

Two pHZ1358 Derivative Vectors for Efficient Gene Knockout in *Streptomyces*

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The deletion of *sti* from the *Streptomyces* plasmid pIJ101 made its derivative pHZ1358 an efficient vector for gene disruption and replacement. Here, pHZ1358 was further optimized by the construction of a derivative plasmid pJTU1278, in which a cassette carrying multiple cloning sites and a *lacZ* selection marker were introduced for convenient plasmid construction in *E. coli*. In addition, the *oriT* region of pJTU1278 was also deleted, generating a vector (pJTU1289) that can be used specifically for PCR-targeting. The efficient usage of these vectors was demonstrated by the deletion of the gene involved in avermectin biosynthesis in *S. avermitilis*.

Keywords: Shuttle vector, pHZ1358, pJTU1278, pJTU1289, conjugation, PCR-targeting

Streptomyces, soil-inhabiting Gram-positive bacteria, occupy a significant niche in biotechnology research because of their ability to produce a wide variety of bioactive compounds. *Streptomyces* are also unusual among prokaryotes as they undergo a complex cycle of morphological differentiation, forming spores, vegetative substrate hyphae, and aerial hyphae during different stages of their life cycle [4]. Although new techniques are continually being developed for different research purposes in this area, gene deletion/disruption remains one of the most efficient and indispensable means.

For gene disruption and replacement in *Streptomyces* species, pHZ1358 [7] has been developed as an efficient pIJ101 [3] derivative vector by the removal of a 574-bp DNA region containing an *sti* (strong incompatibility locus) that causes the accumulation of single-strand plasmids in the host [1, 7]. Further deletion of 36-bp direct repeats makes the plasmid stably replicate as a shuttle vector in *E. coli*.

Here, we report on the construction of two pHZ1358 derivatives, pJTU1278 and pJTU1289, where the former

contains the additional virtues of multiple cloning sites and a *LacZ* selection marker (for convenient construction in *E. coli*), and the latter has a deleted *oriT*, allowing it to be used specifically for PCR-targeting. The usage of pJTU1289 was also demonstrated by the deletion of an avermectin biosynthetic gene.

MATERIALS AND METHODS

Bacterial Strains and Plasmids Used in This Study

DH10B (GIBCO-BRL) and ET12567 (pUZ8002) [5] were used as the *E. coli* host for cloning and conjugation, respectively. *S. avermitilis* NRRL 8165 was used for the gene deletion.

The fosmid vector pCC2FOS™ (EPICENTRE Biotechnologies) was used to construct the genomic library of *S. avermitilis* NRRL 8165 [2]. Plasmid pJTU412 was a gift from Sun *et al.* [7].

The DNA isolation and plasmid manipulation from *E. coli* were performed according to Sambrook and Russell [6].

Construction of pJTU1278 and pJTU1289

For the construction of pJTU1278, pJTU412 was first digested with *KpnI*, blunted with the Klenow fragment of *E. coli* DNA polymerase I, self-ligated to generate pJTU1275, and further digested with *BglII* and *SphI*. The resulting 8.7-kb *BglII*–*SphI* fragment from pJTU1275 was then treated with the Klenow fragment and self-ligated to generate pJTU1276, which was digested with *XbaI*, treated with the Klenow fragment, and self-ligated to generate pJTU1277. A 585-bp PCR fragment from pBluescript II SK(+) using primers LacZ-P1 (5'-AACAATTGCCATTCGCCATTCAGG-3') and LacZ-P2 (5'-AA-CAATTGCCCAATACGCAAACC-3') was digested with *MunI* and inserted into the *EcoRI* site of pJTU1277 to generate pJTU1278. The *LacZ* was in the same direction as the resistant gene *bla* in pJTU1278.

For the construction of pJTU1289, pJTU1278 was digested with *Sse8387I*, and the 8.4-kb *Sse8387I* fragment from pJTU1278 was then self-ligated to generate pJTU1289.

