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Improvement of ClosTron for successive gene disruption in *Clostridium cellulolyticum* using a *pyrF*-based screening system

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Abstract *Clostridium* includes a number of species, such as thermophilic *Clostridium thermocellum* and mesophilic *Clostridium cellulolyticum*, producing biofuels and chemicals from lignocellulose, while genetic engineering is necessary to improve wild-type strains to fulfill the requirement of industrialization. ClosTron system is widely used in the gene targeting of *Clostridium* because of its high efficiency and operability. However, the targetron plasmid present in cell hinders the successive gene disruption. To solve this problem, a *pyrF*-based screening system was developed and implemented in *C. cellulolyticum* strain H10 in this study for efficient targetron plasmid curing. The screening system was composed of a *pyrF*-deleted cell chassis (H10Δ*pyrF*) constructed via homologous recombination and a PyrF expression cassette located in a targetron plasmid containing an erythromycin resistance gene. With the screening system, the gene targeting could be achieved following a two-step procedure, including the first step of gene disruption through targetron transformation and erythromycin selection and the second step of plasmid curing by screening with 5-fluoroorotic acid. To test the developed screening system, successive inactivation of the major cellulosomal exocellulase Cel48F and the scaffoldin protein CipC was achieved in *C. cellulolyticum*, and the efficient plasmid curing was confirmed. With the assistance of the *pyrF*-based screening system, the targetron plasmid-cured colonies can be rapidly selected by one-plate screening instead of traditional days' unguaranteed screening, and the successive gene disruption becomes accomplishable with ClosTron system with improved stability and efficiency, which may promote the metabolic engineering of *Clostridium* species aiming at enhanced production of biofuels and chemicals.

Keywords *Clostridium cellulolyticum* · ClosTron · Targetron Plasmid curing · *PyrF*

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Introduction

Consolidated bioprocessing (CBP) has been considered as one of the most promising approaches for the production of lignocellulosic ethanol, a sustainable biofuel urgently needed for energy conversion (Lynd et al. 2005). Several anaerobic bacteria in the genus *Clostridium*, such as *Clostridium thermocellum* and *Clostridium cellulolyticum*, are potential CBP candidates because they are able to degrade lignicellulose and produce ethanol simultaneously (Higashide et al. 2011; Svetlitchnyi et al. 2013). However, wild-type strains of these microorganisms generally have several drawbacks and thus cannot be used for industrial processes. For example, *C. cellulolyticum* is a mesophilic cellulosome-producing anaerobic microorganism with efficient lignocellulosic degradation and simultaneous pentose

and hexose utilization, but its low ethanol yield and complex by-product pattern limit its industrial application (Desvaux 2005; Li et al. 2012). Thus, targeted genetic engineering of these *Clostridium* strains is obligatory to make them feasible in industrial production of biofuels and related chemicals.

ClosTron is a direct insertion approach developed on the basis of the mobile group II intron from the *ltrB* gene of *Lactococcus lactis* (*Ll.ltrB*) and has been extensively used in the gene targeting of *Clostridium* strains (Heap et al. 2010; Heap et al. 2007; Kuehne et al. 2011), including *C. cellulolyticum* (Cui et al. 2012; Guedon et al. 2002; Higashide et al. 2011; Li et al. 2012). With the ClosTron system, either conditional or unconditional disruption can be achieved depending on the targetron inserting in the sense or antisense orientation of the chromosome DNA chain, respectively. The conditional gene disruption can be partially restored by the RNA splicing and the expression of the reverse transcriptase LtrA from a subsequently transformed targetron plasmid (Frazier et al. 2003; Karberg et al. 2001). Although conditional disruption can be used as synthetic and systems biology approach for bacterial genetic engineering, it makes the recombinant strain unstable and thus not preferred by targeted engineering. In addition, the intracellular targetron plasmid impedes the successive genetic engineering due to the plasmid incompatibility (Uhlin and Nordstrom 1975).

The elimination of targetron plasmid not only alleviates the intracellular expression of LtrA and converts conditional disruption into unconditional, but also enhances the successive gene targeting with ClosTron system. The "conditional disruption" is caused by the stable replication of targetron plasmid in cell, and the problem of plasmid curing in the application of ClosTron system might be bypassed using a non-replicable linear targetron plasmid theoretically. However, this strategy requires high transformation efficiency to support transient expression of the intron RNA and the reverse transcriptase LtrA, which has not been accomplished so far in most Gram-positive bacteria, including *Clostridium* (Cui et al. 2012; Lin et al. 2010; Rattanachaikunsopon and Phumkhachorn 2009). As reported in literatures, the common way to eliminate targetron plasmid in many *Clostridium* strains is to cultivate the transformants in the absence of selection, and the plasmid will be lost spontaneously after several generations of cell reproduction (Chen et al. 2005; Cui et al. 2012; Dong et al. 2010; Shao et al. 2007). This passive method requires a tedious screening process depending on the cell doubling time and, more importantly, the losing frequency of the target plasmid. Plasmids used for genetic manipulation of *C. cellulolyticum* are mostly derived from a plasmid pIM13, and its frequency of plasmid loss is approximately 4.7×10^{-3}/generation. Although the low frequency may be beneficial for the genetic stability, it severely hinders the targeted engineering in this microorganism (Jennert et al. 2000). For instance, Li et al. did not inactivate acetate kinase and phosphoacetyltransferase encoding genes of *C. cellulolyticum* H10 unconditionally because of the failure of plasmid elimination (Li et al. 2012). In our previous work, the plasmid curing in *C. cellulolyticum* H10 was not achieved until seven successive inoculations and cultivations had been performed in fresh medium without antibiotic (Cui et al. 2012), and the whole process could be much longer for successive gene manipulation. Thus, a rapid plasmid curing method will greatly improve the efficiency of successive gene targeting using ClosTron system.

