

Deletion of a hybrid NRPS-T1PKS biosynthetic gene cluster via Latour gene knockout system in *Saccharopolyspora pogona* and its effect on butenyl-spinosyn biosynthesis and growth development

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Summary

Butenyl-spinosyn, a promising biopesticide produced by *Saccharopolyspora pogona*, exhibits stronger insecticidal activity and a broader pesticidal spectrum. However, its titre in the wild-type *S. pogona* strain is too low to meet the industrial production requirements. Deletion of non-target natural product biosynthetic gene clusters resident in the genome of *S. pogona* could reduce the consumption of synthetic precursors, thereby promoting the biosynthesis of butenyl-spinosyn. However, it has

always been a challenge for scientists to genetically engineer *S. pogona*. In this study, the Latour gene knockout system (linear DNA fragment recombineering system) was established in *S. pogona*. Using the Latour system, a hybrid NRPS-T1PKS cluster (~20 kb) which was responsible for phthoxazolin biosynthesis was efficiently deleted in *S. pogona*. The resultant mutant *S. pogona-Δura4-Δc14* exhibited an extended logarithmic phase, increased biomass and a lower glucose consumption rate. Importantly, the production of butenyl-spinosyn in *S. pogona-Δura4-Δc14* was increased by 4.72-fold compared with that in the wild-type strain. qRT-PCR analysis revealed that phthoxazolin biosynthetic gene cluster deletion could promote the expression of the butenyl-spinosyn biosynthetic gene cluster. Furthermore, a TetR family transcriptional regulatory gene that could regulate the butenyl-spinosyn biosynthesis has been identified from the phthoxazolin biosynthetic gene cluster. Because dozens of natural product biosynthetic gene clusters exist in the genome of *S. pogona*, the strategy reported here will be used to further promote the production of butenyl-spinosyn by deleting other secondary metabolite biosynthetic gene clusters.

Introduction

Butenyl-spinosyn is a secondary metabolite produced by *Saccharopolyspora pogona* under aerobic fermentation and exhibits stronger insecticidal activity and a broader pesticidal spectrum than spinosyn (Lewer *et al.*, 2009; Araujo *et al.*, 2019). The butenyl-spinosyn biosynthetic gene cluster consists of 23 ORFs, which are involved in polyketide chain synthesis (*busA, B, C, D, E*) and intramolecular C-C bond formation (*busF, J, L, M*), rhamnose attachment and methylation (*busG, I, K, H*), forosamine synthesis (*busP, O, N, Q, R, S*), and rhamnose synthesis (*glt, gdh, epi, kre*) (Hahn *et al.*, 2006; Huang *et al.*, 2009). The butenyl-spinosyn biosynthetic pathway is similar to that of spinosyn and is completed by the following steps: (i) formation of aglycone by 10 acetyl-CoA and 2 malonyl-CoA with the participation of polyketide

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synthase and bridging enzymes, (ii) formation of pseudoaglycone by glycosylation of aglycone using deoxythymidine diphosphate (dTDP)-rhamnose followed by methylation of the rhamnose and (iii) production of butenyl-spinosyn by glycosylation of pseudoaglycone using dTDP-forosamine.

Notwithstanding its advantages as a biopesticide, the titre of butenyl-spinosyn in the wild-type *S. pogona* strain is too low to meet the industrial production requirements (Rang *et al.*, 2020a,b). In general, the potential productivity of Actinomyces is controlled by genes involved in biosynthetic pathways, regulatory factors and competing metabolic pathways (Dhakal *et al.*, 2017; Kim *et al.*, 2017). Several molecular biology-based methods can be used to remould the genome to promote natural products biosynthesis, such as upregulating the expression of positive regulatory genes, downregulating the expression of negative regulatory genes, and deletion of non-target gene clusters and so on (Yang *et al.*, 2015; Meng *et al.*, 2016; Shen *et al.*, 2017; Li *et al.*, 2018; Rang *et al.*, 2020a,b). Several studies have reported that the regulation of some key regulatory factors, such as polynucleotide phosphorylase gene (*pnp*) and AfsR regulatory gene, could significantly promote butenyl-spinosyn biosynthesis (Li *et al.*, 2018, 2019). However, deletion of non-target gene clusters to increase butenyl-spinosyn production has seldom been used as a strategy in *S. pogona*. The main obstacle is the lack of a simple and highly efficient genetic engineering system in *S. pogona*.

Various technologies for large-fragment DNA deletion have been developed to date, including the Red/ET recombineering system, the Cre/loxP system, the Flp/FRT system and the CRISPR/Cas9 system (Masahito and Katsutoshi, 2013; Liu *et al.*, 2016; Wang *et al.*, 2018; Tao *et al.*, 2018). Although each of these methods has its own unique advantages, they also have limitations, such as host restriction, foreign sequences that remain on the chromosome, and the need for specific enzymes and sequences. Moreover, two or more steps are usually required to obtain the target strain by markerless plasmid-mediated deletion. For example, CRISPR-Cas9 system has been demonstrated as a transformative genome engineering tool for many eukaryotic or prokaryotic organisms. Only a single chimeric guide RNA (gRNA, with features of both crRNA and tracrRNA) and a RNA-guided nuclease (Cas9) are needed for genome editing, but there is a certain off-target effect (Culp *et al.*, 2019). In yeast genome editing, large-fragment DNA deletions have been reported using homologous recombination of linear DNA fragments (Sasaki *et al.*, 2013; Hao *et al.*, 2016). This novel approach relies on the use of the orotidine-5-phosphate decarboxylase gene (*ura4*) or uracil-phosphoribosyl transferase gene (*upp*) as a counter-selectable marker (Céline *et al.*, 2002; Sasaki

et al., 2013). The linear DNA fragment consists of two homologous arms for integration into a targeted chromosomal locus, a counter-selectable marker, and an ~200-bp direct repeat sequence (DRS) to drive the loop-out deletion of the target region and counter-selectable marker. In contrast to CRISPR/Cas9 system, this linear DNA fragment can be generated by only PCR; there is no time-consuming restriction/ligation-dependent cloning and exogenous recombinase treatment required, and the target mutant can be obtained by just a one-step transformation, showing relatively high editing efficiency. In addition, no off-target effect has been reported in the application of this method.

To address the limitations of the existing methods used for editing the *S. pogona* genome and demonstrate that deletion of non-target natural product biosynthetic gene clusters can effectively promote butenyl-spinosyn biosynthesis, herein, we describe a one-step high-efficiency mutation delivery method based on the use of *ura4* as a novel counter-selectable marker. Using this system, we successfully deleted the phthoxazolin biosynthetic gene cluster (cluster 14). Further research found that deletion of this cluster can greatly promote butenyl-spinosyn biosynthesis. Moreover, a TetR family regulatory gene (SP_2655) from the phthoxazolin gene cluster played an important role in the regulation of butenyl-spinosyn biosynthesis. The strategy reported here will facilitate functional genomic research on *S. pogona*, reveal butenyl-spinosyn regulatory mechanisms and improve natural products yields in other actinomycetes.

Results

Screening marker analysis for *S. pogona* genome editing

In the process of genetic transformation, mutants can be screened by drug resistance or nutrient deficiency (Hao *et al.*, 2016; Li *et al.*, 2018). Among these genes used, *ura4* and *upp*, which are involved in the synthesis of uracil nucleotides, are commonly used selection markers in linear-fragment homologous recombination (Céline *et al.*, 2002; Sasaki *et al.*, 2013). The whole-genome sequencing results showed that one copy of the *ura4* gene (SP_8748) and two copies of *upp* genes (SP_7509, SP_8742) were presented in the *S. pogona* genome (NZ_CP031142.1) (Fig. 1A). To simplify the operation process, *ura4* was selected as the candidate selection marker for this study. Its coding product can convert 5-fluoro-orotic acid (5-FOA) into a toxin complex, 5-fluorouracil, which causes cell death (Sasaki *et al.*, 2013). When 5-FOA is added to the culture medium, only the *ura4* deletion strain can grow. To confirm that *ura4* can be used as a screening marker in *S. pogona*, we deleted the whole open reading frame of this gene; the process for construction and verification of the transformants is

shown in Figure 1B–D, and the strain obtained was named *S. pogona-Δura4*. The sensitivity of *S. pogona-Δura4* and *S. pogona* to 5-FOA was determined, and we found that *S. pogona-Δura4* could grow at the 5-FOA concentration tested, whereas *S. pogona* exhibited the opposite effect under the same conditions. Phenotypic analysis showed that there were no differences in growth development and butenyl-spinosyn yield between *S. pogona-Δura4* and *S. pogona* on defined culture medium (Fig. S1). Therefore, *ura4* could be used as the basis for linear-fragment homologous recombination, allowing reverse screening for deletion of gene clusters in *S. pogona*.

