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Genome editing of different strains of *Aureobasidium melanogenum* using an efficient Cre/loxp site-specific recombination system

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ABSTRACT

It has been well known that different strains of *Aureobasidium* spp. can produce commercial pullulan, polymalate, liamocin, intracellular lipids, gluconic acid, siderophore, melanin and various enzymes. In order to fully elucidate their synthetic pathways and regulation, it is necessary to have an efficient gene editing system for genetic modification of *Aureobasidium* spp. In this study, an efficient Cre/loxp site-specific recombination system (pAMGDloxp-1, pAMEXlox-1 and pAMCRE1) was constructed. It was found that they could be successfully used to sequentially delete and express many genes in different strains of *A. melanogenum*. After each round of gene disruption and expression, over 0.5 positive cells per 1000 competent cells and over 49.8 positive transformants per 1.0 μg DNA were achieved. After each round of the antibiotics gene excision by using the Cre-loxp site-specific recombination, over 95.4 % of the antibiotics-resistant cells became sensitive to both hygromycin B and nourseothricin again. This demonstrated that the Cre/loxp site-specific recombination system constructed in this study can efficiently be used to simultaneously delete and express many genes in different strains of *A. melanogenum*. These systems are promising approaches for the easily modifying genomics of the yeast-like fungal strains with enhanced metabolic pathways through multicopy gene deletion and expression.

1. Introduction

The genus ascomycetous *Aureobasidium* spp. is a black yeast-like fungus and a member of the family Saccotheciaceae within the class of the Dothideomycetes. So far, *A. microstictum*, *A. proteae*, *Aureobasidium pullulans*, *A. lini*, *A. namibiae*, *Aureobasidium melanogenum*, *A. leucospermi*, *A. subglaciale*, *A. iranianum*, *A. caulivorum*, *Aureobasidium mangrovei*, *A. thailandense*, *A. pullulans* var. *aubasidan* have been characterized and identified (Nasr et al., 2018). At the same time, the genomic DNAs of many strains of the genus have been sequenced and the sequenced DNAs have been annotated (Chan et al., 2012; Gostincar et al., 2014; Zhang et al., 2019). These yeast-like fungi are largely known as sources of commercial pullulan, polymalate, liamocin, intracellular lipids, gluconic acid, siderophore, melanin and various enzymes (Aung et al., 2019; Chen et al., 2017; Chi et al., 2012, 2016; Garay et al., 2018; Jiang et al., 2017; Li et al., 2007; Ma et al., 2014, 2018; Ni et al., 2009; Wang et al., 2014). The produced pullulan, liamocin, intracellular lipids, siderophore, melanin and alkaline protease can be used to make drug capsule, 5-hydroxy-2-decenoic acid lactone (Massoia lactone), antimicrobial agents, biodiesel and antioxidants (Garay et al., 2018; Jiang et al., 2017; Liu et al., 2018; Wang et al., 2014). This means that different strains of the genus have many potential applications in basic research and biotechnology. However, the exact biosynthesis pathways for pullulan, polymalate, liamocin, siderophore and melanin are still awaited to be elucidated as biosynthesis and regulation of each of the compounds must be encoded by many genes and catalyzed by many corresponding enzymes. At the same time, in order to enhance biosynthesis and regulation of commercial pullulan, polymalate, liamocin, intracellular lipids, gluconic acid, siderophore, melanin and various enzymes, many genes in the yeast-like fungal cells need to be over-expressed or completely deleted. Therefore, it is very important to develop an effective genome editing system for the genus to investigate the function of

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genes or their products and uncover the full synthetic pathways and regulation. In recent years, the CRISPR–Cas system has been widely used in genome editing in fungi, yeasts and bacteria and multigene manipulation can thus be easily achieved using this technique, which makes unnecessary the complex procedure used in any other genome editing techniques (Schuster et al., 2016). However, the commonly used CRISPR/Cas9 systems are protospacer adjacent motif (PAM) dependent, have high off-target effects and its specificity and reliability is still an open debate (Wang et al., 2017a,b; Stella and Montoya, 2016). At the same time, the Cre-*lox*p–mediated recombination system, the use of which is becoming increasingly widespread, is also a powerful gene editing tool that has become a main technique for genetics and cell biology research. Use of the Cre-*lox*p site specific recombination system has greatly expanded our ability to precisely interrogate gene function in a wide variety of organisms from mammalian cells to fungal, yeast and bacterial cells in most recent years (Guldener et al., 1996; Wang et al., 2017a,b). This has been largely due to the simplicity of its use and its adaptability to address diverse biological questions (McLellan et al., 2017). Furthermore, the Cre/loxp system can be used as a tool for generating a recyclable marker system, which allows repetitive rounds of gene deletions and expression and subsequent marker recycling, which is necessary to uncover the whole multigene biosynthesis pathways and their regulation (Nguyen et al., 2018). Therefore, in this study, the Cre/loxp system was used to carry out multigene manipulation in different strains of *A. melanogenum*. Meanwhile, a "FLP/FRT" system, composed of FLP recombinase with corresponding FRT sites, is also rarely used for marker recycling in filamentous fungi, such as *Penicillium chrysogenum*, *Sordaria macrospora*, *Ustilago maydis* and *Acremonium chrysogenum* (Wang et al., 2015). However, this system has not been tried in *Aureobasidium* spp. so far.

To date, it has been demonstrated that most of the compounds and enzymes mentioned above are synthesized by *A. melanogenum*, *A. pullulans* and *Aureobasidium* sp. P6 (Ma et al., 2014, 2018; Zhao et al., 2019), especially by *A. melanogenum* and *A. pullulans* is widely distributed in different environments in this world (Li et al., 2015). So, in this study, an efficient Cre-*lox*p site specific recombination system was developed to knock-out and knock-in different genes in different strains of *A. melanogenum* because of not any other suitable site-specific recombination systems available for this species.

