Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker

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Supplementary Data

Integration of small and medium-sized DNA inserts at the pyrF locus of Clostridium acetobutylicum

The pyrF gene encodes orotidine 5-phosphate decarboxylase, which participates in the same pyrimidine biosynthesis pathway as orotate phosphoribosyltransferase, encoded by pyrE. Inactivation of either pyrF or pyrE will lead to the same FOA$^\text{R}$ phenotype, and both genes are required for uracil prototrophy. These selections can be applied to pyrF in the same way as pyrE. We constructed plasmid pMTL-JH2 according to the same design principles as pMTL-JH12, but targeting the pyrF locus (Supplementary Figure 1A). We transformed C. acetobutylicum cells with pMTL-JH2 DNA, followed the same integration procedure as for pMTL-JH12, and screened four independent FOA-resistant clones. All four clones showed the desired double-crossover recombinant genotype by PCR (Figure 2B), and were thiamphenicol-sensitive, indicating the loss of plasmid DNA. This experiment used the ‘empty’ pMTL-JH2 vector, so the only heterologous sequence delivered to the chromosome was the small lacZ sequence which provides the vector’s multiple cloning site. At time of these experiments, we had not yet demonstrated integration of large DNA inserts. To confirm that pMTL-JH2 could also be used to deliver larger DNA sequences, we constructed four derivatives of pMTL-JH2 by inserting different-

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sized fragments of phage lambda DNA into the allele exchange cassette (Supplementary Figure 1C).

Lambda DNA-HindIII Digest (NEB) is a preparation of phage lambda (cI857ind1 Sam 7) DNA digested to completion with HindIII. The mixture of fragments in this preparation was heated to 60 °C for 5 min to separate the lambda cohesive ends of the fragments derived from the ends of the lambda chromosome, treated with T4 polymerase (NEB) to convert the HindIII cohesive ends and lambda cohesive ends to blunt ends, then individual blunt fragments were purified by agarose gel electrophoresis. Four derivatives of pMTL-JH2 were constructed by ligating the 2.0, 2.3, 4.3 or 6.5 kbp blunt fragment of lambda DNA with pMTL-JH2 DNA linearised using StuI and treated with Antarctic Phosphatase (NEB) to prevent re-circularisation. This positioned the lambda DNA in each pMTL-JH2 derivative between the two regions of homology so that the lambda DNA would be delivered to the chromosome of a double-crossover clone by a successful integration experiment. These plasmids were designated pMTL-JH2-lambda2.0, pMTL-JH2-lambda2.3, pMTL-JH2-lambda4.3 and pMTL-JH2-lambda6.5 accordingly. The derivatives of pMTL-JH2 were each sequenced using the M13F primer to determine the orientation of the insert (because blunt cloning is not directional) which would subsequently inform the design of the screening PCR.

*C. acetobutylicum* cells were transformed with each of the lambda DNA-containing derivatives of pMTL-JH2, and the integration procedure was performed as described in the main text for pMTL-JH12. FOA-resistant clones were screened by PCR and thiamphenicol-sensitivity by replicating. Each of these four inserts was successfully delivered to the *C. acetobutylicum* chromosome.

**DNA integration at the pyrE locus of Clostridium sporogenes and Clostridium difficile**

We constructed plasmids pMTL-JH18 and pMTL-JH27 to target the *pyrE* locus of *C. difficile* 630 and *C. sporogenes* NCIMB 10696 respectively, using a cassette design equivalent to pMTL-JH12 (Figure 1, Table 1). The choice of replicon for pMTL-JH18 and pMTL-JH27 was guided by our recent work [7, 29, and our unpublished observations] which suggests that the pLM13 replicon would limit the growth of *C. sporogenes* NCIMB 10696 colonies when under antibiotic selection for a resistance marker on the plasmid, providing the means to enrich and isolate faster-growing single-crossover clones; while the replicon from pCB102 would be more appropriate to achieve the same in *C. difficile* 630. Both plasmids also include a transfer function to allow conjugation from an *E. coli* donor. We tested pMTL-JH18 in *C. difficile* 630 Δerm [21] and pMTL-JH27 in *C. sporogenes* NCIMB 10696, using equivalent integration procedures as for pMTL-JH12 in *C.
acetobutylicum, but with media and conditions adapted to suit the different hosts.

Plasmids were transferred by conjugation from E. coli CA434 as described previously [7]. Transconjugants (clones which successfully received pMTL-JH27) of C. sporogenes were selected on TYG [30] agar supplemented with 250 µg/ml D-cycloserine to counter-select E. coli and 15 µg/ml thiamphenicol to select for the plasmid-borne resistance marker catP. Cells were sub-cultured twice on the same medium, then sub-cultured twice on TYG agar supplemented with 3 mg/ml 5-fluoroorotic acid (FOA) to select and isolate pyrE-minus clones. Four FOA-resistant colonies were screened by PCR (Supplementary Figure 1A) and screened for thiamphenicol-sensitivity by replica-plating, and the desired genotype was confirmed for all four.

