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The pIT5 Plasmid Series, an Improved Toolkit for Repeated Genome Integration in E. coli
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The pIT5 plasmid series, an improved toolkit for repeated genome integration in *E. coli*

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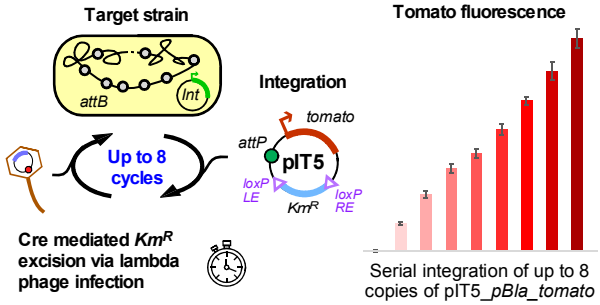
ABSTRACT

We describe a new set of tools for inserting DNA into the bacterial chromosome. The system uses site-specific recombination reactions carried out by bacteriophage integrases to integrate plasmids at up to eight phage attachment sites in *E. coli* MG1655. The introduction of mutant *loxP* sites in the integrating plasmids allows repeated removal of antibiotic resistance genes and other plasmid sequences without danger of inducing chromosomal rearrangements. The protocol for Cre-mediated antibiotic resistance gene removal is greatly simplified by introducing the Cre plasmid by phage infection.



In recent years, genome integration has gained traction for bacterial genetic engineering in biomedicine, biosensing and industrial biotechnology[1-3]. In contrast to plasmid-based gene expression, integration of DNA into the bacterial chromosome minimizes the metabolic burden on host cells, reduces cell-to-cell variation, and eliminates the need for continuous selection with antibiotics[4].

Common methods for chromosomal integration in bacteria include lambda RED based homologous recombineering[5], Tn7 based transposition[6], and phage integrase mediated recombination[7-9]. In addition, variations of these methods have been reported that combine CRISPR-Cas based targeting for improved efficiency[10].

In the CRIM system[7], the target cell is first transformed with a helper plasmid that expresses a bacteriophage integrase (Int) upon temperature induction. Next, a second transformation is performed to introduce the integration plasmid, containing a conditional replication origin (*R6Kγ*) and a phage attachment site (*attP*), permitting efficient and site-specific recombination at the cognate *attB* site on the bacterial chromosome. The major advantage of using phage attachment sites is that these sites have been selected by evolution to minimise effects on host fitness. In addition, as phage integrases have evolved to integrate large phage genomes (e.g lambda is ~50kb), this method can be used to integrate large DNA sequences into a bacterial chromosome.

Following the seminal work by Haldimann and Wanner on the CRIM system, we made a number of modifications to create the pOSIP vectors[8]. The pOSIP system combines the Int expressing helper plasmid and the integration plasmid into a single plasmid to provide a rapid integration protocol that is able to significantly speed up the integration. In addition, the pOSIP vectors also contain purpose-built transcriptional terminators flanking the multiple cloning site (MCS) to avoid effects on and by flanking chromosomal sequences, and a counterselection marker (*ccdB*) and a high copy number *pUC* replicon, both embedded in a MCS to facilitate cloning. Following integration, the majority of the pOSIP ancillary sequence, including the *int* gene, the replication origin, and the antibiotic resistance gene can be removed via FLP-mediated excision at the two engineered *FRT* sites.

Although the pOSIP vectors represent a significant advance over the original CRIM system, both systems remain limited by the number of available attachment sites, and the number of repeated integrations that can be performed. A major drawback of using FLP is that each round of recombination leaves an *FRT* scar in the chromosome. Thus, after a few rounds of genome integration, there will be multiple *FRT* scars scattered around the chromosome, preventing further application of this system due to undesired recombination among FRT scars[8].

Here, we address these shortfalls by presenting a new set of modular, highly scalable integration plasmids that we refer to as pIT5 (IT = Integrating, Terminator protected) (Figure 1). Like CRIM, the pIT5 system is a two-plasmid based system that consists of an Int-expressing helper plasmid and a

dedicated integration plasmid. This is because in general, pre-expression of Int in the target cells gives a higher integration efficiency than the OSIP-expressed Int. The pIT5 plasmids retain all the other key features of the pOSIP plasmids, including the terminator modules, the high copy *pUC* replicon and the *ccdB* counterselection marker (Figure 1a). In addition, the pIT5 integration plasmids are also capable of being "clonetegrated" into the bacterial chromosome by combining the cloning and integration in a single step, bypassing the need for an intermediate cloning strain[8, 11]. Finally, the modularity of pIT5 plasmids means that the choice of antibiotic resistance genes and *attP* sites can be easily reconfigured if necessary (Supplementary Figure 1).

To further increase the scope and scalability of the pIT5 system, 3 additional modifications were introduced. First of all, we have expanded the integration sites to include the *attB* sites of P2-like phages Wphi[12] and D145[13], increasing the number of available sites in *E. coli* MG1655 to 8 (Figure 1c).

Secondly, we replaced the *FRT* sites with a pair of mutant *loxP* sites known as *loxP LE* and *RE*[14]. The mutant *loxP* sites each carry a 5-base change in one of their termini that allows their recombination but which results in a *loxP* scar with mutations in both termini, rendering it insensitive to further Cre mediated recombination (Figure 1b).

However, Cre-*loxP* recombination is less efficient than that of FLP-*FRT*[15]. To improve the efficiency of the recombination protocol, we have also adapted a cosmid packaging method[16] , which allows phage to efficiently package and deliver plasmids, for the rapid delivery of a Cre expression plasmid by λ infection (Figure 1a and Methods in Supporting Information). The Cre cosmid was created by inserting a 224 bp lambda *cos* sequence into the backbone of the pE-FLP plasmid[8] and replacing the *flp* gene with *cre*. Cosmid transduction provides a highly efficient, rapid and cost-effective alternative to transformation. When prepared according to the protocol given here (Supplementary Figure 2), a small-scale (20 mL culture) induction of cosmid phages is enough for over 50,000 transduction reactions. After infection, recombinants are replica-plated on selective and nonselective LB plates (patch plating; Supplementary Figure 3) to screen for loss of antibiotic resistance, which can be confirmed by PCR (Supplementary Figures 4 and 5). The pE-Cre cosmid has a temperature-sensitive replication origin and is cured during the patch plating process.

To demonstrate the utility of the pIT5 system for chromosome integration, a *pBla* promoter driving a *tdTomato* reporter gene was integrated into each of the 8 phage attachment sites (Figure 2) in E4643, a derivative of *E. coli* MG1655 with *lacIZYA* deleted[17]. As shown in Figure 1c, MG1655 has two attachment sites for phage 186, located at 60.02 (site 1) and 69.28 (site 2) centisomes. Integration can occur at either of the two sites, although some integration bias towards 186 #1 site was observed. Simultaneous integration at both 186 attachment sites may also occur in some rare cases.