2. Materials and methods

2.1. Yeast-like fungal strains, media and plasmids

A. melanogenum HN6.2 producing siderophore and esterase (Chi et al., 2012; Chen et al., 2017), *A. melanogenum* P16 producing pullulan (Ma et al., 2014) and *A. melanogenum* 9-1 producing liamocin (Tang et al., 2018) were used to delete and express different genes in them. All the yeast-like fungal strains were grown on a YPD medium containing 20.0 g/L glucose, 20.0 g/L polypeptone and 10.0 g/L yeast extract. The pullulan production medium (Ma et al., 2014), siderophore production medium (Chi et al., 2012), esterase production medium (Chen et al., 2017), liamocin production medium (Tang et al., 2018) were used. The disruptants and transformants obtained in this study were grown in the YPD medium containing 50 μg/mL hygromycin B and both hygromycin B (50 μg/mL) and nourseothricin (50 μg/mL) (Wang et al., 2017a,b). A YNB medium without amino acids was used to grow a lysine mutant. The knock-out vector pFL4A carrying hygromycin B resistance gene (*HPT* gene), knock-in vector pNATX13-NS carrying nourseothricin resistance gene (*NAT* gene) and the plasmid pPWN302 carrying a DNA fragment P_{pgk}-NAT-polyA (Wang et al., 2017a,b) were used to construct deletion and expression plasmids as described below. The ARS (autonomously replicating DNA sequence) element from *Astilago maydis* (Schuster et al., 2016) and the codon-optimized Cre recombinase gene from bacteriophage P1 were synthesized by GenScript (Nanjing, China) and ligated into pUC57, respectively. Therefore, the resulting plasmids were named pUC57-ARS and pUC57-Cre. The plasmid pEGFP carrying the gene encoding enhanced green fluorescent protein (EGFP) was kindly offered by Professor Long-Fei Wu at CNRS, France.

2.2. Construction of deletion, expression and ARS-Cre plasmids

The primers (loxp-1s/loxp-1a) corresponding to the partial loxP sites and the partial nourseothricin resistance gene (Supplementary file 1) were used to PCR amplify a DNA fragment1 (loxp1) from the plasmid pPWN302 as a template. The primer sets (loxp-2s/loxp-2a) were used to PCR amplify another DNA fragment2 (loxp2) from the DNA fragment1 as a template. The DNA fragment2 was digested using *Bam*HI and *Sal*I and the digests (loxp-P_{pgk}-NAT-polyA-loxp) were ligated into the plasmid pFL4A digested with the same enzymes using T_4 DNA ligase, resulting in a plasmid pAMGDlox-1 (accession number: MH453955) (Supplementary file 2A). The DNA fragments loxp-P_{pgk}-NAT-polyA-loxp were obtained by digestion of the pAMGDlox-1 with the enzymes *Bam*HI and *Sal*I (Supplementary file 1) and the digests loxp-P_{pgk}-NAT-polyA-loxp was ligated into the plasmid pNATX13-NS hydrolyzed with the same enzymes using T_4 DNA ligase, yielding a plasmid pAMEXlox-1 (accession number: MH453956) (Supplementary file 2B). The ARS fragment was PCR amplified from the plasmid pUC57-ARS using the primers (ARS-se/ARS-an) (Supplementary file 1) and the codon-optimized P_{gap}-Cre-Cyc1 fragment was PCR amplified from the plasmid pUC57-Cre using the primers (gcc-se/gcc-an) (Supplementary file 1). The synthesized ARS and the codon-optimized P_{gap}-Cre-Cyc1 fragment were digested with enzymes *Bam*HI/*Eco*RI and *Sph*I/*Sal*I and the digests were inserted into the pFL4A digested with the same enzymes, producing a plasmid pAMCRE1 carrying both the ARS and Cre recombinase gene (Accession number: MH453954) (Supplementary file 2C).

2.3. Sequential removal of different genes

To sequentially delete the *SidA* gene encoding L-ornithine-N5-monooxygenase (accession number: FJ769160), a key enzyme in siderophore biosynthesis and the *PPTase* gene (accession number: KX771231.1) encoding phosphopantetheinyl transferase which can modify and activate non-ribosomal peptide synthase (NRPS) for siderophore biosynthesis from *A. melanogenum* HN6.2, 3′-arms and 5′-arms of the *SidA* gene and the *PPTase* gene were PCR amplified from the genomic DNA of *A. melanogenum* HN6.2 using the primers (149-SidA-3-se/149-SidA-3-an, 149-PPT-3-se/149-PPT-3-an, 149-SidA-5-se/149-SidA-5-an and 149-PPT-5-se/149-PPT-5-an) shown in Supplementary file 1. The obtained 3′-arms and 5′-arms were digested with the enzymes *Bam*HI/*Eco*RI, *Pst*I/*Sal*I and *Sph*I/*Sal*I, respectively and the digests were introduced into the pAMGElox-1 digested with the same enzymes, forming the plasmids pAMGDlox-1-ΔSidA and pAMGDlox-1-ΔPPTase (data not shown). The plasmid pAMGDlox-1-ΔSidA and pAMGDlox-1-ΔPPTase were digested with the enzymes *Rco*RI and *Pst*I to release the linear DNA fragments 3′-arm-P_{pgk}-NAT-polyA-5′-arm. The linear DNA fragments carrying 3′-arm and 5′-arm from the *SidA* gene were transformed into the competent cells (10^7 cell/mL) of *A. melanogenum* HN6.2 to obtain a mutant *ΔsidA* based on the methods described by Chi et al. (2012). Then, the plasmid pAMCRE1 was introduced into the mutant *ΔsidA* to excise the *NAT* gene and the introduced plasmid pAMCRE1 was finally lost after cultivation of the disruptants under none selection pressure (without any antibiotics). One of the positive disruptants being sensitive to both

the nourseothricin and hygromycin B was transformed with the linear DNA fragments carrying 3′-arm and 5′-arm from the *PPTase* gene to get a double mutant *ΔsidAΔpptase* in which both the *NAT* gene and *HPT* gene were removed again as described above.

To continuously remove the *GT16* gene (accession number: MG825857.1) encoding glycogenin and the *PUL1* gene (accession number: KM258394.1) encoding pullulan synthase from *A. melanogenum* P16, 3′-arms and 5′-arms of the *GT16* gene and the *PUL1* gene were PCR amplified from the genomic DNA of *A. melanogenum* P16 using the primers (P16-GT16-3-se/P16-GT16-3-an, P16-PUL1-3-se/P16-PUL1-3-an, P16-GT16-5-se/P16-GT16-5-an, P16-PUL1-5-se/P16-PUL1-5-an) shown in Supplementary file 1. The obtained 3′-arms and 5′-arms were digested with the enzymes *Bam*HI/*Eco*RI, *Pst*I/*Sal*I and *Sph*I/*Sal*I, respectively and the digests were introduced into the pAMGDlox-1 digested with the same enzymes, forming the plasmids pAMGDlox-1-ΔGT16 and pAMGDlox-1-ΔPUL1 (data not shown). The single mutant *Δgt16* and the double mutant *Δgt16Δpul1* without both the *HPT* and *NAT* genes were obtained using the same methods as described above.

