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One-Step Cloning and Chromosomal Integration of DNA

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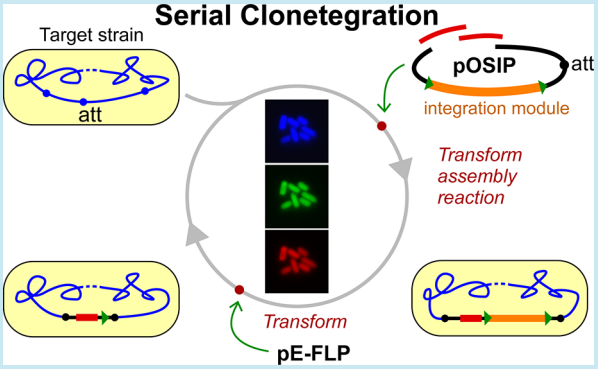
Supporting Information

ABSTRACT: We describe "clonetegration", a method for integrating DNA into prokaryotic chromosomes that approaches the simplicity of cloning DNA within extrachromosomal vectors. Compared to existing techniques, clonetegration drastically decreases the time and effort needed for integration of single or multiple DNA fragments. Additionally, clonetegration facilitates cloning and expression of genetic elements that are impossible to propagate within typical multicopy plasmids.



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Heterologous expression from a host cell chromosome, rather than plasmids, can reduce metabolic burdens and obviate the need for selectable markers in maintaining designer DNA sequences within an evolving bacterial population. Chromosomal integration of DNA is thus critical in synthetic biology, biotechnology, and metabolic engineering.[1,2] However, while existing techniques such as CRIM,[3] recombineering,[4−7] and Tn7-based integration[8,9] are useful and popular, they are also time-consuming. For example, CRIM requires multiple rounds of DNA transformation, while recombineering involves numerous steps and can take 1−2 weeks to complete.[4,6] We simplified the cloning of DNA sequences into prokaryotic chromosomes via three types of improvements to the well-known CRIM system, in which integration is mediated by bacteriophage integrases. The resulting method is quick and simple, enabling the bacterial chromosome to be used as a practical and powerful replacement to traditional plasmid vectors.

First, we developed new vectors that reduce the length and complexity of the integration protocol. In the CRIM system (Figure 1a, black trace), the target cell is initially transformed with a helper plasmid expressing a bacteriophage integrase (steps A1-2). A second transformation (step 4) introduces the CRIM plasmid containing the cloned insert and the "*attP*" site, a sequence of DNA necessary for site-specific recombination at the corresponding "*attB*" site on the bacterial chromosome. To test whether efficient integration could be achieved with a single transformation, we combined the integrase-expressing and the integration plasmids into a single vector (Figure 1b). The resulting hybrid vector, One-Step Integration Plasmid (pOSIP), integrates at high efficiency, thus bypassing two protocol steps typically requiring overnight incubation (Figure 1a, left-most green trace).

Restriction sites at key locations enable easy modification of the pOSIP backbone. We took advantage of this architecture to quickly construct five plasmid variants expressing a tyrosine integrase from either phage 186, HK022, lambda, phi80, or P21 or the serine integrase from phiC31. To our knowledge, our integration system is the first to use the efficient integrase from phage 186. pOSIP further contains two useful features not present in the CRIM plasmids (Figure 1b). First, forward and reverse transcription terminators flank the Multiple Cloning Site (MCS) to insulate integrated OSIP plasmids from transcription in chromosomal regions flanking the integration site and vice versa (Supplementary Figure 1). Second, a counter-selectable cassette containing the toxic *ccdB* gene in the middle of the MCS facilitates cloning by eliminating cells transformed only with the parental (unmodified) OSIP vector.[10]

Second, we sought to further simplify the integration protocol. Integration efficiency of most pOSIPs is sufficiently high to produce large numbers of integrants from pOSIP-insert cloning mixtures, instead of purified (miniprepped) plasmid DNA. This new one-step procedure, which we call "clonetegration" (simultaneous cloning and integration), effectively treats chromosomes as large cloning vectors. Like standard plasmid cloning, clonetegration requires the initial assembly of one or multiple DNA fragments into pOSIP using one's favorite cloning technique, such as Gibson assembly,[11] Clonetech In-Fusion(R),[12] or traditional restriction digest and ligation. The