After Cre/*loxP* excision, all strains displayed stable tdTomato expression in the absence of antibiotic selection when streaked on a solid agar plate (Figure 2a). Quantitative liquid-based fluorescence assays revealed some slight variations in tomato expression levels among the 8 strains (Figure 2b and Supplementary Figure 6). Consistent with the literature[18], our results showed that integration at sites closer to the *E. coli* replication origin (*oriC*) displayed higher fluorescence intensities, with up to ~1.6 fold difference between the two extremes (Wphi vs. Phi80). The *tdTomato* reporter was also integrated into BL21(DE3), an *E. coli* B strain commonly used for protein overproduction. A similar tdTomato expression pattern was obtained in this strain, albeit with higher overall fluorescence intensities (Supplementary Figure 6), presumably because BL21(DE3) is deficient in Lon and OmpT proteases[19], leading to higher tdTomato levels. Of note, BL21(DE3) only carries a single phage 186 attachment site (site 2) and the lambda attachment site in BL21(DE3) is already occupied, being used to express T7 RNAP.

To demonstrate the ability of the pIT5 system for repeated integrations, we also made 7 independent strains with 2 copies of *pBla-tdTomato* and strains with increasing number of integrated tomato reporters from 3 copies to 8 copies (Figure 2b). The result showed a close to linear relationship between fluorescence intensities and the number of integrated *pBla-tdTomato* modules, with the cells harbouring 8 copies of tomato reporters giving ~7.5 fold higher fluorescence intensity than the average of the strains expressing a single copy of integrated reporter (Figure 2b).

To increase the scope of the pIT5 system, we also designed 4 self-contained, inducible gene expression modules that can be integrated into the bacterial chromosome. The salicylate, vanillic acid, and cumate inducible systems were designed based on the sensor reporters of Meyer et al[20], while the IPTG induction system was constructed by inserting a high affinity *lacOid* operator at +1 position of the *lacUV5* promoter (Supplementary Figure 1). When integrated, all 4 modules exhibited over 100-fold induction in the presence of inducers (Figure 2c). The response curves for each inducible system were fitted using a standard Hill function (Figure 2c). In our system, the salicylate induction system gave the highest fold induction and is most sensitive to the change in inducer concentration (Hill coefficient of 2.6), while the IPTG induction module was the most tightly controlled in the absence of an inducer and showed a more graded response.

While we used reporter gene assays to demonstrate the practicality of the pIT5 system, it is anticipated that the pIT5 system will have broad applications in synthetic biology and metabolic engineering. The pIT5 system allows further genome engineering for strains already harbouring *FRT* scars that are normally prevented from further FLP manipulation. In addition, due to the site-specific nature of the integration and the high integration efficiency, this system also provides a means of reconstruction of large metabolic pathways in *E. coli* using multiple attachment sites. The pIT5 system can also be deployed to other bacteria if suitable phage attachment sites are present. To this end, it has been shown that site-specific integration also works for *Salmonella* at phage 186 attachment site[8].

Supporting Information

Complete Methods, Supplementary Figures 1 to 6 and Supporting References. This material is available free of charge via the Internet at http:// pubs.acs.org.

Author Contributions

N.H., I.B.D., and K.E.S. designed research; N.H. performed research; N.H., I.B.D., and K.E.S. analysed data; and N.H., I.B.D., and K.E.S. wrote the paper.

Notes

The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1. The pIT5 chromosomal integration system. (a) An overview of the pIT5 integration protocol (see Supporting Information for details). This process can be iterated 8 times to generate strains with multiple insertions. (b) Recombination between *loxP LE* and *RE* generates a non-functional *loxP* scar. (c) Location of phage attachment sites in the *E. coli* MG1655 chromosome. The newly included attachment sites are in blue. The *oriC* (red) is the *E. coli* replication origin.

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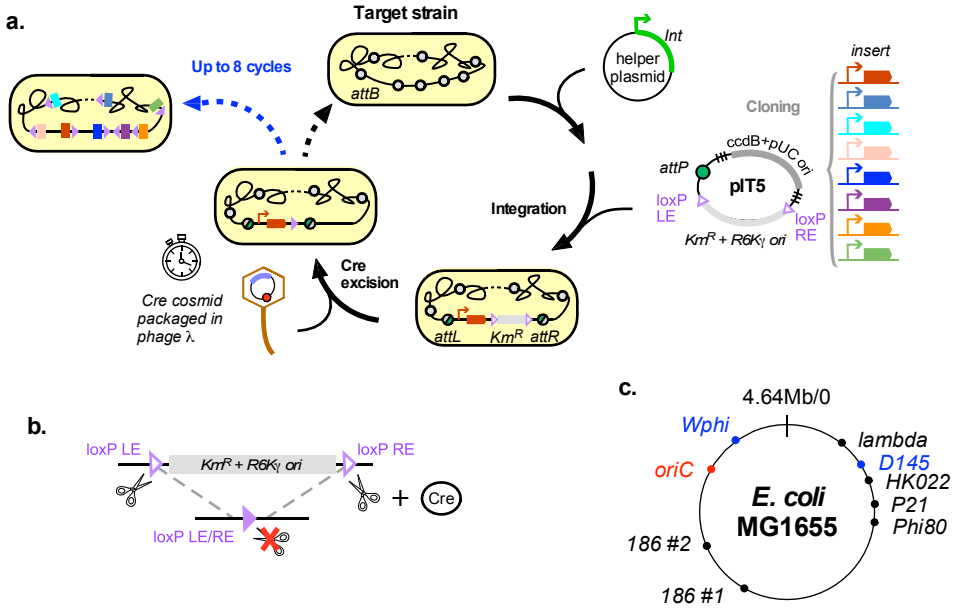