PyrF (i.e., orotidine 5-phosphate decarboxylase) is an essential enzyme in the pyrimidine biosynthesis and can also be the target for the antimetabolite 5-fluoroorotic acid (FOA). Thus, the targeted inactivation and ectopic expression of *pyrF* have been widely used as convenient selection and counterselection marker in genetic engineering of various microorganisms (Boeke et al. 1984; Heap et al. 2012; Sato et al. 2005; Suzuki et al. 2012; Tripathi et al. 2010). In this study, we applied the *pyrF*-based selection system in *C. cellulolyticum* strain H10 to improve the ClosTron system by promoting the plasmid curing. By use of this selection system, the transformed targetron plasmid could be rapidly eliminated by screening with FOA, and the following gene disruption could be performed with no disturbance of the targetron previously constructed. Two essential cellulosomal proteins in *C. cellulolyticum* H10, CipC and Cel48F, were successfully disrupted using the developed screening system, and a triple mutant H10Δ*pyrF*::Cel48F235s::CipC117a was constructed via successive targeted disruptions. This is the first *pyrF*-based screening system developed for the enhancement of plasmid curing during targeted genetic manipulation, which will support directed engineering of various *Clostridium* and other microorganisms for biorefinery.

Materials and methods

Bacterial strains and cultivation

The bacterial strains and plasmids used in this study were listed in Table 1. *Escherichia coli* strains were grown aerobically on a rotary shaker (180 rpm) at 37 °C in Luria-Bertani (LB) broth or on a LB plate with 1.5 % (wt/vol) agar and supplemented with ampicillin (100 mg l^{-1}) when desired. *C. cellulolyticum* H10 strains were routinely grown anaerobically at 34 °C in modified GS-2-rich medium (Cui et al. 2012) or modified VM medium (Jennert et al. 2000) with 5.0 g l^{-1} cellobiose or avicel as carbon source, and 20 mg l^{-1} erythromycin was supplemented for recombinant strains. The VM medium was defined as uracil free by replacing yeast extract with trace elements ($FeSO_4{\cdot}7H_2O$ 5 mg, $ZnSO_4{\cdot}7H_2O$ 1.44 mg, $MnSO_4{\cdot}7H_2O$ 1.12 mg, $CuSO_4{\cdot}5H_2O$ 0.25 mg,

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
Strains		
E. coli		
DH5α	*f80dlacZΔM15*, *Δ(lacZYA-argF)U169*, *deoR, recA1*, *endA1*, *hsdR17* (r_k^-, m_k^+), *phoA*, *supE44*, l^-, *thi-1*, *gyrA96*, *relA1*	Invitrogen
C. cellulolyticum		
H10	ATCC35319, wild-type stain	ATCC
H10Δ*pyrF*	Derived from H10, *ccel_0614* disrupted via homologous recombination	This study
H10::PyrF402s	Derived from H10, *ccel_0614*::PyrF402s	
H10::CipC117a	Derived from H10, *ccel_0728*::CipC117a	This study
H10::Cel48F235s	Derived from H10, *ccel_0729*::Cel48F235s	This study
H10Δ*pyrF*::pIMP1	Derived from H10Δ*pyrF*, *ccel_0614* disrupted, containing plasmid pIMP1	This study
H10Δ*pyrF*::pGZ102	Derived from H10Δ*pyrF*, containing plasmid pGZ102	This study
H10Δ*pyrF*::CipC117a	Derived from H10Δ*pyrF*, *ccel_0614* disrupted, *ccel_0728*::CipC117a	This study
H10Δ*pyrF*::Cel48F235s	Derived from H10Δ*pyrF*, *ccel_0614* disrupted, *ccel_0729*::Cel48F235s	This study
H10Δ*pyrF*::Cel48F235s::CipC117a	Derived from H10Δ*pyrF*::Cel48F235s, *ccel_0614* disrupted, *ccel_0728*::CipC117a, *ccel_0729*::Cel48F235s	This study
Plasmids		
pIMP1	Em^R, Ap^R, *E. coli-C. cellulolyticum* shuttle vector	Lab storage
pGZ5	Derived from pIMP1, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *pyrF* disruption cassette	This study
pGZ102	Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, P_{fd}-PyrF expression cassette	This study
pSY6	Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *ptb* promoter, *L. lactis* Ll.Ltr intron, *ltrA*	Shao et al. (2007)
pGZ-*pyrF*	Derived from pSY6, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *ptb* promoter, PyrF402s intron	This study
pGZ-*cel48F*	Derived from pSY6, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *ptb* promoter, Cel48F235s intron	This study
pGZ-*cipC*	Derived from pSY6, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *ptb* promoter, CipC117a intron	This study
pGZ-PyrF	Derived from pSY6, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, P_{fd}-PyrF expression cassette	This study
pGZ-PyrF-*cel48F*	Derived from pGZ-PyrF, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *ptb* promoter, Cel48F235s intron	This study
pGZ-PyrF-*cipC*	Derived from pGZ-PyrF, Em^R, Ap^R, *E. coli–C. cellulolyticum* shuttle vector, *ptb* promoter, CipC117a intron	This study

$Na_2B_4O_7$ 0.2 mg, $Mo_7(NH_4)_6O_{24}{\cdot}4H_2O$ 1.0 mg, $NiCl_2$ 0.04 mg, $CoCl_2$ 0.02 mg, HBO_3 0.03 mg, Na_2SeO_3 0.02 mg, 10 M HCl 0.5 ml/l) and vitamins (D-biotin 0.08 μM, pyridoxamine 0.02 μM, cyanocobalamin 0.001 μM, ρ-aminobenzoic 0.15 μM, thiamine 0.9 μM, L-alanine 0.22 μM) (Higashide et al. 2011; Jennert et al. 2000). Uracil (80 μg ml^{-1}) and FOA (500 μg ml^{-1}) (dissolved in DMSO) were added when indicated (Tripathi et al. 2010). All *C. cellulolyticum* and *E. coli* strains were stored at −80 °C in 25 % sterile glycerol.