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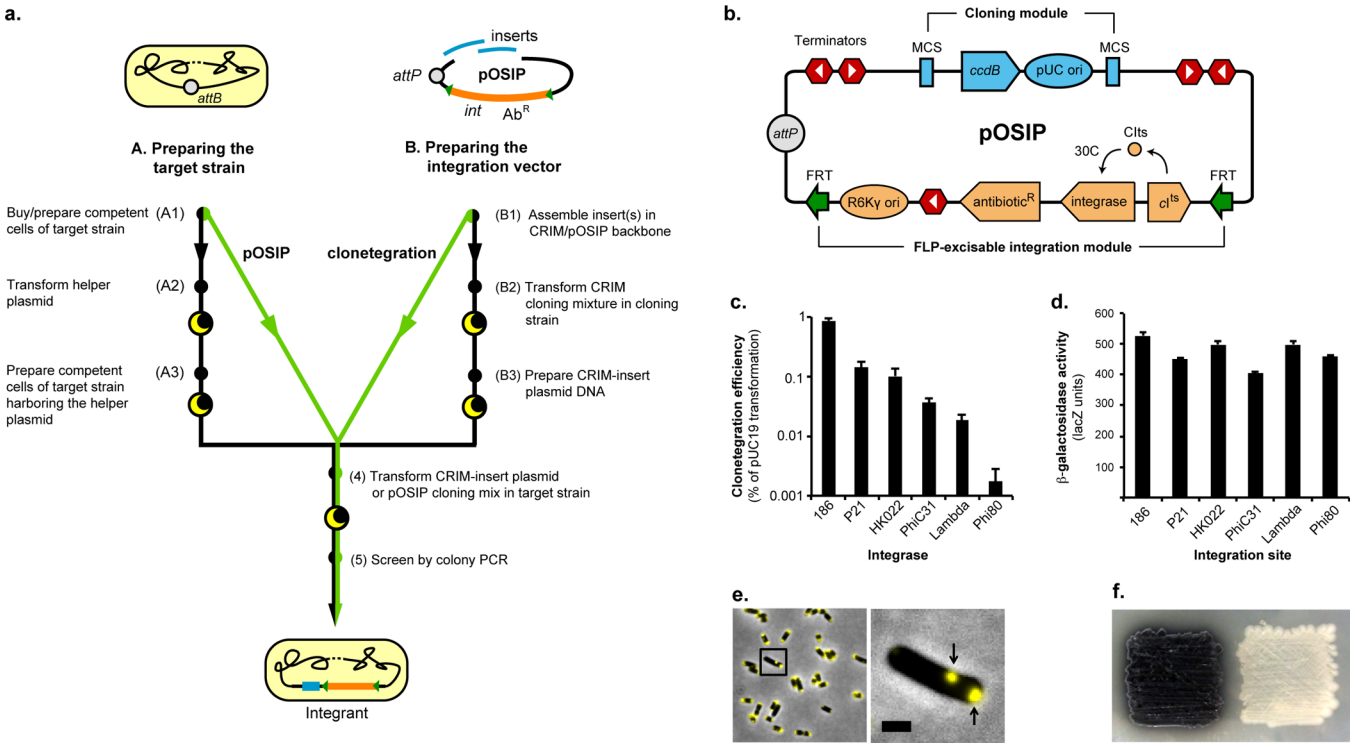

Figure 1. One step cloning and integration ("clonetegration"). (a) Clonetegration with pOSIP (green trace) requires fewer steps and is faster compared to chromosomal integration by CRIM (black trace). (b) pOSIP is composed of two functional modules. Cloning results in the replacement of the "insert module" with the DNA fragment to be integrated. The heat-inducible "integration module" allows expression of the integrase and selection of integrants by antibiotic resistance. The integration module can be removed postintegration by expressing FLP recombinase. (c) Clonetegration enables integration of a ~4.6-kb lacZ cassette at high efficiency (mean ± SEM; n = 3 independent cultures per integrase/integration site). (d) Clonetegration of *lacZ* cassette into MG1655 Δ*lacIZYA* at different chromosomal loci results in qualitatively similar β-galactosidase activity (mean ± SEM; n = 4 independent cultures per integration site). (e,f) Sequences that cannot be cloned on medium-copy number plasmids can be assembled directly in the chromosome. Clonetegration of a *tsr-venus* expression cassette results in bright, membrane-localized fluorescent foci in NEB 5-alpha (e, arrows). Scale bar = 1 μm. Clonetegration of a *vioE-ABDE* expression cassette results in dark pigmentation (f, left), seen here on bacterial cells streaked on a square area on an LB-plate; in comparison, the parental strain produces a beige color (f, right).

resulting cloning mixtures are then directly transformed into chemically or electro-competent cells (Figure 1a, right-most green trace). Integration of properly assembled pOSIP-insert molecules occurs during post-transformation outgrowth. Verification of integration can be performed by colony PCR using sets of primers we optimized from the original CRIM set (Supplementary Tables 1, 2; Supplementary Figure 1).