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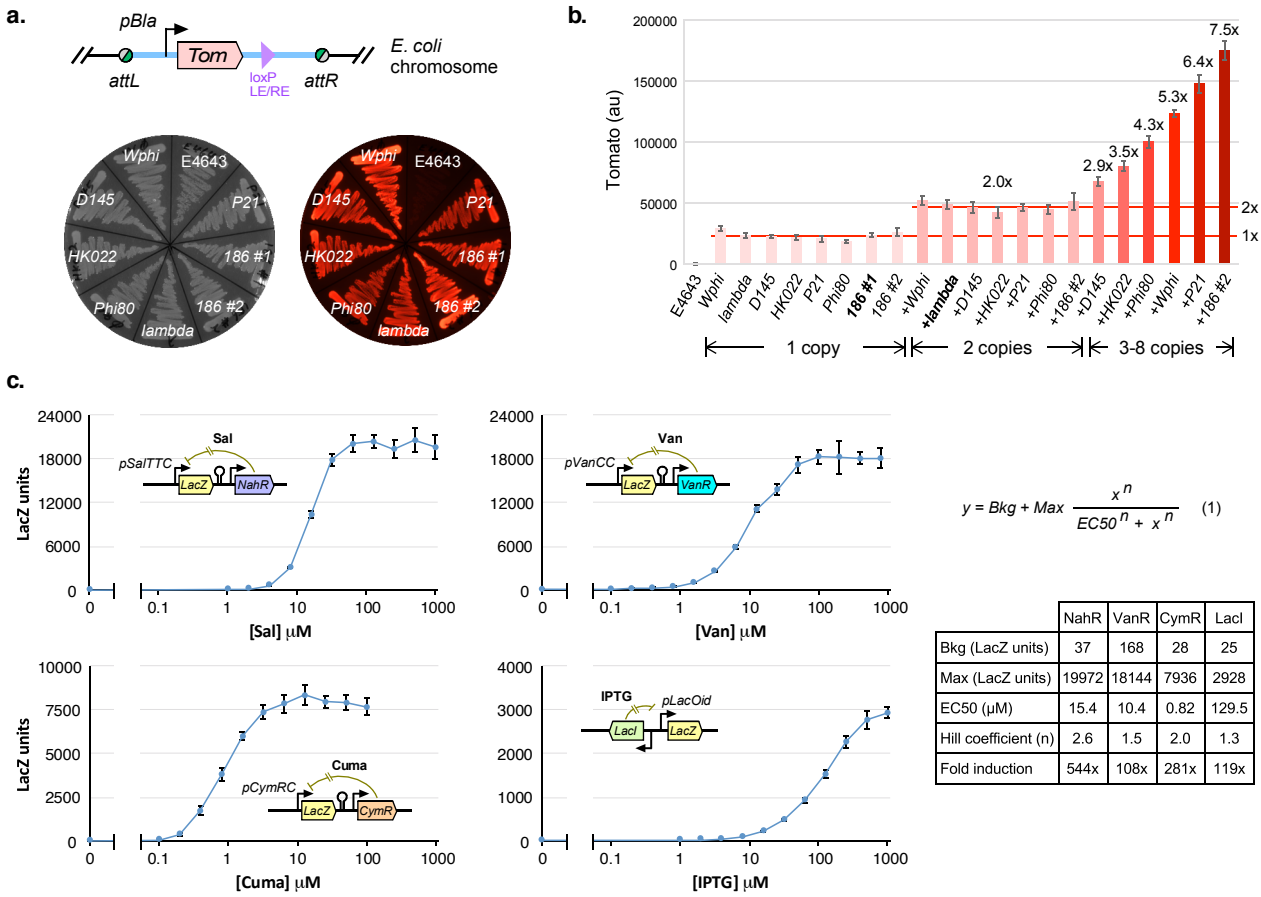

	NahR	VanR	CymR	LacI
Bkg (LacZ units)	37	168	28	25
Max (LacZ units)	19972	18144	7936	2928
EC50 (μM)	15.4	10.4	0.82	129.5
Hill coefficient (n)	2.6	1.5	2.0	1.3
Fold induction	544x	108x	281x	119x

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Supporting Information

The pIT5 plasmid series, an improved toolkit for repeated genome integration in *E. coli*

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METHODS

General Strains and Media

E4643 is a derivative of *E. coli* strain K-12 F⁻ MG1655 *rph*⁺ with the *lacIZYA* (MG1655:360,527–366,797) region removed[1]. BL21(DE3) is *E. coli* strain B F⁻ *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*⁺]$_{K\text{-}12}$.

The cosmid phage packaging strain is CY2120, which is a λ-resistant derivative of BW25113 *Δ9 λcI857 Sam7 Δcos::FRT*[2]. Strain GT7 is CY2120 transformed with the pE_Cre cosmid for rapid delivery of the Cre expressing plasmid by λ infection.

The unmodified pIT5 plasmids, which carry the *ccdB* counterselection marker and a high copy number *pUC* replicon, are propagated in DB3.1= *gyrA462 endA1 ∆(sr1-recA) mcrB mrr hsdS20 glnV44 (=supE44) ara14 galK2 lacY1 proA2 rpsL20 xyl5 leuB6 mtl1* (Invitrogen). The edited pIT5 plasmids replicate by their *R6Kγ* origin and are propagated in the Pir-expressing (*pir*⁺) strain E4644 = EC100D (Epicentre) F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d(*lacZΔM15*) Δ*lacX74 recA1 endA1 araD139 Δ(ara-leu)7697 galU galK* λ⁻ *rpsL Str*^{R} *nupG pir*⁺(DHFR). All other plasmids can be propagated in a standard cloning strain such as DH5α (New England Biolabs).

LB (1% Bacto-tryptone, 1% NaCl, and 0.5% yeast extract, pH 7.0) is used for routine growth of strains. M9MM (1 x M9 salts [6.78 g of NaH_2PO_4, 3 g of KH_2PO_4, 1 g NH_4Cl and 0.5 g NaCl/L H_2O], 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01 mM $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, and 0.4 % glycerol) is used for LacZ and liquid-based quantitative fluorescent assays. Antibiotics used were (μg/mL for selection of integrated copy/plasmid copies): Ap – ampicillin (-/100), Cm – chloramphenicol (20/30), Km – kanamycin (20/50), Sp – spectinomycin (20/50), Tc – tetracycline (3/20). Stock concentrations of all inducers were at 1M. Vanillic acid (Van; Sigma-Aldrich Cat. 94770) and cumic acid (Cuma; Sigma-Aldrich Cat. 268402) were dissolved in 100% ethanol. Sodium salicylate (Sal; VWR Chemicals Cat. 28065.230) and isopropyl-β-D-galactopyranoside (IPTG; BioVectra Cat. 1882) were dissolved in H_2O.

DNA Construction

DNA constructions used commercial DNA synthesis (Integrated DNA Technologies), restriction enzyme-based cloning, and isothermal Gibson assembly.

The detailed plasmid maps for all pIT5 plasmids can be found in Supplementary Figure 1. The nomenclature of the pIT5 plasmid series is as following: the first letter after “pIT5” denotes the resistance marker (K – Km, C – Cm, S – Sp, T – Tc) and the second letter indicates the phage attachment site (L – lambda, D – D145, H – HK022, T – P21, P – Phi80, O – 186, W – Wphi). For example, pIT5-KL carries the kanamycin resistance gene and the lambda *attP* site. For quantitative fluorescent assays, the *ccdB* and *pUC* replicon of pIT5 was replaced with a *tdTomato* reporter gene[3] expressed from a constitutive *pBla* promoter.