Plasmid construction

pGZ5 and pGZ102 were constructed basing on pIMP1 for *pyrF* disruption via homologous recombination and PyrF expression in *C. cellulolyticum*, respectively (Table 1). For pGZ5, 1.8 kb *pyrF* disruption cassette was obtained by splicing by overlapping extension polymerase chain reaction (SOE PCR) (Horton et al. 1989). In detail, the 5′ and 3′ flanking regions of *pyrF* were firstly amplified with primer set pyrF-U-1/pyrF-U-2 and pyrF-D-1/pyrF-D-2 (Table S1), which resulted in fragments with 885 and 937 bp, respectively. These two fragments were then ligated by overlap PCR with primer set pyrF-U-1/pyrF-D-2. The *pyrF* disruption cassette was subsequently digested with *Pst*I and *Xma*I and cloned between the corresponding sites of pIMP1 to generate pGZ5 (Fig. 1a). To construct pGZ102, the intact *pyrF* gene was amplified from the genome DNA of *C. cellulolyticum* with primer set pyrF-U/pyrF-D, and the constitutive ferredoxin promoter P_{fd} from *Clostridium pasteurianum* was synthesized (Guedon et al. 2002). The promoter sequence and the *pyrF* gene were ligated via overlap

PCR to construct the P_{fd}-PyrF expression cassette, and the plasmid pGZ102 was finally obtained by cloning the cassette into the *Xma*I restriction site of pIMP1 following the standard procedure (Sambrook et al. 1989) (Fig. 1a).

Targetron plasmids were constructed by replacing the 353-bp *Xho*I +*BsrG*I targeting region of plasmid pSY6 in this study (Shao et al. 2007). DNA target sites and corresponding intron retargeting primers were designed as described (Cui et al. 2012) (Table S1).The targeting regions for *pyrF*, *cipA*, and *cel48F* were obtained by SOE PCR with primer set Ccelxxxx-IBS/EBS universal, Ccelxxxx-EBS2/Ccelxxxx-EBS1d, and Ccelxxxx-IBS/Ccelxxxx-EBS1d successively according to previous report (Cui et al. 2012), where xxxx indicated the target gene number (Table S1 and Fig. 1b). The linearization of targetron plasmid pGZ-*pyrF* was performed by double digestion with *Hind*III and *Xma*I, and the fragment containing *pyrF* targetron was gel purified and used for transformation directly. pGZ-PyrF-derived targetrons contained the P_{fd}-PyrF expression cassette which was cloned into the *Xma*I site of pSY6 (Table 1 and Fig. 1b). A synonymous mutation (GT**A**-GT**C**, V199V) in *pyrF* gene had been achieved to delete the *Bsr*GI site before the cloning of targeting regions in pGZ-PyrF to eliminate the potential disturbance during the following targetron construction. The

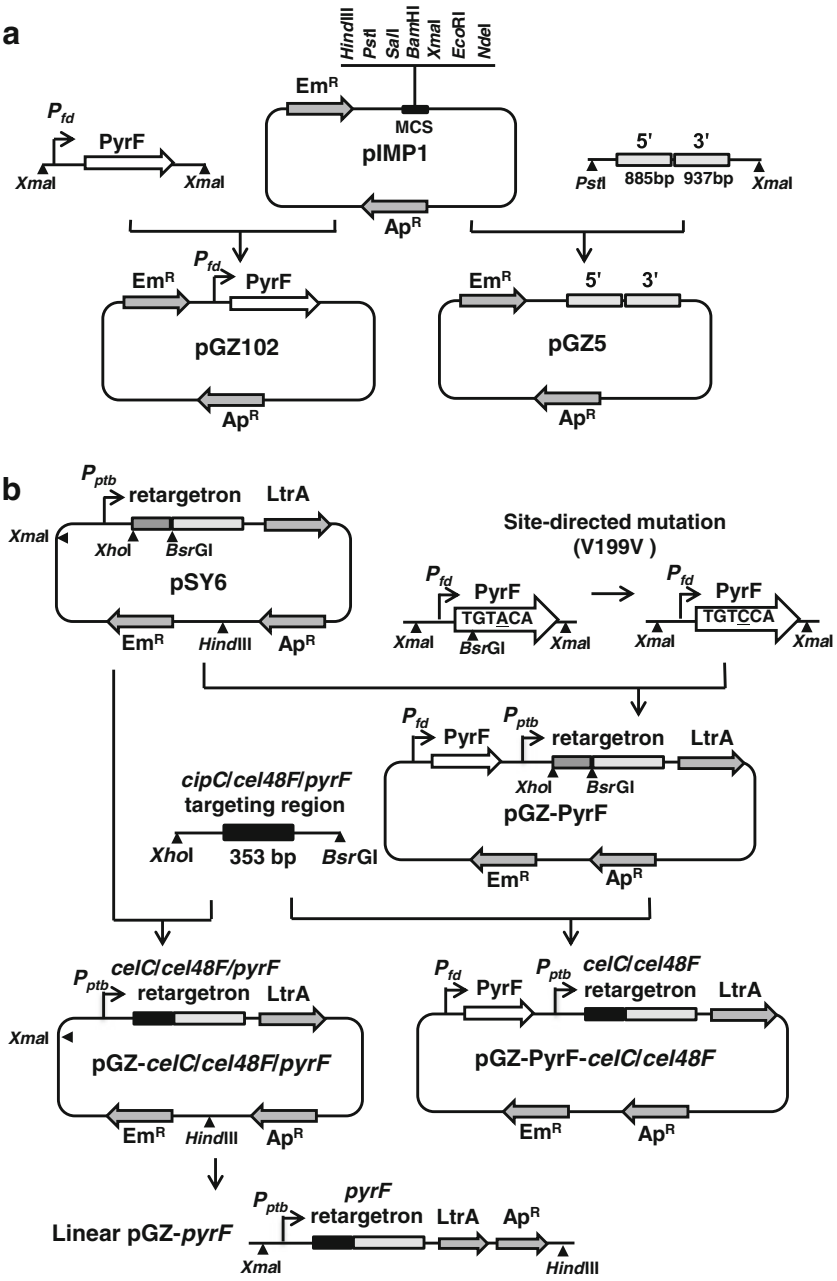

Fig. 1 Schematic representation of the plasmid construction involved in the *pyrF*-based screening system. **a** Construction of pGZ102 and pGZ5 on the basis of pIMP1. Both plasmids contained an ampicillin resistance gene (Ap^R) and an erythromycin resistance gene (Em^R). pGZ102 harbored a PyrF expression cassette controlled by a constitutive ferredoxin promoter (P_{fd}), and pGZ5 harbored the 5′ and 3′ flanking regions of the gene *pyrF* for gene disruption via homologous recombination. **b** Construction of targetron plasmids on the basis of pSY6. All targetron plasmids contained the targetron region and the reverse transcriptase LtrA expressed under the control of *ptb* promoters (P_{ptb}), and the targeting region was amplified by SOE PCR with primers listed in Table S1. The mutated site of gene *pyrF* was *underlined*. All relevant restriction sites were indicated by *solid triangles*

