Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-023-12779-8

METHODS AND PROTOCOLS

Digital PCR: a tool in clostridial mutant selection and detection

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Received: 30 June 2023 / Revised: 29 August 2023 / Accepted: 6 September 2023

Abstract

The ClosTron mutagenesis system has enabled researchers to efficiently edit the clostridial genome. Since site-specific insertion of the mobile *ClosTron* insert may cause errors, validation is key. In this paper we describe the use of digital PCR (dPCR) as an alternative tool in selecting clostridial mutant strains. *Clostridium perfringens* chitinase mutant strains were constructed in which the mobile *ClosTron* intron was inserted into one of the chitinase genes. On-target insertion of the mobile intron was validated through conventional PCR. In order to confirm the absence of off-target insertions, dPCR was used to determine the amount of the *ClosTron* intron as well as the amount of a reference gene, located in close proximity to the interrupted gene. Subsequently, mutant strains containing an equivalent amount of both genes were selected as these do not contain additional off-target mobile *ClosTron* inserts. The outcome of this selection procedure was confirmed through a validated PCR-based approach. In addition to its application in mutant selection, dPCR can be used in other aspects of clostridial research, such as the distinction and easy quantification of different types of strains (wildtype vs. mutant) in complex matrices, such as faecal samples, a process in which other techniques are hampered by bacterial overgrowth (plating) or inhibition by matrix contaminants (qPCR). This research demonstrates that dPCR is indeed a high-throughput method in the selection of clostridial insertion mutants as well as a robust and accurate tool in distinguishing between wildtype and mutant *C. perfringens* strains, even in a complex matrix such as faeces.

Key points

- *Digital PCR as an alternative in ClosTron mutant selection*
- *Digital PCR is an accurate tool in bacterial quantification in a complex matrix*
- *Digital PCR is an alternative tool with great potential to microbiological research*

Keywords Digital PCR · ClosTron mutagenesis · Mutant selection

Introduction

The *Clostridium* genus harbours over 250 species including diverse strains of medical, environmental or bio-industrial relevance. Most members of the *Clostridium* genus are harmless non-pathogenic bacteria that can be found throughout the environment, on plants and in animals (on skin and on mucosal surfaces). The exploitation of members of the *Clostridium* genus in industrial fermentation processes is promising through their ability to produce chemical commodities such as biofuels from renewable biomass. The saccharolytic mesophiles *C. beijerinckii*, *C. acetobutylicum* and *C. saccharobutylicum* (recently reclassified as *C. beijerinckii*) are able to convert sugar into the solvents acetone, butanol and ethanol (Jang et al. [8]; Lee et al. [14]; Liao et al. [16]; Little et al. [17]). Various *Clostridium* species are able to convert additional substrates including steel mill waste gases, pre-treated waste lignocellulosic material, and waste glycerol, producing fermentation by-products including acetate, butyrate, hydrogen, 2,3-butanediol and

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Published online: 14 September 2023

isopropanol (Harzevili and Hiligsmann [5]). In addition to the species relevant in biofuel production, other members of the genus are involved in cellulosic and hemicellulosic biomass degradation (*C. cellulolyticum*), carbon fixation (*C. carboxidivorans*, *C. ljungdahlii*), platform chemical production (*C. pasteurianum*) or act as an anticancer therapeutic (*C. novyi*) (Joseph et al. [9]). Furthermore, many clostridial pathogens have been identified as the causative agents of an array of enteric, histotoxic and neurologic diseases in humans and animals. The most well-known clostridial pathogens are *C. difficile*, *C. tetani*, *C. perfringens* and *C. botulinum*. Understanding the pathogenesis of such diseases is of major importance for the health and welfare of animals and humans alike.

In this context, strain engineering has evolved dramatically with gene deletion being a main tool in gaining new insights in the pathogenesis of infections. The development of knock-out mutants has enabled researchers to verify or disprove the role of proposed virulence factors during *C. perfringens* pathogenesis, such as α-toxin, NetB, collagen adherence locus and the Arg-like quorum sensing system (Chen et al. [3]; Keyburn et al. [11]; Wade et al. [27]; Yu et al. [30]). Also getting rid of proteins impairing the industrial production process of biofuels can be an important application of targeted gene deletions. In the mid-2000s, the ClosTron technique was developed, a group II intron-directed mutagenesis system for *Clostridium*. The exact mode-of-action has been described previously (Heap et al. [6]; Jang et al. [7]; Kuehne and Minton [12]; Lee et al. [14]; Shao et al. [21]). In short, this genome editing tool kit allows to inactivate genes through the insertion of a mobile ClosTron intron into the bacterial DNA sequence. The ClosTron delivery plasmid contains a group II intron encoding region with an antibiotic resistance gene, inactivated through an orientation-specific phage td group I intron. The RNA transcript binds to LtrA, a protein encoded on the plasmid backbone, forming a ribonuclear protein complex (RNP). After splicing out of the td intron, the RNP complex binds to the specific targeted site of the target gene. The RNA is inserted into the nicked DNA strand, after which the opposite DNA strand is synthesised through the reverse transcriptase activity of LtrA. Host nucleases degrade the remaining RNA, followed by complementary strand formation by DNA polymerase. Finally, the gaps are sealed by host ligases. Despite the specific targeting of the ClosTron mutagenesis system, off-target insertions may occur (Little et al. [17]; Mohr et al. [18]; Shen [22]). It is hypothesised that non-specific integration is caused by high sequence similarity to the desired site or continuous expression of the ClosTron elements (Liao et al. [16]). The targeting specificity has been improved by precise management of the intron RNA expression and intron-encoded protein through an inducible expression system (Zhang et al. [31]). Despite the great improvements in recent years, confirmatory analysis is crucial since unintentional gene mutagenesis may impact research outcomes. The lack of off-target insertions is often validated through southern blotting, PCR-based approaches and whole genome sequencing (Cooksley et al. [4]). Despite its wide usage, southern blotting requires a large amount of DNA and is considered cumbersome, labour-intensive and time-consuming. Kwon and Ricke ([13]) described a PCR-based approach identifying the transposon-flanking sequences using subsequent restriction digest, Y-shaped linker ligase and PCR amplification using linker-based and insertion-based primers. Although often used, protocol optimisation can require significant effort and results are sometimes questionable. The exact location of the off-target insertion can be identified using whole genome sequencing, although this technique is considered expensive for mutant selection.