Gene Deletion of *aveA4* (*S. avermitilis* HYL16)

The 4,671-bp *BamHI* fragment containing the portion of *aveA4* from cosmid 14A7 of the *S. avermitilis* NRRL 8165 genome library was cloned into pJTU1289 to generate pJTU1314. The *aac(3)IV* and

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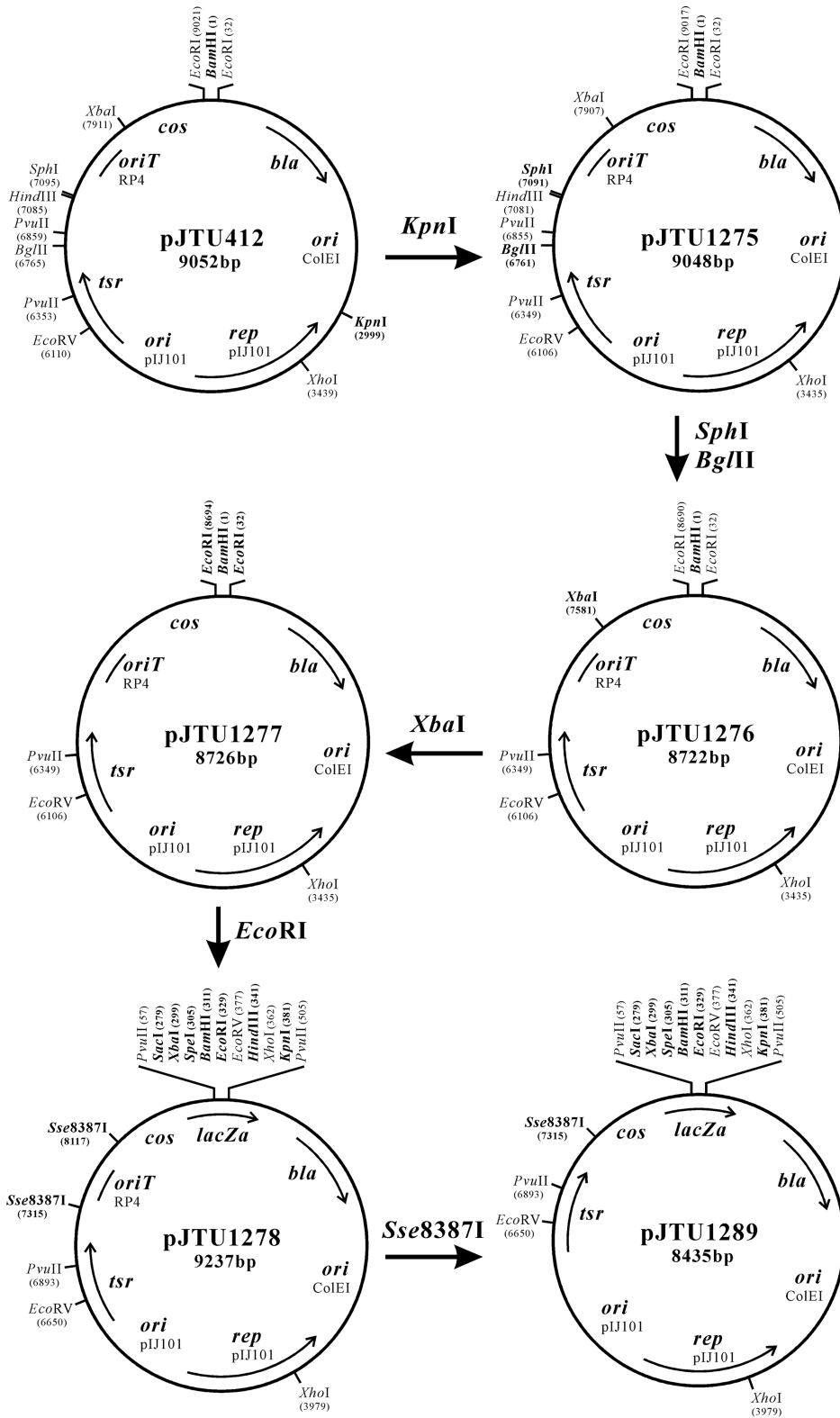