mutation was performed by PCR of pGZ-PyrF with primer set *BsrG*I-U/*BsrG*I-D using the Phusion™ High-Fidelity DNA Polymerase (NEB, Beijing, People's Republic of China) according to the manufacturer's protocol (Table S1). The PCR product was treated with *Dpn*I (NEB, Beijing, People's Republic of China) for 3 h at 37 °C to remove the plasmid template before transformation to *E. coli* DH5α. The site mutation was verified by sequencing (Sangon, Beijing, People's Republic of China).

Electrotransformation of *C. cellulolyticum*

Competent cells of *C. cellulolyticum* were prepared for electroporation according to a previous report (Cui et al. 2012) with the following modifications: (a) cells were grown to late exponential phase ($OD_{600\ nm}$ 0.5–0.7) and chilled on ice for 30 min before centrifugation, and 10 mg ml^{-1} of glycine was used to weaken the cell wall when needed; (b) 0.5–1 μg methylated DNA was loaded into a 0.2- or 0.4-cm cuvette (Bio-Rad) with 200 or 500 μl cell suspension, respectively, which had been kept on ice for 20 min prior to electroporation; (c) both commercial electroporation apparatuses (BTX ECM630 and Bio-Rad Gene Pulser Xcell) and custom-built pulse generation system MT01-3KV (0.2 cm cuvette, 1.2 kV, 2,000 Hz, 40 square pulses, 10 % duty cycle) were applied for electroporation. All manipulations were performed under anaerobic conditions. Transformation efficiency was determined by transforming pIMP1 into the wild-type strain H10, and colonies grown on agar plates with erythromycin were counted for calculation (transformants per microgram DNA). Three replicates were used to calculate the average value and standard error.

Disruption and complementation of *pyrF* in H10

The disruption of *pyrF* was performed via either homologous recombination or ClosTron method by transforming pGZ5 or pGZ-*pyrF* into the wild-type strain H10, and the obtained mutants were named as H10Δ*pyrF* or H10::PyrF402s, respectively. Transformants were screened on GS-2 medium plate with 20 μg ml^{-1} erythromycin. The obtained colonies were suspended in 100 μl sterile distilled water and then plated on solid GS-2 medium supplemented with 500 μg ml^{-1} FOA. The FOA-resistant *pyrF*-disruptant was tested by colony PCR using primers Ccel0614-1 and Ccel0614-2 (Table S1), and those yielded PCR products of 0.5 or 2.4 kb were verified as *pyrF*-inactivated mutant after sequencing (Figs. S1 and S2). PyrF complementation was achieved by transforming plasmid pGZ102 into H10Δ*pyrF* strain after plasmid curing following a successive inoculation approach (Cui et al. 2012), and pIMP1 was also transformed in parallel as control. Erythromycin-resisting transformants of pGZ102 and pIMP1 were identified as H10Δ*pyrF*::pMTC102 and H10Δ*pyrF*::pIMP1, respectively (Table 1).

Disruption of *cipC* and *cel48F* in H10 and H10Δ*pyrF*

The disruption of *cipC* and *cel48F* in *C. cellulolyticum* was achieved by using corresponding targetron plasmids listed in Table 1. pGZ-*cel48F* and pGZ-*cipC* were used for gene disruptions on the basis of wild-type strain H10. Double mutants H10Δ*pyrF*::Cel48F235s and H10Δ*pyrF*::CipC117a were constructed by transforming pGZ-PyrF-*cel48F* and pGZ-PyrF-*cipC* into H10Δ*pyrF*, respectively. Triple mutant H10Δ*pyrF*::Cel48F235s::CipC117a was constructed on the basis of H10Δ*pyrF*::Cel48F235s using targetron pGZ-PyrF-*cipC* after plasmid curing. All targetron transformants were selected on plate with erythromycin. The disruption of *cipC* and *cel48F* was confirmed by colony PCR giving 1.2 kb PCR product with primer sets Ccel0728-1/Ccel0728-2 and Ccel 0729-1/Ccel 0729-2, respectively. For double and triple mutants, disruption of *pyrF* was simultaneously investigated by PCR with Ccel0614-1 and Ccel0614-2 in multiple mutants (Table S1 and Fig. 2b).

Plasmid curing

For plasmid curing, transformants of H10-derived mutants (i.e., H10Δ*pyrF*, H10::Cel48F235s, and H10::CipC117a) were inoculated and cultivated in liquid or solid GS-2 medium without erythromycin alternately for five to seven times as previously described (Cui et al. 2012). Transformants of H10Δ*pyrF*-derived strains (i.e., H10Δ*pyrF*::pMTC102, H10Δ*pyrF*::pIMP1, H10Δ*pyrF*::Cel48F235s, H10Δ*pyrF*::CipC117a, and H10Δ*pyrF*::Cel48F235s::CipC117a) were screened using solid GS-2 medium supplemented with 500 μg ml^{-1} of FOA to eliminate the plasmid. For recombinant strains with targetron plasmids, a preliminary cultivation was performed in 5 ml GS-2 medium with erythromycin for 36 h prior to the FOA screening to enhance the targetron insertion.

All transformants were finally inoculated into GS-2 medium with erythromycin, and the plasmid curing was verified if no growth was observed.