This research focusses on the use of dPCR as a high-throughput alternative to validate the correct insertion in ClosTron mutants (experiment I). dPCR is a robust PCR technique that enables absolute quantification of its targets by partitioning the samples into numerous partitions. Each partition functions as an individual entity containing all elements to perform the PCR reaction: template molecule, primers, fluorescent probes, dNTPs, DNA polymerase and potentially additional salts. Each partition undergoes the PCR amplification. In the end fluorescence signals are measured in each partition, labelling them either as positive or negative. By assuming that the distribution of target molecules in the partitions follows the Poisson distribution, the possibility that a partition contains more than one molecule is accounted for and the absolute quantity can be derived from the fraction of negative to positive partitions (Vogelstein and Kinzler [25]). Digital PCR results in an improved precision and accuracy even in the presence of inhibitors and more accurate quantification when amplification efficiency is low (Kanagal-Shamanna [10]; The dMIQE Group and Huggett [24]). In addition to its clinical applications, dPCR has been used in various aspects of microbiology, including viral and bacterial quantification, validation of whole-virus or plasmid DNA reference material and the detection of antimicrobial drug resistance in bacterial strains (Salipante and Jerome [20]). During this research, *C. perfringens* mutant strains were constructed using the ClosTron mutagenesis system directed towards either the chitinase A (*chiA*) or chitinase B (*chiB*) gene. In order to rule out potential off-target insertions of the mobile ClosTron intron, a dPCR assay was developed in which the amount of mobile intron was compared to the amount of a reference gene. A side-by-side comparison was made with Y-linked PCR data in order to validate the results. In addition to detection of off-target insertions, the use of dPCR in other ClosTron mutant-related research was evaluated in which the technique was used as

an absolute quantification method to distinguish between wildtype and mutant strains in mixed samples, even in a complex matrix such as faeces (experiment II).

Material and methods

Construction of C. perfringens ClosTron mutants

The pathogenic *C. perfringens* type G strain CP56 was used as the wildtype strain (Belgian Coordinated Collections of Microorganisms—LMG 33101). Two putative chitinases (ChiA and ChiB) have been identified as potential pathogen-specific virulence factors important during early necrotic enteritis pathogenesis (Lepp et al. [15]). Either gene *chiA* (GenBank accession number F8UNI5) or *chiB* (GenBank accession number F8UNI4) was inactivated by insertion in the pathogenic *C. perfringens* strain CP56 using the ClosTron mutagenesis system, as previously described (Heap et al. [6]; Kuehne and Minton [12]) (www.clostron.com). ClosTron intron-targeting regions were designed to insert at the 389 bp and 613 bp gene position for *chiA* and *chiB* (on the sense strand), respectively, using the Perutka algorithm implemented at www.clostron.com. The intron containing the target regions and inactivated antibiotic resistance gene were synthesised and cloned into ClosTron plasmid pMTL007C (ATUM, Newark, CA, USA). The chemically competent *E. coli* CA434 strain was transformed with the plasmid, serving as a conjugal donor strain (Wang et al. [28]) and cultured overnight in Luria-Bertani (LB) medium (Merck Life Science, Overijse, Belgium) containing 25 µg/ml kanamycin and 12.5 µg/ml chloramphenicol. The cells were pelleted by centrifugation (1500*g*, 2 min) and washed repeatedly with PBS. Thereafter the pellet was resuspended in 200 µl of an overnight culture of *C. perfringens* CP56. The conjugal mixture was plated onto a non-selective brain heart infusion broth (BHI) agar plate (Fisher Scientific, Merelbeke, Belgium) and incubated anaerobically for 4 h in order to allow conjugal transfer of the plasmid from the *E. coli* donor strain to the *C. perfringens* recipient strain. The growth from the non-selective plate was harvested, resuspended in PBS and plated onto a selective BHI agar plate containing 30 U/ml polymyxin B sulphate (PMB) and 100 µg/ml sodium sulfadiazine (SDZ), inhibiting *E. coli* overgrowth and therefore favouring *C. perfringens*, and 15 µg/ml thiamphenicol, favouring growth of strains containing the plasmid. After overnight anaerobic incubation, single colonies were streaked to purity on BHI plates containing 15 µg/ml thiamphenicol, after which they were transferred to BHI plates containing 10 µg/ml erythromycin. Integration of the intron into the target site whilst losing the plasmid is selected through erythromycin resistance and thiamphenicol sensitivity, respectively.

Validation of on-target ClosTron insertion

In order to validate the correct site of insertion of the ClosTron cassette, colonies obtained through the ClosTron mutagenesis were tested by PCR (Fig. 1) using a forward primer targeting the mobile ClosTron intron (FW_EBS universal, 5′ CGAAATTAGAAACTTGCGTTCAGT AAAC 3′) and a reverse primer downstream of the respective insertion site (Rev_chiA_selection, 5′ GTTTTG TCCTTCTTGGTTCTG 3′, 73 bp amplicon; Rev_chiB_ selection, 5′ CAATCAATATCAACAAAGTCCATATTG 3′, 117 bp amplicon). The fragments were PCR amplified using the BioMix™ DNA polymerase according to the manufacturers' instructions (Bioline, London, UK). The PCR reaction procedure consisted of initial denaturation 3 min at 95°C, 35 amplification cycles (30 s at 95°C, 30 s at 50°C and 90 s at 72°C) and final elongation 12 min at 72°C. For every mutant type (mutants with insertion in either *chiA* or *chiB*), three positive colonies were selected for subsequent analysis to exclude potential off-target insertions, using dPCR.

DNA extraction from pure bacterial culture

Pure *C. perfringens* wildtype or mutant cultures were grown overnight in BHI at 42°C in an anaerobic chamber. DNA of the pure cultures was extracted, as previously described by Pitcher et al. ([19]). In short, 1 g of bacterial culture was resuspended in 15 ml Tris-buffer (10 mM Tris, 100 mM EDTA, pH 8). The pellet was recovered after centrifugation (10 min, 10,000*g*) and resuspended in 3 ml enzyme solution (200 µg/ml RNase A, 25 mg/ml lysozyme in TE buffer (10 mM Tris, 300 mM EDTA, pH 8)). After 1 h incubation at 37°C, 200 µg/ml proteinase K was added to the mixture. After an additional incubation of 15 min at 37°C, 15 ml GES (5 M guanidine thiocyanate, 0.1 mM EDTA, 35 mM sarkosyl) was supplemented and the mixture was left on ice for 10 min. Subsequently, 0.5 M ammonium acetate was added and the mixture was left for an additional 10 min on ice. Afterwards, 2.5 volumes of cold chloroform/isoamyl alcohol (24:1) were added and the mixture was centrifuged at 12,000*g* for 30 min. The upper phase was mixed with 0.6 volumes of isopropanol. Precipitating DNA strands were collected using a sterile glass rod, washed with pure EtOH and air dried. The DNA was resuspended in 5 ml TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8). To further enhance the DNA purity, a second purification step was performed. After adding 100 µg/ml RNase A, the mixture was incubated for 1 h at 37°C. Afterwards, 0.3 mM acetate-EDTA and 0.6 volumes of chloroform/isoamyl alcohol (24:1) were added. The mixture was centrifuged for 20 min at 12,000*g*. The upper phase was mixed with 0.6 volumes of isopropanol.