We quantified the efficiency of clonetegration using a 4.6kb lacZ-expression cassette assembled into each of our six pOSIP plasmids (Figure 1c). All trials gave *lacZ* positive integrants in MG1655 Δ*lacIZYA* (Supplementary Figure 2). Expression from the integrated constructs is similar across all integration sites, suggesting that surrounding chromosomal sequences had minimal influence on our terminator protected insert (Figure 1d). The phage 186 integrase performed best, with a clonetegration efficiency ~500-fold better than that of the poorest-performing integrase, Phi80. Phage 186 integrase might therefore be suggested for the most challenging sequences targeted for chromosomal integration. Of note, we observed that reactions catalyzed by phage 186 integrase result in integration not only within the *tRNIleY* gene[13] but sometimes into an alternative site within the *tRNIleX* gene (39% probability; n = 72 colonies; Supplementary Figure 3). No colonies harbored integrants at both chromosomal loci. We designed new PCR validation primers for users needing to distinguish between these two integration locations (Supplementary Tables 1, 2). Finally, we successfully performed clonetegration in *Salmonella typhimurium*, a bacterium receiving renewed interested in synthetic biology,[14] demonstrating that clonetegration is generalizable to other prokaryotes (Supplementary Figure 4).

In the CRIM protocol, one must first clone target sequences in a multicopy plasmid prior to integration (Figure 1a, steps B1−3). By removing this plasmid intermediate step, clonetegration can also enable integration of sequences that are lethal when present at multiple copies within a cell. We encountered such toxic sequences ourselves while working on separate research projects. In one such endeavor, we wanted to use the protein fusion Tsr-Venus as a sensitive, single-molecule fluorescent reporter of gene expression.[15] However, we were unable to clone a *tsr-venus* expression cassette in pOSIP or in the popular pBR322 vector (Methods in Supporting Information). In contrast, clonetegration of our toxic Tsr-Venus expression cassette into NEB 5-alpha was successful, resulting in cells with the expected pattern of yellow fluorescence foci at polar and midcell membrane positions[15] (Figure 1e). As a second example, we were exploring the construction of colorimetric reporters using a large (~6 kb) four-gene dark green pigment-producing cassette (*ABDE*) from the *vioE* operon. While we were unable to clone a *vioE-ABDE* expression cassette in pOSIP using standard methods,

