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The ClosTron: Mutagenesis in *Clostridium* refined and streamlined

John T. Heap, Sarah A. Kuehne, Muhammad Ehsaan, Stephen T. Cartman, Clare M. Cooksley, Jamie C. Scott, Nigel P. Minton *

BBSRC Sustainable Bioenergy Centre, School of Molecular Medical Sciences, Centre for Biomolecular Sciences, The University of Nottingham, University Park, Nottingham, NG7 2RD, UK

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ABSTRACT

The recent development of the ClosTron Group II intron directed mutagenesis tool for *Clostridium* has advanced genetics in this genus, and here we present several significant improvements. We have shown how marker re-cycling can be used to construct strains with multiple mutations, demonstrated using FLP/FRT in *Clostridium acetobutylicum*; tested the capacity of the system for the delivery of transgenes to the chromosome of *Clostridium sporogenes*, which proved feasible for 1.0 kbp transgenes in addition to a marker; and extended the host range of the system, constructing mutants in *Clostridium beijerinckii* and, for the first time, in a B1/NAP1/027 'epidemic' strain of *Clostridium difficile*. Automated intron design bioinformatics are now available free-of-charge at our website http://clostron.com; the out-sourced construction of re-targeted intron plasmids has become cost-effective as well as rapid; and the combination of constitutive intron expression with direct selection for intron insertions has made mutant isolation trivial. These developments mean mutants can now be constructed with very little time and effort for the researcher. Those who prefer to construct plasmids in-house are no longer reliant on a commercial kit, as a mixture of two new plasmids provides unlimited template for intron re-targeting by Splicing by Overlap Extension (SOE) PCR. The new ClosTron plasmids also offer blue–white screening and other options for identification of recombinant plasmids. The improved ClosTron system supersedes the prototype plasmid pMTL007 and the original method, and exploits the potential of Group II introns more fully.

1. Introduction

The eubacterial genus *Clostridium* encompasses organisms of both medical and industrial importance. These include the pathogens *Clostridium tetani*, *Clostridium perfringens*, *Clostridium botulinum* (Hatheway, 1990) and *Clostridium difficile* (Brazier, 2008); and the solvent-producers *Clostridium acetobutylicum* (Papoutsakis, 2008) and *Clostridium thermocellum* (Lynd et al., 2005). Research both to counter pathogens and exploit useful strains has been hindered by a history of limited genetic methods, especially for mutagenesis. A modest number of directed mutants of *Clostridium* strains had been reported prior to 2007, almost all constructed by homologous recombination (summarized in Heap et al., 2009a). Of these, most were either 'single-crossover' strains in which an integrated plasmid serves as an insertional mutagen, or 'double crossover' strains in which an introduced alternative allele is exchanged with the wild-type allele. The former type of mutant is inherently unstable, while the latter type has proven difficult to isolate, partly because the use of negative selection markers, commonly relied upon to facilitate allele exchange in other organisms, has not been established in *Clostridium*.

To address the need for reliable mutagenesis, we recently developed the ClosTron (Heap et al., 2007), a Group II intron directed mutagenesis system for *Clostridium*. Bacterial Group II introns are a relatively newly-characterized type of mobile element (Lambowitz and Zimmerly, 2004) which can be used for the directed construction of stable mutants thanks to two key properties. Firstly, intron target specificity is determined mainly by base-pairing between the target site DNA and intron RNA, which can be rationally modified. Secondly, intron mobility requires the presence of an intron-encoded protein (IEP), which can be provided transiently during mutagenesis and subsequently removed to ensure the stability of the strain; a strategy analagous to the stabilization of mini-transposon insertions by removal of the transposase. Like most other Group II intron mutagenesis systems, the ClosTron plasmid pMTL007 contains a mini-intron derivative of the Ll.LtrB intron from *Lactococcus lactis*, ideal for rational intron re-targeting and loss of the IEP gene *ltrA* (Karberg et al., 2001). Other elements of pMTL007 facilitate conjugal transfer of the plasmid into *Clostridium* spp., its subsequent replication and maintenance, expression of the intron and IEP, and specific selection of clones containing an insertion.

The ClosTron system has transformed our research, and we have distributed pMTL007 to many other laboratories, some of which have already begun to publish studies using ClosTron mutants (Emerson et al., 2009; Kirby et al., 2009; Twine et al., 2009; Underwood et al., 2009). Despite its usefulness, the system and procedures we described

* Corresponding author. Tel.: +44 115 84 67458; fax: +44 115 82 32120.
E-mail addresses: john.heap@nottingham.ac.uk (J.T. Heap), nigel.minton@nottingham.ac.uk (N.P. Minton).

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previously did not fully exploit the potential of Group II introns, and were limited in the range of host strains and applications for which they were suitable. Here we addressed these limitations and also thoroughly optimized the mutagenesis procedure. The result is a notably faster, less labor-intensive, more flexible, and more broadly-applicable approach to directed mutagenesis in *Clostridium*.

2. Materials and methods

Details of plasmid construction including oligonucleotides are provided in the Supplementary material online.

2.1. Bacterial strains, growth and plasmid transfer

Bacterial strains, culture conditions and plasmid transfer procedures were as previously described (Heap et al., 2009b).

Sporulation of *Clostridium difficile* R20291 was measured by enumerating the titre of heat-resistant colony-forming units (CFU) formed in BHIS broth (brain heart infusion supplemented with 5 g/l yeast extract and 1 g/l L-cysteine, (Sorg and Sonenshein, 2008)). Sporulation cultures were inoculated with 1% their volume of an exponential phase overnight starter culture, then incubated for 5 days at 37 °C to allow spore development. Samples were heated to 60 for 30 min to inactivate vegetative cells, then serially-diluted and plated on BHIS agar supplemented with 0.1% taurocholate to enumerate heat-resistant CFU ml^{-1}.