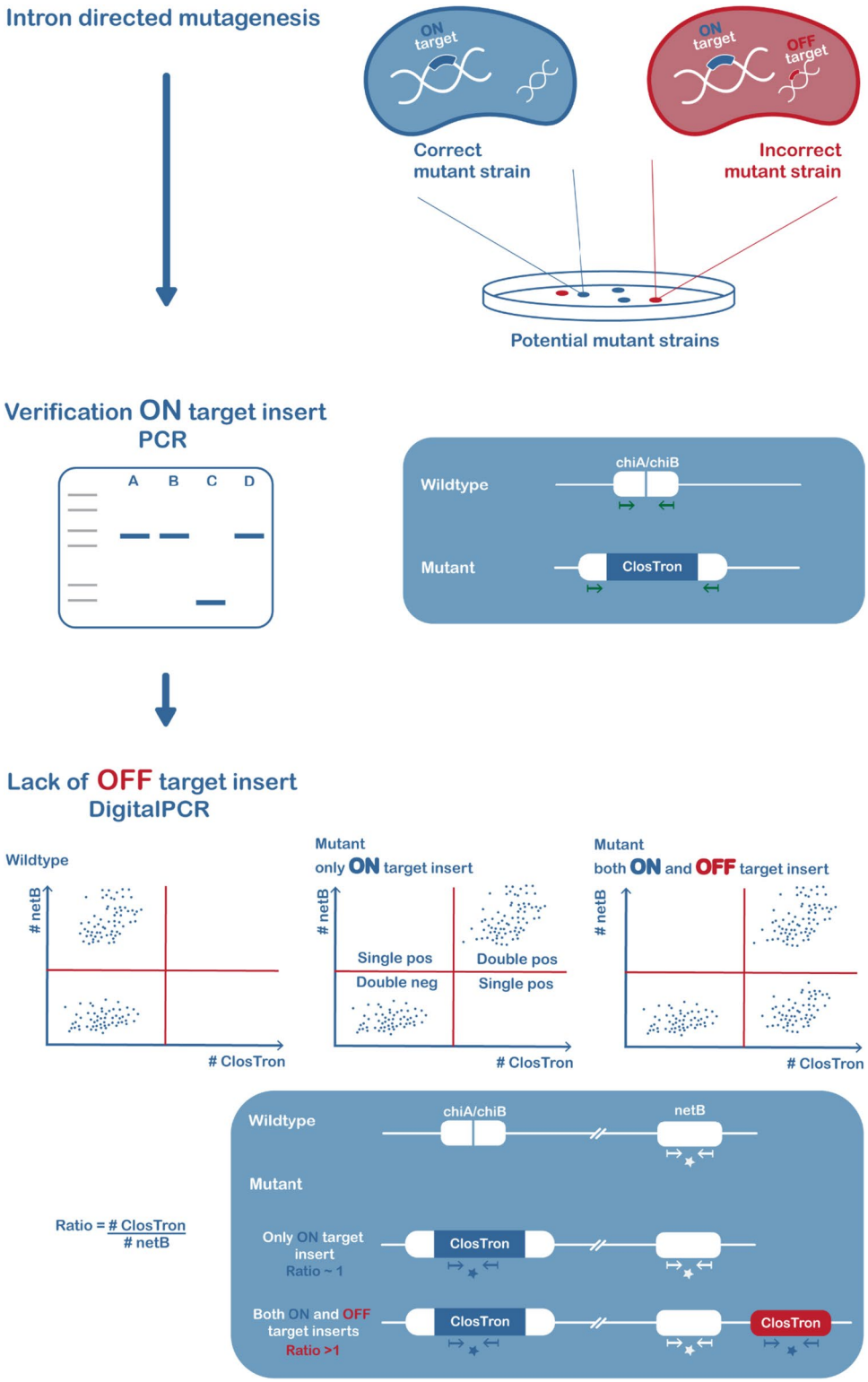

Fig. 1 Schematic representation of the workflow followed during the mutant selection process. First, mutants in *chiA* or *chiB* gene were constructed using the ClosTron mutagenesis system, resulting in strains having either a single on-site insert (blue) or an additional off-target mobile *ClosTron* intron (red). The on-target insertion was verified using a traditional PCR using primers flanking the insert site. Subsequently, the absence of off-target inserts was confirmed using the digital PCR assay in which the amount of the mobile intron was compared to the amount of the reference gene *netB* in close proximity. During dPCR, the DNA sample is first partitioned after which the PCR reaction is carried out for every partition separately. One partition is represented on the plot by a single dot. Primers are represented by arrows (green: flanking site of on-target insertion; white: *netB* specific; blue: *ClosTron* specific). Probes are represented by stars (white: HEX; blue: FAM)

Precipitated DNA was collected using a sterile glass rod, washed with pure EtOH and air dried. The DNA was resuspended in 1 ml SSC buffer (15 mM NaCl, 1.6 mM citric acid, 4.5 mM NaOH, pH 8) and stored at −20°C until subsequent analysis. In order to obtain DNA from a mix of bacteria, equal amounts of wildtype and mutant overnight cultures were mixed, followed by the above-mentioned DNA extraction procedure.

DNA extraction of spiked faeces

Faecal samples were collected from healthy broilers not suffering from necrotic enteritis, an enteric disease caused by *C. perfringens* type G. Overnight cultures of wildtype CP56, mutant CP56Δ*chiA* and mutant CP56Δ*chiB* strains were grown in brain heart infusion broth (BHI). For mixed samples, equal volumes of wildtype and the respective mutant overnight culture were combined. The faecal sample was divided into equal parts of 100 mg and spiked with 100 µl of bacterial culture, either the pure strain or a mix. DNA was extracted using the CTAB method. In short, the spiked faeces were mixed with 500 mg RNase-free 0.1 mm zircon/silica beads, 500 µl CTAB buffer (5% hexadecyltrimethylammonium bromide, 0.35 M NaCl, 120 mM K_2HPO_4) and 500 µl phenol-chloroform-isoamyl alcohol mixture (25:24:1). The mixture was homogenised using the TissueLyser® (2 repeats of 90 s at 22.5 Hz) and centrifuged for 10 min at 8000 rpm. After transferring into a new tube, phenol was removed from the supernatant by adding an equal volume of chloroform-isoamyl alcohol (24:1) followed by centrifugation for 10 s at 16,000*g*. Nucleic acids were precipitated from the aqueous phase by adding 2 volumes of PEG-6000 solution (30% polyethylene glycol, 1.6 M NaCl). After a 2-h incubation period, the mixture was centrifuged for 20 min at 13,000*g*. The pellet was washed twice using 1 ml 70% ethanol. Following air drying, the pellet was resuspended in 50 µl RNase-free water. All samples were diluted to a final concentration of 1 ng/µl and stored at −20°C until subsequent analysis. Quality was checked using NanoDrop.

