid pJScav (Table 1 and Fig. 1A). Two *amyE* integration target sequences were introduced into pBeloBAC11, the leading (5′) sequence *amyE1* and the trailing (3′) sequence *amyE2* (Figs. 1A–D and 2). The *amyE1* and *amyE2* are 521 bp and 1029 bp long, respectively. Linear DNA inserted between *amyE1* and *amyE2* sequences will integrate into *B. subtilis* by double-crossover recombination events between the chromosomal *amyE* of *B. subtilis* and *amyE1,2* sequences (Overkamp et al., 2013). Furthermore, we introduced two genetic circuits (*ven* and *cat*) into pBeloBAC11 between two flanking *amyE* integration sequences.

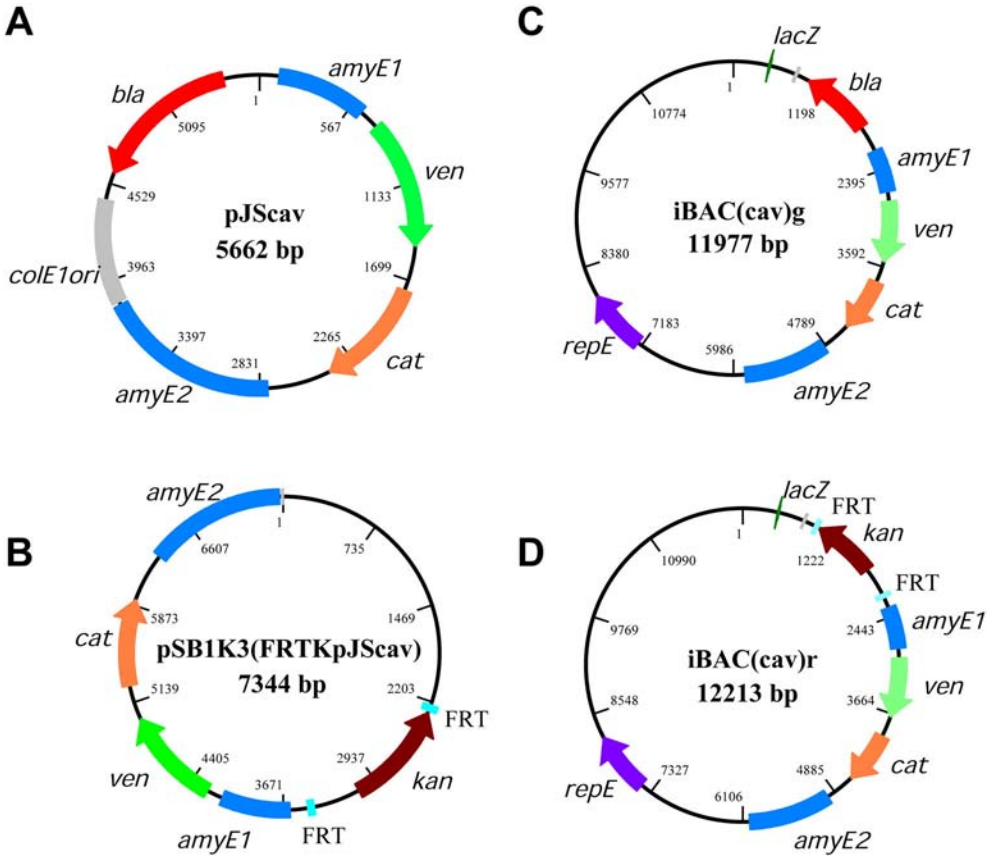

Fig. 1. Plasmid and BAC maps. Figure shows the maps of the key plasmids and BACs used in this study. (A) pJScav: source of the *amyE* locus integration target sequences and *cat* and *ven* genetic circuits (B) pSB1K3(FRTKpJScav): intermediate for the λ red recombineering-based approach of iBAC(cav)r construction. (C) iBAC(cav)g: iBAC(cav) generated by Gibson Isothermal Assembly harbours ampicillin resistance gene. (D) iBAC(cav)r: iBAC(cav) constructed using λ red recombineering harbours kanamycin resistance gene.