Phthoxazolin gene cluster deletion based on linear-fragment homologous recombination

It was previously reported that linear-fragment homologous recombination was successfully used for large-fragment DNA knockout in yeast and *Bacillus subtilis* (Céline *et al.*, 2002; Hao *et al.*, 2016). To prove that this editing system is also applicable in *S. pogona*, the phthoxazolin gene cluster was selected as a modification target; this cluster belongs to a hybrid NRPS-PKS gene cluster, based on the result of antiSMASH prediction. The deletion region of the phthoxazolin gene cluster spans a 20-kb region on the *S. pogona* chromosome, including 28 ORFs (Fig. 2A). To generate the expected mutant, the linear DNA fragment (UA_{C14}-*PermE-ura4*-DRS_{C14}-DA_{C14}) with two homologous arms, a *ura4* gene (containing *PermE* promoter) and a DRS, was constructed by fusion PCR (Fig. 2B and C). Then, UA_{C14}-*PermE-ura4*-DRS_{C14}-DA_{C14} was transferred into *S. pogona-Δura4* through protoplast transformation, and finally, approximately 100 transformants were obtained on each plate (Fig. 2D). The resulting mutant, named *S. pogona-Δura4-Δc14*, was verified by PCR with the primers Fc14_{DRS}/Rc14_{down} for multiple independent transformants, and a 1.8-kb band was observed; no band was observed for the original strain *S. pogona-Δura4* (Fig. 2E, Fig. S2A). These results confirmed that the genome-editing system with *ura4* as the screening marker could be used to construct recombinant strains in *S. pogona*, and *ura4* can be used repeatedly to screen the transformants.

Phthoxazolin gene cluster deletion can significantly promote the butenyl-spinosyn biosynthesis

To examine the effect of phthoxazolin gene cluster deletion on butenyl-spinosyn production in greater detail, butenyl-spinosyn production of *S. pogona-Δura4-Δc14* and *S. pogona* was detected (Fig. 3A). HPLC detection results showed that there were two obvious chromatographic peaks at a maximum absorption wavelength of

250 nm (the characteristic wavelength of butenyl-spinosyn), one at 5.4 min and the other at 8.0 min, and their peak areas were significantly different between *S. pogona-Δura4-Δc14* and *S. pogona*. In particular, the peak area of the peak from *S. pogona-Δura4-Δc14* at 8.0 min was significantly higher than that of the peak from *S. pogona*. Moreover, the accumulation curve showed that the chromatographic peak tended to stabilize on the 6th day (Fig. 3B). Meanwhile, a substance with an *m/z* = 650 was identified in the eluent collected at 5.4 min through LC-MS/MS analysis, while a substance with an *m/z* = 617 was identified in the eluent collected at 8.0 min, and the two substances were identified as a rhamnose ion fragment with an *m/z* = 189 by secondary mass spectrometry analysis (Figs S3 and S4). Therefore, the presence of butenyl-spinosyn in the collected eluents was determined by LC-MS/MS analysis and identification of characteristic ions. Compared with the structures of butenyl-spinosyn derivatives reported by Paul *et al.*, these two substances were identified as the butenyl-spinosyn components spinosyn α c (5.4 min) and α d (8.0 min) (Fig. 3C) (Lewer *et al.*, 2009). The main difference between these two substances is that the C21 and C17 of spinosyn α c are connected to buta-1,3-dienyl and hydroxyethoxy group, respectively, while the corresponding positions of spinosyn α d are connected to but-1-enyl and ethoxy group.

The total areas calculated for these two peaks of *S. pogona-Δura4-Δc14* and *S. pogona* were 2417.66 ± 225.87 mAU*s and 512.47 ± 78.15 mAU*s respectively. The butenyl-spinosyn yield of *S. pogona-Δura4-Δc14* was significantly higher, by 4.72-fold, than that of *S. pogona*. As previously mentioned, butenyl-spinosyn biosynthesis involves 23 genes (*busA-busS*, *gtt*, *gdh*, *epi* and *kre*). Next, we analysed the transcription levels of these 23 genes by qRT-PCR and found that transcription of fifteen biosynthetic genes (*busB*, *C*, *D*, *F*, *L*, *M*, *G*, *H*, *I*, *N*, *O*, *P*, *Q*, *R*, *S*), as well as the rhamnose synthetic genes (*gtt*, *gdh*, *epi* and *kre*), was significantly stimulated in *S. pogona-Δura4-Δc14* (fold change > 2.0, *P* < 0.01) (Fig. 4A).

Phthoxazolin gene cluster deletion can change strain growth and glucose consumption pattern

In general, the production of natural products is affected by biomass. We performed growth kinetics analysis to determine whether *S. pogona-Δura4-Δc14* exhibited phenotypic changes. Within the first 84 h of fermentation, *S. pogona* showed logarithmic growth and went through a brief stationary period (2 days), followed by entry into a recession period at 6 days. However, *S. pogona-Δura4-Δc14* exhibited a long logarithmic growth phase during the test period, which caused it to enter the stationary

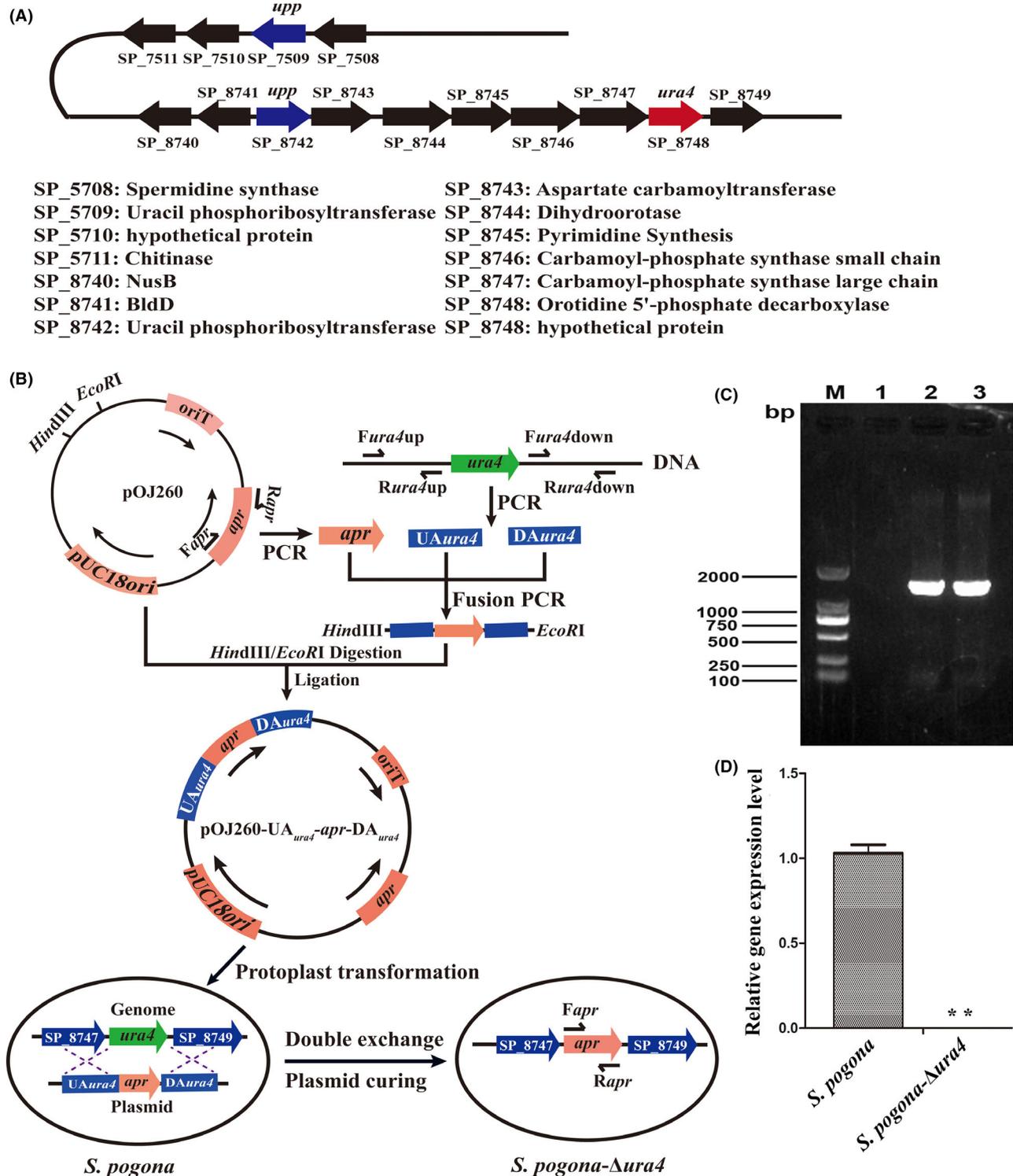


Fig. 1. Construction of the mutant *S. pogona-Δura4* for the Latour system. A. Distribution of the nutrient deficiency-related genes *ura4* and *upp* in the *S. pogona* genome. One *ura4* and two *upp* genes are present throughout the *S. pogona* genome. B. Construction process of *S. pogona-Δura4*. C. PCR identification of *S. pogona-Δura4*. Lane M: DL2000 DNA marker; lane 1: *S. pogona*, lanes 2 and 3: *S. pogona-Δura4*. *S. pogona* genomic DNA was used as the control. For *S. pogona-Δura4* (lanes 2 and 3), the tested transformants showed a 1.3-kb PCR band, whereas *S. pogona* showed no band of the same place (lane 1). D. qRT-PCR verification of *ura4* in *S. pogona-Δura4* and *S. pogona*. The cells of the different strains were inoculated into SEM and cultured at 30°C for 4 days. Total RNA was then isolated and used for qRT-PCR assays. The *S. pogona* was used as the control. 16S rRNA served as the normalization control. The statistical significance of the differences between the values of *S. pogona-Δura4* and the control strain is indicated (** $P < 0.01$, Student's *t*-test, two tailed). Error bars were calculated from four independent determinations of mRNA abundance in each sample.