To repeatedly remove the *PKS1* gene (accession number: KU290362) encoding polyketide synthase that catalyzes biosynthesis of 3,5-dihydroxydecanoic acid and the *MSN2* gene (accession number: XP013341874.1) encoding a transcriptional activator from *A. melanogenum* 9-1, 3′-arms and 5′-arms of the *PKS1* gene and the *MSN2* gene were PCR amplified from the genomic DNA of *A. melanogenum* 9-1 using the primers (612-PKS1-3-se/612-PKS1-3-an, 612-MSN2-3-se/612-MSN2-3-an, 612-PKS1-5-se/612-PKS1-5-an, 612-MSN2-5-se/612-MSN2-5-an) shown in Supplementary file 1. The obtained 3′-arms and 5′-arms were digested with the enzymes *Bam*HI/*Eco*RI, *Pst*I/*Sal*I and *Sph*I/*Sal*I, respectively and the digests were inserted into the pAMGDlox-1 digested with the same enzymes, forming the plasmids pAMGDlox-1-ΔPKS1 and pAMGDlox-1-ΔMSN2 (data not shown). The single mutant *Δpks1* and the double mutant *Δpks1Δmsn2* without both the *NAT* gene and *HPT* gene were got using the same methods as described above.

2.4. Multigene expression

To sequentially express the *CT2* gene (accession number: NW_006383037) encoding cutinase from *Chaetomium thermophilum* (Wang et al., 2012) and the *EGFP* gene from the plasmid pEGFP in *A. melanogenum* HN6.2, the *CT2* gene and the *EGFP* gene were PCR amplified from the cDNA of *C. thermophilum* and the plasmid pEGFP using the primers (CT2-1-se/CT2-2-an, GFP-se/GFP-an) shown in Supplementary file 1. The obtained *CT2* gene and *EGFP* gene were digested with the enzymes *Sac*I/*Kpn*I, *Sac*I/*Sal*I, respectively and the digests were ligated into the plasmid pAMEXlox-1 digested with the same enzymes, forming the plasmids pAMEXlox-1-CT2 and pAMEXlox-1-EGFP (data not shown). The plasmids pAMEXlox-1-CT2 and pAMEXlox-1-EGFP were digested with the enzymes *Rco*RI and *Sma*I to release the linear DNA fragments 26S-rDNA-loxp-P_{pgk}-NAT-polyA-loxp-CT2-P_{TEF}-18S-rDNA and 26S-rDNA-loxp-P_{pgk}-NAT-polyA-loxp-EGFP-P_{TEF}-18S-rDNA. The former linear DNA fragments were transformed into the competent cells of *A. melanogenum* HN6.2 to obtain a single transformant 149-CT2 following the procedures described by Chi et al. (2012). Then, the plasmid pAMCRE1 was introduced into the transformant 149-CT2 to excise the *NAT* gene and the plasmid pAMCRE1 was finally lost after cultivation of the transformants under none selection pressure (without any antibiotics). One of the transformants being sensitive to both the nourseothricin and hygromycin B was transformed with the latter linear DNA fragments carrying the EGFP gene to get a double transformant 149-CT2-GFP (carrying both the *CT2* gene and the *EGFP* gene) in which both the *NAT* gene and *HPT* gene were removed again as described above.

To continuously express the *PUL1* gene encoding pullulan synthase and the *EGFP* gene in *A. melanogenum* P16, the *PUL1* gene was PCR amplified from the genomic DNA of *A. melanogenum* P16 using the primers (P16-PUL1-se/P16-PUL1-se) shown in Supplementary file 1. The PCR products were digested with the enzymes *Afl*II/*Mlu*I and the digests were introduced into the plasmid pAMEXlox-1digested with the same enzymes, yielding the plasmid pAMEXlox-1-PUL1 (data not shown). The plasmid pAMEXlox-1-PUL1 was digested with the enzymes *Rco*RI and *Sma*I to release the linear DNA fragments 26S-rDNA-loxp-P_{pgk}-NAT-polyA-loxp-PUL1-P_{TEF}-18S-rDNA. The linear DNA fragments were transformed into the cells of the P16 strain to obtain a single transformant P16-PUL1. The single transformant P16-PUL1 without the *NAT* gene and the double transformant P16-PUL1-GFP (carrying both the *PUL1* gene and the *EGFP* gene) without both *HPT* and *NAT* genes were acquired using the same methods as described above.

To consecutively express the *MSN2* gene mentioned above and the *EGFP* gene in *A. melanogenum* 9-1, the *MSN2* gene was PCR amplified from the genomic DNA of *A. melanogenum* 9-1 using the primers (612-BMSN2-se/612-BMSN2-se) shown in Supplementary file 1. The PCR products were digested with the enzymes (*Mlu*I/*Kpn*I) and the digests were linked into the plasmid pAMEXlox-1 digested with the same enzymes, forming the plasmid pAMEXlox-1-MSN2 (data not shown). The plasmid pAMEXlox-1-MSN2 was digested with the enzymes *Rco*RI and *Sma*I to release the linear DNA fragments 26S-rDNA-loxp-P_{pgk}-NAT-polyA-loxp-MSN2-P_{TEF}-18S-rDNA. The linear DNA fragments were transformed into the cells of the 9-1 strain to obtain a single transformant 9-1-MSN2. The transformant 9-1-MSN2 without the *NAT* gene and the transformant 9-1-MSN2-GFP (carrying both the *MSN2* gene and the *EGFP* gene) without both *HPT* and *NAT* genes were obtained using the same methods as described above.

2.5. Estimation of transformation efficiency and excision rate of the antibiotics resistance gene

Before each transformation, the competent cell numbers (about 10^6 cells/mL) in the transformation mixture were exactly calculated under a microscope. After each transformation, the colony numbers on each plate with nourseothricin were also quantitatively measured. Finally, the transformant numbers per 1000 competent cells added to the mixture and the positive transformant numbers per 1.0 μg of the linear DNA fragments added to the mixture were calculated. Each experiment was carried out in triplicate.

After the PAMCRE1 was introduced into each disruptant or transformant, the cells were grown in the YPD medium with hygromycin B at 28 °C for 2 d. Then, 100 colonies of each strain were transferred into the YPD plates, the YPD plates with nourseothricin and the YPD plates with hygromycin B by the replica plating and the plates were incubated at 28 °C for 2 d. Finally, the numbers of the formed colonies on each plate were measured and excision rate of the antibiotics resistance gene by the pAMCRE1 was calculated. Each experiment was carried out in triplicate. All the data obtained were subjected to One-way Analysis of Variance (ANOVA) (JeffWu and Hamada, 2000). *P* values were calculated by Student's t-test ($n = 3$). *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL).