Digital PCR

The primers and probes used for dPCR were designed using the PrimerQuest tool provided by IDT (Integrated DNA Technologies; Table 1). High efficiency of the primer sets was

Table 1 Overview of primers and probes used for dPCR analysis for mutant selection (experiment I) or to distinguish between wildtype and mutant strain CP56Δ*chiA* or CP56Δ*chiB* (experiment II). Primer efficiency was determined using qPCR with a serial dilution of the standard fragments (entire *ClosTron* or *netB* gene)

	Primer/probe name	Purpose	Sequence (5′ to 3′)	Length amplicon (bp)	Efficiency (%)
Mutant selection	FW_ClosTron	Primers/probe specific for ClosTron insert	GCACACTCAAGTCTCGATTCA	105	98.5
	REV_ClosTron		GTGGTATGGCGGGTAAGTTT		
	Probe_ClosTron		/56-FAM/TGCCAGCGG/ZEN/AATGCTTTCATCCTA/3IABkFQ		
	FW_netB	Primers/probe specific for netB gene	CACTTGTTGACGAAAGTTTGTTTG	136	93.6
	Rev_netB		GCTTTAGCATTAACAGCACCTAAA		
	Probe_netB		/5HEX/TCCTCGCCA/ZEN/TTGAGTAGTTTCCCA/3IABkFQ		
Distinguish between wildtype and CP56Δ*chiA*	FW_WT/mut.chiA	FW upstream insertion site *chiA*	TGGATTTGATGGAGTAGACATTGA		
	REV_WT(chiA)	REV downstream insertion site *chiA*	TGTCCTTCTTGGTTCTGAAAGAT	97	99
	Probe_WT(chiA)	Probe spanning insertion site (empty)	/5HEX/TCCACAAGC/ZEN/CAATCAGGGAATCCA/3IABkFQ/		
	REV_mut	REV binds to ClosTron insert	GGTGCAAACCAGTCACAGTA	107	97.6
	Probe_mut.chiA	Probe spanning insertion site with ClosTron	/56-FAM/AGCCAATCA/ZEN/GGTGAAGTAGGGAGGTA/3IABkFQ/		
Distinguish between wildtype and CP56Δ*chiB*	FW_WT/mut.chiB	FW upstream insertion site *chiB*	TCTTTAGGTGGATGGTCAAAGTC		
	REV_WT(chiB)	REV downstream insertion site *chiB*	TTCCCAATCAATATCAACAAAGTCC	135	96.1
	Probe_WT(chiB)	Probe spanning insertion site (empty)	/5HEX/AGCCGCAAC/ZEN/TCCTTCAATAAGAGCT/3IABkFQ/		
	REV_mut	REV binds to ClosTron insert	GGTGCAAACCAGTCACAGTA	95	97.1
	Probe_mut. chiB	Probe spanning insertion site with ClosTron	/56-FAM/TAGCGTGAA/ZEN/GTAGGGAGGTACCGC/3IABkFQ/		

ensured through qPCR (Tables S1–S3 using the MIQE checklist (Bustin et al. [2])). All dPCR experiments were performed using the three-colour crystal digital PCR™ with Naica® system supplied by Stilla® technologies (Villejuif, France). Each reaction was performed in 25 µl reaction mixture containing 5 µl Naica® multiplex PCR mix, 100 nM fluorescein, 1 µM of each primer, 250 nM of each probe and 1 µl DNA. The concentration of DNA isolated from pure bacterial cultures was 0.6 ng/µl (diluted in nuclease-free water), whereas 1 ng/µl of DNA was used when working with spiked faecal samples. Partitioning was performed at 40°C. DNA was denatured for 10 min at 95°C. Forty PCR cycles consisted each of 15 s denaturation at 95°C followed by 30 s of annealing at 60°C. The Sapphire chips were scanned using the Prism3 instrument and the Crystal Reader Software. Data were analysed using the Crystal Miner Software supplied by Stilla®, and the concentration of the target region was quantified using its integrated Poisson statistics. The amount of partitions in each cluster was quantified by drawing polygons around the selected area (experiment I) or by setting the linear threshold manually (experiment II). dPCR data is represented according to the Digital MIQE guidelines (Table S4) (The dMIQE Group and Huggett [24]). The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Experiment I: clostridial mutant selection

During the first experiment, potential off-target insertions of the mobile *ClosTron* intron were analysed in the mutant strains harbouring the on-site insertion (Fig. 1). Using dPCR, the amount of intron was compared to the amount of the *netB* gene, a gene located on the pCP1netB plasmid, in close proximity to the chitinase genes. Equal amounts of both genes indicate a single insertion and thus the absence of off-target insertions. The primers and probes were designed using the PrimerQuest tool provided by IDT (Integrated DNA Technologies) and are shown in Table 1. The DNA was extracted from pure bacterial cultures of both the wildtype and mutant strains with previously confirmed on-target ClosTron insertion (CP56Δ*chiA*_31, CP56Δ*chiA*_51, CP56Δ*chiA*_69, CP56Δ*chiB*_19, CP56Δ*chiB*_27 and CP56Δ*chiB*_32), as previously described. DNA was diluted to a final concentration of 0.6 ng/µl. The ratio is determined by dividing the amount of *ClosTron* by the amount of *netB*. The confidence interval is quantified by using generalised linear mixed models, as described elsewhere (Vynck et al. [26]). Since no strains harbouring additional off-target insertions could be generated, an alternative control was used to strengthen our research. Bacterial strains were isolated during the intermittent phase of the ClosTron mutagenesis system in which they did not lose the ClosTron plasmid yet and therefore contained double the amount of the mobile insert compared to the reference gene.