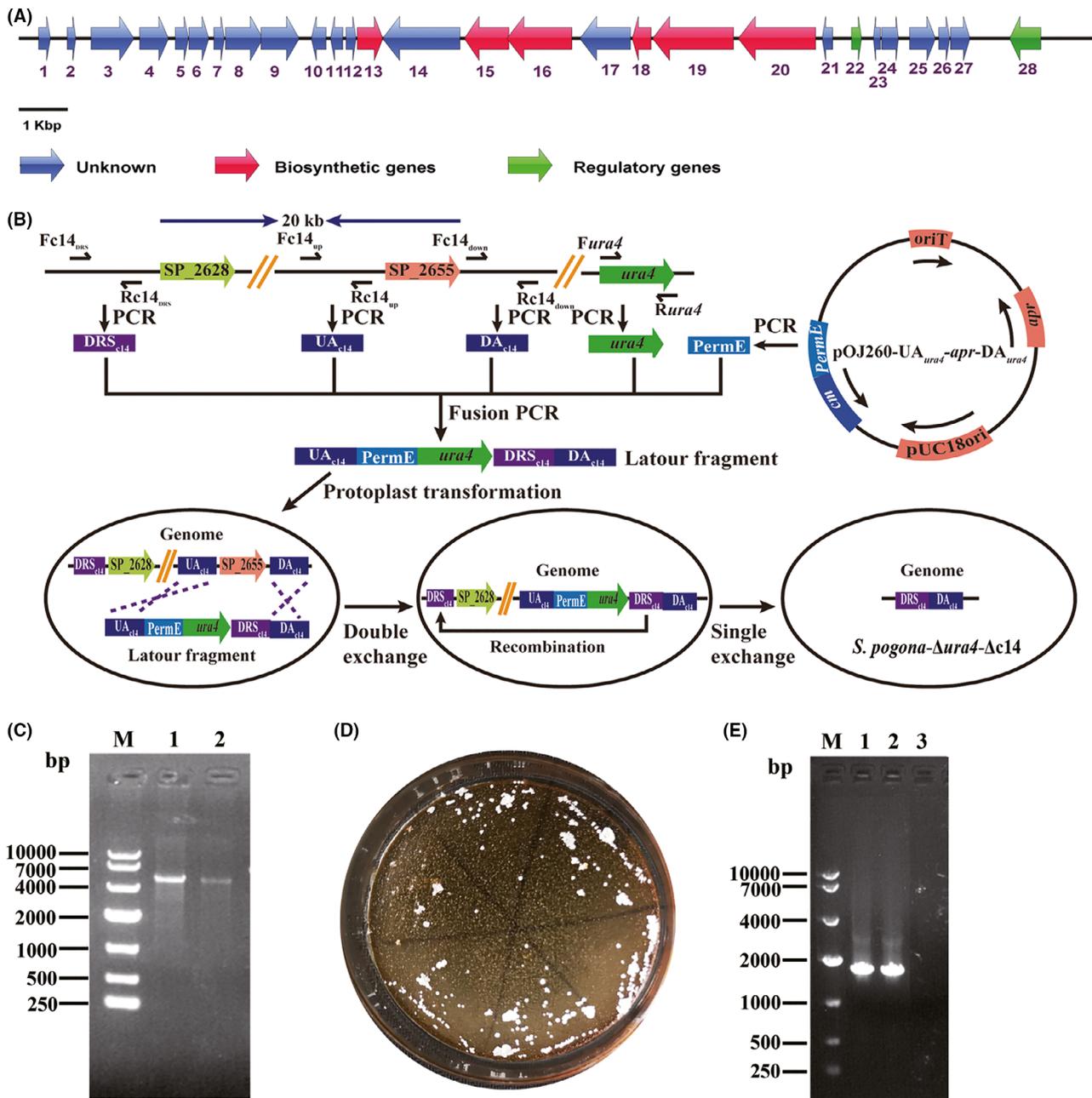


Fig. 2. Latour system construction and large-scale deletion of phthoxazolin gene cluster. **A.** Analysis of the gene composition of the phthoxazolin gene cluster deletion region. The blocking region covers a size of 20 kb, including 28 ORFs. **B.** Schematic diagrams of large-scale deletions using Latour fragments. The Latour fragment, indicated by the box, consists of five regions: UA_{c14} and DA_{c14} , indicated by dark blue box, are upstream and downstream region of the targeted site of recombination for integration of the Latour fragment into the chromosome; $ura4$, indicated by the green box, is the counter-selectable marker; $PermE$, indicated by the cyan box, is the promoter for expression of $ura4$; DRS_{c14} , indicated by the purple-violet box, is the direct repeat sequence for deletion of the target region and $ura4$. **C.** Electrophoretic analysis of the Latour fragment obtained by fusion PCR. Lane M: DL5000 DNA marker; lanes 1 and 2: Latour fragment. **D.** Transformants number of *S. pogona-Δura4-Δc14* obtained based on Latour system. **E.** Electrophoresis of DNA to confirm large-scale deletion of the phthoxazolin gene cluster. Lane M: DL10000 DNA marker; lanes 1 and 2: *S. pogona-Δura4-Δc14*, lane 3: *S. pogona-Δura4*. The *S. pogona-Δura4* genomic DNA was used as the control. For *S. pogona-Δura4-Δc14* (lanes 1 and 2), the tested transformants showed a ~1.8-kb PCR band, whereas *S. pogona-Δura4* showed no band of the same size (lane 3).

period on the 9th day, but the final strain density far exceeded that of *S. pogona* (Fig. 3D). Glucose consumption kinetics analysis showed that the glucose

consumption rate was consistent with the growth tendency of these strains. For the wild-type strain, the glucose concentration rapidly decreased during the