Four inducible pIT5 plasmids were made, each containing a different phage attachment site and a terminator-protected promoter that is responsive to one of the four regulators (CymR, NahR, VanR, and LacI) placed *in cis* under the control of a constitutive *pLacIq* promoter (Supplementary Figure 1b). For LacZ assays, a *lacZ* reporter gene was PCR amplified and cloned into the unique *XbaI* site downstream of the inducible promoters.

The lambda, HK022, P21, and Phi80 helper plasmids are from Haldimann and Wanner[4]. Additional helper plasmids (for 186, D145, and Wphi) were made using the same vector backbone (Supplementary Figure 1c).

The pE_Cre cosmid was made by inserting a 224 bp lambda *cos* sequence (lambda coordinates 48426-48502 and 1-147) into the *BstXI* site of the pE-FLP plasmid[5], and replacing the *flp* gene with *cre* (Supplementary Figure 1d).

The lambda, Phi80, and P21 helper plasmids are available from Addgene: pHelper_L (=pInt-ts, #66076), pHelper_P (=pAH123, #66077), and pHelper_T (=pAH121, #164901). All the other plasmids will be deposited in Addgene.

Integrating pIT5 Plasmids

The recipient strain is first transformed with a helper plasmid that expresses a phage integrase for the intended target site under control of the lambda CI*ts* repressor. The helper plasmid expresses Int at temperatures above 30 °C and carries a temperature-sensitive pSC101 *repA101* gene needed for its maintenance. For transformation and integration, these cells are grown at 30 °C in LB + Ap 100 µg/mL to OD_{600} ~0.4-0.6, transferred to 39 °C for 20 min, before being made competent and transformed with corresponding pIT5 plasmid by electroporation, with selection for integrants on LB + Km 20 µg/mL plates at 37 °C. Note that all standard pIT5 plasmids carry a *kmR* resistance gene; however, other antibiotic resistance genes can also be used. We have made available alternative pIT5 plasmids that carry chloramphenicol, spectinomycin, or tetracycline resistance genes (Supplementary Figure 1a).

Cre/*loxP* Excision and Patch Plating

An isolated colony from the integration plate is resuspended in 5 µL of LB, mixed with 5 µL of Cre expressing cosmid phage stock (~3 x 10^5 cosmid transducing units/mL), and incubated at 37 °C for 30mins for phage infection. One millilitre of fresh LB is then added, and the mixture is incubated at 30 °C for 6 hrs before 100 µL of 1/10 diluted culture is plated on LB + Ap 100 µg/mL plates and incubated at 30 °C overnight (Supplementary Figure 3a). The plasmid can replicate at 30 °C and expresses Cre constitutively from the P2 phage *pE* promoter. Fifty well-isolated colonies are then replica-picked onto LB and LB + Km 20 µg/mL plates, and incubated at 37 °C overnight (Supplementary Figure 6b). Colonies which grow on non-selection plates but not on Km plates are re-streaked onto a new LB plate, and incubated at 37 °C to ensure complete curing of the pE_Cre

cosmid, followed by PCR verification of the excision (see below). On average, the successful rate for Cre/*loxP* excision is ~30%, regardless of the attachment site. Thus, although 50 colonies have been patched in the example provided (Supplementary Figure 3b), patching 20 colonies is generally sufficient. Once the *loxP* flanked *KmR* gene and *R6Kγ* replicon is removed, the cells can be used for another round of integration.

Note that rather than using cosmid transduction, integrants can also be made competent and the pE_Cre plasmid transformed by standard transformation protocols, and selecting on LB + Ap 100 µg/mL plates at 30 °C overnight.

PCR Verification

Integrant and excision colonies are validated by PCR with a mix of 4 primers (Supplementary Figures 4 and 5). Successful integration produces 2 bands corresponding to the *attL* and *attR* regions. Excision of the antibiotic resistance module and the *R6Kγ* replicon by Cre/*loxP* removes the binding site for one of the primers, leading to the loss of the *attR* band. The primer sequences for all *att* sites are given in Supplementary Figure 4, and the expected PCR product sizes and the example gel photos are given in Supplementary Figure 5.

Cosmid Packaging and Titering

Cosmid packaging and titering was performed as previously described[6]. An overnight culture of GT7 was prepared by resuspending a colony from the streak plate in 3 mL LB with antibiotic (Ap, 100 µg/ml) and incubating at 30 °C overnight. 200 µL overnight culture was subcultured in 20 mL LB, and grown at 30 °C with shaking until OD_{600} ~0.4. Lambda phage packaging was induced via heat induction at 42 °C for 20 mins followed by incubation at 37 °C for 3 hrs. The culture was then transferred to a 50 mL tube and centrifuged at 3,220 ×g for 10 mins. The cell pellet was washed once with 2 mL lambda dilution buffer (10 mM $MgSO_4$, 20 mM Tris-HCl pH 7.5, 100 mM NaCl), resuspended again in 2 mL lambda dilution buffer and transferred into an Eppendorf tube for lysis with 20 ul of chloroform. After 15 min incubation at 37 °C with shaking, 5 mg of DNase I (GmbH) was added, and the mixture was incubated at 37 °C for a further 5 mins before centrifugation at 16,100 ×g for 5 mins. The supernatant, which contains pE_Cre cosmid packaged in lambda phages, was diluted 1:10 with lambda dilution buffer and stored at 4°C.

Cosmid-transducing particles were titered by a ‘drip’ assay in which 10µl of serial dilutions of the phage stock (1:10 in lambda dilution buffer) were mixed with equal volumes of early log phase (OD_{600} ~0.4) E4643, incubated at 37 °C for 30 mins before applying the whole mixture to one side of an LB + Ap 100 µg/mL plate and tipping the plate to produce a drip down the plate, with colonies counted after incubation at 30 °C overnight. A typical undiluted cosmid preparation gives ~3 x 10^6 colonies per mL.