Experiment II: distinguishing between wildtype and mutant strains

During the second experiment, dPCR was used as a detection technique to distinguish between wildtype and mutant *C. perfringens* strains (Fig. 2). Based on experiment I, strains CP56Δ*chiA*_69 and CP56Δ*chiB*_27 were selected to represent both types of mutants, respectively. In order to detect the wildtype strain, forward and reverse primers were designed adjacent to the insertion site. The same forward primer was combined with a reverse primer able to anneal to the mobile *ClosTron* insert, in order to detect the mutant strain. Specificity was ensured through probe design with HEX- and FAM-fluorescent probes being able to anneal overlapping the insertion site to detect the wildtype and mutant strain, respectively. An overview of the selected primer/probe combinations is given in Table 1. First, the assay was performed on DNA isolated from pure bacterial cultures, as previously described. In order to evaluate the effect of a complex faecal matrix, the assay was performed on DNA extracted from broiler faeces spiked with bacteria, either wildtype, mutant or an equal mix of both strains, as previously described. All assays were performed in triplicate. Negative controls were included, in which DNA extraction was performed using fresh BHI culture. DNA extraction and subsequent dPCR assay was performed in triplicate.

Y-linked PCR

In order to validate the dPCR assay, the absence of off-target insertions was verified using the PCR-based approach described by Kwon and Ricke ([13]). In short, 3 µg of linker 2 (5′ TGTCCCCGTACATCGTTAGAACTACTCGTACCA TCCACAT 3′) was phosphorylated using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. After 20 min of enzyme denaturation at 65°C, an equal amount of linker 1 (5′ TTTCTG CTCGAATTCAAGCTTCTAACGATGTACGGGGACACA TG 3′) was added to the mixture. The Y linker was formed after a 2-min incubation at 95°C followed by slowly cooling down the mixture to room temperature. Genomic DNA was obtained by the method of Pitcher et al. ([19]) as previously described. The DNA was digested using NlaIII (New England Biolabs), according to the manufacturer's instructions. After denaturation of the enzyme, 50 ng of digested DNA was ligated to 1 µg of Y linker using T4 DNA ligase (New England Biolabs), according to manufacturer's instructions. Two microlitres of the reaction mixture was used as template for PCR amplification.

Both a Y linker-specific forward primer (5′ CTGCTCGAA TTCAAGCTTCT 3′) and ClosTron-specific reverse primer (5′ CGAACAATAACAGAGCCGTAT 3′) were designed, resulting in an on-target PCR fragment of 170 bp or 310 bp in

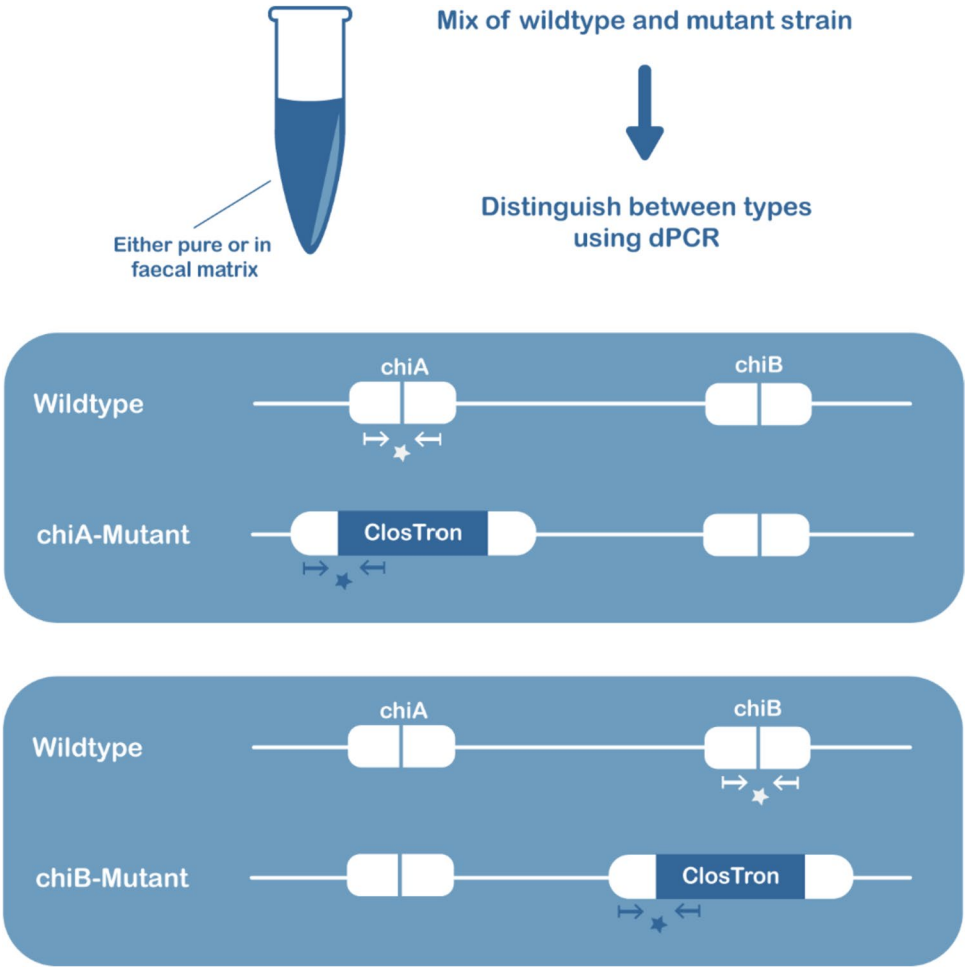

Fig. 2 Schematic representation of the primers and probes used during the digital PCR assay in order to distinguish between the wildtype and mutant strain (either CP56ΔchiA or CP56ΔchiB). Primers are represented by arrows (white: flanking the insert site in the respective gene c*hiA* or *chiB*; blue: forward primer upstream of the insert site and reverse primer located in the ClosTron insert). Probes are represented by stars (white: HEX; blue: FAM)

chiA mutants and *chiB* mutants, respectively. Additional PCR fragments indicate non-specific off-target insertions. The PCR reaction procedure consisted of initial denaturation 3 min at 95°C, 35 amplification cycles (30 s at 95°C, 30 s at 60°C and 1 min at 72°C) and final elongation 12 min at 72°C. The PCR products were visualised on a 2% agarose gel.