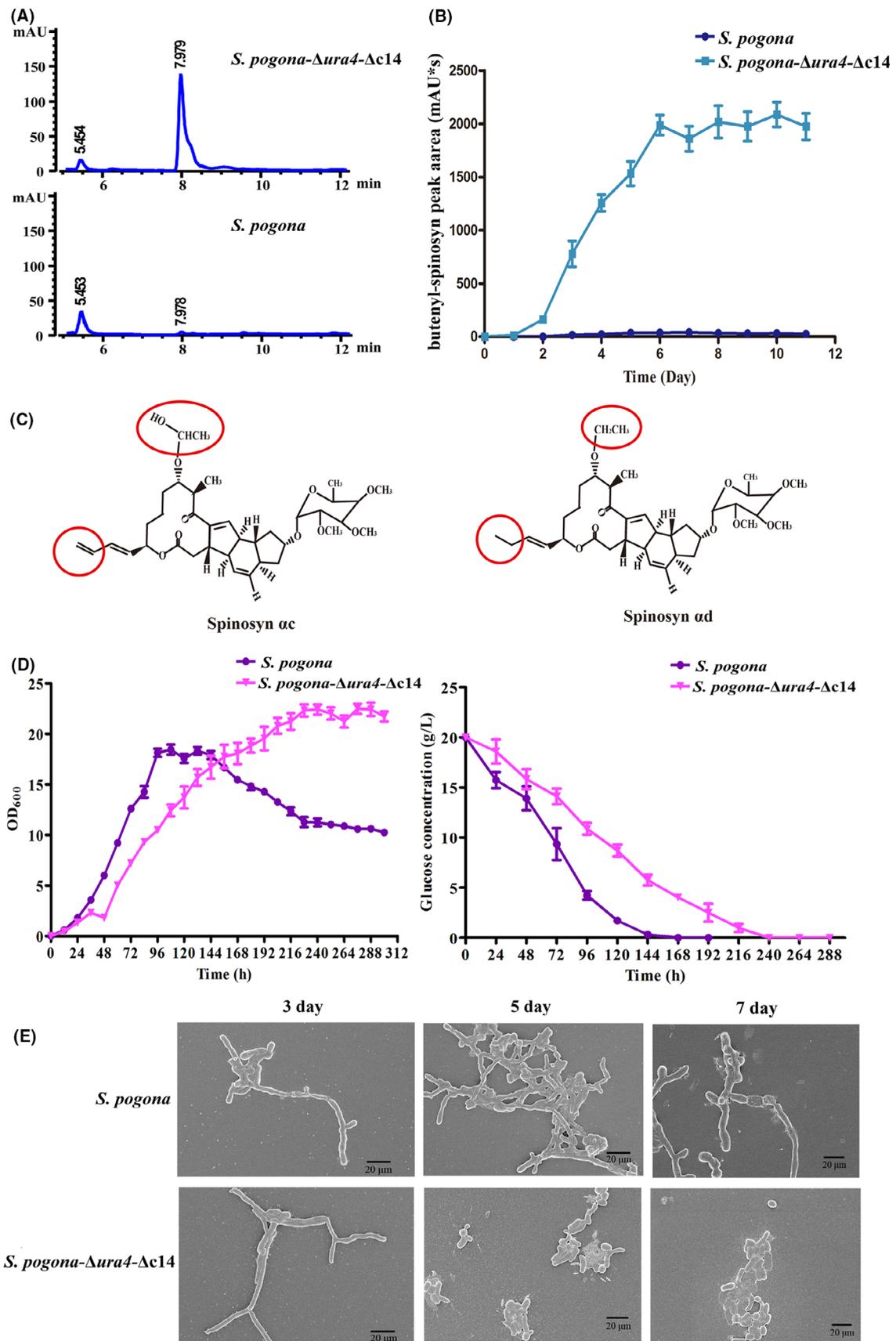


Fig. 3. Effects of phthoxazolin gene cluster blocking on strain growth development and butenyl-spinosyn biosynthesis. A. Comparison of butenyl-spinosyn production in *S. pogona-Δura4-Δc14* and *S. pogona* by HPLC analysis. Blocking the phthoxazolin gene cluster greatly increased the peak area of the chromatographic peak appearing at 8 min, which was identified by mass spectrometry as a derivative of butenyl-spinosyn. B. Yield curve of butenyl-spinosyn at 8.0 min. C. The substances were identified in the eluents collected at 5.4 min and at 8.0 min respectively. Left: spinosyn α c (5.4 min); right: spinosyn α d (8.0 min). The main differences between spinosyn α c and α d are whether C21 and C17 exhibit buta-1,3-dienyl formation and hydroxylation modification. D. Comparison of the growth kinetics (left) and glucose consumption kinetics (right) of *S. pogona-Δura4-Δc14* and *S. pogona*. E. Scanning electron micrographs of *S. pogona-Δura4* and *S. pogona*. Mycelial morphology comparison between *S. pogona-Δura4* and *S. pogona* on the 3rd, 5th and 7th days. Bar, 20 μ m.

logarithmic phase, and the glucose was almost completely exhausted at 144 h. However, the glucose consumption rate of *S. pogona-Δura4-Δc14* was obviously weaker than that of *S. pogona*, and this strain required more time (240 h) than the wild-type strain to complete the glucose consumption under the same conditions. When *S. pogona* enters the stationary phase, there remains enough glucose in the extracellular environment of *S. pogona-Δura4-Δc14* for central carbon metabolism, so *S. pogona-Δura4-Δc14* can continue to grow (Fig. 3D).

To investigate the effect of phthoxazolin gene cluster deletion on exogenous glucose utilization efficiency, qRT-PCR was used to analyse the transcriptional levels of genes related to glucose transport. Based on the *S. pogona* whole-genome data, three genes (SP_0153, SP_0154 and SP_0155) involved in sugar transport, encoding sugar ABC transporters, were selected as detection targets. On the 2nd day, the transcription levels of these three genes in *S. pogona-Δura4-Δc14* were significantly lower than those in *S. pogona* (fold change < 0.67, $P < 0.05$). However, on the 4th day, the transcription levels of SP_0153 and SP_0155 (but not SP_0154) in *S. pogona-Δura4-Δc14* were significantly higher than those in *S. pogona* (fold change > 1.5, $P < 0.01$), which ultimately led to differences in glucose consumption (Fig. 4B, Fig. S5). These results suggest that phthoxazolin gene cluster deletion can affect glucose metabolism by controlling the differential expression of the sugar transport system and then changing the strain growth pattern.

Phthoxazolin gene cluster deletion can accelerate mycelium fragmentation

The cellular morphological difference between *S. pogona* and *S. pogona-Δc28* was also observed by scanning electron microscopy (SEM). *S. pogona* and *S. pogona-Δura4-Δc14* strains showed branching filaments on the 3rd day. On the 5th day, *S. pogona* mycelia exhibited vigorous growth, but *S. pogona-Δura4-Δc14* mycelia showed a short rod shape and no branches (Fig. 3E). On the 7th day, *S. pogona* showed mycelial rupture and reduced branching. The *amfC* gene has been reported to be associated with mycelium formation (Yonekawa *et al.*, 1999). We analysed the expression pattern of this

gene by qRT-PCR and found that *amfC* expression decreased in *S. pogona-Δura4-Δc14* compared with *S. pogona* (5.2-fold, $P < 0.01$) (Fig. 4C, Fig. S5), indicating that phthoxazolin gene cluster deletion caused abnormal expression of genes related to mycelium formation, which eventually led to premature mycelial rupture.

SP_2655 from the phthoxazolin gene cluster, as a TetR family regulatory factor, can repress spinosyn α d biosynthesis

The qRT-PCR results showed that phthoxazolin gene cluster deletion can increase the transcription of butenyl-spinosyn biosynthetic genes. To further reveal the relationship of the phthoxazolin gene cluster and butenyl-spinosyn biosynthesis, the gene composition of the phthoxazolin gene cluster deletion region was analysed in detail, and it was found that this deletion region contains two regulatory genes, which encode a cold shock protein (SP_2649) and TetR family regulatory factor (SP_2655) respectively (Table S2). Generally, TetR family transcription factors can regulate diverse physiological functions in actinomycetes, such as growth development, natural product biosynthesis and osmotic stress (Jiang *et al.*, 2017). The members of this family are often employed as negative regulators that inhibit the expression of target genes (Deng *et al.*, 2013). Therefore, we speculated that SP_2655 might repress butenyl-spinosyn biosynthesis.

To test this hypothesis, the complemented strain *S. pogona-Δura4-Δc14::SP_2655* was constructed, and its butenyl-spinosyn production was evaluated. The process of construction and verification of the transformants is shown in Fig. 5A–C. As expected, the chromatographic peak of *S. pogona-Δura4-Δc14::SP_2655* was no longer apparent at 8.0 min but its peak area was still higher than that of *S. pogona* (Fig. 5D). The accumulation curve showed that its chromatographic peak area is lower than that of *S. pogona-Δura4-Δc14* during the whole fermentation period (Fig. 5E). In addition, the growth kinetics of *S. pogona-Δura4-Δc14::SP_2655* showed significant changes compared with *S. pogona-Δura4-Δc14*. Its main growth characteristic was that it has a higher growth rate in the logarithmic phase and quickly enters the stationary phase. Even compared with *S. pogona*, it also showed a higher growth rate and cell