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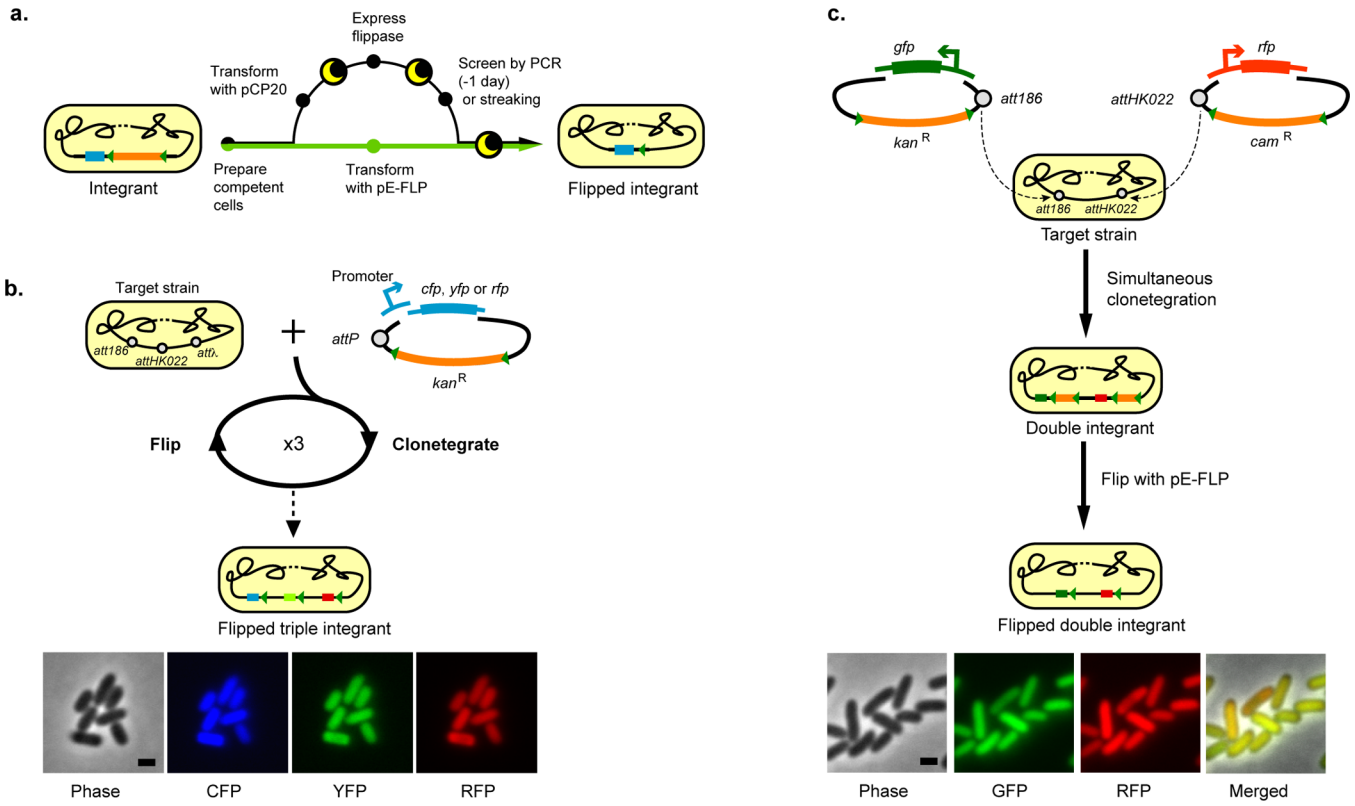

Figure 2. Serial and parallel clonetegration. (a) The pE-FLP plasmid enables simple one-step excision of the integration module from pOSIP integrants (green trace). In contrast, excision with pCP20 requires multiple steps performed over 2−3 days (black trace). (b) Clonetegration is scalable to multiple integration sites. Three fluorescent protein (FP) expression cassettes were clonetegrated in successive rounds into NEB 5-alpha. The integration module and kanamycin resistance marker, flanked by FRT sites, were excised using pE-FLP after each round of clonetegration. The resulting bacterial strain expresses mTurquoise CFP (blue), Venus YFP (green), and mRuby2 RFP (red) from phages 186, HK022, and lambda integration sites, respectively. A phase contrast image is shown for reference. Scale bar = 1 μm. (c) Clonetegration can be performed concurrently with two independent DNA fragments. A Clover GFP (green) expression cassette was clonetegrated into phage 186 integration site in NEB 5-alpha using a pOSIP encoding a kanamycin resistance marker (kan^R). In parallel, an mRuby2 RFP (red) expression cassette was clonetegrated into phage HK022 integration site with a chloramphenicol resistance marker (cam^R). The integration modules and antibiotic resistance markers of both pOSIP vectors, flanked by FRT sites, were removed in a single step using pE-FLP. A phase contrast image and a merged image from all channels are shown for reference. Scale bar = 1 μm.

clonetegration into NEB 5-alpha successfully produced dark green colonies (Figure 1f).

Third, we aimed to improve the ease of constructing strains with multiple integrated sequences. Like CRIM, our system results in integration of the entire pOSIP plasmid. The ability to remove the integration module, including the antibiotic marker (e.g., kan^R), would make our system scalable by allowing integration of additional kanamycin-resistant pOSIPs into other chromosomal loci. To enable construction of such marker-less strains,[16] we engineered all pOSIPs with FRT sites flanking the integration module (Figure 1b), allowing excision of these sequences following expression of the FLP recombinase.[17]

To test FLP-mediated excision, we transformed chromosomal integrants with the popular FLP-expressing plasmid pCP20.[18] We followed the standard pCP20 protocol (Figure 2a, black trace). First, we transformed the target strain with pCP20 and grew cells overnight at 30 °C on ampicillin plates. Next, we restreaked individual colonies and induced FLP expression by overnight growth at 37 °C; like our integrase expression cassette, FLP expression in pCP20 is under control of the thermosensitive transcription factor lambda CIts. Growth is performed on LB without antibiotics because pCP20, which contains a temperature-sensitive replicon, is not propagated at 37 °C. Lastly, we screened the colonies on the resulting LB plates for FLP-mediated excision by PCR and by streaking for loss of antibiotic resistance.