Results

Validation of on-target ClosTron insertions

Two types of *C. perfringens* mutant strains, CP56Δ*chiA* and CP56Δ*chiB*, were developed using the ClosTron mutagenesis system resulting in insertional inactivation of either gene *chiA* or *chiB*, respectively. In order to validate the correct site of insertion of the ClosTron cassette, the obtained colonies were tested by PCR using a FW primer targeting the mobile ClosTron intron and a REV primer downstream of the insertion site (Figure S1). All colonies of the *chiA* mutant strain and 12/17 colonies of the *chiB* mutant strain were positive for the amplification of the 117 or 73-bp fragment, indicating the presence of the on-target insertion. For every mutant type, three colonies with confirmed on-target insertion (CP56Δ*chiA*_31, CP56Δ*chiA*_51, CP56Δ*chiA*_69, CP56Δ*chiB*_19, CP56Δ*chiB*_27 and CP56Δ*chiB*_32) were randomly selected for analysis to detect potential off-target insertions, using dPCR.

dPCR as a tool to confirm the lack of off-target insertions in clostridial mutants

Despite the specific targeting of the ClosTron system, off-target insertions of the *ClosTron* insert may occur. Therefore, confirmatory analysis is crucial. Using dPCR, the presence of a single intron insertion was determined in the selected mutant strains harbouring the on-target insertion (CP56Δ*chiA*_31, CP56Δ*chiA*_51, CP56Δ*chiA*_69, CP56Δ*chiB*_19, CP56Δ*chiB*_27 and CP56Δ*chiB*_32), thereby excluding potential off-target insertions of the ClosTron intron. Both the amount of mobile ClosTron intron and the amount of the *netB* gene, a reference gene located on the same plasmid, were quantified and compared. Equal amounts of both genes indicate the absence of

off-target insertions. When the ClosTron insertion site and the reference gene are in close proximity to each other, theoretically, all partitions should either be double positives (*ClosTron*+ and *netB*+) or double negatives. Nevertheless, DNA shearing originating from the DNA extraction process or mixture preparation can result in breaks between the targeted (FAM) and reference gene (HEX). In this experimental setup, there are roughly 6–7 kB between the ClosTron insertion place and the *netB* gene. As a result, DNA breakage leads to different clusters of partitions: double positives (*ClosTron*+ *netB*+), double negatives (*ClosTron*− *netB*−) and single positives (either *ClosTron*+ *netB*− or *ClosTron*− *netB*+, due to DNA break between the two genes) (Table 2). The ratio of the amount of total ClosTron positive partitions (*ClosTron*+ *netB*− and *ClosTron*+ *netB*+) on total *netB* positive partitions (*ClosTron*− *netB*+ and *ClosTron*+ *netB*+) was approximately 1 for all strains, indicating that both genes were present in an equal amount. Based on the dPCR results, all strains were identified as mutant strains without off-target insertions. This lack of off-target insertions also was verified in all strains using a previously described PCR-based approach (Figure S2). Since no strains harbouring additional off-target insertions could be generated, *chiA* mutant strains that still contain the ClosTron plasmid were used as an alternative control. Theoretically, the ratio of total ClosTron positive partitions on total *netB* positive partitions should be approximately 2. Indeed, the ratio of all control strains was between 1.8 and 2, strengthening our statement that our dPCR assay is indeed able to detect additional insertions of the intron and thus an alternative tool to select mutant strains.

Digital PCR as a tool to distinguish between wildtype and mutant strains in experimental samples

Two dPCR assays were developed to distinguish between the wildtype and mutant strain (either CP56Δ*chiA* or CP56Δ*chiB*). Specificity of both assays was assessed using DNA obtained from overnight bacterial cultures of either the wildtype strain, the mutant strain or an equal mix of both (Table 3). For both the CP56Δ*chiA*-targetting and the CP56Δ*chiB*-targetting primer-probe combination, wildtype DNA resulted in a gene-specific signal, without any significant ClosTron-specific signal. In addition, dPCR on the DNA obtained from pure cultures of the mutant strains, either CP56Δ*chiA* or CP56Δ*chiB*, using their respective primer/probe mixtures, resulted in a ClosTron-specific signal, without any gene-specific signal. In mixed DNA samples, an equivalent amount of gene-specific signal compared to the ClosTron-specific signal could be detected. The specificity of the assay was also assessed using spiked faeces, a complex matrix potentially impairing detection. In accordance to using bacterial overnight cultures, similar results were obtained using DNA obtained from spiked faecal samples, indicating high specificity of both assays.

Discussion

Strain engineering is a crucial tool in current and future clostridial research. The ClosTron method, a group II intron-directed mutagenesis system, enables researchers to efficiently produce mutant strains in members of the

Table 2 Results of the clostridial mutant selection using dPCR. Polygons were positioned around the single positive (*ClosTron*+ *netB*−; *ClosTron*− *netB*+) and double positive (*ClosTron*+ *netB*+) droplets in order to quantify and compare the amounts. The respective concentration of the signals was determined. The ratio of the amount of total ClosTron positive partitions (*ClosTron*+ *netB*− and *ClosTron*+ *netB*+) on total *netB* positive partitions (*ClosTron*− *netB*+ and *ClosTron*+ *netB*+) was calculated. A ratio near the value of 1 indicates the absence of off-target ClosTron insertions. Controls were defined as chiA mutant strains containing an additional plasmid with ClosTron

		Concentration (copies/µl)			Ratio $\frac{ClosTron}{netB}$	*Confidence interval (95% LL; 95% UL)*
		Clos+ *netB*−	*Clos*− *netB*+	*Clos*+ *netB*+		
WT	**CP56**	0.07	376.7	0	0.002	
Mutant	**CP56ΔchiA_31**	130.6	122.1	486.3	1.01155	[0.98055; 1.0436]
	CP56ΔchiA_51	175	148.2	61.4	1.1152	[1.06325; 1.1697]
	CP56ΔchiA_69	1.41	0.9	10931	1.00005	[0.8862; 1.1285]
	CP56ΔchiB_19	111	123.5	1357	0.99325	[0.97145; 1.01545]
	CP56ΔchiB_27	41.3	43.8	4065	0.99955	[0.9742; 1.0256]
	CP56ΔchiB_32	49.8	54.8	3647	0.9989	[0.9751; 1.02325]
Control	**CP56ΔchiA + plasmid_A**	71.1	19.1	31.1	2.03586	
	CP56ΔchiA + plasmid_B	228	51.6	141.6	1.91304	
	CP56ΔchiA + plasmid_C	155.6	30.9	110	1.88503	
	CP56ΔchiA + plasmid_D	232	41.4	196	1.80286	

Table 3 Assessment of the specificity of dPCR assay used to distinguish between wildtype (CP56) and mutant (CP56ΔchiA_69 or CP56ΔchiB_27) strains using DNA isolated from pure overnight cultures of the different strains (either a single strain or a mix of both wildtype and mutant) or spiked faecal samples. All assays were performed in triplicate. Given value is the concentration (copies/µl) with its respective standard deviation. Wildtype strain is detected using primers flanking the insertion site (ClosTron−). The mutant is detected using a forward primer upstream of the insertion site and a reverse primer located in the ClosTron insert (ClosTron+)