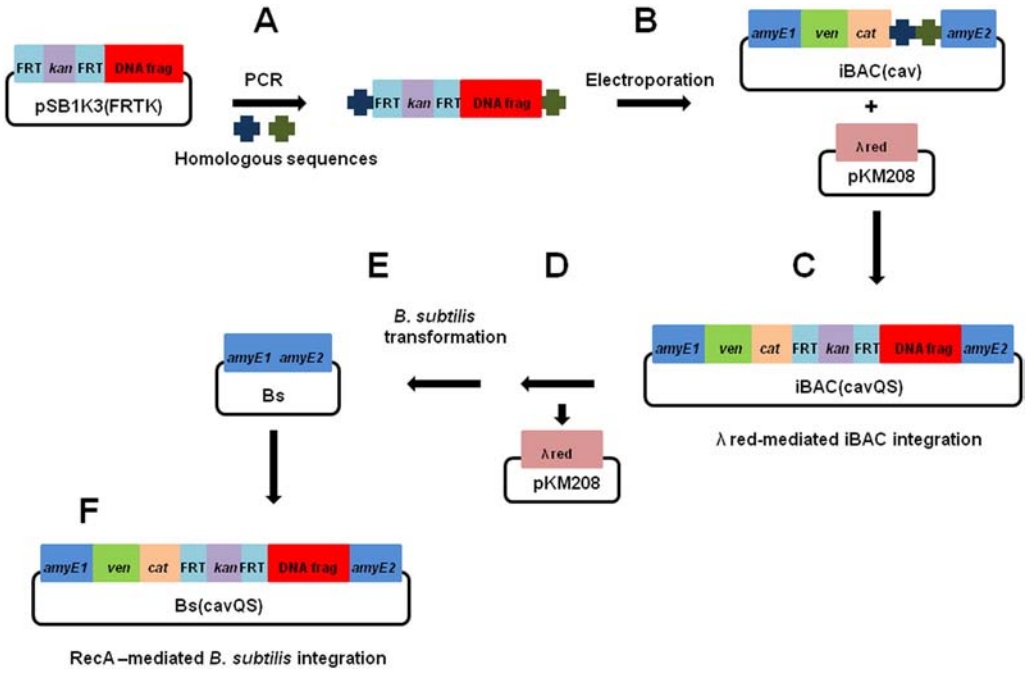

Fig. 2. iBAC-mediated chromosomal integration strategy. Figure shows the main steps of the iBAC-mediated DNA integration into *B. subtilis* chromosome (A) PCR amplification of the DNA fragment and kanamycin resistance cassette flanked by sequences homologous to the integration target locus on iBAC(cav). FRT sites in the kanamycin resistance cassette allow flipping out of the kanamycin marker by FLP recombinase. (B) Electroporation of the DNA fragment into *E. coli* harbouring iBAC(cav) and pKM208-bourne IPTG-inducible λ red system. (C) Integration of the DNA fragment into iBAC(cav) by λ red system-mediated homologous recombination. (D) Curing out of pKM208. (E) Transformation of *B. subtilis* with iBAC(cav) harbouring the DNA fragment. (F) Integration of the DNA fragment into *B. subtilis* chromosome by homologous recombination.

The genetic circuit *ven* consists of the constitutive Ppen promoter, RBS and the yellow fluorescent protein-encoding *mVenus* (Nagai et al., 2002; Yansura and Henner, 1984). The genetic circuit *cat* harbours the chloramphenicol resistance-encoding gene under the control of the constitutive *Bacillus*-specific promoter from the Registry of Standard Biological Parts (http://parts.igem.org/Main_Page). The genetic circuits *ven* and *cat* introduced into pBeloBAC11 are isolated on both ends with terminators from the Registry of Standard Biological Parts. The engineered iBAC(cav) allows rapid selection of transformants by *Bacillus*-specific chloramphenicol resistance and *mVenus* expression.