Fig. 1. Construction of pJTU1278 and pJTU1289. pJTU1278 and pJTU1289 were optimized from pJTU412. After two rounds of digestion, blunting with polymerase, and re-ligation using *KpnI* and *BglII*–*SphI*, respectively, *KpnI* and *HindIII* were eliminated from the vector. A multiple cloning site (*SacI*, *XbaI*, *SpeI*, *BamHI*, *EcoRI*, *HindIII*, *KpnI*) and *lacZ* gene (for clone selection in *E. coli*) were then inserted. *EcoRV* and *XhoI* can also be used for cloning. *ori^{pIJ101}*, *ori^{ColEI}*, and *oriT* were kept for the shuttle between *Streptomyces* and *E. coli*. The vector can also be used for cosmid library construction.

oriT cassettes were amplified from the pIJ733 disruption cassette using the primers aveA4-T1 (5'-ACTCCCGCCTGCACGACGCC-ACTCCCCAGCCCACAAGGATTCGGGGATCCGTCGACC-3') and aveA4-T2 (5'-GCGGAGCCATGGTGGCCATCGAGGCGTCC-GAGGACGAGATGTAGGCTGGAGCTGCTTC-3'). The resulting PCR product was then used to replace the 1,150-bp DNA region of *aveA4* in pJTU1314 to generate pJTU1317, and then in strain NRRL 8165 to generate *S. avermitilis* HYL16. A comparison of the wild-type and mutant *S. avermitilis* HYL16 was conducted through fermentation, extraction, and an LC-MS analysis.

Cultivation of *S. avermitilis*

The *Streptomyces* culture conditions were as described by Kieser *et al.* [4]: An SFM solid medium was used for the *S. avermitilis* sporulation, fermentation, and conjugation between *E. coli* and *Streptomyces*. A TSB culture supplemented with 10.3% sucrose and 1% yeast extract was used for the growth of mycelia for the isolation of total DNA. Thiostrepton and apramycin were used at a concentration of 12.5 µg/ml and 15 µg/ml in the solid and liquid medium, respectively, when necessary for the screening of conjugants.

Analysis of Avermectin

After 7 days of growth on an SFM agar plate, the mycelia of *S. avermitilis* and the *S. avermitilis* HYL16 mutants were collected and extracted with ethanol. The extract was concentrated under reduced pressure to yield an oily substance, which was further extracted with 1 ml of methanol. The methanol extract was analyzed directly using an HPLC-MS Agilent 1100 equipped with an Agilent ZORBAX SB-C18 (2.1×150 mm) column and using a linear gradient program of acetonitrile/H₂O: 60% acetonitrile over 3 min, 60%–

90% over 5 min, 90% over 2 min, 90%–100% over 2 min, and constant 100% acetonitrile over 3 min at a flow rate of 0.5 ml/min. An iontrap mass spectrometer was operated with an electrospray ionization source in the positive-ion mode, where the drying gas flow was 8 l/min, the nebulizer pressure was 30 psi, the drying gas temperature was 325°C, and the fragmentation amplitude was varied between 1.0 and 1.8 V.

RESULTS AND DISCUSSION

Construction of pJTU1278 and pJTU1289

Derived from pIJ101, pHZ1358 is an efficient *Escherichia coli*/*Streptomyces* shuttle vector for targeted gene deletion and disruption. However, a further improvement of pHZ1358 would be removing the 36-bp direct repeats, which often cause a problem of plasmid recombination in *E. coli* [7].

For the convenience of gene cloning, a multiple cloning site is needed. Therefore, the vector optimization was started from the pHZ1358 derivative pJTU412. By digesting with *KpnI* and then blunt-ending by filling-in to eliminate the *KpnI* site in pJTU412, followed by further digestion with *BglII*–*SphI*, blunt-ending by filling-in, and re-ligation to eliminate the *HindIII* site, a cassette carrying multiple cloning sites was introduced, along with the selection marker *lacZ* gene with a PCR fragment from pBluescript II SK(+) (Fig. 1). Successful cloning was confirmed by restriction digestion and sequencing (Fig. 2).