2.6. Confirmation of the deleted and integrated genes on genomic DNAs

One loop of the cells of each disruptant and transformant obtained above and their wild type strains was inoculated into 50.0 mL of the YPD medium in a 250-mL flask and aerobically

cultivated for 10 h, respectively. The cells were collected and washed with sterile distilled water by centrifugation at 5000×*g* and 4 °C for 10 min. The genomic DNAs were extracted as described above and used as the template for PCR. The DNA fragments with the deleted and integrated genes were PCR amplified using the primers shown in Supplementary file 1. PCR reactions and conditions were performed as described above and the PCR products obtained were separated in an agarose gel. The sizes of the PCR products were estimated using the Automated Documentation and Analysis System (Gene-Genius, USA). The PCR products were sequenced by Shanghai Sangon Company.

2.7. Extraction and quantitative measurement of siderophore, pullulan and liamocin

Extraction and quantitative measurement of siderophore were done according to the methods described by Lu et al. (2019). Extraction and quantitative measurement of pullulan were performed based on the procedures described by Ma et al. (2014). Extraction and quantitative measurement of liamocin were conducted following the methods described by Tang et al. (2018).

2.8. Determination of cutinase activity

The cutinase activity (esterase activity) was assayed according to the methods described by Chen et al. (2017). One unit of the esterase activity was defined as the amount of enzyme that released 1.0 μM of *ρ*-nitrophenol from *ρ*-NPB per minute under the assay conditions used in this study.

2.9. Microscopy

The yeast-like fungal cells were observed under blue light with an Olympus U-LH100HG fluorescent microscope having a 100× oil immersion objective and under normal light with an Olympus U-LH100HG phase contrast microscope equipped with 100× oil immersion objective. Images were recorded using the cellSens Standard software.

2.10. Determination of the transcriptional level of various genes by fluorescent real-time PCR

Each disruptant and transformant obtained above and their wild type strains were grown aerobically in at 28 °C and 160 rpm for 72 h. The cultures were centrifuged at 8000× *g* and 4 °C for 10 min and the pellets obtained were used as the samples for total RNA isolation. The total RNA was purified by a RNAprep pure Tissue Kit (TIANGEN, China). Reverse transcription was performed using a PrimeScript RT reagent Kit (TaKaRa, Japan) according to the manufacturer's protocol. The fluorescent real-time RT-PCR assay was carried out in triplicate on a 96-well plate in a 20 μL reaction volume per well containing 9 μL of SYBR Green PCR Master Mix (TIANGEN, China), 0.5 μL of 1:10 diluted cDNA, and 200 nM of each forward and reverse primer (Liu et al., 2011). All the primers used for the fluorescent real-time PCR were shown in Supplementary file 1. The relative expression quantity was calculated using the formula $RATE = 2^{-DDCt}$. The data obtained from the real-time PCR analysis were subjected to One-way Analysis of Variance (ANOVA) (JeffWu and Hamada, 2000). *P* values were calculated by Student's t-test ($n = 3$). *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Simultaneous and sequential gene knock-out by the Cre/loxP site-specific recombination in different strains of A. melanogenum

It has been well confirmed that *A. melanogenum* HN6.2 can produce a considerable amount of siderophore (1.1 mg/L) fusigen (Wang et al., 2009) and the biosynthesis of the fusigen usually commences with the N^5-hydroxylation of L-ornithine catalyzed by an L-ornithine-N5-monooxygenase and assembly to the final siderophore is catalyzed by nonribosomal peptide synthetases (NRPSs) (Haas, 2003). Therefore, the key L-ornithine-N5-monooxygenase encoded by the *SidA* gene is involved in the first step of the fungal siderophore biosynthesis. In addition, phosphopantetheinyl transferases (PPTases) catalyze the posttranslational modification of the NRPSs with a peptidyl carrier protein (PCP) domain, leading to their conversion to the active form during the biosynthesis of lysine, polyketides and nonribosomal peptides (Kim et al., 2013). In this case, abolishment of the gene encoding PPTase will render a mutant of *A. melanogenum* HN6.2 unable to synthesize fusigen and lysine. As expected, removal of the *SidA* gene caused the mutant *ΔsidA* not to synthesize any siderophore (Table 1) while its wild type strain HN6.2 could produce 0.8 ± 0.01 mM of siderophore (Table 1). After the *NAT* gene was removed by the Cre recombinase and the pAMCRE1 was lost, the *PPTase* gene in the mutant *ΔsidA* was successfully deleted as described in Materials and methods, the obtained *sidAΔpptase* mutant in which the *NAT* gene was removed by the Cre recombinase and the pAMCRE1 was lost could not both synthesize any siderophore and grow in the YNB medium without lysine, either (Table 1). Our data also showed that the *sidA* gene in the mutant *ΔsidA* and both the *SidA* gene and the *PPTase* gene in the mutant *ΔsidAΔpptase* were totally knocked-out (Fig. 1A).

In our previous study (Ma et al., 2012, 2014), it was found that *A. melanogenum* P16 could produce over 60.0 g/L pullulan. Although its biosynthesis pathway is still unknown, the pullulan synthase (Pul1) which function is still unclear was found to be involved in pullulan biosynthesis (Ma et al., 2012). Due to the similarity between chemical structures of pullulan and glycogen molecules, the glycogenin1 encoded by the *GT16* gene on which α-1,4-glucan is synthesized may also be related to pullulan biosynthesis. Using the same methods mentioned above, it was found that the *Δgt16* in which the single *GT16* gene was deleted, the *NAT* gene was excised by the Cre-Loxp site-specific recombination system and the pAMCRE1 was lost only could synthesize and secret a small amount of pullulan (1.7 ± 0.1 g pullulan/g of cell dry weight) while the double mutant *Δgt16Δpul1* in which both the *GT16* gene and the *PUL1* gene were abolished, the *NAT* gene was excised by the Cre-Loxp site-specific recombination system and the pAMCRE1 was lost could not synthesize any pullulan (Table 1), suggesting that the *GT16* gene and the *PUL1* gene could play an important role in pullulan biosynthesis *A. melanogenum* P16. In contrast, their wild type strain P16 could synthesize 2.2 ± 0.1 g pullulan/g of cell dry weight (Table 1). Supplementary file 3 also showed that the colonies of the double mutant *Δgt16Δpul1* became rough, matt and dried and the surface of the colonies was changed to be ruffle and irregular while the colonies of its wild type strain P16 were smooth and slimy due to the presence of the produced pullulan, suggesting that the produced pullulan could determine colony morphology of *A. melanogenum* P16. It can be seen from Fig. 1B that the *GT16* gene in the single mutant *Δgt16* and both the *GT16* gene and the *PUL1* gene in the double mutant *Δgt16Δpul1* were completely removed.