Transconjugants of C. difficile 630Δerm containing pMTL-JH18 were selected on BHI agar supplemented with 250 µg/ml D-cycloserine and 8 µg/ml cefoxitin to counter-select E. coli, and 15 µg/ml thiamphenicol to select for the plasmid-borne resistance marker catP. Thiamphenicol-resistant C. difficile colonies containing pMTL-JH18 all grew quickly, at a rate similar to the wild-type on unsupplemented medium. Apparently the replication defect of pMTL-JH18 does not substantially limit the growth of C. difficile colonies on BHI agar supplemented with thiamphenicol, meaning that enrichment for single-crossover clones is not easily achieved. Consequently we were not able to obtain single-crossover clones using thiamphenicol, nor double-crossover clones using FOA. However, we have noted that the extent of the replication defect of some plasmids is dependent upon the size of the plasmid (Heap, Cartman, Ehsaan, Ng and Minton, unpublished observations). To attempt to exacerbate the replication defect of pMTL-JH18, a 6.5 kbp fragment of phage lambda DNA was inserted into the allele exchange cassette (in the same way as described above for pMTL-JH2) increasing the size of the plasmid from 5.9 kbp to 12.5 kbp, and the integration experiment was repeated. The thiamphenicol-resistant C. difficile colonies obtained showed a variety of growth rates, and the faster-growing colonies (corresponding to single-crossover clones) were enriched by sub-culturing twice on the same medium. Cells were then sub-cultured twice on CDM [31] agar supplemented with 2 mg/ml FOA and 5 µg/ml uracil to select and isolate pyrE-minus clones. FOA-resistant colonies were easily obtained, and five independent clones were screened as before by PCR (Supplementary Figure 1B) and thiamphenicol-sensitivity, and confirmed to be recombinants.

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Supplementary Figure 1: DNA integration at the pyrF locus of *C. acetobutylicum*.

(A) Selection of stable double-crossover clones using pMTL-JH2. The first recombination event (plasmid integration) is mediated by the long region of homology between pMTL-JH2 and *pyrZ/pyrD*. Single-crossover clones are obtained on medium containing thiamphenicol. The second recombination event (plasmid excision) is mediated by the short region of homology between pMTL-JH2 and an internal fragment of *pyrF*. Double-crossover clones are selected using FOA.

(B) PCR screening of four candidate double-crossover clones using primers Cac-pyrI-sF1 and M13F which anneal where indicated in (A). MW, 2-Log DNA Ladder (NEB) molecular weight marker; 1–4, candidate clones; WT, wild-type *C. acetobutylicum* genomic DNA control. All four candidates show the expected 549 bp band.

(C) Homologous recombination cassette of pMTL-JH2 and four derivatives containing different fragments of lambda DNA, shown to scale.
Supplementary Figure 2:

(A) PCR screening of four FOA-resistant candidate clones of *C. sporogenes* for DNA integration at the *pyrE* locus using pMTL-JH27. Primers Csp-pyrD-sF2 and M13F anneal to the chromosome and lacZ insert respectively, resulting in a 541 bp PCR product from double-crossover clones. MW, 2-Log DNA Ladder (NEB) molecular weight marker; plasmid, pMTL-JH27 plasmid DNA control; WT, wild-type *C. sporogenes* genomic DNA control; 1–4, candidate clones. All four candidates show the expected 558 bp band.

(B) PCR screening of five FOA-resistant candidate clones of *C. difficile* for DNA integration at the *pyrE* locus using pMTL-JH18::lambda6.5 (pMTL-JH18 with the 6.5 kbp lambda DNA insert). Primers Cdi630-pyrD-sF1 and lambda6.5-sF2 anneal to the chromosome and lambda insert respectively, resulting in a 623 bp PCR product from double-crossover clones. MW, 2-Log DNA Ladder (NEB) molecular weight marker; plasmid, pMTL-JH18::lambda6.5 plasmid DNA control; WT, wild-type *C. sporogenes* genomic DNA control; 1–5, candidate clones. All five candidates show the expected 623 bp band.
| Oligonucleotide | Binding site | Purpose | Sequence 5′ to 3′ |
|----------------|--------------|---------|------------------|
| M13F           | lacZ in integration vectors | Plasmid-specific reverse primer | TGTAAAACGACGGCCAGT |
| lacZa-sF2      | lacZ in integration vectors | Plasmid-specific forward primer | ACTGGCCGTCGTGTTTACACGTCG |
| Cac-pyrI-sF1   | pyrI of *C. acetobutylicum* | Chromosome-specific forward primer | TGTGATGAAATATAGAAGCCAGG |
| CAC0026-sF2    | CAC0026 of *C. acetobutylicum* | Chromosome-specific forward primer | TAGCCCAATTGTATTTTGACTTCTTTAAATAAATACCTG |
| Cdi630-pyrD-sF1| pyrD of *C. difficile* | Chromosome-specific forward primer | TAGAGAGAATAATAAAAGTTAGACGAAATAAGAG |
| Csp-pyrD-sF2   | pyrD of *C. sporogenes* | Chromosome-specific forward primer | GAAGACTTTAGAAAATTATATGAAAGAAG |
| Cac-thl-sF1    | thl of *C. acetobutylicum* | Chromosome-specific forward primer | ACTTGCTAAGATAGTCTTTAGGTTCAGCAG |
| Cac-pyrD-sR1   | pyrD of *C. acetobutylicum* | Chromosome-specific reverse primer | AGCCATATCCTAATATTCTCTTCCATTAG |
| Cac-hydA-sR2   | hydA of *C. acetobutylicum* | Chromosome-specific reverse primer | TTGATGATTTTGACTTACATG |
| Cdi630-CD0189-sR3 | CD0189 of *C. difficile* | Chromosome-specific reverse primer | CCAAGCTCTATGACAGCAGCTAGTTTCAG |
| Csp-3233-sR4   | CBO3233 homolog of *C. sporogenes* | Chromosome-specific reverse primer | AATAGTGTCCAAAGCACTTG |
| Cac-atpB-sR1   | atpB of *C. acetobutylicum* | Chromosome-specific reverse primer | ATGATACTGTATTGAAACCTTTCTAAAGG |

**Supplementary Table 1:**

List of oligonucleotide primers. PCR screening was performed using appropriate combinations of primers as described in the text and figure legends. Validated chromosome-specific reverse primers for each locus targeted in this study are also listed to provide researchers with further screening options.