

The kanamycin resistance cassette in the CFT073 $\Delta cftR$ cell was excised using FLP recombinase encoded by the pCP20 plasmid, following the protocol established by Datsenko and Wanner. However, PCR analysis indicated that the cassette remained intact (Figure S3-1 A). To improve FLP-mediated excision, colonies were re-streaked daily for three consecutive days on agar plates supplemented with carbenicillin and chloramphenicol and incubated at 30°C, followed by a final incubation at 42°C. Despite these measures, PCR results continued to show retention of the kanamycin resistance cassette (Figure S3-1 B), suggesting that the FLP recombination under these conditions was ineffective.

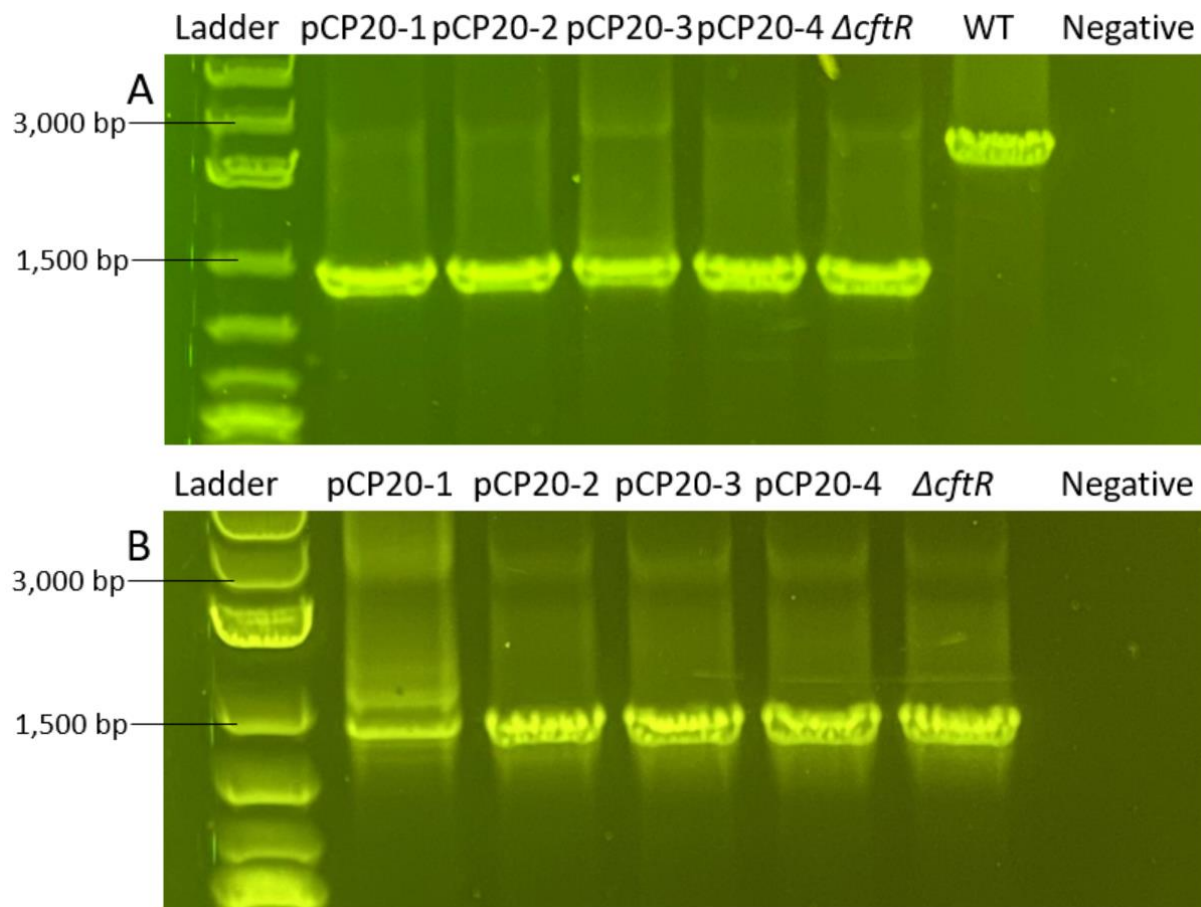


Figure S3-1. Assessment of FLP recombinase-mediated excision of the kanamycin resistance cassette. **A:** Initial excision attempt following the standard Datsenko and Wanner protocol. Lanes pCP20-1 to pCP20-4 show PCR products indicating the cassette remained intact from four individual colonies. Ladder: 1 kb Plus DNA ladder (NEB). WT: PCR with CFT073 wild type chromosomal DNA; $\Delta cftR$: PCR with CFT073 $\Delta cftR$ chromosomal DNA (positive control); Negative: PCR with no-template DNA (negative control). **B:** Excision attempt after an optimized regimen of three consecutive daily re-streaks. Lanes pCP20-1 to pCP20-4 again show the intact cassette. Ladder: 1 kb Plus DNA ladder (NEB); $\Delta cftR$: PCR with CFT073 $\Delta cftR$ chromosomal DNA (positive control); Negative: PCR with no-template DNA (negative control). The results demonstrated the persistence of the kanamycin resistance cassette under both standard and optimized FLP recombination conditions.