Growth analysis of *C. cellulolyticum* wild-type and recombinant strains

Growth analysis of *C. cellulolyticum* strains was preceded in 200 ml anaerobic bottle at 34 °C with 5 g l^{-1} cellobiose or 5 g l^{-1} Avicel as sole carbon source. Cell growth in cellobiose was monitored by measuring the optical density at 600 nm with a UV–Vis spectrophotometer. The cell growth in Avicel was

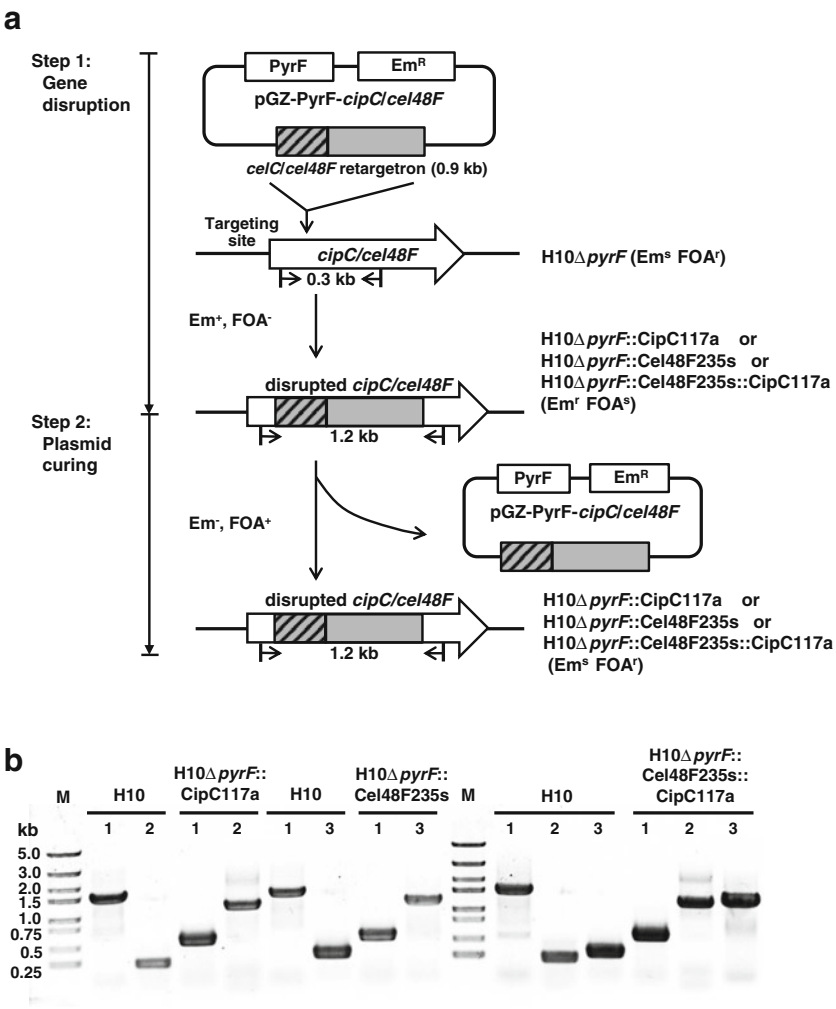

Fig. 2 Schematic representation and PCR confirmation of the two-step procedure of successive gene disruption using the *pyrF*-based screening system. **a** Schematic representation. *Gray boxes* indicated targetron sequences with 0.9 kb, in which the 353-bp targeting region in the targetron was represented by *slashes*. *Em* erythromycin, *FOA* 5-fluoroorotic acid. Screening with or without Em/FOA was indicated with the *superscript* "+" or "–," respectively. The phenotypes of resistance (*r*) or sensitivity (*s*) to Em/FOA of the obtained recombinant strains were represented in *parentheses*. **b** PCR confirmation of the recombinant strains. Colonies of *C. cellulolyticum* H10, H10Δ*pyrF*::CipC117a, H10Δ*pyrF*::Cel48F235s, and H10Δ*pyrF*::Cel48F235s::CipC117a were used as templates for PCR with primer sets Ccel0641-1/Ccel0641-2 (*1*), Ccel0728-1/Ccel0728-2 (*2*), and Ccel0729-1/Ccel0729-2 (*3*). *M* DNA markers

estimated by measuring the total cellular protein (Desvaux et al. 2000; Pavlostathis et al. 1988). In detail, cell pellets of 1.5 ml culture were collected by centrifugation at 10,000×*g* for 10 min and were washed twice with 0.9 % (*w*/*v*) NaCl. The pellets were then resuspended in 0.2 ml of 0.2 M NaOH and stood still in a boiling water bath for 5 min. The hydrolyzed samples were centrifuged (10,000×*g*, 10 min) after cooling in ice, and the supernatants were directly used for quantification of the total cellular protein using Bradford method (Bradford 1976). For calculation of colony-forming unit (CFU), a dilution series of both wild-type and recombinant strains were prepared after cultivation in GS-2 medium for 24 h with cellobiose as a carbon source. One hundred microliters of 10^3 to 10^6 dilutions was plated on different solid media (GS-2, GS-2 + FOA, VM, and VM + uracil) containing erythromycin and cultivated for 5 days. The plates that contained 50–150 colonies were selected for colony counting, basing on which the CFU per microliter was calculated by multiplying the corresponding dilution fold and dividing the sample volume.

Quantification of transcripts in *C. cellulolyticum* wild-type and recombinant strains

Total RNAs of *C. cellulolyticum* wild-type and recombinant strains were isolated using TRIzol reagent (Invitrogen, Beijing, People's Republic of China). Reverse transcription of RNA was performed with specific primers (Table S1) and SuperScript III First-Strand Synthesis Supermix (Invitrogen, Beijing, People's Republic of China). Both obtained cDNA and corresponding RNA were used as templates, and the DNA-free RNA was confirmed when no PCR product was observed in the RNA sample. Quantitative reverse transcriptase PCRs (qRT-PCR) of cDNAs were performed with gene-specific primers using a Real-Time PCR System LightCycler R480 (Roche, Shanghai, People's Republic of China). qPCR reactions were performed using a FastStart Universal SYBR Green Master (Rox) according to the manufacturer's protocol (Roche, Shanghai, People's Republic of China). The comparative *Ct* method was used to calculate the

abundance of specific transcript in samples by quantifying the 16S rRNA gene in parallel as control.