Minimal Medium LacZ Assays

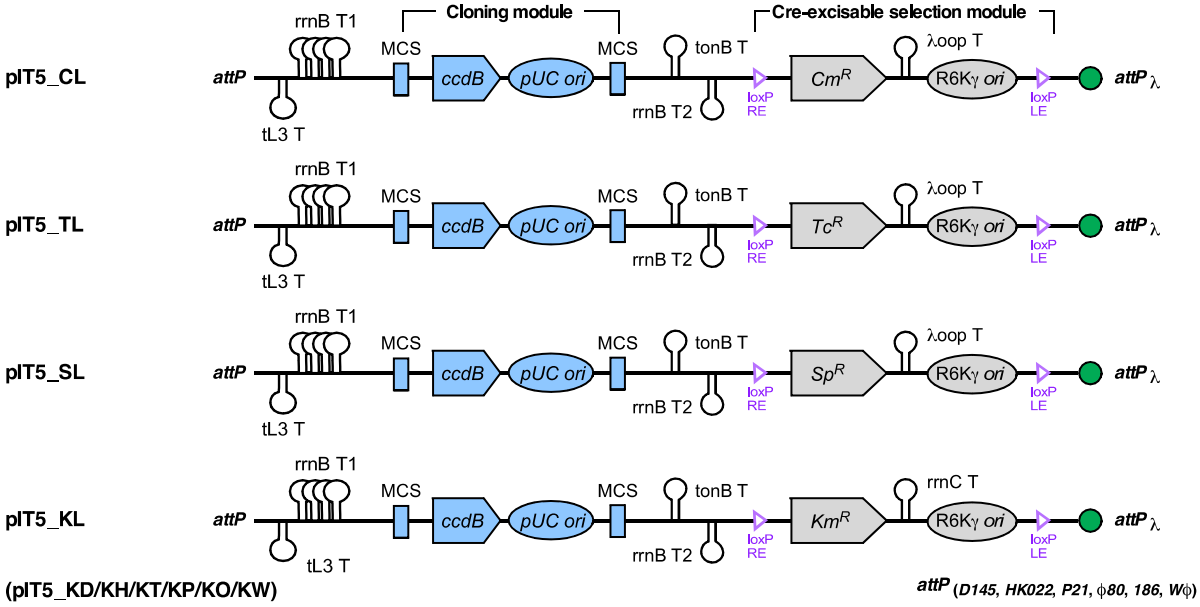
Microtiter plate-based LacZ assays were carried out as previously described[7]. Briefly, cultures were grown in M9MM in 96-well microtitre plates. After reaching mid-log phase, 5 µL of culture was added

to 195 µL pre-warmed assay buffer in fresh microtitre plate, consisting of 88 µL TZ8 buffer, 60 µL 4 mg/mL *o*-nitrophenyl-β-D galactoside (Sigma-Aldrich Cat. N1127) in TZ8, 2 µL 10 mg/mL chicken egg white lysozyme (Sigma-Aldrich Cat. L6876, 40,000 units/mg) in TZ8, 4 µL 20 mg/mL polymyxin B (Sigma-Aldrich Cat. P-4932) in H_2O, 6 µL H_2O and 35 µL M9MM. TZ8 buffer is 100 mM Tris-HCl pH 8.0, 1 mM $MgSO_4$, 10 mM KCl. The plate was incubated at 28°C in the plate reader, with OD_{414} readings taken every 2 min for 1 hr. Enzyme activity was determined as the slope of the line of best fit of OD_{414} versus time (readings with OD_{414}>2.5 were ignored). LacZ units were calculated as 200,000 × (OD_{414}/min)/(OD_{600} x 5 µL). Assays were performed in quadruplicate from independent colonies and repeated on at least two different days (n>=8).

Quantitative Fluorescent Assays

Bacterial cultures (100 µL) were grown in M9MM in 96-well microtitre plates. After reaching late-log phase, fluorescence intensities were measured using a VICTOR X5 plate reader (PerkinElmer) equipped with 544/15 nm excitation and 590/20 nm emission filters (PerkinElmer Cat. 1420-503 and 1420-544). The final tomato fluorescence units were expressed as (culture fluorescence – background fluorescence from culture media)/OD_{600}. Cell autofluorescence is negligible at these wavelengths. Assays were performed in quadruplicate from independent colonies and repeated on two different days (n=8).

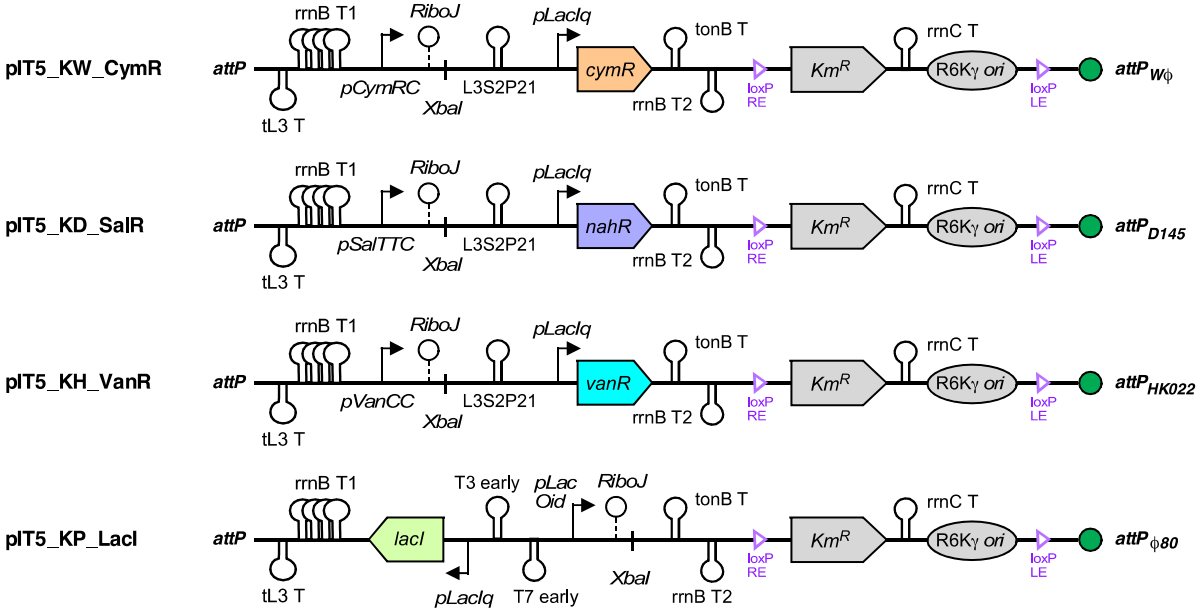
a. Basic pIT5 vectors



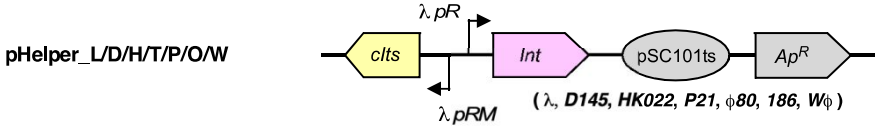
(pIT5_KD/KH/KT/KP/KO/KW)

attP (*D145, HK022, P21, ϕ80, 186, Wϕ*)