	Origin of DNA	Pure cultures		Spiked faeces	
		ClosTron+	ClosTron−	ClosTron+	ClosTron−
Distinguish between CP56 and CP56Δ*chiA*	**CP56**	0.1 ± 0.1	630.4 ± 38.9	0	285.8 ± 80.1
	CP56ΔchiA	571.2 ± 269.2	0	374.6 ± 239.4	0
	CP56 + CP56ΔchiA	140.2 ± 31.5	119.9 ± 28.5	138.7 ± 43.9	164.8 ± 21.5
	Negative control	0	0	0.02 ± 0.02	0
Distinguish between CP56 and CP56Δ*chiB*	**CP56**	0	589.6 ± 34.1	0.1 ± 0.1	324.2 ± 115.3
	CP56ΔchiB	632.9 ± 213.7	0	393.9 ± 153.7	0 ± 0
	CP56 + CP56ΔchiB	120.6 ± 4.8	111.7 ± 4.8	269.5 ± 78.2	251.2 ± 58.1
	Negative control	0	0	0	0

Clostridium genus. Despite the specific targeting of the ClosTron mutagenesis system, off-target insertions may occur (Little et al. [17]; Mohr et al. [18]; Shen [22]). Despite the great improvements in specificity throughout recent years, confirmatory analysis is crucial in order to provide high-quality research since unintentional gene mutagenesis may impact research outcomes. The absence of off-target insertions is often confirmed through southern blotting, PCR-based approaches or sometimes whole genome sequencing (Cooksley et al. [4]). However, these methods have their restrictions.

dPCR, a PCR-based method resulting in absolute quantification, has potential as an alternative approach for mutant selection. During our research, a dPCR assay was developed for detecting potential off-target integration of the mobile *ClosTron* intron and assessed using *C. perfringens* mutant strains with verified on-target insertion. Therefore, the amount of the mobile intron was compared to the amount of a reference gene, located on the same plasmid as the targeted gene. Indeed, all investigated strains displayed an equal amount of both genes, indicating single *ClosTron* on-site insertion. These results were validated using a previously described PCR-based approach, indicating that the selection of strains using dPCR was indeed successful. Considering the ease of the technique, its applicability in high-throughput strain selection is high. On the downside, this dPCR technique is unable to directly verify on-target insertions and confirm the absence of off-target insertions in a single assay, stressing the need for an additional pre-PCR step. Potentially, a combination of two dPCR assays could be used to circumvent this problem, one assay using primers across the insert site and a second assay using internal primers to assess additional off-target insertions. However, this approach requires mode optimisation and could hamper the high-throughput advantage of dPCR. Careful selection of the reference gene is of great importance when using dPCR for mutant selection. When the targeted gene is located on chromosomal DNA, the reference gene should be of this type as well, since DNA extraction methods have a variable efficiency for different types of DNA (plasmid vs. chromosomal) (Becker et al. [1]). DNA shearing during the extraction protocol or the PCR mixture preparation has to be minimised, whilst guaranteeing mixture homogenisation (Yoo et al. [29]). An increased distance between the target and reference gene could increase the chance for breakage of the DNA strand in between both genes. This is the case for genes located on the chromosome as well as genes located on large plasmids.

The distinction between wildtype and mutant strains is often made using selective plating of the bacteria, although its labour intensity and potential overgrowth by other bacteria are great disadvantages when working with faecal samples. Alternatively, enumeration of the amount of both bacteria has been established using qPCR. Nevertheless, the need for a standard curve and potential inhibition when detecting small amounts of a gene are limiting factors to this technique (Smith and Osborn [23]). The second aim of this research was assessing the use of dPCR to distinguish between wildtype and mutant strains in complex samples such as faeces. Two types of dPCR assays were developed to distinguish between either wildtype strain CP56 and mutant strain CP56Δ*chiA* or wildtype strain CP56 and mutant strain CP56Δ*chiB*, respectively. The specificity of both assays was assessed using DNA derived from pure cultures as well as using faecal samples spiked with the respective bacterial strains. Indeed, both primer/probe mixtures displayed high specificity towards the different types of bacteria. In addition, both assays were able to distinguish between both types of bacteria (wildtype vs. mutant) using DNA derived from mixed equal amounts of both types of bacteria, either as pure bacterial cultures or spiked in a faecal matrix. Variations in the detected concentrations could be explained by variations

in bacterial growth, DNA extraction procedures and measurement of DNA concentrations. This research indicates that dPCR is an alternative technique to enumerate the number of certain bacteria in complex samples, although sensitivity assays should be performed beforehand. Primer/probe design is crucial during this process, since faecal samples contain a lot of contamination as well as commensal enteric bacteria, complicating primer design. In addition, cross reaction between the primer sets should be limited.

In conclusion, we have shown that dPCR is a promising technique to use in clostridial mutant selection, although reference gene selection, primer design and choosing the appropriate DNA extraction method are of great importance. In addition, if high specificity is guaranteed, dPCR can be used in other types of clostridial research that needs differentiation between wildtype and mutant strains, even in complex faecal samples. As a result, dPCR has to be considered an alternative tool with great potential to microbiological research.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-023-12779-8.

Author contributions ED designed the assays and conducted the experiments under the supervision of FVI, RD and EG. CC performed the DNA extraction procedures. Digital PCR was conducted at the Department of Morphology under the supervision of WDS. All authors read and approved the manuscript.

Funding The researcher Dierick Evelien was supported by Research Foundation Flanders FWO (Fonds Wetenschappelijk Onderzoek Vlaanderen) under grant number 12X8622N.

Data availability The datasets generated during and/or analysed during the study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This article does not contain any studies with live animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