Results

Improvement of the transformation efficiency of *C. cellulolyticum* H10

To test the strategy of using non-replicable linear targetron plasmids to eliminate the plasmid curing problem, the transformation efficiency of *C. cellulolyticum* H10 was improved. According to the previous reports, the transformation efficiency of *C. cellulolyticum* was approximately 10^2 CFU μg^{-1} DNA (Jennert et al. 2000; Tardif et al. 2001) and was then increased to the order of 10^3 by using the cell-weakening agent glycine (Cui et al. 2012). A novel custom-built pulse generator MT01-3KV (0.2 cm cuvette, 1.2 kV, 2,000 Hz, 40 square pulses, 10 % duty cycle) was developed in this study and resulted in >80 times higher of transformation efficiency in comparison with the commercial pulsers (i.e., 0.93×10^2 and 1.16×10^2 CFU μg^{-1} DNA for BTX ECM630 and BioRad Xcell, respectively, and 9.7×10^3 CFU μg^{-1} DNA for MT01-3KV). The transformation efficiency was further improved up to 5.3×10^4 CFU μg^{-1} DNA when the competent cells were pretreated with 10 mg ml^{-1} glycine, which should be the highest transformation efficiency reported so far according to our knowledge.

Construction of the *pyrF*-based screening system

Based on the high efficient transformation method, we first tested whether non-replicable linear targetron plasmids could be used for gene targeting in *C. cellulolyticum* H10. Both linearized and circular plasmid pGC-*pyrF* which contained a *pyrF* targetron PyrF402s were transformed into H10 to disrupt the gene *pyrF* (Table 1). After the screening on the GS-2 plate with erythromycin, no transformant was obtained for linear plasmid; in contrast, numerous colonies were observed for the circular pGZ-*pyrF*, and the disruption of *pyrF* was confirmed by colony PCR after FOA screening (Fig. S2). This result indicated that the gene targeting with non-replicable linear targetron could not be accomplished under current conditions, and a new strategy is required to solve the plasmid-curing problem.

Subsequently, a *pyrF*-based screening system, consisting of a *pyrF*-inactivated mutant as chassis and a PyrF expression cassette carried by targetron plasmids, was developed to enhance the plasmid curing. To obtain the *pyrF*-inactivated strain H10Δ*pyrF*, the plasmid pGZ5 was transformed into the wild-type strain H10. pGZ5 contained the 5′ and 3′ flanking regions of *pyrF*, with which the intact *pyrF* gene in the genome DNA of H10 could be deleted via homologous recombination. All of the 48 tested colonies were verified as *pyrF*-inactivated mutant after sequencing, indicating a deletion frequency of 100 % (Fig. S1). The plasmid curing of H10Δ*pyrF* was achieved after seven times continuous inoculation and cultivation in antibiotic-free GS-2 medium, after which the PyrF complementation was accomplished by transforming PyrF expression plasmid pGZ102 into H10Δ*pyrF*. pGZ102 was constructed by inserting a PyrF expression cassette under the control of a constitutive promoter P_{fd} into pIMP1, which was also transformed into H10Δ*pyrF* as negative control. The phenotypes of FOA resistance and uracil auxotroph were subsequently investigated in H10, H10Δ*pyrF*, H10Δ*pyrF*::pGZ102, and the control H10Δ*pyrF*::pIMP1 (Table 2). H10 and H10Δ*pyrF*::pGZ102, which contained the wild-type or complemented PyrF, showed similar phenotypes of FOA sensitivity (FOA^s), while the *pyrF*-inactivated strains H10Δ*pyrF* and H10Δ*pyrF*::pIMP1 were all uracil auxotrophs and resistant to FOA (FOA^r). This indicated that the FOA^r or FOA^s phenotype of *C. cellulolyticum* H10 could be flexibly controlled using the constructed *pyrF*-based screening system.

Enhancement of the plasmid curing by using the *pyrF*-based screening system

Both H10Δ*pyrF*::pGZ102 and H10Δ*pyrF*::pIMP1 were used to test whether the frequency of plasmid curing could be improved with the application of the developed *pyrF*-based screening system (Table 1). According to the screening principle of the *pyrF*-based screening system, colonies in which the plasmid pGZ102 was cured should be easily selected by FOA screening due to the constitutively expressed PyrF, while H10Δ*pyrF*::pIMP1 should not be influenced. Because the resistance to erythromycin was introduced by the transformed plasmid, after plasmid curing, H10Δ*pyrF*::pGZ102 should be sensitive to the antibiotic and H10Δ*pyrF*::pIMP1 should maintain the resistance. Several colonies of H10Δ*pyrF*::pGZ102 and H10Δ*pyrF*::pIMP1 growing in GS-2 + FOA solid medium were obtained, and seven of each were subsequently inoculated into fresh GS-2 medium with erythromycin. No growth of H10Δ*pyrF*::pGZ102-derived strains was observed indicating that pGZ102 had been eliminated during FOA screening. In contrast, all H10Δ*pyrF*::pIMP1-derived strains grew well in the presence of antibiotic. This result suggested that continuous inoculation and cultivation would be needed to cure plasmid from H10Δ*pyrF*::pIMP1, while the developed screening system could achieve the plasmid curing rapidly by one-step FOA screening in *C. cellulolyticum* recombinant strains.

Table 2 Phenotypes of the wild-type and recombinant strains of *C. cellulolyticum* H10

Strain or genotype	Colony-forming unit (CFU)[a]/μl×10[5]			
	GS-2	GS-2 + FOA	VM	VM + uracil[c]
H10	2.1±0.4	0.00005±0.00002	2.9±1.9	3.2±2.6
H10Δ*pyrF*	2.8±0.8	1.5±0.5	0	2.1±0.7
H10Δ*pyrF*::pIMP1	4.8±2.5	3.5±0.3	0	2.3±0.2
H10Δ*pyrF*::pGZ102	2.7±0.9	0.00005±0.00004	2.5±0.4	3.4±1.3

[a] The CFU numbers were calculated based on three independent setups, and the average values and standard errors were given

Successive disruption of *cipC* and *cel48F* in *C. cellulolyticum* H10 by use of the *pyrF*-based screening system