Tang et al. (2018) reported that *A. melanogenum* 9-1 could synthesize and secret over 40 g/L liamocin and a polyketide synthase (PKS) may be involved in its biosynthesis. We also found that the transcriptional activator MSN2 may be associated with

Table 1
Products, cell growth, colony morphology and cell morphology of different disruptants and their wild type strains.

Strain	Siderophore (mM)	Siderophore production medium (+Lysine)	Siderophore production medium (−Lysine)	Pullulan/cell dry weight (g/g)	Liamocin (g/l)	Colony morphology	Cell morphology
HN6.2	0.8 ± 0.1	+	+	0	0	normal	Yeast cells
ΔsidA	0**	+	+	0	0	normal	Yeast cells
ΔsidAΔpptase	0**	+	−	0	0	normal	Yeast cells
P16	0	+	+	2.2 ± 0.1	0	sticky	Yeast cells
Δgt16	0	+	+	1.7 ± 0.1*	0	sticky	Yeast cells
Δgt16Δpul1	0	+	+	0**	0	dried	Yeast cells
9−1	0	+	+	0	35.8 ± 1.2	sticky	Yeast cells
Δpks1	0	+	+	0	0**	normal	Yeast cells
Δpks1Δmsn2	0	+	+	0	0**	dried	filamentous

+: growth, −: No growth.
Data are given as means ± SD, n = 3. *, ($P < 0.05$) means different compared with that of the wild-type strain, **, ($P < 0.01$) means significantly different compared with that of the wild type strain.

regulation of liamocin biosynthesis. Using the same methods mentioned above, we found that the single mutant *Δpks1* in which the *PKS1* gene was deleted and the *NAT* gene in the genomic DNA was excised by the Cre-Loxp site-specific recombination system and the pAMCRE1 was lost could not synthesize and secret any liamocin while the double mutant *Δpks1Δmsn2* in which both the *PKS1* gene and the *MSN2* gene were abolished and the *NAT* gene in the genomic DNA was excised by the Cre-Loxp site-specific recombination system and the pAMCRE1 disappeared could not synthesize any liamocin, either, but the double mutant cells became filamentous (Table 1, Supplementary file 4), suggesting that the *MSN2* gene also could determine cell morphology of *A. melanogenum* 9-1. Furthermore, the colony of *A. melanogenum* 9-1 was smooth and slimy while that of the double mutant *Δpks1Δmsn2* was rough (Supplementary file 4). In contrast, *A. melanogenum* 9-1 could produce 35.8 ± 1.2 g/L of liamocin (Table 1). However, it is still completely unknown how the cell morphology of *A. melanogenum* 9-1 is determined by the *MSN2* gene. It can be seen from Fig. 1C that the *PKS1* gene in the *Δpks1* mutant and both the *PKS1* gene and the *MSN2* gene in the *Δpks1Δmsn2* mutant were completely abolished.

The results in Table 2 demonstrated that after the first round of the gene disruption for each strain, over 0.7 positive transformants per 1000 competent cells and over 68.5 positive transformants per 1.0 μg of added DNA fragments were gained and after the second round of the gene disruption for each strain, over 0.6 positive transformants per 1000 competent cells and over 58.7 positive transformants per 1.0 μg of added DNA fragments were obtained, suggesting that after each round of the gene disruption, the transformation efficiency was only slightly reduced. The results in Table 2 also showed that after the first round of the antibiotics gene excision by using the cre-loxp site-specific recombination, over 96.4 % of the colonies became sensitive to both hygromycin B and nourseothricin and after the second round of the antibiotics gene excision by using the same cre-loxp site-specific recombination, over 95.9 % of the colonies became sensitive to both hygromycin B and nourseothricin, indicating that loss rate of both the two antibiotics genes through the loxP recombination mediated by Cre recombinase was very high and efficiency.

3.2. Simultaneous and sequential gene knock-in by the Cre/loxP site-specific recombination in different strains of A. melanogenum

In addition to siderophore, *A. melanogenum* HN6.2 also produce esterase (Chen et al., 2017). Analysis of the genome of *C. thermophilum*, a thermophilic fungus, found that its genomic DNA contained a cutinase gene. The cutinase which is also one kind of esterase can hydrolyze the ester bonds of the plant polymer cutin, a variety of plastic polymers including poly(ethylene terephthalate) (PET) and poly(butylene succinate) (PBS), insoluble triacylglycerols and low-molecular-weight soluble esters (Chen et al., 2013). In this case, overexpression of the *CT2* gene encoding cutinase from *C. thermophilum* (Wang et al., 2012) in *A. melanogenum* HN6.2 will enhance esterase activity. As expected, expression of the *CT2* gene caused a transformant 149-CT2 to produce 262.2 ± 7.5 U/mL of esterase activity while *A. melanogenum* HN6.2 only produced 203.6 ± 6.0 U/mL of esterase activity (Table 3). After the *NAT* gene was cleaned by the Cre recombinase and the pAMCRE1 was lost, the *EGFP* gene in the transformant 149-CT2 was successfully expressed as described in Materials and Methods. The obtained double transformant 149-CT2-EGFP in which the *NAT* gene was removed by the Cre recombinase and the pAMCRE1 was lost could yield 245.2 ± 4.4 U/mL of esterase activity and all the yeast cells of the double transformant 149-CT2-EGFP became green under UV light (Table 3; Supplementary file 5A2). Our data also showed that the transcriptional levels of the *CT2* gene in the transformant 149-CT2 and both the *CT2* gene and the *EGFP* gene in the double transformant 149-CT2-EGFP were greatly enhanced (Table 4).

Similarly, the *PUL1* gene mentioned above was expressed in *A. melanogenum* P16. The results in Table 3 showed that a transformant P16-PUL1 was able to produce 2.6 ± 0.2 g pullulan/g of cell dry weight while *A. melanogenum* P16 only produced 2.2 ± 0.1 g pullulan/g of cell dry weight. After the *NAT* gene was removed by the Cre recombinase and the pAMCRE1 was lost, the *EGFP* gene in the transformant P16-PUL1 was successfully expressed as described in Materials and methods. The obtained double transformant P16-PUL1-GFP in which the *NAT* gene was removed by the Cre recombinase and the pAMCRE1 was lost could yield 2.5 ± 0.3 g pullulan/g of cell dry weight and all the yeast cells of the double transformant P16-PUL1-GFP also became green under UV light (Table 3; Supplementary file 5B2). It was also shown that the transcriptional levels of the *PUL1* gene in the transformant P16-PUL1 and both the *PUL1* gene and the *EGFP* gene in the double transformant P16-PUL1-GFP were greatly enhanced (Table 4). The reason for this was still unclear.