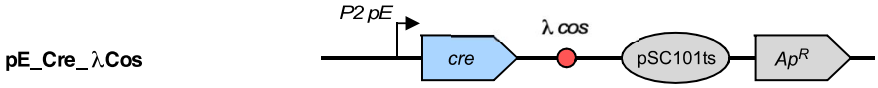
b. Inducible pIT5 vectors



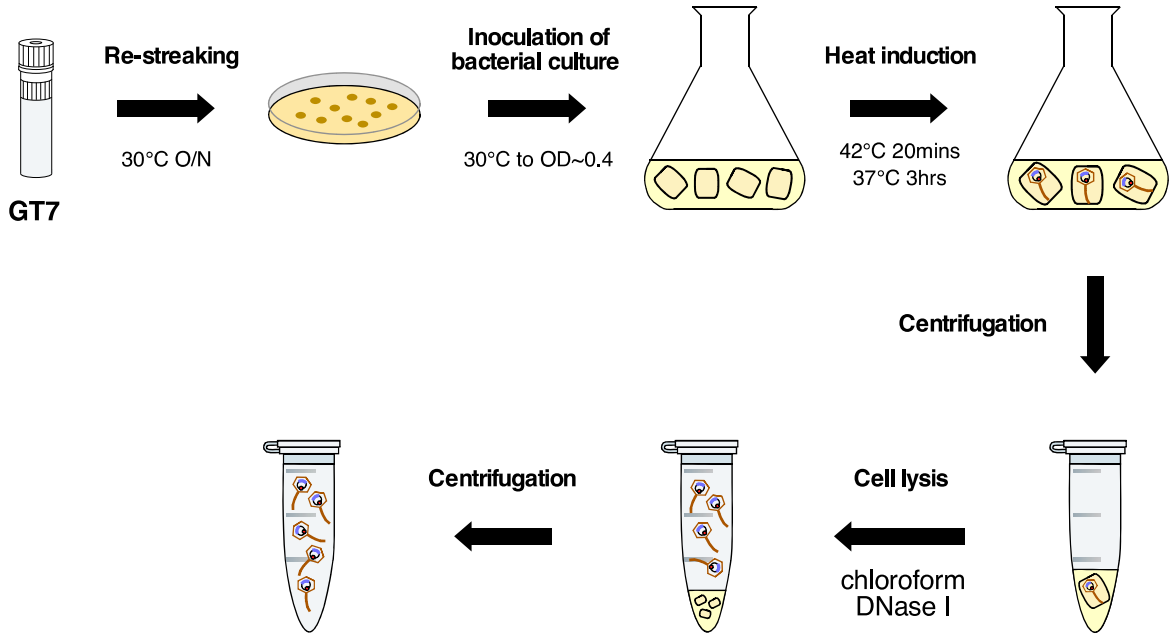
c. Helper plasmids



d. Cre expressing Cosmid

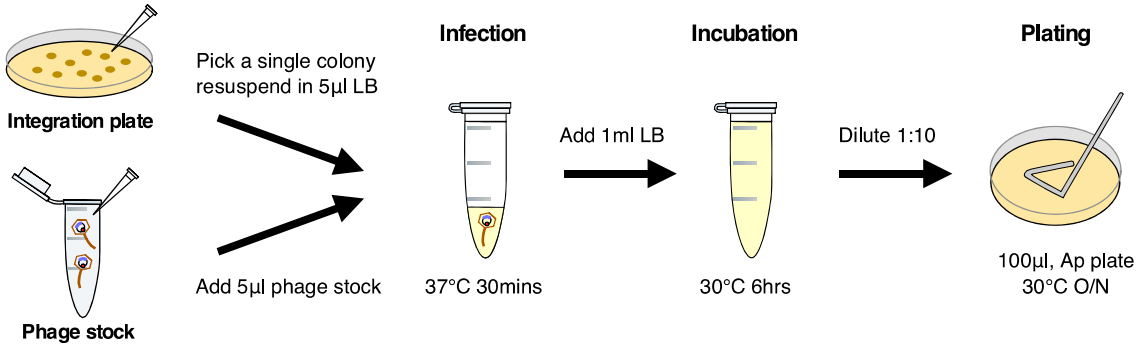


Supplementary Figure 1: Details of the plasmids used in this study.

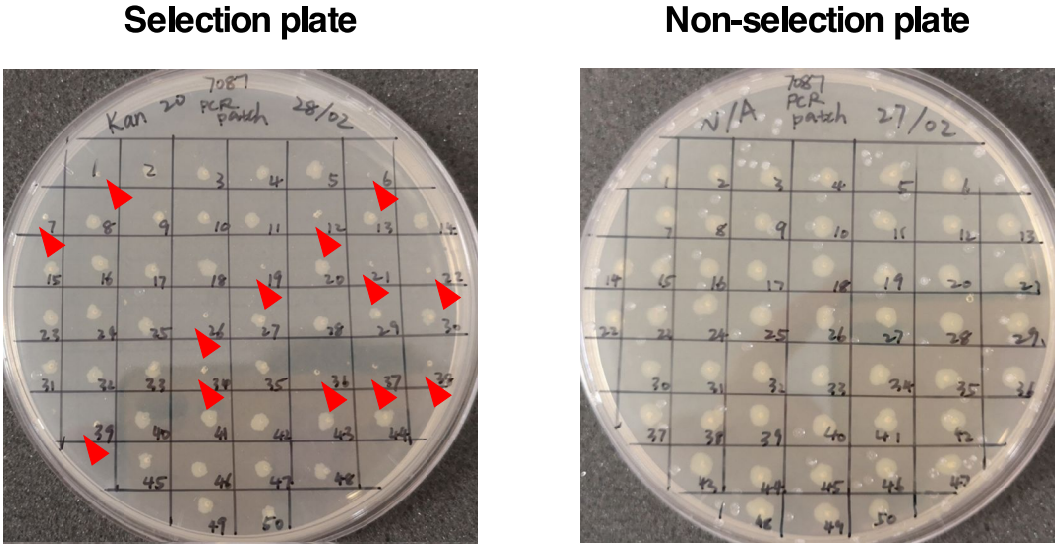


Supplementary Figure 2: The pE_Cre cosmid packaging protocol. See text for details.

a.