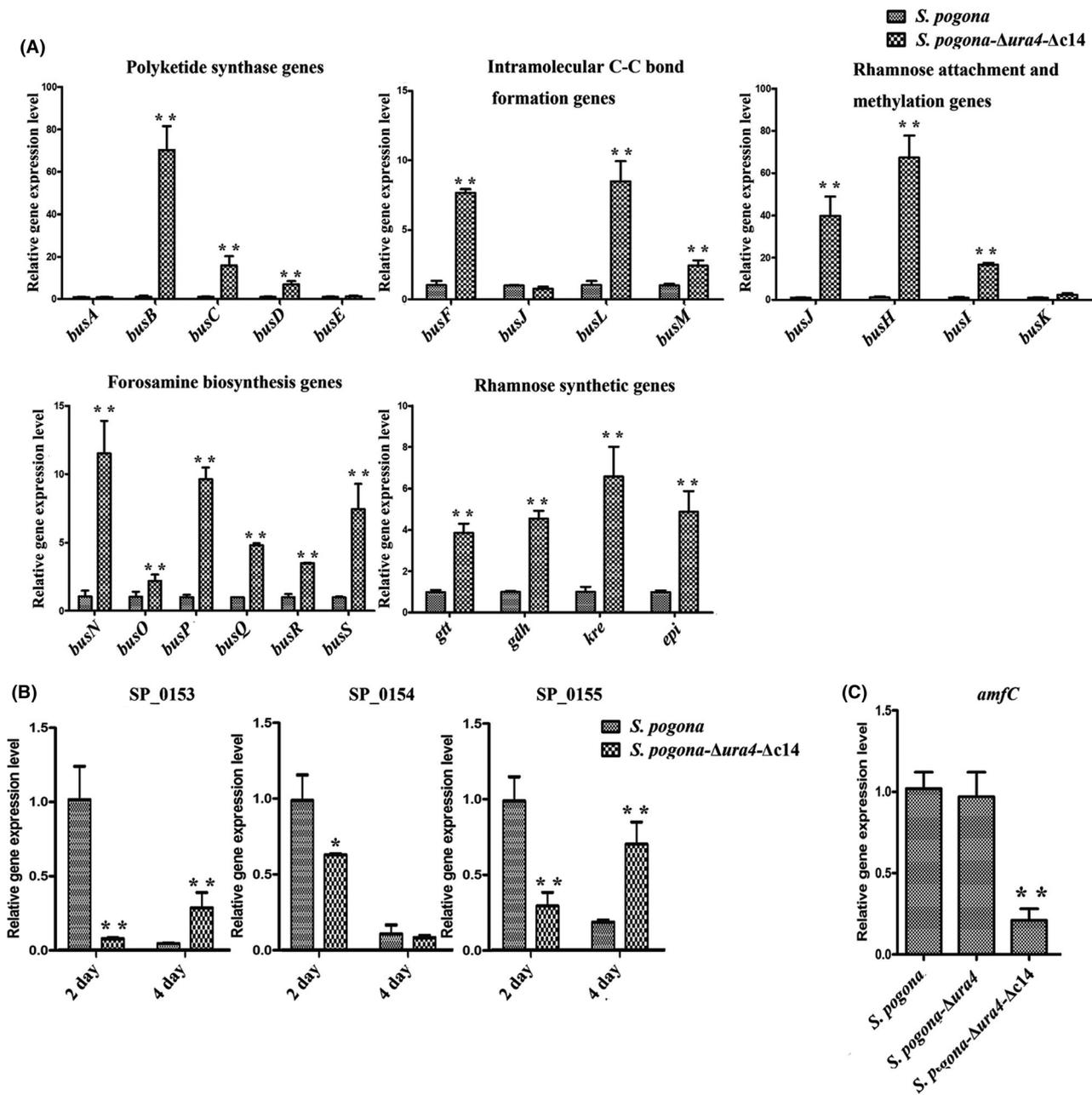


Fig. 4. Effects of phthoxazolin gene cluster blocking on the transcriptional levels of the butenyl-spinosyn biosynthetic gene cluster, mycelium formation protein-encoding gene (*amfC*) and sugar transport-related genes. A. Relative transcript level analysis of 23 genes involved in butenyl-spinosyn biosynthesis. The cells of the different strains were cultured in SFM and incubated at 30°C for 8 days. Total RNA was then isolated and used for qRT-PCR assays. *S. pogona* was used as the control. 16S rRNA served as the normalization control. B. Relative transcript levels analysis of SP_0153, SP_0154 and SP_0155, which are involved in sugar transport. The cells of the different strains were cultured in SFM and incubated at 30°C for 2 or 4 days. Total RNA was then isolated and used for qRT-PCR assays. *S. pogona* was used as the control. 16S rRNA served as the normalization control. C. Relative transcript levels analysis of *amfC*, which is involved in mycelium formation. The cells of the different strains were cultured in SFM and incubated at 30°C for 5 days. Total RNA was then isolated and used for qRT-PCR assays. *S. pogona* was used as the control. 16S rRNA served as the normalization control. The statistical significance of the differences between the values of *S. pogona-Δura4-Δc14* and the control strain is indicated (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test, two tailed). Error bars were calculated from four independent determinations of mRNA abundance in each sample.

density (Fig. 5F), suggesting SP_2655 was required for strain normal growth. To further prove the possibility that SP_2655 regulates butenyl-spinosyn production, we

constructed the SP_2655 deletion mutant *S. pogona-Δura4-ΔSP_2655* by Latour system and measured its production, and found that the chromatographic peak

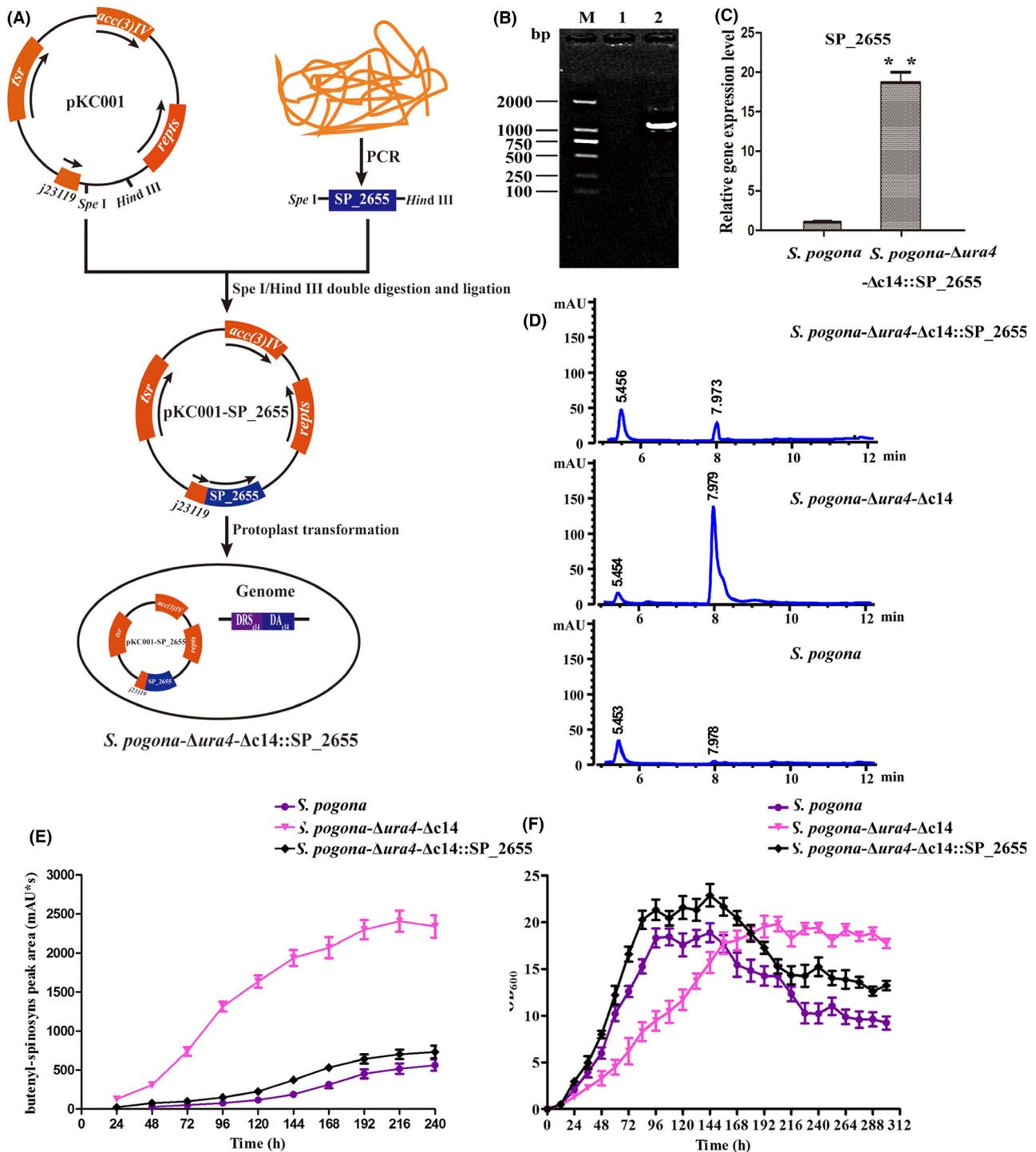


Fig. 5. Individual expression of *SP_2655* in *S. pogona-Dura4-Δc14* and its effect on butenyl-spinosyn biosynthesis. A. Construction process of *S. pogona-Dura4-Δc14*::*SP_2655*. B. PCR identification of *S. pogona-Dura4-Δc14*::*SP_2655*. Lane M: DL2000 DNA marker; lane 1: *S. pogona*, lane 2: *S. pogona-Dura4-Δc14*::*SP_2655*. *S. pogona* genomic DNA was used as the control. For *S. pogona-Dura4-Δc14*::*SP_2655* (lane 2), the tested transformants showed a ~1.0 kb PCR band, consistent with the size of the *tsr* gene, whereas *S. pogona* showed no band of the same size (lane 1). (C) Relative transcript level analysis of *SP_2655* in *S. pogona-Dura4-Δc14*::*SP_2655* and *S. pogona*. The cells of the different strains were cultured in SFM and incubated at 30°C for 4 days. Total RNA was then isolated and used for qRT-PCR assays. *S. pogona* was used as the control. 16S rRNA served as the normalization control. The statistical significance of the differences between the values of the *S. pogona-Dura4-Δc14*::*SP_2655* and control strains is indicated (***P* < 0.01, Student's *t*-test, two tailed). Error bars were calculated from four independent determinations of mRNA abundance in each sample. D. Detection of butenyl-spinosyn production in *S. pogona-Dura4-Δc14*::*SP_2655* by HPLC analysis. E. Cumulative yield comparison of butenyl-spinosyn in *S. pogona* and its derivative. F. Growth kinetics analysis in *S. pogona* and its derivative.