We used two different approaches, Gibson Isothermal Assembly (Gibson et al., 2009; Merryman and Gibson, 2012) and λ red recombineering (Datsenko and Wanner, 2000; Sabri et al., 2013; Ublinskaya et al., 2012) to assemble iBAC(cav). In the former approach, PCR amplified pBeloBAC11 backbone and the genetic circuits *ven* and *cat* flanked by *amyE* integration target sites in addition to the ampicillin resistance encoding gene *bla* (Fig. 1A) were joined to generate iBAC(cav)g (Fig. 1C). The construction of iBAC(cav)g was verified by diagnostic PCR with flanking primers for each part and sequencing. Furthermore, *E. coli* harbouring correctly assembled iBAC(cav)g grew on a selective medium containing chloramphenicol and expressed *mVenus* encoding the yellow fluorescent protein. The second iBAC(cav)r (Fig. 1D) was generated by λ red recombineering. λ red recombineering allows integration by incorporating sequences at both ends of the DNA fragment that are homologous to the integration target site. The inhibition of the host's RecBCD exonuclease V by the Gam protein of the λ red system allows proteins Bet and Exo contact with the homologous sequences at the ends of the DNA fragment to promote recombination. We used our developed streamlined λ red recombination approach (Juhas et al., 2014a) to construct iBAC(cav)r (Fig. 1D). First, we generated plasmid pSB1K3(FRTKpJScav) (Fig. 1B) by introducing the DNA fragment harbouring the genetic circuits *ven* and *cat* flanked by *amyE* integration target sites into pSB1K3(FRTK) (Juhas et al., 2014a). Then, we integrated the DNA fragment into pBeloBAC11 using λ red system-mediated homologous recombination. We used these two approaches to introduce different resistance markers, ampicillin and kanamycin into iBAC(cav)g into iBAC(cav)r, respectively. The construction of plasmid pSB1K3(FRTKpJScav), iBAC(cav)g and iBAC(cav)r was confirmed by PCR with flanking primers and sequencing. The integration and diagnostic flanking primers used are listed in Supplementary Table 1. Construction of iBAC(cav)g and iBAC(cav)r was further verified by pulsed field gel electrophoresis (PFGE) (Supplementary Fig. 1). DNA bands obtained in the PFGE experiment after digesting iBAC(cav)g and iBAC(cav)r with BamHI and XhoI restriction endonucleases had the correct molecular weight (Supplementary Fig. 1).

3.2. iBAC-mediated B. subtilis chromosomal integration strategy

The general strategy of our iBAC-mediated chromosomal integration strategy is shown in Fig. 2. First, the DNA fragment to be integrated is cloned into plasmid pSB1K3(FRTK) (Juhas et al., 2014a) next to the kanamycin resistance cassette. The kanamycin resistance gene in the cassette is flanked by FRT (flippase recognition target) sites. Second, the PCR amplified DNA fragment with the kanamycin resistance cassette is electroporated into *E. coli* harbouring iBAC(cav) and pKM208-bourne λ red system that mediates homologous recombination into iBAC(cav). Third, iBAC(cav) harbouring DNA fragment is transformed into *B. subtilis* and DNA fragment is integrated into the target *amyE* locus (Yang et al., 2013) of the *B. subtilis* chromosome by RecA-mediated homologous recombination (Fig. 2) (Yadav et al., 2014; Shi et al., 2013). Transformants are identified by *Bacillus*-specific chloramphenicol resistance and *mVenus* expression and confirmed by diagnostic PCR and sequencing. λ red recombineering has many advantages over other DNA assembly methods, such as Gibson Isothermal Assembly (Juhas and Ajioka, 2016). We used λ red recombineering approach mainly due to its potential to assemble very large DNA fragments. The FRT sites in the kanamycin resistance cassette introduced into iBAC(cav) allow flipping out of the kanamycin marker from iBAC(cav) by flippase (FLP) recombinase and subsequent integration of other DNA fragments.