Furthermore, after the *MSN2* gene was introduced into the competent cells of the *Δpks1Δmsn2* mutant of *A. melanogenum* 9-1 mentioned above, the yeast cell morphology of the transformant 9-1-MSN2 was totally restored (Table 3) while the *Δpks1Δmsn2* mutant still kept filamentous (Table 3). After the *NAT* gene was cleaned by the Cre recombinase and the pAMCRE1 was lost, the *EGFP* gene in the transformant 9-1-MSN2 was successfully expressed as described in Materials and methods. The obtained

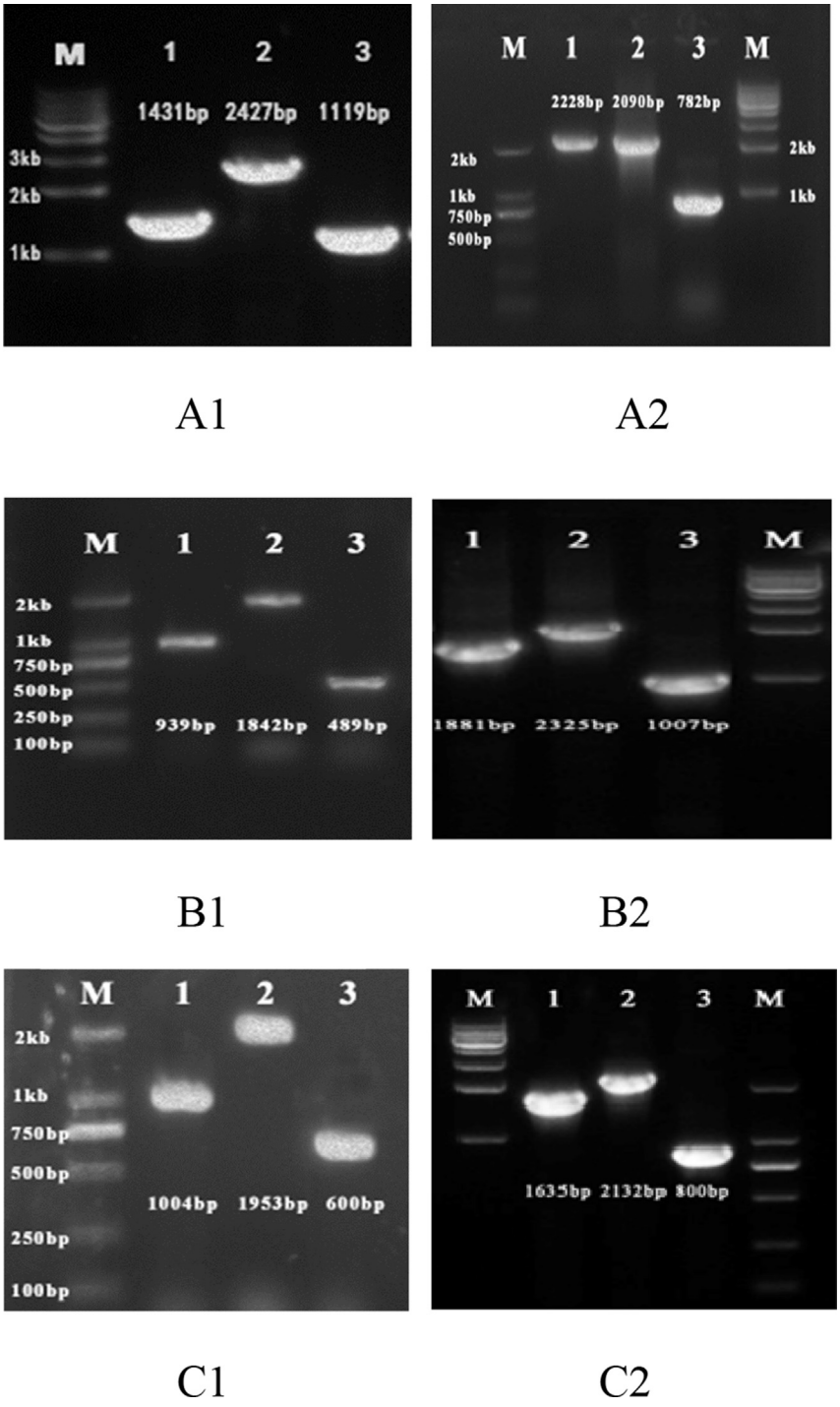

Fig. 1. Confirmation of the inserted antibiotic resistance gene by PCR. Lane 1 in (A1): The PCR products from the *SidA* gene in the wild type strain HN6.2 using 149-SidA-5-se/149-SidA-3-an in Supplementary file 1; Lane 2 in (A1): The PCR products from the inserted *NAT* gene in the mutant *ΔsidA* using 149-SidA-5-se/149-SidA-3-an in Supplementary file 1; Lane 3 in (A1): The PCR products from the excised *NAT* gene in the mutant *ΔsidA* in which the pAMCRE1 was lost using 149-SidA-5-se/149-SidA-3-an in Supplementary file 1. Lane 1 in (A2): The PCR products from the *PPTase* gene in the wild type strain HN6.2 using primers 149-PPT-5-se/149-PPT-3-an in Supplementary file 1; Lane 2 in (A2): The PCR products from the inserted *NAT* gene in the mutant *ΔsidAΔpptase* using the primers 149-PPT-5-se/149-PPT-3-an in Supplementary file 1; Lane 3 in (A2): The PCR products from the excised *NAT* gene in the mutant *ΔsidAΔpptase* in which the pAMCRE1 was lost using the primers 149-PPT-5-se/149-PPT-3-an in Supplementary file 1. Lane 1 in (B1): The PCR products from the *GT16* gene in the wild type strain P16 using the primers P16-GT16-5-se/P16-GT16-3-an in Supplementary file 1; Lane 2 in (B1): The PCR products from the inserted *NAT* gene in the mutant *Δgt16* using the primers P16-GT16-5-se/P16-GT16-3-an in Supplementary file 1; Lane 3 in (B1): The PCR products from the excised *NAT* gene in the mutant *Δgt16* in which the pAMCRE1 was lost using the primers P16-GT16-5-se/P16-GT16-3-an in Supplementary file 1. Lane 1 in (B2): The PCR products from the *PUL1* gene in the wild type strain P16 using the primers P16-PUL1-5-se/P16-PUL1-3-an in Supplementary file 1; Lane 2 in (B2): The PCR products from the inserted *NAT* gene in the mutant *Δgt16Δpul1* using the primers P16-PUL1-5-se/P16-PUL1-3-an in Supplementary file 1; Lane 3 in (B3): The PCR products from the excised *NAT* gene in the mutant *Δgt16Δpul1* in which the pAMCRE1 was lost using the primers P16-PUL1-5-se/P16-PUL1-3-an in Supplementary file 1. Lane 1 in (C1): The PCR products from the *PKS1* gene in the wild type strain 9-1 using the primers 612-PKS1-5-se/612-PKS1-3-an in Supplementary file 1; Lane 2 in (C1): The PCR products from the inserted *NAT* gene in the mutant *Δpks1* using the primers 612-PKS1-5-se/612-PKS1-3-an in Supplementary file 1; Lane 3 in (C1): The PCR products from the excised *NAT* gene in the mutant *Δpks1* in which the pAMCRE1 was lost using the primers 612-PKS1-5-se/612-PKS1-3-an in Supplementary file 1. Lane 1 in (C2): The PCR products from the *MSN2* gene in the wild type strain 9-1 using the primers 612-MSN2-5-se/612-MSN2-3-an in Supplementary file 1; Lane 2 in (C2): The PCR products from the inserted *NAT* gene in the mutant *Δpks1Δmsn2* using the primers 612-MSN2-5-se/612-MSN2-3-an in Supplementary file 1; Lane 3 in (C2): The PCR products from the excised *NAT* gene in the mutant *Δpks1Δmsn2* in which the pAMCRE1 was lost using the primers 612-MSN2-5-se/612-MSN2-3-an in Supplementary file 1.