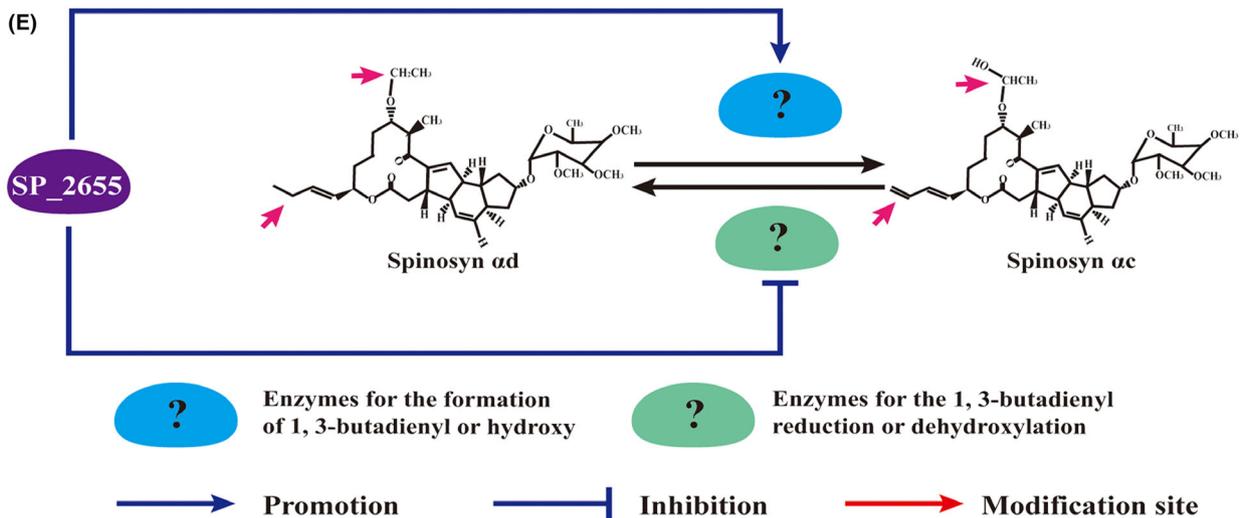
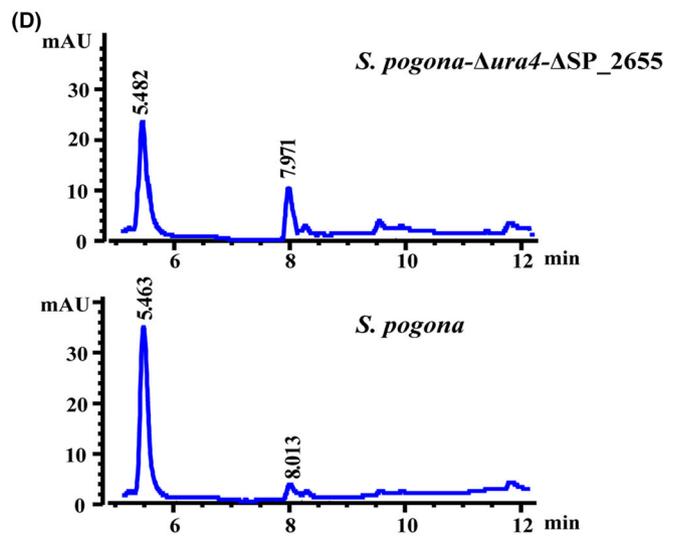
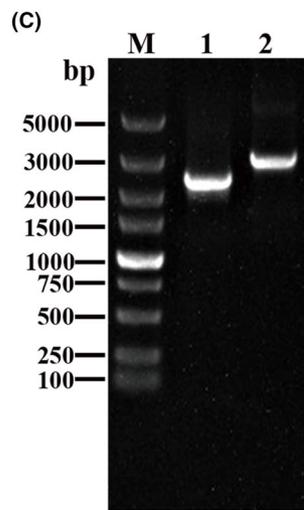
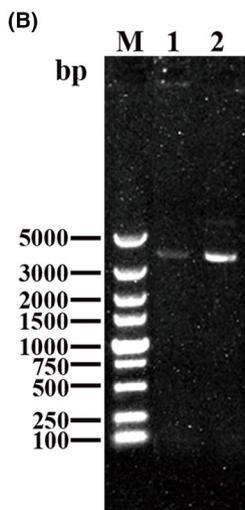
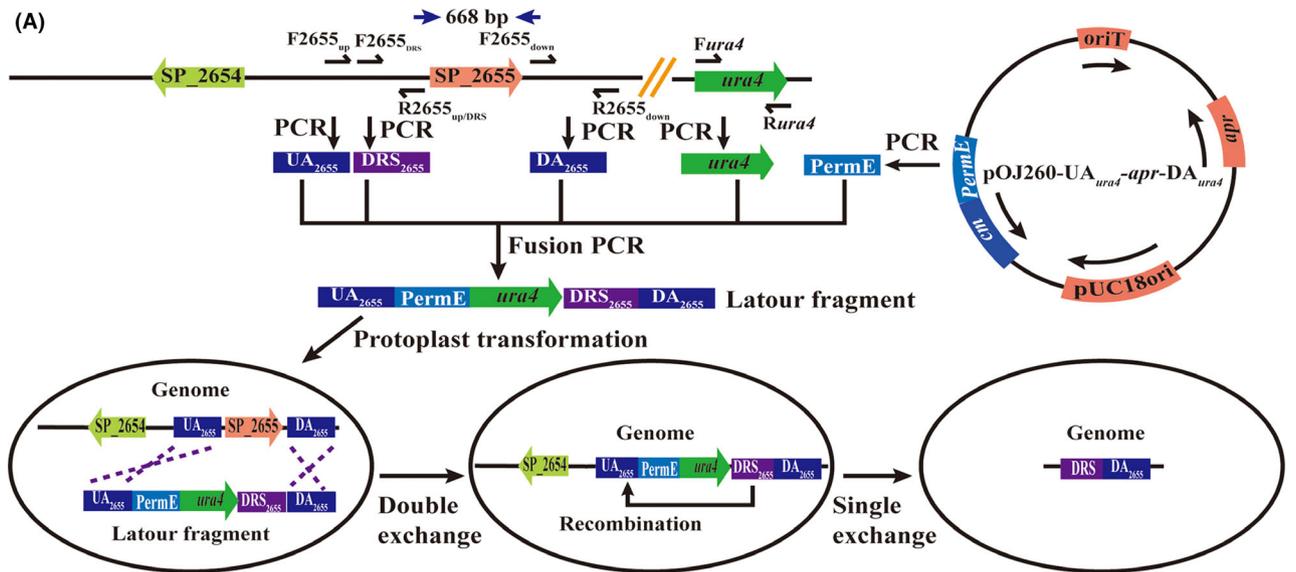


Fig. 6. Effects of SP_2655 deletion on butenyl-spinosyn biosynthesis. A. Schematic diagrams of large-scale deletions using Latour fragments. The Latour fragment, indicated by the box, consists of five regions: UA₂₆₅₅ and DA₂₆₅₅, indicated by dark blue box, are upstream and downstream region of the targeted site of recombination for integration of the Latour fragment into the chromosome; *ura4*, indicated by the green box, is the counter-selectable marker; *PerME*, indicated by the cyan box, is the promoter for expression of *ura4*; DRS₂₆₅₅, indicated by the purple-violet box, is the direct repeat sequence for deletion of the target region and *ura4*. B. Electrophoretic analysis of the Latour fragment obtained by fusion PCR. Lane M: DL5000 DNA marker; lanes 1 and 2: Latour fragment. (C) PCR identification of *S. pogona-Δura4-ΔSP_2655*. Lane M: DL5000 DNA marker; lane 1: *S. pogona-Δura4-ΔSP_2655*, lane 2: *S. pogona-Δura4*. The *S. pogona-Δura4* genomic DNA was used as the control. For *S. pogona-Δura4-ΔSP_2655* (lane 1), the tested transformants showed a ~2.3-kb PCR band, whereas *S. pogona-Δura4* showed no band of the same size (lane 2). (D) Comparison of butenyl-spinosyn production in *S. pogona-Δura4-ΔSP_2655* and *S. pogona* by HPLC analysis. (E) Possible regulatory model of SP_2655 in the mutual conversion between spinosyn α c and α d.

area at 8.0 min was increased 8.7-fold compared with *S. pogona*, indicating SP_2655, as a TetR family regulatory factor, was likely to involve in the negative regulation of spinosyn α d biosynthesis (Fig. 6A–D, Fig. S2B).

In addition, the production of spinosyn α c was increased to a certain extent in the mutant *S. pogona-Δura4-Δc14::SP_2655* compared with the mutant *S. pogona-Δura4-Δc14*. As mentioned above, the main differences between spinosyn α c and α d are whether C21 and C17 exhibit buta-1,3-dienyl formation and hydroxylation modification. It is likely that some enzymes are involved in these specific site modifications of butenyl-spinosyn, while SP_2655 plays a very important role in regulating the functions of these enzymes. Based on the structural differences between spinosyn α c and α d, a conjecture was proposed that SP_2655 is likely to control the mutual conversion of spinosyn α c and α d by positively regulating the expression of enzymes for the formation of buta-1,3-dienyl and hydroxy groups or negatively regulating the expression of enzymes for buta-1,3-dienyl reduction and dehydroxylation (Fig. 6E). To identify the genes that encode these enzymes and SP_2655, the detailed regulatory mechanism needs to be further studied.