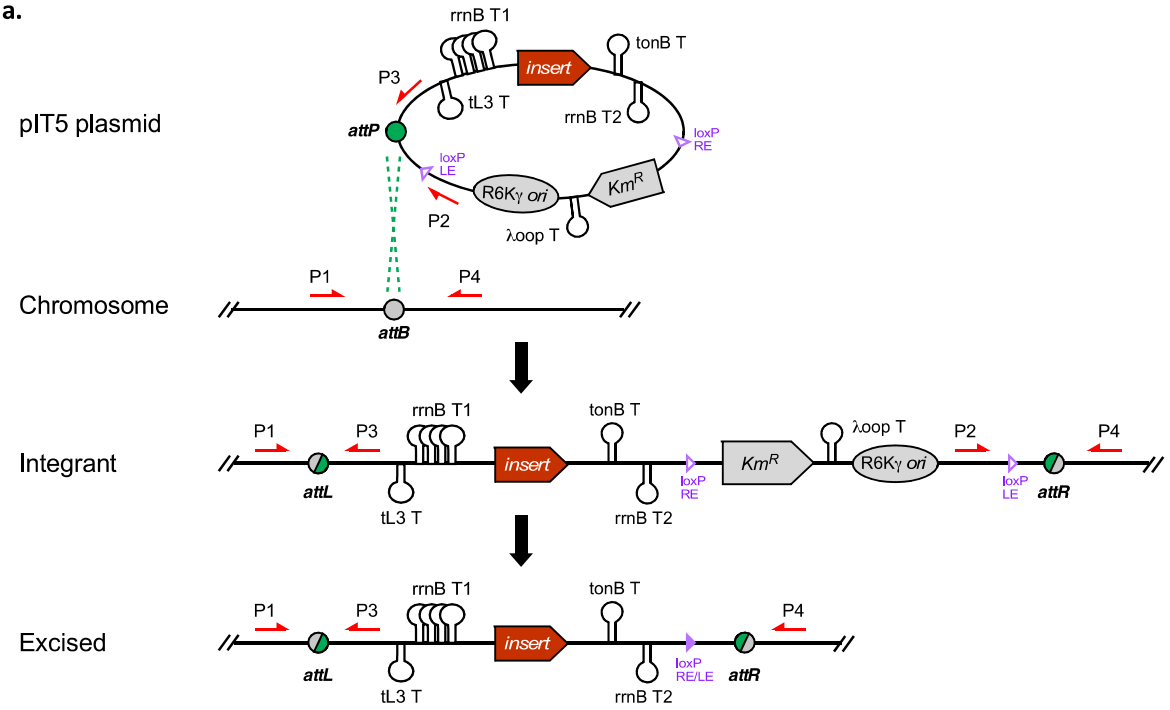


b.



Supplementary Figure 3: Rapid delivery of pE_Cre cosmid via lambda phage infection. (a) A flowchart for Cre/*loxP* excision protocol, see text for details. (b) An example of patch plating. In this example, 50 colonies were replica-plated onto LB + Km 20 µg/mL and plain LB plates, respectively. Thirteen of the 50 colonies tested were Km sensitive (i.e. 26%, indicated by red arrow heads).

a.

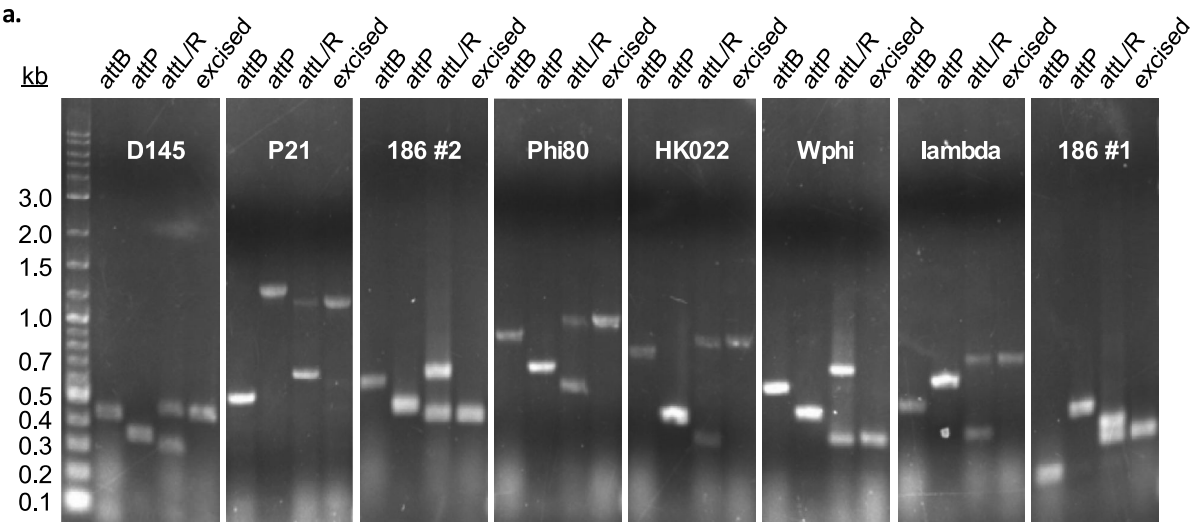


b.

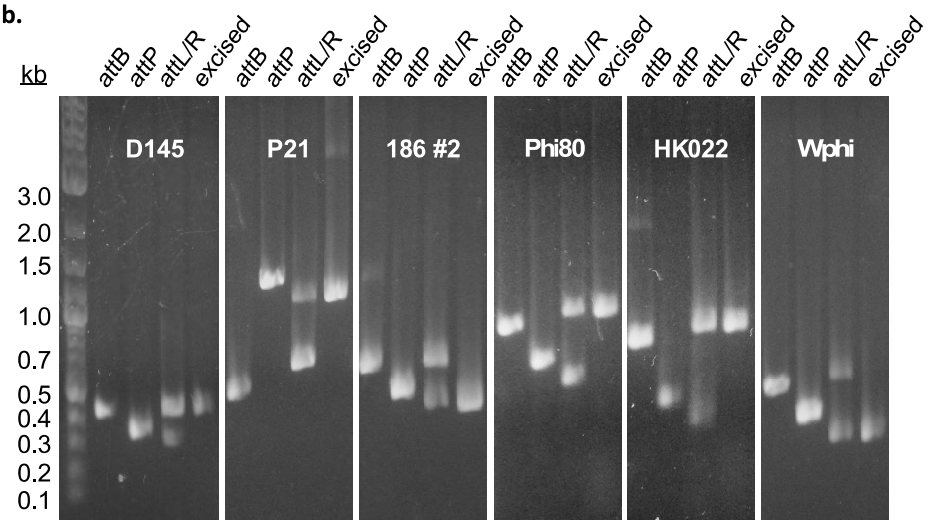
Integration site	P1	P2	P3	P4
D145	ACCAGTCACGA ACAAACTCAGC	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	AGCGTACAATA AAGGCTCCACG
P21 (MG1655) *	ATCGCCTGTAT GAACCTG	ACTTAACGGC TGACATGG	GGGAATTAATT CTTGAAGACG	TAGAACTACCA CCTGACC
186 #2	TCCGGAATGC CTGCATTG	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	CCCTGGAGCC AAAATATCC
Phi80	GATTTGAGCGA GCAACTGTACC	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	TTCGAGATCAG CAGGCGTAAC
HK022	GGAATCAATG CCTGAGTG	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	GGCATCAACA GCACATTC
Wphi	GTTGAAGCTATT GAGTAGTAGC	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	GAGATATCAGAT GTTGAATACCCG
Lambda	CGCCAAAAGC CAATGCCAGC	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	TCTGGTCTGG TAGCAATG
186 #1	CTCATTCGAAA CCACCCACCG	ACTTAACGGC TGACATGG	ACGAGTATCG AGATGGCA	GATCATCATGT TTATTGCGTGG
P21 (BL21) #	ATCGCCTGTAT GAACCTG	ACTTAACGGC TGACATGG	GGGAATTAATT CTTGAAGACG	CCCATATGGGC TCAAGTAAAGG