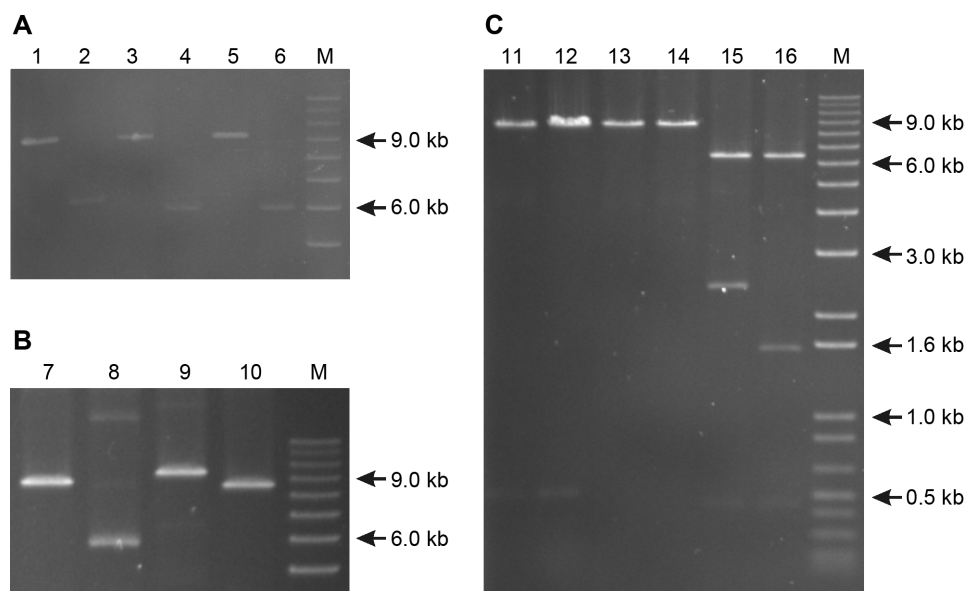


Fig. 2. Confirmation of successful cloning of pJTU1278 and pJTU1289.

The successful construction of pJTU1275 was indicated by the loss of the *KpnI* restriction site at 2,999-bp (lane 2), whereas pJTU412 was linearized by *KpnI* (lane 1). The loss of the *BglII* and *HindIII* sites confirmed the successful cloning of pJTU1276 (lanes 4, 6), whereas pJTU1275 was linearized by *BglII* and *HindIII* (lanes 3, 5). The cloning of pJTU1276, pJTU1277, pJTU1278, and pJTU1289 was confirmed with *XbaI* (B). The loss of the *XbaI* site confirmed the successful cloning of pJTU1277 (lane 8), whereas pJTU1267, pJTU1278, and pJTU1289 were linearized by *XbaI* (lanes 7, 9, 10). All the clones were confirmed with *PvuII* (C). Lanes 11–16 show the digestion of pJTU412, pJTU1275, pJTU1267, pJTU1277, pJTU1278, and pJTU1289 with *PvuII*, separately.