Both *cipC* and *cel48F* are major cellulosomal genes that are located in the *cip-cel* cluster in *C. cellulolyticum* H10, encoding a scaffoldin protein and a family 48 cellulase, respectively (Maamar et al. 2006). Therefore, they were selected as targets to test the *pyrF*-based screening system in successive gene targeting. The gene targeting was firstly performed following the reported ClosTron method without the application of the screening system developed in this study (Cui et al. 2012). Targetron plasmids pGZ-*cel48F* and pGZ-*cipC* containing no PyrF expression cassette were transformed into the wild-type strain H10 to inactivate gene *cel48F* and *cipA*, respectively (Table 1). The resulted mutant strains H10::Cel48F235s and H10::CipC117a were confirmed by colony PCRs (Fig. S1), and the plasmid curing was subsequently performed by continuous inoculation and cultivation under no selection stress according to the reported procedure (Cui et al. 2012). However, the descendants of both H10::Cel48F235s and H10::CipC117a remained to be resistant to erythromycin after five times inoculation (data not shown). This indicated that the targetron plasmids were not successfully cured, and neither H10::Cel48F235s nor H10::CipC117a can be used for further gene disruption with ClosTron system.

With the application of the *pyrF*-based screening system, two steps will be were followed to obtain the desired mutant. The first step is the targeted gene disruption achieved by transforming PyrF-expressing targetron plasmids into the chassis H10Δ*pyrF*, and the second step is the plasmid curing basing on the FOA screening (Fig. 2a). Accordingly, targetron plasmids pGZ-PyrF-*cel48F* and pGZ-PyrF-*cipC* containing the PyrF expression cassette were subsequently constructed and transformed into H10Δ*pyrF*, and the obtained transformants were verified as double mutants H10Δ*pyrF*::CipC117a and H10Δ*pyrF*::Cel48F235s, respectively, according to the colony PCR and sequencing (Fig. 2b). Both H10Δ*pyrF*::CipC117a and H10Δ*pyrF*::Cel48F235s were then cultivated in erythromycin-added medium to enhance the targetron insertion and screened by FOA to eliminate the targetron plasmids as described in "Materials and methods." Colonies grown on FOA plates were then inoculated into liquid medium with erythromycin to confirm the elimination of the targetron plasmids, and no growth was observed. This indicated that the plasmid curing was successfully accomplished for either H10Δ*pyrF*::CipC117a or H10Δ*pyrF*::Cel48F235s using the *pyrF*-based screening system.

To test whether the successive gene targeting could be performed by using the screening system, the disruption of *cipC* was subsequently performed on the basis of the plasmid-cured H10Δ*pyrF*::Cel48F235s using the targetron plasmid pGZ-PyrF-*cipC* following the two-step procedure described above (Fig. 2a). According to colony PCR and sequencing results, the simultaneous disruption of gene *pyrF*, *cel48F*, and *cipC* was achieved, resulting in triple mutant H10Δ*pyrF*::Cel48F235s::CipC117a with the phenotype of erythromycin sensitivity (Fig. 2b), which indicated that the targetron plasmid was eliminated and further targeted gene disruption could be performed accordingly.

The influence of *cel48F* and *cipC* disruption on cellulose utilization and gene transcription of *cip-cel* cluster in *C. cellulolyticum*

The growth patterns of wild-type strain H10 and recombinant strains (H10Δ*pyrF*, H10Δ*pyrF*::CipC117a, and H10Δ*pyrF*::Cel48F235s) were analyzed by determining the cell turbidity or the concentration of cellular protein of the cultures with cellobiose or Avicel as the sole carbon source, respectively. H10Δ*pyrF* and H10 showed similar growth patterns with either cellobiose or Avicel as sole carbon source, which indicated that the disruption of *pyrF* did not influence the growth pattern of H10. With cellobiose as carbon source, all recombinant strains grew similarly to H10 (Fig. 3), whereas a lack of growth was observed using Avicel as carbon source in *cipC* or *cel48F*-disrupted strains. The biomass of H10Δ*pyrF*::Cel48F235s was only one fifth of that of H10 after 10 days of cultivation (Fig. 3), while almost no growth of H10Δ*pyrF*::CipC117a was observed even after 30 days of cultivation (data not shown). This indicated that the disruption of *cel48F* and *cipC* severely affected the cellulose utilization of *C. cellulolyticum.*

The stem loop-like structure of the intron RNA may affect the co-transcribed genes behind (Lambowitz and Zimmerly

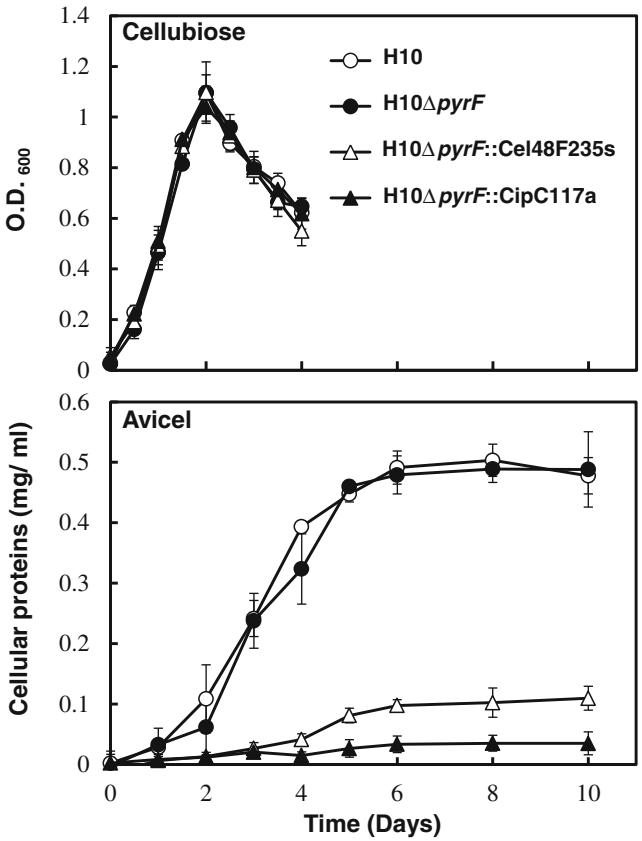

Fig. 3 Growth patterns of *C. cellulolyticum* H10, H10Δ*pyrF*, H10Δ*pyrF*::CipC117a, and H10Δ*pyrF*::Cel48F235s with cellobiose or avicel as the carbon source. When growing in cellobiose, the optical density at 600 nm was measured to determine the cell growth, while the total cellular protein was monitored when avicel was used as the carbon source