Supplementary Figure 4: Validation of pIT5 integrants by PCR. (a) Location of the primer binding sites. Colony PCR screening of single integrants uses a mixture of 4 primers listed in part (b). Primers P2 and P3 give an *attP* band using the pIT5 plasmid as a template, primers P1 and P4 produces an *attB* band with unintegrated parental strain, a single integrant produces two bands, one from P1 and P3 primer pairing (*attL*), and one from P2 and P4 primers (*attR*). Excision of the *loxP* flanked sequence removes the binding site for P2 primer, leading to the loss of the *attR* band. (b) Primer sequences. Two separate primer sets are designed for P21 for strains MG1655 and BL21(DE3) (marked with * and # respectively), as MG1655 carries a defective e14 prophage at the P21 site.

a.



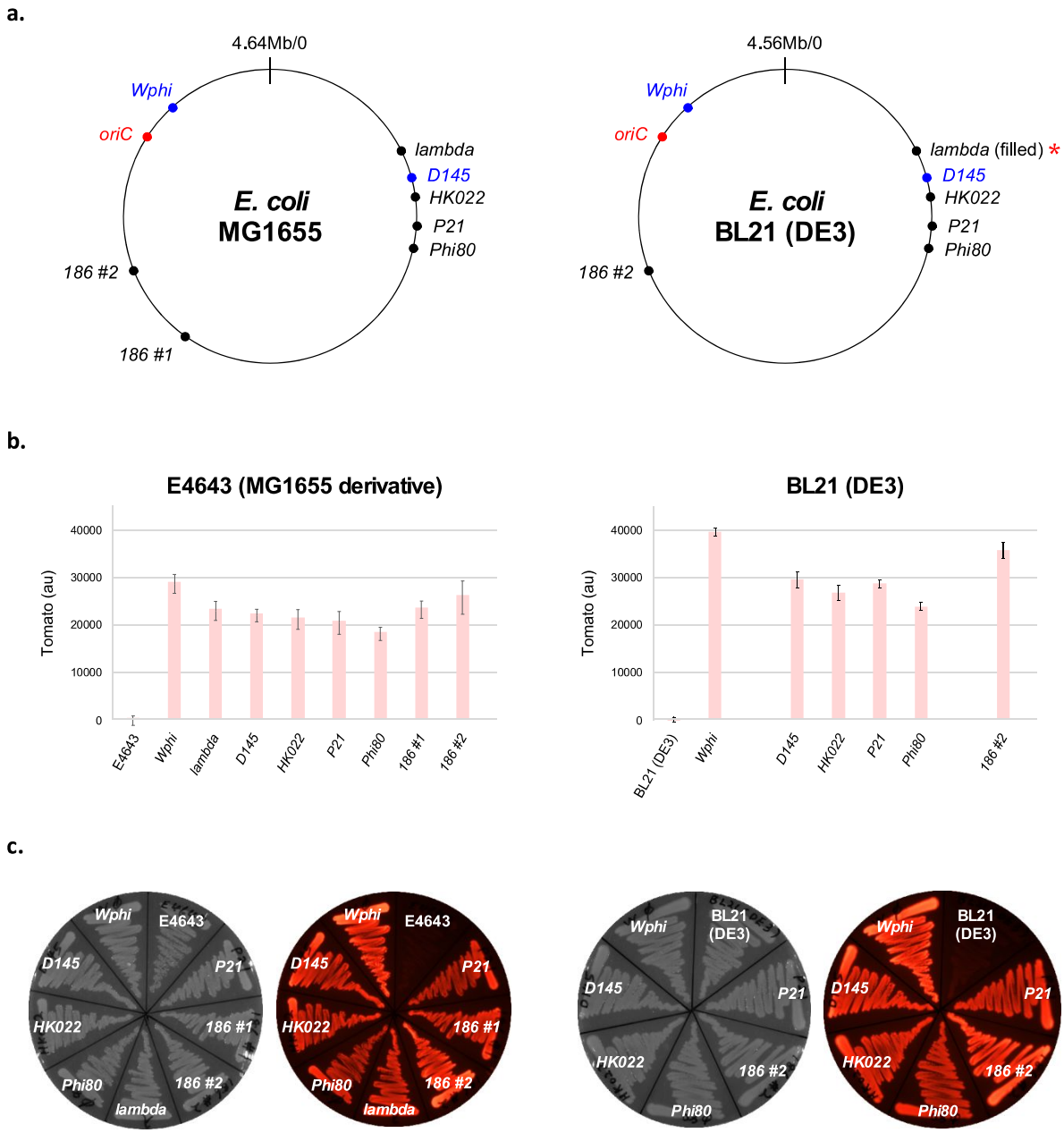
b.



c.

Integration site	attB (bp)	attP (bp)	attL/R (bp)	excised (bp)
D145	436	353	322, 467	467
P21 (MG1655) *	506	1226	622, 1110	1110
186 #2	601	476	429, 648	429
Phi80	869	639	968, 540	968
HK022	740	427	343, 824	824
Wphi	510	395	309, 596	309
Lambda	434	546	314, 666	666
186 #1	241	476	328, 389	328
P21 (BL21) #	478	1226	622, 1082	1082

Supplementary Figure 5: The expected PCR results. Example of colony PCR results for pIT5 integration and excision at each of the attachment site in MG1655 (a) and BL21(DE3) (b). (c) The expected PCR product sizes. The expected sizes are slightly different between MG1655 and BL21(DE3) at P21 site due to the use of different primer sequences (Supplementary Figure 4b).



Supplementary Figure 6: Comparison between MG1655 and BL21(DE3). (a) Location of phage attachment sites in the *E. coli* MG1655 (left) and BL21(DE3) (right). The relative positions of the attachment sites are similar except that in *E. coli* BL21(DE3), only a single phage 186 attachment site (site 2) is present, and the lambda attachment site is unavailable, as it is used to express T7 RNAP. The newly identified attachment sites are in blue the *E. coli* replication origin (*oriC*) is in red. (b) A *pBla-tdTomato* reporter was integrated into each of the available attachment sites in *E. coli* E4643 and BL21(DE3) and assayed by liquid based fluorescent assays. The expression pattern was very similar between the two strains except the overall fluorescence intensities is higher in BL21(DE3). Data are mean ± 95% confidence limits (Student's t), n = 8. (c) Each of the *pBla-tdTomato* stable integrants was also streaked on a solid agar plate in the absence of antibiotic selection and visualized with a fluorescent imager. Both the bright field image and the fluorescent image are shown.

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