The standard protocol produced excision of pOSIP integration modules in less than 5% of pCP20-transformed cells (Supplementary Figure 5); CIts-controlled modules, present on both pOSIP and pCP20, may be interfering with one another. We thus developed a new FLP expression system that relies neither on temperature induction nor lambda CIts. Because the pCP20 protocol is time-consuming, we also sought to design a new procedure so that excision can be performed in a single day, rather than the 2 or 3 days required when using pCP20.

We achieved both goals by driving FLP expression via pE, a strong constitutive promoter from phage P2[19] (Supplementary Figures 5 and 6; Supplementary Table 3). In contrast to pCP20, pE-FLP successfully catalyzed excision of the integration and propagation modules from integrants with 100% efficiency. Also, pE-FLP is active immediately after transformation, thus avoiding the overnight heat-induction step of pCP20. Given the high efficiency of pE-FLP, screening colonies for excision of the antibiotic marker and neighboring sequences is typically not necessary. Because pE-FLP retains the temperature-sensitive replicon of pCP20, blocking postflip-

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ping propagation of the plasmid is achieved by simply growing integrants at 37 °C. In short, pE-FLP reduced the excision protocol to a mere transformation step.

We confirmed that our overall protocol, including pE-FLP-mediated vector backbone excision, does indeed enable scalable clonetegration: we successfully integrated expression cassettes for cyan, yellow, and red fluorescent proteins at separate chromosomal loci via three separate rounds of clonetegration (Figure 2b, Supplementary Figure 7). Each time, integration was mediated by a pOSIP vector encoding a kanamycin resistance marker. As needed to allow reuse of the kanamycin resistance marker, we excised the integration module from integrants via flippase expression from pE-FLP. Similarly, we serially clonetegrated four 4.6-kb lacZ expression cassettes, giving 18.4 kb of integrated sequences (Supplementary Figure 8).

In the protocols described above, each expression cassette must be integrated via a separate round of clonetegration. We sought to increase the rate at which multicassette integrants could be constructed. Since each OSIP plasmid expresses its own integrase, cotransformation should allow integration at multiple chromosomal sites simultaneously. Indeed, dual integration of two fluorescent-protein-expressing cassettes was successful in standard commercially available chemically competent cells, without requiring further optimization of the standard protocol (Figure 2c, Supplementary Figure 7b).

While all clonetegration experiments described here were successful, further improvements to clonetegration efficiency could be useful for certain applications such as library construction or integration of very large sequences. Such improvements might be obtained by increasing the expression level of our pOSIP integrases, screening new phage integrases for higher integration efficiency, or transforming DNA using electroporation. As with all cloning procedures, best laboratory practice is to sequence the region of interest from the final clonetegrated strain to ensure that no errors have been introduced.

In part because of the laborious nature of current procedures to integrate DNA fragments into prokaryotic chromosomes, plasmids remain the most popular expression vectors. The technique we describe here, clonetegration using pOSIP, is simple and rapid, can facilitate cloning of toxic sequences, and is amenable to automation. We have integrated up to four expression cassettes at independent chromosomal loci in successive rounds and two cassettes in the same round. We anticipate that clonetegration with pOSIP will become a valuable technique facilitating genetic engineering with difficult-to-clone sequences and rapid construction of synthetic biological systems.

■ ASSOCIATED CONTENT

Supporting Information

Complete Methods, Supplementary Tables 1 to 3, Supplementary Figures 1 to 8 and Supporting References. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

[⊥]These authors contributed equally to this work. F.S.-P., L.C., I.B.D., and K.E.S. designed research; F.S.-P., L.C., D.G.P., I.B.D., and K.E.S. performed research; F.S.-P., L.C., I.B.D., D.E., and K.E.S. analyzed data; and F.S.-P., L.C., I.B.D., D.E., and K.E.S. wrote the paper.

Notes

The authors declare no competing financial interest.

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