Sporulation of *Clostridium beijerinckii* NCIMB 8052 was measured by enumerating the titre of heat-resistant colony-forming units formed in CBM broth (O'Brien and Morris, 1971) supplemented to final concentrations of 50 g/l glucose and 5 g/l $CaCO_3$. Sporulation cultures were inoculated with 2% their volume of an exponential phase overnight starter culture, then incubated for 3 days at 37 °C to allow spore development. Samples were heated to 70 for 15 min to inactivate vegetative cells, then serially-diluted and plated on CGM agar (Hartmanis and Gatenbeck, 1984) to enumerate heat-resistant CFU ml^{-1}.

2.2. Conventional intron re-targeting

We constructed two plasmids (pMTL20IT1 and pMTL20IT2) which can be mixed together and then used as a template for SOE PCR, allowing intron re-targeting in one step. Generally, when SOE PCR is used to introduce nucleotide changes at the region of overlap between two PCR products, the two partial-length PCR amplification reactions must be performed separately and the purified products subsequently combined in a second step using the outermost primers (Ho et al., 1989). Failure to separate the amplification reactions in this way results in a mixture of PCR products from which the desired species is not necessarily easily purified. The Sigma-Aldrich TargeTron kit includes a special intron template which can be used in this way, but the small quantity is easily exhausted; whereas pMTL20IT1 and pMTL20IT2 provide an unlimited source.

Several options are available for screening modular ClosTron plasmids re-targeted by the conventional SOE PCR method, as these plasmids contain a small 'stuffer' fragment, including a *lacZα* ORF, which is replaced when the intron is re-targeted (Fig. 1). Clones containing successfully re-targeted modular ClosTron plasmids can be distinguished from those containing the parental plasmid by a white rather than blue colony color on plates supplemented with Xgal. The presence of an insert of the correct size can be verified by colony PCR using primers spofdx-seq-F1 and pMTL007-R1, which gives a product of 440 bp from the parental plasmid, or 548 bp after re-targeting. Restriction analysis using SacI and/or BglII can also be used to screen for re-targeting, as both these restriction endonucleases cut twice in all modular ClosTron plasmids prior to re-targeting, and only once after re-targeting. Finally, primers spofdx-seq-F1 and pMTL007-R1 are used to check the PCR product-derived portion of re-targeted plasmids by sequencing.

2.3. Cargo sequence delivery

Integration frequencies in *Clostridium sporogenes* NCIMB 10696 were determined for introns containing 'cargo' fragments of various sizes. Plasmid pMTL007C-E2::Csp-pyrF-595s, or a cargo-containing derivative, was transferred to *Clostridium sporogenes* NCIMB 10696 by conjugation as previously described (Heap et al., 2009b). The day after conjugation, a single transconjugant colony was used to inoculate 1 ml of TYG broth supplemented with 250 g/ml cycloserine and 7.5 g/ml thiamphenicol. After overnight anaerobic incubation at 37 °C, serial dilutions of the culture were spread onto plates supplemented with only 250 g/ml cycloserine to enumerate the total number of colony-forming units per ml (CFU ml^{-1}) and onto plates supplemented with both 250 g/ml cycloserine and 2.5 g/ml erythromycin to determine the number of erythromycin-resistant CFU ml^{-1}. Integrants per cell (integration frequency) was determined as erythromycin-resistant CFU ml^{-1} per total CFU ml^{-1}.

3. Results

3.1. A modular ClosTron plasmid design facilitates construction of derivatives

In order to test and improve the scope and utility of ClosTron mutagenesis, we needed to construct several new plasmid variants. The four types of change likely to be most useful in constructing ClosTron plasmid variants are replacement of the Gram-positive origin of replication, replacement of the plasmid marker, replacement or removal of the intron marker, and addition of 'cargo' sequence to be delivered by the intron. None of these alterations can be easily made to the prototype ClosTron plasmid pMTL007 in a single cloning step, as suitable restriction sites for cloning are absent or not unique (Heap et al., 2007). In order to address issues of this type in our recombinant work generally, we recently developed the pMTL80000 modular system for *Clostridium–E. coli* shuttle plasmids (Heap et al., 2009b). This system provides a convenient, rapid and reliable way to construct and modify plasmids using a choice of standardized components (http://clostron.com/pMTL80000.php). To bring these advantages to the construction of ClosTron plasmid variants, we re-constructed the ClosTron system in the pMTL80000 modular format (Fig. 1).

Useful changes are easily made to modular ClosTron plasmids using the appropriate combinations of restriction sites, as indicated in Fig. 1. We have now made numerous variant ClosTron plasmids for different purposes, and some of the most useful are listed in Table 1. Later sections of this report describe examples of why the different variants are necessary, and how they facilitate useful applications.

3.2. Streamlined ClosTron mutagenesis procedure

The typical ClosTron mutagenesis procedure is logically separated into four steps: Intron design, plasmid construction, plasmid transfer and mutant isolation; each described below. Here we present a substantially improved procedure which is much less labor-intensive, more robust and faster than previously described (Heap et al., 2007). Intron insertion sites and plasmid names are designated in the established way (Karberg et al., 2001; Heap et al., 2007, and Table 3).

3.2.1. Step one: re-targeted intron design

The 'rules' governing target site recognition by the Ll.LtrB intron have been studied in detail. Alternative sites to which the intron could be re-targeted (by making appropriate changes to the intron sequence) can be identified in almost any gene using a computer algorithm (Perutka et al., 2004). Although this algorithm is published, implementing it