Table 2
Transformation efficiency and excision rate during multigene disruption.

Wild type strain	Mutants	Target gene	Positive transformants per 1000 competent cells	Positive transformants per 1.0 μg of added DNA	Excision rate
HN6.2	*ΔSidA*	*SidA*	0.8 ± 0.1	76.8 ± 9.0	(96.4 ± 2.0) %
	ΔSidA Δpptase	*SidA PPTase*	0.6 ± 0.1	66.6 ± 7.2	(97.5 ± 1.0) %
P16	*ΔGT16*	*GT16*	0.8 ± 0.1	78.4 ± 5.8	(97.0 ± 1.2) %
	ΔGT16Δpul1	*GT16* and *PUL1*	0.6 ± 0.1	66.8 ± 7.6	(96.4 ± 1.0) %
9−1	*Δpks1*	*PKS1*	0.7 ± 0.1	68.5 ± 4.8	(98.0 ± 1.7) %
	Δpks1Δmsn2	*PKS1*and *MSN2*	0.6 ± 0.2	58.7 ± 4.1	(95.9 ± 2.4) %

Data are given as means ± SD, n = 3. Each plate had 10^5 competent cells.

Table 3
Products, colony morphology, cell morphology and fluorescent of transformants and their wild type strains.

Strain	Esterase (U/ml)	Pullulan/cell dry weight (g/g)	Colony morphology	Cell morphology	Fluorescent of cells
HN6.2	203.6 ± 6.0	0	normal	Yeast cells	−
149-CT2	264.4 ± 13.5**	0	normal	Yeast cells	−
149-CT2-EGFP	245.2 ± 4.4**	0	normal	Yeast cells	+
P16	0	2.2 ± 0.48	normal	Yeast cells	−
P16-PUL1	0	2.6 ± 0.8*	normal	Yeast cells	−
P16-PUL1-GFP	0	2.5 ± 0.1*	normal	Yeast cells	+
Δpks1Δmsn2	0	0	dried	Filamentous	−
9-1-MSN2	0	0	sticky	Yeast cells	−
9-1-MSN2-GFP	0	0	sticky	Yeast cells	+

+: fluorescent, −: no fluorescent; NA: not assayed.
Data are given as means ± SD, n = 3. *, (P < 0.05) means different compared with that of the wild type strain; **, (P < 0.01) means significantly different compared with that of the wild type strain.

double transformant 9-1-MSN2-GFP in which the *NAT* gene was removed by the Cre recombinase and the pAMCRE1 was lost still kept filamentous, but all the yeast cells of the double transformant 9-1-MSN2-GFP became green under UV light (Table 3; Supplementary file 5C2). It was also shown that the expression of the *MSN2* gene in the transformant 9-1-MSN2 and both the *MSN2* gene and the *EGFP* gene in the double transformant 9-1-MSN2-GFP was significantly promoted (Table 4).

The results in Table 5 demonstrated that after the first round of the gene expression for each strain, over 0.5 positive transformants per 1000 competent cells and over 49.8 positive transformants per 1.0 μg of added DNA fragments were achieved and after the second round of the gene expression for each strain, over 0.8 positive transformants per 1000 competent cells and over 81.6 positive transformants per 1.0 μg of added DNA fragments were acquired, suggesting that after each round of the gene expression, the transformation efficiency was almost not changed. The results in Table 4 also showed that after the first round of the antibiotics gene excision by using the Cre-loxp site-specific recombination, over 95.4 % of the colonies became sensitive to both hygromycin B and nourseothricin and after the second round of the antibiotics gene excision by using the cre-loxp site-specific recombination, over 95.9 % of the colonies became sensitive to both hygromycin B and nourseothricin, indicating again that loss rate of both of the two antibiotics genes through the loxP recombination mediated by Cre recombinase was very high and efficiency. This again demonstrated that the cre-loxp site-specific recombination systems constructed in this study could be easily used to carry out simultaneous and sequential gene knock-in in different strains of *A. melanogenum*.

Table 4
Relative Transcriptional levels of different genes in different transformants and their wild type strains.

Strains	Relative transcriptional level of genes (%)			
	CT2	*PUL1*	*MSN2*	*EGFP*
HN6.2	0	NA	NA	0
149-CT2	100*	NA	NA	0
149-CT2-EGFP	88.3 ± 5.6	NA	NA	100*
P16	NA	100	NA	0
P16-PUL1	NA	13,380.7 ± 89.7**	NA	0
P16-PUL1-GFP	NA	12,199.5 ± 81.7**	NA	100
9−1	NA	NA	100*	0
Δpks1Δmsn2	NA	NA	0	0
9-1-MSN2	NA	NA	125.7 ± 9.6*	0
9-1-MSN2-GFP	NA	NA	99.7 ± 7.0	100*

Data are given as means ± SD, n = 3. *, (P < 0.05) means different compared with that of the control strain,**, (P < 0.01) means significantly different compared with that the control strain; NA: not assayed.