To experimentally verify the putative EcoCFTII recognition site (5'-CACAG-3'), the plasmid pACYC177 was selected as a model substrate. This plasmid was considered suitable because, although the CACAG sequence occurs frequently in DNA, pACYC177 contains only three instances of it. Critically, removing these sites was not predicted to disrupt essential genetic elements such as the kanamycin resistance gene or the origin of replication. An inverse PCR strategy was employed to construct a modified version of pACYC177 with partial deletion of CACAG sites. However, these attempts were unsuccessful, as agarose gel electrophoresis did not reveal PCR products of the expected size (Figure S3-2). This failure is likely attributable to the formation of stable secondary structures, such as hairpins, within the pACYC177 template DNA, which can interfere with polymerase during amplification.

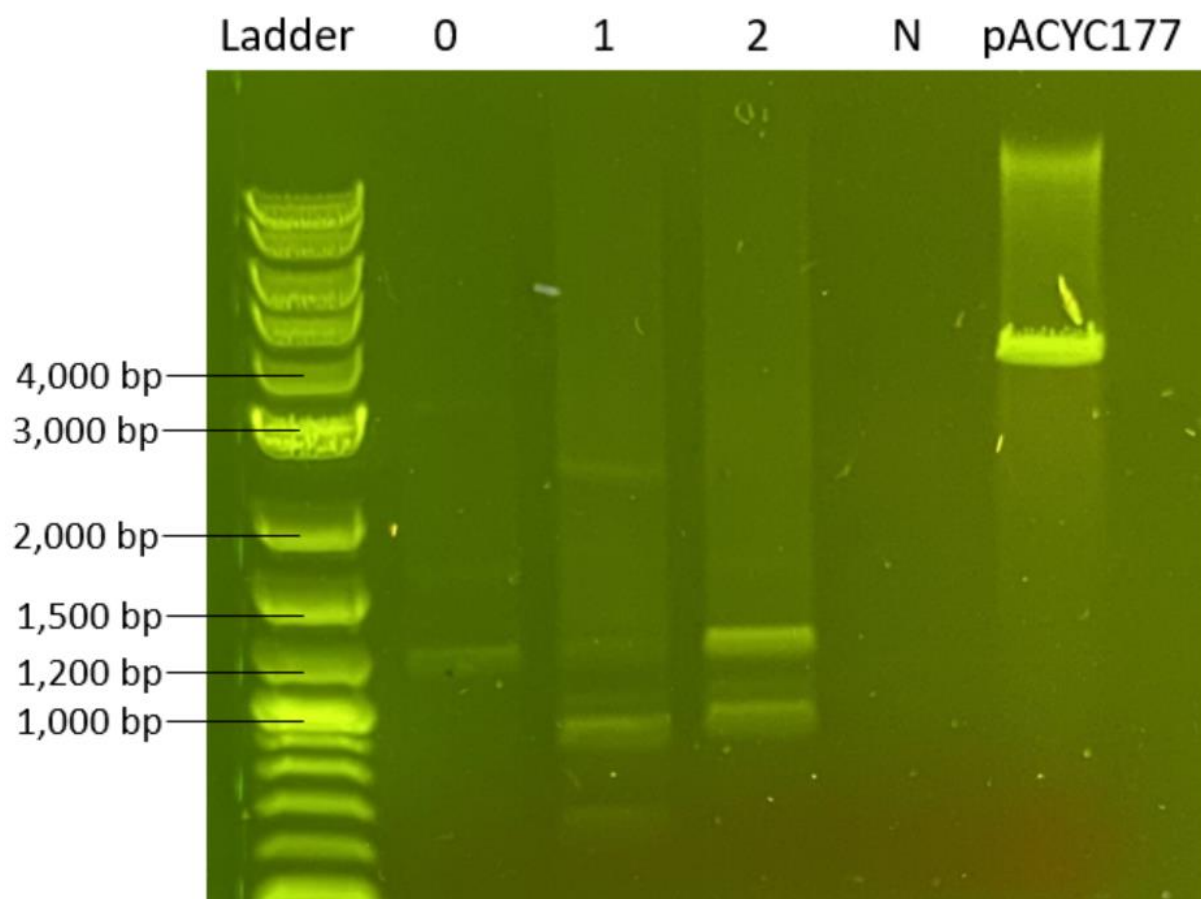


Figure S3-2. Analysis of inverse PCR products for the construction of CACAG-deficient pACYC177 variants. Lanes labelled '0', '1', and '2' correspond to inverse PCR products intended to generate pACYC177 derivatives containing zero, one, and two CACAG sites, with expected sizes of 2,713 bp, 2,718 bp, and 2,754 bp, respectively. The parental pACYC177 plasmid digested with *Hind*III (expected size: 3,941 bp) was included as a control. Ladder: 1 kb Plus DNA ladder (NEB); N: PCR with no-template DNA (negative control).