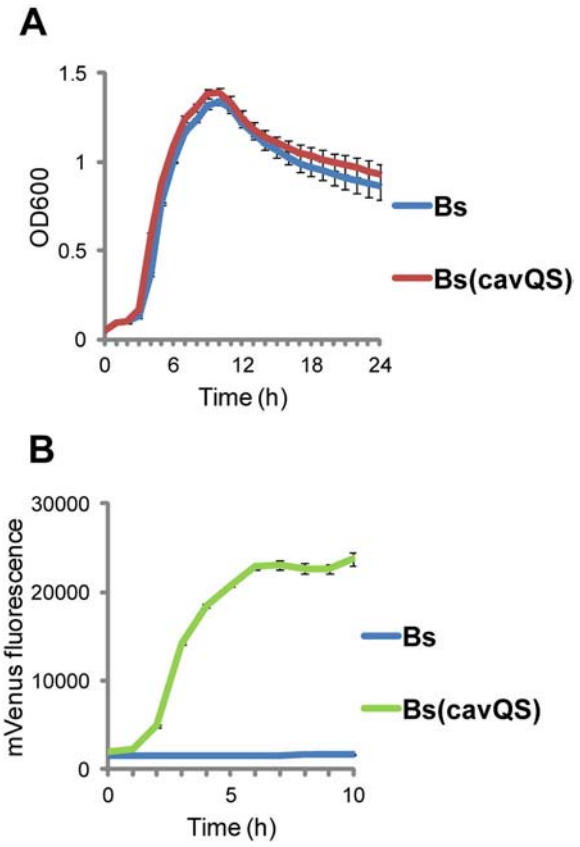

Fig. 3. Growth and *mVenus* expression of *B. subtilis* with chromosomal integration. (A) Integration of DNA fragment into *amyE* locus of the *B. subtilis* chromosome does not have negative impact on growth. (B) Quantitative verification of the integrated genetic circuit *ven* expression by measuring *mVenus* fluorescence over time with the microplate reader (Fluostar Omega, BMG Labtech, UK). The initial absorbance of the analyzed strains at 600 nm (OD600) was 0.05. The experiments were carried out in triplicate, error bars represent standard errors. Bs: *B. subtilis* wild type strain 168, Bs(cavQS): engineered *B. subtilis* strain 168 with 10 kb DNA fragment from *E. coli*.

3.3. iBAC-mediated integration of DNA fragment from E. coli into B. subtilis

The constructed iBAC(cav) (Fig. 1C and D) can be used for the integration of any DNA fragment into *B. subtilis* chromosome. To confirm its suitability for chromosomal integration, we cloned 10 kb DNA fragment from *E. coli* K12 MG1655 into iBAC(cav). The constructed iBAC(cavQS) harbouring cloned 10 kb DNA fragment was transformed into the recipient *B. subtilis* as described in Section 2. The exact position within the *amyE* locus where chromosomal integration occurred is shown in Supplementary Fig. 2. Integration of the DNA fragment into the targeted *amyE* region was verified by diagnostic PCR and sequencing (Supplementary Fig. 3 and Supplementary Table 1). We chose *amyE* locus as integration target mainly because it is well-characterized, non-essential and widely conserved in a number of *B. subtilis* strains (Voigt et al., 2009; Jeong et al., 2015; Juhas et al., 2011, 2012a, 2012b, 2014b; Juhas, 2015b, 2015c; Lajoie et al., 2013; Glass, 2012). To verify the non-essentiality of *amyE*, we compared the growth of the engineered *B. subtilis* strain Bs(cavQS) harbouring integrated DNA fragment with that of the wild type (Bs) (Fig. 3A). The integration generated viable *B. subtilis*. The growth rate of the engineered *B. subtilis* strain Bs(cavQS) was not affected compared to the wild type (Fig. 3A). The integration of the genetic circuit *cat* with the 10 kb DNA fragment allowed selection of transformants by *Bacillus*-specific chloramphenicol resistance. The integration of the genetic circuit *ven* allowed rapid identification of transformants by fluorescent protein *mVenus* expression. Besides qualitative confirmation by visual observation, the expression of the genetic circuit *ven* was confirmed also quantitatively by measuring *mVenus*-induced fluorescence over time with the microplate reader (Fig. 3B).

3.4. iBAC chromosomal integration efficiency

The transformation efficiency is considered to be among the main bottlenecks in the directed *B. subtilis* evolution (Vojcic et al., 2012). The *B. subtilis* transformation efficiency is dependent on the strain and method employed and was shown to vary from about 100–200 transformants per μg DNA for transformation on solid media to about 1.4×10^7 for transformation of *B. subtilis* protoplasts in the presence of polyethylene glycol (Hauser and Karamata, 1994; Akamatsu and Taguchi, 2001; Chang and Cohen, 1979).