Discussion

Butenyl-spinosyn is one of the most promising biological pesticides with a very large potential market; however, its biosynthetic yield in wild-type *S. pogona* is too low to meet the requirements for industrial production and agricultural application (Dhakal *et al.*, 2017; Li *et al.*, 2018). Metabolic engineering remains the most effective way to promote the natural product biosynthesis, such as positive regulatory gene overexpression, negative regulatory gene deletion, non-target gene cluster deletion and so on (Liu *et al.*, 2017; Tao *et al.*, 2019). Due to the low efficiency of genome editing, metabolic engineering of *S. pogona* has been achieved at only the single-gene level to date (Li *et al.*, 2018, 2019; He *et al.*, 2020). The whole-genome sequencing results showed 28 gene clusters in the *S. pogona* genome. The activation of these gene clusters is likely to increase substrate and energy consumption, and to eventually interfere with strain

growth development and butenyl-spinosyn biosynthesis. The non-target gene cluster deletion will provide useful clues for improvement of the yield of butenyl-spinosyn biosynthesis. Therefore, a suitable and efficient large-fragment DNA editing method should be developed for editing of the *S. pogona* genome.

In this study, linear-fragment homologous recombination with *ura4* as a counter-selectable marker was successfully applied for large-fragment DNA deletion in *S. pogona*. This system has been demonstrated to be effective for genome editing in many organisms, including *Schizosaccharomyces pombe*, *B. subtilis* and *Saccharomyces cerevisiae* (Céline *et al.*, 2002; Sasaki *et al.*, 2013; Hao *et al.*, 2016). However, there is no related report on the use of this system for genome editing in *S. pogona*. We tried to apply this system to edit the genome of *S. pogona*. The gene *ura4* was also selected as the candidate counter-selectable marker in our study, and we confirmed that deletion of this gene did not affect strain growth development and butenyl-spinosyn biosynthesis but made *S. pogona* resistant to 5-FOA. Then, this editing system was successfully used for the deletion of phthoxazolin gene cluster and SP_2655 in *S. pogona*. During the construction of the engineered strains, we found that several features of this method make it more suitable for genome editing of *S. pogona* than other existing methods (Luo *et al.*, 2016; Li *et al.*, 2018). Compared with homologous recombination based on pOJ260 plasmid, recombinant DNA molecules can be generated just by PCR without the need for time-consuming restriction/ligation. No foreign sequence was introduced after the modification of the target gene or gene cluster. Compared with ribosome engineering, this method can modify specific sites directionally. In addition, the linear recombination fragment enters the cell through protoplast transformation rather than via inefficient conjugation transfer, which increases the knockout efficiency of the phthoxazolin gene cluster or SP_2655, preliminarily verifying its applicability in *S. pogona* genome editing. Therefore, it could be concluded that this one-step mutation delivery system can be adapted to, at least, other *Saccharopolyspora* species.

Many studies have demonstrated that deletion of non-target natural product biosynthesis gene clusters can

reduce the consumption of substrates, precursors, energy and reducing power by other unnecessary metabolic pathways, increasing the flow of precursors and energy to the target natural product biosynthesis pathway and finally promoting target natural product biosynthesis (Meng *et al.*, 2016; Liu *et al.*, 2018). Our study also confirmed that deletion of the phthoxazolin gene cluster can significantly promote spinosyn α d biosynthesis, and the total peak areas of butenyl-spinosyn increased by 4.72-fold compared with those observed for the wild-type strain. Compared with other yields previously reported, for example, polynucleotide phosphorylase gene (Li *et al.*, 2018), AfsR regulatory gene (Li *et al.*, 2019) or Sp1418 (He *et al.*, 2020) was overexpressed in *S. pogona*, the butenyl-spinosyn yield was just improved by 1.92-fold, 1.17-fold or 2.26-fold compared with the wild-type strain, respectively, indicating that gene cluster deletion could improve the production of butenyl-spinosyn more than single-gene level engineering. In the mutant *S. pogona- Δ ura4- Δ c14*, almost all the tested genes (*busB*, *C*, *D*, *F*, *L*, *M*, *G*, *H*, *I*, *N*, *O*, *P*, *Q*, *R*, *S*, *gtt*, *gdh*, *epi* and *kre*) involved in butenyl-spinosyn biosynthesis were significantly upregulated compared with those in the wild-type strain, suggesting that deletion of the phthoxazolin gene cluster can stimulate the transcription of the *bus* gene cluster. These results indicate that deletion of non-target gene clusters can be an effective strategy to improve the synthesis of target natural products.

A TetR family transcriptional factor, SP_2655, was functionally characterized in *S. pogona* and was shown to significantly repress spinosyn α d biosynthesis. To further reveal the mechanism by which phthoxazolin gene cluster deletion can effectively promote butenyl-spinosyn biosynthesis, we analysed the gene organization and structure of the phthoxazolin gene cluster deletion region through antiSMASH analysis and found that there were two regulatory genes in addition to the core structural genes (Blin *et al.*, 2017, 2019). These two regulatory genes (SP_2649 and SP_2655) encode cold shock protein A and a TetR family transcriptional factor respectively. Overexpression of SP_2655 can significantly repress spinosyn α d production in the mutant *S. pogona- Δ ura4- Δ c14*; however, the production of spinosyn α d increased significantly after SP_2655 was deletion in wild-type strain, indicating that SP_2655 is a TetR family transcriptional factor that negatively regulates spinosyn α d biosynthesis. As another regulatory factor, cold shock protein A is an RNA chaperone that binds single-stranded nucleic acids and destabilizes stem loop structures in nascent mRNA at low temperature, thus allowing transcription of downstream genes to continue under cold stress conditions (Wang *et al.*, 2014; Wang *et al.*, 2016; Caruso *et al.*, 2018). Whether deletion of SP_2649 can also cause related phenotypic changes in

S. pogona needs to be further verified by genetic modification. Currently, there are few studies on the regulatory network of butenyl-spinosyn biosynthesis, and the discovery of these two regulatory factors can provide important clues for further understanding the regulatory mechanism of butenyl-spinosyn biosynthesis.

In summary, we have developed a simple and efficient method to perform large-fragment DNA deletion and successfully deleted the phthoxazolin gene cluster in *S. pogona* for the first time, greatly promoting butenyl-spinosyn biosynthesis. This genome-editing system provides an alternative strategy for other non-target gene clusters of *S. pogona* in the future. Moreover, our study reveals an important new role for SP_2655 in the control of butenyl-spinosyn biosynthesis. The strategy reported here will be useful for revealing butenyl-spinosyn regulatory mechanisms and improving antibiotic production in other actinomycetes.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The media and culture conditions used in this study are described in the Supplementary Materials and Methods.

Construction of the S. pogona- Δ ura4

The primers used in this study are listed in Table S1. To construct the Δ ura4 mutant, the double exchange replacement of the targeted gene was performed as described previously (Yang *et al.*, 2015). The ~ 1.5 kb upstream and downstream fragments (UA_{ura4} and DA_{ura4}) flanking the *ura4* gene were PCR-amplified using the primer pairs of *Fura4*_{up}/*Rura4*_{up} and *Fura4*_{down}/*Rura4*_{down} respectively. Plasmid pOJ260 was used to amplify apramycin resistance gene (*apr*) with primer pair *Fapr*/*Rapr*. The 5'-end of *Rura4*_{up} and *Fura4*_{down} has a 30–35 nt overlapping sequence, respectively, allowing the joining of UA_{ura4}, *apr* and DA_{ura4} in a subsequent fusion PCR. The resulting ~ 4.3 kb fusion fragment was digested with *Hind* III and *Eco*R I and cloned into the corresponding restriction sites of pOJ260, yielding pOJ260-UA_{ura4}-*apr*-DA_{ura4}. Then, this plasmid was transformed into *S. pogona* by protoplast transformation (Supplementary Materials and Methods). Transformants were selected directly on R5 plates containing 50 μ g ml⁻¹ apramycin. Since pOJ-260 is a suicide plasmid, it cannot be replicated in *S. pogona* and will eventually be lost as the strain grows. Therefore, the chromosome structure at the *ura4* locus of several Apra^R colonies was analysed by PCR using *Fapr* and *Rapr*, and the mutant that was successfully verified was named *S. pogona- Δ ura4*.

Construction of linear recombination DNA fragment for deleting target gene cluster or gene

The linear recombination DNA fragment for deleting phthoxazolin gene cluster was amplified from *S. pogona* genome using several primers (Table S1). The ~ 1.5 kb upstream and downstream fragments (UA_{C14} and DA_{C14}) were obtained using the primer pairs Fc14_{up}/Rc14_{up} and Fc14_{down}/Rc14_{down} respectively. The intact open reading frame (ORF) of *ura4* gene was obtained using the primer pair *Fura4/Rura4*. The ~ 500 bp DRS_{C14} was obtained using the primer pair Fc14_{DRS}/Rc14_{DRS}. In addition, the strong promoter *PermE* was obtained using the primer pair F_{PermE}/R_{PermE} and plasmid pOJ260-*cm-PermE* as template to overexpress *ura4* gene. Rc14_{up}, R_{PermE}, Fc14_{DRS} and Rc14_{DRS} have a 30–35 nt overlapping sequence. The PCR products of UA_{C14}, *PermE*, *ura4*, DRS_{C14} and DA_{C14} were fused to deletion cassettes by multistage fusion PCR and finally obtained the ~ 4.3 kb linear recombination DNA fragment, named UA_{C14}-*PermE-ura4*-DRS_{C14}-DA_{C14}.