4. Discussion

So far, the Cre-loxp system has been used to remove selection markers in *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, *Candida albicans*, *Hansenula polymorpha*, *Cryptococcus neoformans*, *Xanthophyllomyces dendrorhous*, *Aspergillus* spp., *Trichoderma* spp., *Neurospora* spp., *Neotyphodium* spp. and so on (Zhao et al., 2019). In these yeasts, episomal plasmids and/or inducible promoters are employed to control the transient expression of the Cre recombinase gene. However, both episomal plasmids and stringent inducible promoters are unavailable in many other yeasts (Cao et al., 2017). Zhang et al. (2019) used a Cre-loxp system, in which the transient expression of the Cre recombinase was controlled by a genetically unstable vector independent of episomal plasmids and inducible promoters. The plasmid pSbCre used in *Saccharomyces boulardii* to generate a recyclable marker system contained the Cre gene that was under the regulation of the galactose-inducible *GAL1* promoter (Wang et al., 2015). The recombinase gene (*Cre*) on a plasmid pXACre11 for making a recyclable marker system in *Aspergillus nidulans* was placed under the control of the *A. nidulans xlnA* (xylanase A) gene promoter, thus providing a means to switch on (xylose induction) or off (glucose repression) recombinase expression (Forment et al., 2006). A multicopy autonomously replicating plasmid, pGK423-Cre was utilized to express Cre recombinase under the control of the constitutive *PGK1* promoter (Nakamura et al., 2016). It should be noted that in

Table 5
Transformation efficiency and excision rate during multigene expression.

Wild type strain	Transformants	Gene	Positive transformants per 1000 competent cells	Positive transformants per 1.0 μg of added DNA	Excision rate
HN6.2	149-CT2	*CT2* gene	0.5 ± 0.0	49.8 ± 2.9	(95.9 ± 2.9) %
	149-CT2-EGFP	*CT2* gene and *EGFP* gene	0.8 ± 0.1	83.7 ± 8.9	(98.5 ± 1.0) %
P16	P16-PUL1	*PUL1* gene	0.6 ± 0.1	80.9 ± 7.61	(95.4 ± 3.9) %
	P16-PUL1-GFP	*PUL1* gene and *EGFP* gene	0.9 ± 0.1	87.2 ± 5.5	(95.9 ± 1.7) %
9–1	9-1-MSN2	*MSN2* gene	0.7 ± 0.0	79.7 + 10.9	(96.9 ± 2.6) %
	9-1-MSN2-GFP	*MSN2* gene and *EGFP* gene	0.8 + 0.1	81.6 ± 4.9	(95.9 ± 1.8) %

Data are given as means ± SD, n = 3. Each plate had 10^5 competent cells.

the present study, it can be cleanly observed that a constitutive promoter of 3-phosphate glycerol dehydrogenase gene in *A. melanogenum* was used to drive the transient expression of the Cre recombinase and a terminator of cytochrome C gene in *S. cerevisiae* was used to terminate expression of the Cre recombinase in the pAMCRE1 (Supplementary file 2C). At the same time, the ARS element from *A. maydis* (Schuster et al., 2016) was also used to construct the pAMCRE1 to keep its temporary stability in the transformant cells in order to make the produced Cre recombinase effectively cut and remove the antibiotics resistance gene and afterwards to allow the plasmid pAMCRE1 to be lost under no selection pressure (without any antibiotics). Indeed, a self-replicating plasmid containing an *U. maydis* ARS element was also used to construct a Cre-loxp site specific recombination system by Schuster et al. (2016). Without selection (without any antibiotics), such plasmids are rapidly lost and this feature was chosen to be able to limit potential long term toxic effects of the Cre recombinase (Schuster et al., 2016).

The results in Tables 1 and 2 and Fig. 1 showed that the Cre/loxp site-specific recombination systems constructed in this study could be used to remove different genes in different strains of *A. melanogenum* and loss rate of both the two antibiotics genes through the loxP recombination mediated by Cre recombinase was very high and efficiency. It has been reported that the gene targeting efficiency in the yeast *K. marxianus* by using a Cre-*lox*P system for multiple gene disruption, *i.e.*, the number of white colonies that verified the integration of the disruption cassette was 34 % for the first and 15 % for the second round of transformation (Ribeiro et al., 2007). Overnight incubation in a galactose containing medium caused about 20–30 % yeast to loss the hydromycin B resistance gene through the loxP recombination mediated by Cre recombinase which production was induced by galactose in the medium (Wang et al., 2015). About 24 h of incubation in a YPD medium would cause about 50–60 % *S. boulardii* to loss their pSbCre (Wang et al., 2015). Recombinase mediated marker rescue was at efficiencies of 70–80 % in *A. nidulans* using a plasmid pXA-Cre11 (Forment et al., 2006). This indicated that loss rate of both the two antibiotics genes through the loxP recombination mediated by the Cre recombinase constructed in this study was very high and efficiency compared to any other Cre-loxp site-specific recombination systems. This meant that the Cre-loxp site-specific recombination systems constructed in this study could be easily used to carry out simultaneous and sequential gene knock-out in different strains of *A. melanogenum*.

Tables 3–5 also revealed that the Cre/loxp site-specific recombination systems constructed in this study could be used to express different genes in different strains of *A. melanogenum* and loss rate of both the two antibiotics genes through the loxP recombination mediated by the Cre recombinase was also very high and efficiency.

As mentioned above, most of the useful products are produced by the widely distributed *A. melanogenum* (Li et al., 2015) and it is very meaningful to have efficient Cre/loxp site-specific recombination systems obtained in this study. If the promoters on all the plasmids are changed to those in a new host species, for example in *A. pullulans*, this system can be applied to other *Aureobasidium* species, such as *A. pullulans*. At the same time, it is necessary to test if the ARS and Cre gene could work in other *Aureobasidium* species, such as in *A. pullulans*.

5. Conclusions

The Cre/loxp site-specific recombination systems constructed in this study can easily and efficiently be used to simultaneously and sequentially delete and express many genes in different strains of *A. melanogenum*. These systems are promising approaches for the easily modifying genomics of the yeast-like fungal strains with enhanced metabolic pathways through multicopy gene deletion and expression.

The ethical statement

The manuscript completely complies to the Ethical Rules applicable for this journal and no human and animal samples were used in this study.

Availability of data and material

A complete list of the plasmids and the yeast strains we constructed, together with the DNA sequences of each genetic part (promoters, coding sequences, and terminators) used in this work and further experimental data are provided in the Supplementary files.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.06.001.

List of Abbreviation

CRISPR–Cas	clustered regularly interspaced short palindromic repeats-Cas
PPTase	phosphopantetheinyl transferase
NRPS	Non-ribosomal peptide synthase
PCP	peptidyl carrier protein

YNB	Yeast nitrogen base
HPT	hygromycin B resistance gene
NAT	Nourseothricin resistance gene
EGFP	enhanced green fluorescent protein
PCR	polymerase chain reaction
YPD	yeast polypeptone dextrose
PET	poly(ethylene terephthalate)
PBS	poly(butylene succinate)
ARS	artificial replication sequence
PKS	polyketide synthase

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