2011). *cipC* and *cel48F* are the first two genes in *cip-cel* cluster which contains major cellulosomal genes in *C. cellulolyticum* H10. To test whether the disruption of these two genes affected the following genes in *cip-cel* cluster, the transcription level of all 12 genes in the cluster was analyzed in H10Δ*pyrF*::CipC117a and H10Δ*pyrF*::Cel48F235s via qRT-PCR (Fig. S3). In *cipC*-disrupted strain, the transcript abundance of all 12 genes was reduced for 10^2 to 10^5 times compared with that of H10Δ*pyrF*. This result indicated that the disruption of *cipC* inhibited the transcription of the whole cluster, and the *cip-cel* cluster was consecutively transcribed which was consistent with the previous report (Abdou et al. 2008). Interestingly, the transcription of all genes in *cip-cel* cluster was also repressed for 10^2 to 10^3 times in e*cel48F*-disrupted strain, including *cipC*, although *cipC* was transcribed ahead of *cel48F* in *cip-cel* cluster according to previous reports (Abdou et al. 2008; Maamar et al. 2006). This result indicated that there might be a hitherto unknown regulation mechanism behind the expression and assembling of the cellulosomal components in *C. cellulolyticum*.

Discussion

Plasmid curing is a common problem of genetic manipulation of many microorganisms, and several methods by using chemical agents, elevated temperature, ultraviolet light, plasmid incompatibility, and microwave have been reported to promote the plasmid elimination (Berzin et al. 2013; Brana et al. 1983; Hovi et al. 1988; Spengler et al. 2006). Here, we suggest a new strategy using a *pyrF*-based screening system to promote the plasmid curing and has demonstrated its application in the efficient and successive gene targeting of *C. cellulolyticum* with the ClosTron system. Because the group II intron-based targetron technology could be used widely in *Clostridium* strains, and the *pyrF*-based screening system has been developed in more microorganisms besides *Clostridium*, our new strategy of plasmid curing could be easily extended to other microorganisms.

Another strategy to solve the plasmid curing problem is using linear plasmid, which requires high transformation efficiency to support the transient gene expression or homologous recombination. In this study, we found that the high transformation efficiency with 5.3×10^4 transformants μg^{-1} DNA still cannot satisfy the requirement of transient function of the ClosTron system. It has been reported that gene targeting through homologous recombination with suicide vectors demands a transformation efficiency of over 10^5 transformants μg^{-1} DNA (Cui et al. 2012). The function of the ClosTron system, including the expression and gene targeting of the intron RNA and the reverse transcriptase LtrA, is generally more complex than the homologous recombination procedure via allele exchange. Thus, we proposed that the transformation efficiency should be further increased for at least one order of magnitude to meet the requirement of genetic engineering with non-replicable ClosTron plasmid.

The *pyrF*-based genetic tool has been widely used for genetic engineering via homologous recombination (Boeke et al. 1984; Chung et al. 2013; Liu et al. 2011; Tripathi et al. 2010). Here, we applied this screening tool in ClosTron-based gene targeting of *C. cellulolyticum* for rapid plasmid curing. By using this screening system, the targeted gene disruption could be completed following a two-step procedure (Fig. 2a), and the obtained recombinant strain could be ensured as targetron free. The *pyrF*-inactivated chassis was initially constructed via homologous recombination, so it might be still a problem to eliminate the plasmid with the homologous arms. In this study, the plasmid curing of H10Δ*pyrF* was not achieved until seven times successive inoculation under no selection stress. This problem could be solved by using a second plasmid backbone with a different antibiotic gene for the following genetic manipulation on the basis of the chassis cell to avoid the plasmid incompatibility, in case the plasmid curing could not be easily accomplished. For example, the

pNW33N-derived plasmid with the chloramphenicol-resistant gene has been used in the gene manipulation of *C. thermocellum* (Olson et al. 2010; Tripathi et al. 2010), and it might be used as an alternative plasmid.

With the application of the developed screening system, three multiple mutants (H10Δ*pyrF*::CipC117a, H10Δ*pyrF*::Cel48F235s, and H10Δ*pyrF*::Cel48F235s::CipC117a) were successfully constructed. The function of either CipC or Cel48F in *C. cellulolyticum* has been investigated via random mutation or RNAi (Maamar et al. 2004; Perret et al. 2004). Our results supported the previous conclusion that the disruption of either CipC or Cel48F inhibited the cellulose degradation significantly (Fig. 3). The transcription pattern of the whole *cip-cel* cluster was also investigated since it was reported that this cluster was consecutively transcribed (Abdou et al. 2008). The results indicated that the disruption of either *cipC* or *cel48F* blocked up the transcription of the whole cluster, and the phenotypes of H10Δ*pyrF*::CipC117a and H10Δ*pyrF*::Cel48F235s should be caused by the inactivation of the whole *cip-cel* cluster instead of the disruption of single gene. To knock-out a single gene in a cluster, an endogenous promoter region should be inserted into the chromosome DNA right behind the inserted intron sequence to support the transcription of the following genes, and the "knock-in" of the promoter could be potentially accomplished by using targetron as the DNA cargo (Kuehne et al. 2011), while the efficiency of this knock-in method should be investigated in further study since the integration efficiency of the intron could be dramatically reduced when carrying extra sequences (Enyeart et al. 2013; Jia et al. 2011).

In conclusion, the *pyrF*-based screening system was successfully developed in this study to solve the problem of targetron plasmid curing demonstrated in *C. cellulolyticum*. The conditional disruption of the targetron transformants could be converted into unconditional efficiently, and the successive gene targeting was achievable. Considering that PyrF is widely found in microbes as an essential enzyme in the pyrimidine biosynthesis, the new strategy of plasmid curing developed in this study based on the *pyrF*-based screening system could be extended to other plasmid-mediated engineering of various microorganisms for plasmid curing.

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