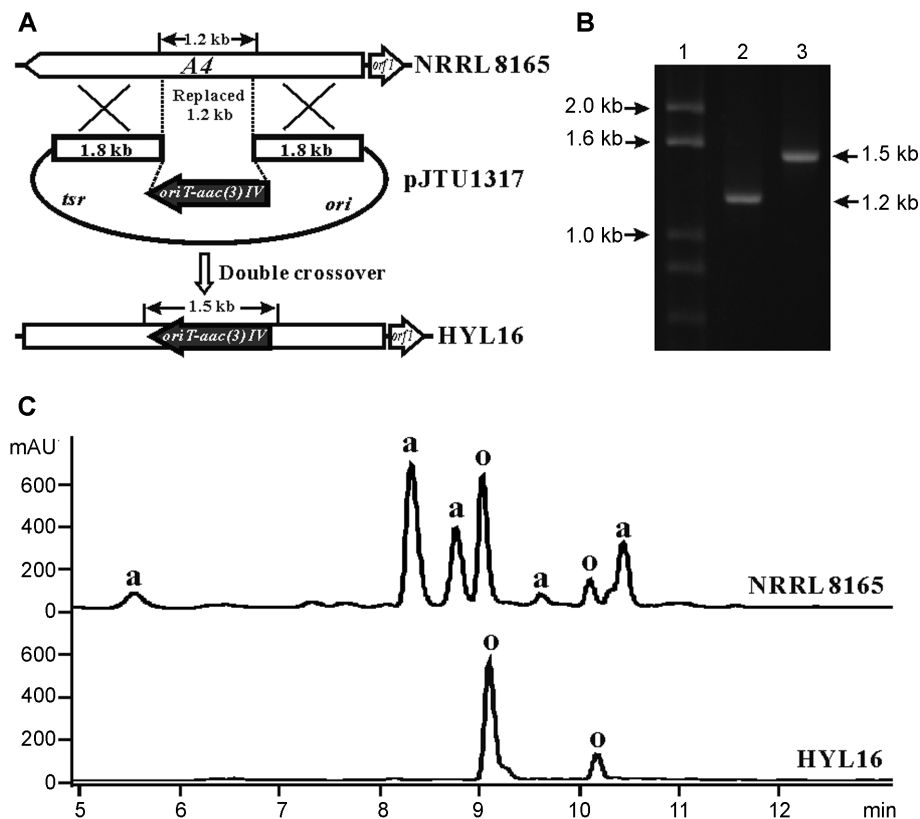


Fig. 3. Deletion of the *aveA4* gene.

The 1.2-kb region of *aveA4* was replaced with the *oriT-tac(3)IV* cassette using a double crossover (A). The successful construction of the *S. avermitilis* HYL16 was confirmed by PCR (B) with a 1.5-kb PCR product as expected, in contrast to the 1.2-kb DNA fragment from the wild type strain. Comparison of metabolites between the wild-type strain and the mutant *S. avermitilis* HYL16 showed that the avermectin corresponding to “a” peaks of the wild-type strain NRRL 8165 disappeared in *S. avermitilis* HYL16. Oligomycin corresponding to “o” peaks still produced in HYL16 (C).

Based on the above manipulations, the pHZ1358 derivative pJTU1278 contained the following characteristics: *ori*, *rep* from pIJ101, and resistant gene *tsr* for operation in *Streptomyces*; *ori* (ColE1), *lacZ*, and resistance gene *bla* for manipulation in *E. coli*; and *oriT* for conjugation from *E. coli* to *Streptomyces*. In addition, owing to its segregational instability from its progenitor pHZ1358, it can be used for efficient gene deletion/disruption in *Streptomyces*. Since a single *cos* was included, it can also be used for cosmid library construction.

For the specific purpose of PCR-targeting, an additional optimization of pJTU1278 was carried out to generate pJTU1289, where the 0.8-kb Sse83871 fragment containing *oriT* was deleted.

Deletion of *aveA4*

The usage of pJTU1278 and pJTU1289 as gene disruption vectors was demonstrated by the deletion of a gene essential for avermectin biosynthesis in *S. avermitilis* MA-4680. As shown in Fig. 3, the 1,150-bp DNA region of *aveA4* was replaced by the *oriT-tac(3)IV* cassette to generate *S. avermitilis* HYL16. The successful construction of *S.*

avermitilis HYL16 was confirmed using a PCR (Fig. 3B), and the successful deletion of *aveA4* was confirmed by a comparison of the production of metabolites between the wild-type and the mutant *S. avermitilis* HYL16 using an HPLC analysis. Clearly, the production of avermectin “a” was completely abolished in *S. avermitilis* HYL16, in sharp contrast to the wild-type strain, whereas the oligomycin “o” was still produced in *S. avermitilis* HYL16 at the same level as that in the wild-type strain NRRL 8165 (Fig. 3C).

In addition to the *S. avermitilis* tested in this study, the two plasmids pJTU1278 and pJTU1289 were also successfully used for gene deletion in several other *Streptomyces* strains, including *S. nanchangensis*, *S. hygrosopicus*, *Streptomyces* sp. FR-008, and *Actinosynnema pretiosum*, confirming their efficiency as gene deletion/disruption vectors.

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