References

1. Becker L, Steglich M, Fuchs S, Werner G, Nübel U (2016) Comparison of six commercial kits to extract bacterial chromosome and plasmid DNA for MiSeq sequencing. Sci Rep 6(1):28063. https://doi.org/10.1038/srep28063
2. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time pcr experiments. Clin Chem 55(4):611–622. https://doi.org/10.1373/clinchem.2008.112797
3. Chen Y, McClane BA, Fisher DJ, Rood JI, Gupta P (2005) Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. AEM 71(11):7542–7547. https://doi.org/10.1128/AEM.71.11.7542-7547.2005
4. Cooksley CM, Zhang Y, Wang H, Redl S, Winzer K, Minton NP (2012) Targeted mutagenesis of the *Clostridium acetobutylicum* acetone–butanol–ethanol fermentation pathway. Metab Eng 14(6):630–641. https://doi.org/10.1016/j.ymben.2012.09.001
5. Harzevili FD, Hiligsmann S (2017) In: Harzevili FD, Hiligsmann S (eds) *Microbial fuels*. CRC Press. https://doi.org/10.1201/9781351246101
6. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP (2007) The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods 70(3):452–464. https://doi.org/10.1016/j.mimet.2007.05.021
7. Jang IJA, Choi SY, Lee JI, Lee SY (2014) Metabolic engineering of *Clostridium acetobutylicum* for butyric acid production with high butyric acid selectivity. Metab Eng 23:165–174. https://doi.org/10.1016/j.ymben.2014.03.004
8. Jang KWJ, Im JA, Palaniswamy S, Yao Z, Lee HL, Yoon YR, Seong HJ, Papoutsakis ET, Lee SY (2023) Efforts to install a heterologous Wood-Ljungdahl pathway in *Clostridium acetobutylicum* enable the identification of the native tetrahydrofolate (THF) cycle and result in early induction of solvents. Metab Eng 77:188–198. https://doi.org/10.1016/j.ymben.2023.04.005
9. Joseph RC, Kim NM, Sandoval NR (2018) Recent developments of the synthetic biology toolkit for *Clostridium*. Front Microbiol 9. https://doi.org/10.3389/fmicb.2018.00154
10. Kanagal-Shamanna R (2016) Digital PCR: principles and applications. Clin Appl PCR:43–50. https://doi.org/10.1007/978-1-4939-3360-0_5
11. Keyburn AL, Bannam TL, Moore RJ, Rood JI (2010) NetB, a pore-forming toxin from necrotic enteritis strains of *Clostridium perfringens*. Toxins 2(7):1913–1927. https://doi.org/10.3390/toxins2071913
12. Kuehne SA, Minton NP (2012) ClosTron-mediated engineering of *Clostridium*. Bioengineered 3(4):247–254. https://doi.org/10.4161/bioe.21004
13. Kwon YM, Ricke SC (2000) Efficient amplification of multiple transposon-flanking sequences. J Microbiol Methods 41(3):195–199. https://doi.org/10.1016/S0167-7012(00)00159-7
14. Lee SY, Jang YS, Lee JY, Lee J, Park JH, Im JA, Eom MH, Lee J, Lee SH, Song H, Cho JH, Seung DY (2012) Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. MBio 3(5). https://doi.org/10.1128/mBio.00314-12
15. Lepp D, Roxas B, Parreira VR, Marri PR, Rosey EL, Gong J, Songer JG, Vedantam G, Prescott JF (2010) Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PLoS One 5(5):e10795. https://doi.org/10.1371/journal.pone.0010795
16. Liao C, Seo SO, Celik V, Liu H, Kong W, Wang Y, Blaschek H, Jin YS, Lu T (2015) Integrated, systems metabolic picture of acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum*. PNAS 112(27):8505–8510. https://doi.org/10.1073/pnas.1423143112
17. Little GT, Willson BJ, Heap JT, Winzer K, Minton NP (2018) The butanol producing microbe *Clostridium beijerinckii* NCIMB 14988 manipulated using forward and reverse genetic tools. Biotechnol J 13(11):1700711. https://doi.org/10.1002/biot.201700711
18. Mohr G, Hong W, Zhang J, Cui G, Yang Y, Cui Q, Liu Y, Lambowitz AM (2013) A targetron system for gene targeting in thermophiles and its application in *Clostridium thermocellum*. PLoS ONE 8(7):e69032. https://doi.org/10.1371/journal.pone.0069032
19. Pitcher DG, Saunders NA, Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8(4):151–156. https://doi.org/10.1111/j.1472-765X.1989.tb00262.x
20. Salipante SJ, Jerome KR (2020) Digital PCR—An emerging technology with broad applications in microbiology. Clin Chem 66(1):117–123. https://doi.org/10.1373/clinchem.2019.304048
21. Shao L, Hu S, Yang Y, Gu Y, Chen J, Yang Y, Jiang W, Yang S (2007) Targeted gene disruption by use of a group II intron

(targetron) vector in *Clostridium acetobutylicum*. Cell Res 17(11):963–965. https://doi.org/10.1038/cr.2007.91
22. Shen A (2019) Expanding the *Clostridioides difficile* genetics toolbox. J Bacteriol 201(14). https://doi.org/10.1128/JB.00089-19
23. Smith CJ, Osborn AM (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 67(1):6–20. https://doi.org/10.1111/j.1574-6941.2008.00629.x
24. The dMIQE Group, Huggett JF (2020) The digital MIQE guidelines update: minimum information for publication of quantitative digital PCR experiments for 2020. Clin Chem 66(8):1012–1029. https://doi.org/10.1093/clinchem/hvaa125
25. Vogelstein B, Kinzler KW (1999) Digital PCR. PNAS 96(16):9236–9241. https://doi.org/10.1073/pnas.96.16.9236
26. Vynck M, Vandesompele J, Nijs N, Menten B, De Ganck A, Thas O (2016) Flexible analysis of digital PCR experiments using generalized linear mixed models. Biomol 9:1–13. https://doi.org/10.1016/j.bdq.2016.06.001
27. Wade B, Keyburn AL, Haring V, Ford M, Rood JI, Moore RJ (2016) The adherent abilities of *Clostridium perfringens* strains are critical for the pathogenesis of avian necrotic enteritis. Vet Microbiol 197:53–61. https://doi.org/10.1016/j.vetmic.2016.10.028
28. Wang S, Hong W, Dong S, Zhang Z-T, Zhang J, Wang L, Wang Y (2018) Genome engineering of *Clostridium difficile* using the CRISPR-Cas9 system. Clin Microbiol Infect 24(10):1095–1099. https://doi.org/10.1016/j.cmi.2018.03.026
29. Yoo H-B, Lim H-M, Yang I, Kim S-K, Park S-R (2011) Flow cytometric investigation on degradation of macro-DNA by common laboratory manipulations. J Biophys Chem 02(2):102–111. https://doi.org/10.4236/jbpc.2011.22013
30. Yu Q, Lepp D, Mehdizadeh Gohari I, Wu T, Zhou H, Yin X, Yu H, Prescott JF, Nie S-P, Xie M-Y, Gong J (2017) The Agr-like quorum sensing system is required for pathogenesis of necrotic enteritis caused by *Clostridium perfringens* in poultry. Infect Immun 85(6). https://doi.org/10.1128/IAI.00975-16
31. Zhang J, Liu Y-J, Cui G-Z, Cui Q (2015) A novel arabinose-inducible genetic operation system developed for *Clostridium cellulolyticum*. Biotechnol Biofuels 8(1):36. https://doi.org/10.1186/s13068-015-0214-2