The building process of single-gene deletion mutant was similar to that of gene cluster, and the detailed process was as follows. The ~ 1.0 kb upstream and downstream fragments (UA₂₆₅₅ and DA₂₆₅₅) were obtained using the primer pairs F2655_{up}/R2655_{up} and F2655_{down}/R2655_{down} respectively. The intact open reading frame (ORF) of *ura4* gene was obtained using the primer pair *Fura4/Rura4*. The ~ 500 bp DRS was obtained using the primer pair F2655_{DRS}/R2655_{DRS}. The strong promoter *PermE* was obtained using the primer pair F_{PermE}/R_{PermE} and plasmid pOJ260-*cm-PermE* as template to overexpress *ura4* gene. R2655_{up}, R_{PermE}, F2655_{DRS} and R2655_{DRS} have a 30–35 nt overlapping sequence. The PCR products of UA₂₆₅₅, *PermE*, *ura4*, DRS₂₆₅₅ and DA₂₆₅₅ were fused to deletion cassettes by multistage fusion PCR and finally obtained the ~ 3.9 kb linear recombination DNA fragment, named UA₂₆₅₅-*PermE-ura4*-DRS₂₆₅₅-DA₂₆₅₅.

Finally, the UA_{C14}-*PermE-ura4*-DRS_{C14}-DA_{C14} and UA₂₆₅₅-*PermE-ura4*-DRS₂₆₅₅-DA₂₆₅₅ were transformed into the original strain *S. pogona-Δura4* by protoplast transformation, respectively, and cultured in CSM medium at 30°C for 2 days. A 100 μl bacterium solution was spread on 5-FOA screening medium and incubated at 30°C until colony formation. The 5-FOA resistant strains were testified by PCR using the corresponding primer pairs and sequencing (Sangon Biotech, Shanghai), and the mutant that was successfully verified was named *S. pogona-Δura4-Δc14* or *S. pogona-Δura4-ΔSP_2655*.

Construction of *S. pogona-Δura4-Δc14::SP_2655*

To construct the SP₂₆₅₅ complementation strain, the 639 bp SP₂₆₅₅ was obtained using the primer pair

F2655/R2655 and *S. pogona* genome as template. The PCR-amplified product was digested with *Hind* III and *Spe* I and cloned into the corresponding restriction sites of pKC001, which contained a synthetic promoter j23119 and a *tsr* gene, yielding pKC001-SP₂₆₅₅. Then, this plasmid was transformed into mutant *S. pogona-Δura4-Δc14* by protoplast transformation. Transformants were selected directly on R5 plates containing 5 μg ml⁻¹ thiostrepton. The chromosome structure at the *tsr* locus of several thiostrepton resistance colonies was analysed by PCR using *Ftsr* and *Rtsr*, and the mutant that was successfully verified was named *S. pogona-Δura4-Δc14::SP_2655*.

Cultivation profile analysis of the *S. pogona* and its derivative strains

The cell cultures were collected in SFM at 10th day to determine the butenyl-spinosyn production using HPLC 1290 system. The sample processing and testing methods were described previously (Li *et al.*, 2018) with minor modifications. The fermentation broth was mixed with acetone at a 1 : 1 volumetric ratio for 48 h. Cultures were centrifuged at 9000 r.p.m. for 15 min, and the supernatants were filtered through 0.22 μm Millipore filters. After filtration, a 10 μl sample was loaded onto a C18 column (AQ12S05-1546WT, YMC, Kyoto, Japan) and gradient eluted with the elution buffer at 1.0 ml min⁻¹. The elution buffer composed of solvent A (water) and solvent B (methanol and acetonitrile at a 1 : 1 volumetric ratio). Each chromatographic separation lasted 25 min with the following gradient: 2% B (2 min hold) ramped to a final mobile phase concentration of 100% B for 15 min (5 min hold). The detection wavelength was set at 250 nm during the analysis. MS analysis was performed using an Thermo LTQ XL Ion Trap mass spectrometer in positive electrospray ionization mode (Thermo Scientific, Waltham, MA, USA). The 15 μl sample (Chromatographic peak collected) was loaded onto a C18 column (LAGV-25005-102130, Thermo Scientific) and gradient eluted with the elution buffer at 300 μl min⁻¹. The elution buffer composed of solvent A (water, 0.1% formic acid, v/v) and solvent B (methanol, 0.1% formic acid, v/v). Each chromatographic separation lasted 20 min with the following gradient: 50% B (2 min hold) ramped to a final mobile phase concentration of 100% B for 10 min (5 min hold). The mass analyser was scanned over a mass range of 80 to 800 m/z. Data analysis and instrument operations were performed under Xcalibur software control. Other phenotypic features such as growth curve, glucose consumption and mycelium morphology were also analysed, and the detailed operation is described in the Supplementary Material and Methods.

RNA isolation, cDNA synthesis and quantitative reverse transcription PCR

Total RNAs of the *S. pogona* and its derivative strains were separately isolated by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined by measuring the ratio of OD₂₆₀ nm to OD₂₈₀ nm. DNase treatment and cDNA synthesis were carried out by RNase-free DNase 1 (Invitrogen) and a High-capacity cDNA Archive kit (Fermentas) according to each manufacturer's instructions. The real-time qPCR amplification was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA), as previously described (Yang *et al.*, 2014). The sequences of the primers used in qRT-PCR were developed with Primer Premier 5.0 and are listed in Table S3. Transcript generated from the 16S rRNA gene was used for normalization. Results were shown as the means of four replicate experiments.

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Author contributions

L.Q.X. conceived the project. L.Q.X. and J.R. generated the concepts and designed the research. J.R. and Y.L.L. performed mutant construction. L.C., L.S., Z.Q.Y., Q.Q.W. and Y.M.Z. performed HPLC and data analysis. H.C.H., Y.L., Y.J.S., Y.W.L., Q.J.X. and S.B.H. performed LC-MS/MS and data analysis. J.R., Q.J.X., L.Q.X. and X.Z.D. wrote the manuscript. All authors discussed the results and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Strains, plasmids and primers used in this study.

Table S2. Gene composition analysis of the phthoxazolin biosynthetic gene cluster blocking region.

Table S3. qRT-PCR primers used in this study.

Fig. S1. Effects of *ura4* deletion on strain growth development and butenyl-spinosyn biosynthesis. (A) Growth curve. (B) Comparison of butenyl-spinosyn production in mutant *S. pogona-Δura4* and wild-type *S. pogona* by HPLC analysis. (C) Scanning electron microscope of the mutant *S. pogona-Δura4* and wild-type *S. pogona*. Bar, 20 μm.

Fig. S2. Latour system mediated single gene cluster and gene deletions. (A) Identification of the mutant *S. pogona-Δura4-Δc14*. Eleven randomly selected transformants were verified by PCR and the original strain *S. pogona-Δura4* genomic DNA was used as a control. All eleven clones showed 1.8 kb amplicons, the expected size of the mutant *S. pogona-Δura4-Δc14*. Lane M: DL5000 DNA marker; lane 1: *S. pogona-Δura4*, lanes 2–12: *S. pogona-Δura4-Δc14*. (B) Identification of the mutant *S. pogona-Δura4-ΔSP_2655*. Ten randomly selected transformants were verified by PCR and the original strain *S. pogona-Δura4* genomic DNA was used as a control. All ten clones showed 2.3 kb amplicons, the expected size of the mutant *S. pogona-Δura4-ΔSP_2655*. Lane M: DL5000 DNA marker; lane 1: *S. pogona-Δura4*, lanes 2–11: *S. pogona-Δura4-ΔSP_2655*.

Fig. S3. MS and MS/MS analysis of collected eluents at 5.4 min. The MS identification result showed that the m/z of this substance is 650 (M + H)⁺. The MS/MS identification result further showed that (M + H)⁺ ions at m/z = 650

contained a rhamnose ion fragment of 189 molecular mass, which was confirmed as a spinosyn α .

Fig. S4. MS and MS/MS analysis of collected eluents at 8.0 min. The MS identification result showed that the m/z of this substance is 617 ($M + H$)⁺. The MS/MS identification result further showed that ($M + H$)⁺ ions at $m/z = 617$ contained a rhamnose ion fragment of 189 molecular mass, which was confirmed as a spinosyn

Fig. S5. A model depicting the roles of the phthoxazolin gene cluster in butenyl-spinosyn biosynthesis, mycelial morphology and sugar transport. I, butenyl-spinosyn biosynthesis; II, mycelial morphology; III, sugar transport. Different colored boxes indicate the samples at specific time points selected for qRT-PCR analysis.