To analyse the iBAC-mediated *B. subtilis* integration efficiency we measured efficiency of chromosomal integration of the engineered iBAC(cavQS) into *amyE* locus (Fig. 4). Transformation of *B. subtilis* using circular DNA often tends to produce Campbell-type integration (Young, 1983) at single homologous region. This type of integration is not suitable for *B. subtilis* integration due to its high instability. To mitigate against the undesired Campbell-type integration, we performed the *B. subtilis* chromosomal integration with iBAC(cavQS) linearised by restriction digest and with PCR amplified DNA fragment located between *amyE1* and *amyE2* integration sequences of iBAC(cavQS), in addition to the uncut iBAC(cavQS). The integrations using linear DNA fragments occur by double-crossover recombination events between the chromosomal *amyE* of *B. subtilis* and *amyE1,2* sequences. Chromosomal integrations were confirmed by diagnostic PCR and the integration efficiencies were calculated from the number of colony-forming units (CFU) per microgram of transformed DNA. Notably, the integration efficiencies differed between the DNA templates (Fig. 4). The integration efficiency with the uncut iBAC(cavQS) was approximately 15 times higher than integration using iBAC(cavQS) linearised by restriction digest and PCR amplified DNA fragment. This could be due to Campbell-type integration of the circular iBAC(cavQS). The chromosomal integration efficiency of the uncut iBAC(cavQS) (1.5×10^4 transformants/μg electroporated DNA) was comparable to that achieved with the small plasmid pVE18 using electroporation method in a previous study (Brigidi et al., 1990; Vojcic et al., 2012).

4. Conclusions

Novel strategies combining the advantages of *B. subtilis* with the efficient and reliable DNA assembly and editing tools of *E. coli* are crucial for *B. subtilis* engineering efforts. The integration of DNA edited in *E. coli* into *B. subtilis* chromosome is crucial for the development of a

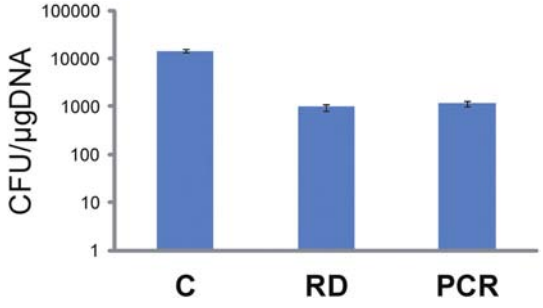

Fig. 4. iBAC-mediated chromosomal integration efficiency. Figure shows *B. subtilis* chromosomal integration efficiency calculated from the number of the colony forming units per μg of electroporated DNA for circular and linear iBAC(cavQS). C: uncut iBAC(cavQS), RD: iBAC(cavQS) linearised by restriction digest, PCR: PCR amplified DNA fragment from iBAC(cavQS). The mean and standard errors were calculated from three independent replicates.

robust synthetic biology toolkit. We developed a simple and flexible BAC-mediated method for DNA integration into the *B. subtilis* chromosome. Our method benefits from the DNA assembly and editing tools available in *E. coli*, such as λ red recombineering (Datsenko and Wanner, 2000). We constructed iBAC(cav) by introducing sequences homologous to the *amyE* integration target locus on the *B. subtilis* chromosome and genetic circuits *cat* and *ven*. The genetic circuits *ven* and *cat* allow easy identification of transformants with *B. subtilis*-specific chloramphenicol resistance and *mVenus* expression.

To test the engineered iBAC(cav)-mediated system we integrated 10 kb DNA fragment from *E. coli* into the *B. subtilis* chromosome. The chromosomal integration efficiency using iBAC(cav)-mediated system is comparable to that achieved with plasmids by electroporation; however, iBAC(cav) provides much larger cloning capacity. Although BGM can accommodate stable integration of even larger DNA fragments (Itaya et al., 2005, 2008; Iwata et al., 2013; Kaneko et al., 2005, 2009), previous integration methods required two landing pad sequences to determine the cloned region. The landing pad sequences, correctly aligned in an *E. coli* pBR322 plasmid were introduced into a pBR sequence locus of the *B. subtilis* chromosome prior to cloning (Kaneko et al., 2003; Itaya et al., 2000). Our iBAC(cav)-mediated system provides an alternative to other *B. subtilis* chromosome integration approaches. It does not require introduction of the landing pad sequences integrated at the target site before cloning and allows easy identification of transformants by fluorescent protein expression. Our system will facilitate rational design of synthetic biology devices in *B. subtilis*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.03.017.

Conflicts of interest

There are no conflicts of interest associated with this manuscript.

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