Conjugation of \( \varphi \)BT1-derived integrative plasmid pDZL802 in *Amycolatopsis mediterranei* U32

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**ABSTRACT**

The genus *Amycolatopsis* is well known for its ability to produce antibiotics, and an increasing number of valuable biotechnological applications, such as bioremediation, biodegradation, bioconversion, and potentially biofuel, that use this genus have been developed. *Amycolatopsis mediterranei* is an industrial-scale producer of the important antibiotic rifamycin, which plays a vital role in antimycobacterial therapy. Genetic studies of *Amycolatopsis* species have progressed slowly due to the lack of efficient transformation methods and stable plasmid vectors. In *A. mediterranei* U32, electroporation and replicable plasmid vectors have been developed. Here, we establish a simple and efficient conjugal system by transferring integrative plasmid pDZL802 from ET12567 (pUZ8002) to *A. mediterranei* U32, with an efficiency of \( 4 \times 10^{-5} \) CFU per recipient cell. This integrative vector, based on the \( \varphi \)BT1 int-attP locus, is a stable and versatile tool for *A. mediterranei* U32, and it may also be applicable to various other *Amycolatopsis* species for strain improvement, heterologous protein expression, and synthetic biology experiments.

**KEYWORDS**

Amycolatopsis; conjugal transfer system; integrative plasmid; metabolic engineering; natural products

**Introduction**

The genus *Amycolatopsis*, a member of the phylogenetic group nocardioform actinomycetes, is well known for its ability to produce antibiotics, including rifamycin, vancomycin, and balhimycin. Recently, other *Amycolatopsis* species with different important applications, such as *Amycolatopsis tucumanensis* for bioremediation, *Amycolatopsis* M3–1 and *Amycolatopsis* HT-32 for biodegradation, and *Amycolatopsis* sp. CGMCC 1149 and *Amycolatopsis* sp ATCC 39116 for bioconversion, have been discovered. Additionally, analysis of the *A. mediterranei* genome has shown that it has a potential application in biofuel production.\(^1\) Furthermore, with the development of sequencing techniques, more members of the genus *Amycolatopsis* have been identified.\(^2\)–\(^5\)

To study the biosynthetic and regulatory mechanisms underlying these bioactive applications, stable and reliable genetic tools and methods are needed. Thus far, three indigenous plasmids, pMEA100,\(^6\) pMEA300,\(^7\) and pA387,\(^8\) have been reported in *Amycolatopsis* species. pMEA100 and pMEA300, which exist in integrated or freely replicating forms, are not suitable for molecular genetic studies.\(^9\) pULVK2A\(^10\) and pDXM32,\(^11\) which are derivatives of pA387, are replicable plasmids that are routinely used in gene cloning. Meanwhile, several methods for the introduction of DNA into different *Amycolatopsis* species were successfully developed.\(^9\) These plasmids and transfer systems have greatly facilitated studies of *Amycolatopsis* species.

*Amycolatopsis mediterranei* U32 is an industrial-scale rifamycin SV producer, and its genome sequence has been determined.\(^3\) Ding et al.\(^11\) developed an efficient transformation method, using electroporation, for gene disruption and complementation in *A. mediterranei* U32. Here, a simple and efficient method of transferring the integrative plasmid pDZL802 into...
U32 via intergeneric *Escherichia coli*-mycelia conjugation was established.

**Methods and materials**

**Bacterial strains and plasmids**

All strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α2 (F− φ80 lacZΔM15Δ (lacZY-argF) U169 endA1 recA1hsdR17 (r−, m+K) deoR thi-1 susE44 λ− gyrA96 relA1) was used as the host strain for cloning. *E. coli* strain ET12567 (pUZ8002),13,14 a methylation-defective strain (dam−13::Tn9 dcm-6 hsdM Chl), was used as the donor in intergeneric conjugation, while *A. mediterranei* U32 was used as the recipient. *Sarcina lutea* was used as the indicator microorganism for the antibacterial activity assays. Plasmid pBC-Am contains an apramycin resistance gene flanked by two *Sma*I sites for selection in *E. coli* and *A. mediterranei* U32. Plasmid pRT802 contains the φBT1 integrase and a kanamycin resistance gene.15 The plasmid pBC-Am was digested with *Sma*I to obtain the *aac*(3)IV fragment, which was ligated into the *Msc*I-*Sma*I sites of pRT802 to yield the apramycin-resistant plasmid pDZL802 used for conjugation (Fig. 1).

**Media and culture conditions**

*E. coli* strains and *S. lutea* were cultured in Luria broth (LB) medium at 37°C with shaking at 200 rpm. *A. mediterranei* U32 strains were cultured in Bennet medium16 at 30°C with shaking at 200 rpm for the extraction of genomic DNA and the analysis of growth and rifamycin SV production. YEME medium17 and modified YEME medium (MYM, YEME supplemented with 5 mM MgCl₂, 5 mM CaCl₂, 10 g glycine l⁻¹, 0.6 g KNO₃ l⁻¹, and 220 g sucrose l⁻¹) were used to grow *A. mediterranei* U32 recipient cells11 at 30°C with shaking at 200 rpm. When required, antibiotics were added at the following concentrations (μg/ml⁻¹): kanamycin (Kan), 40; chloramphenicol (Chl), 34; apramycin (Apr), 50. Solid mannitol soya flour (MS)17 medium containing 10 mM MgCl₂ and 10 mM CaCl₂ was used in the conjugation experiments.

**Conjugal transfer experiments**

Conjugal transfer between *E. coli* and *A. mediterranei* U32 was performed as described by Kieser et al.17 with minor modifications. ET12567 (pUZ8002/pDZL802) was grown to an OD₆₀₀ range of 0.4–0.5, washed twice with fresh LB to remove residual antibiotics, and then resuspended in fresh LB. *A. mediterranei* U32 competent cells (5 μl, approximately 2 × 10⁹ cells) were added to 500 μl of 2×YT broth and heat shocked at 45°C for 10 min. Donor cells (approximately 10⁹ cells) were mixed with recipient cells, and the bacteria were pelleted by centrifugation. The pellets were resuspended, and the mixtures were placed onto 1 cm²

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**Table 1. Strains and plasmids used in this study.**

| Strain or plasmid | Relevant characteristics | Ref. or source |
|-------------------|--------------------------|----------------|
| *E. coli* DH5α    | F− recA lacZΔM15         | MBI            |
| ET12567/pUZ8002   | dam− dcm                | Lab stock      |
| *A. mediterranei* U32 | A high yield producer of rifamycin SV | Lab stock |
| U32-DZL802        | U32 with pDZL802 integrated into the chromosome | This study |
| pRT802            | *E. coli-Streptomyces* shuttle plasmid, encoding φBT1 int and attP, resistant to kanamycin | Lab stock |
| pDZL802           | Apramycin-resistant version of pRT802 | This study |
| pBC-Am            | Donor of *aac*(3)-IV; the 1.5-kb HindIII/EcoRI fragment of pULVK2A was inserted between the corresponding sites in vector pBC-SK(−) (Stratagene) to produce pBC-Am; Aprar Cmr | Lab stock |
sterilized filter papers on MS plates, and then incubated for 20–24 h at 30°C. The mixtures were washed with 500 μl of sterilized water and spread on MS plates. These plates were overlaid with 1 ml of water containing 0.5 mg of nalidixic acid and 1.25 mg apramycin and were incubated at 30°C for several days.

**Determination of the pDZL802 integration site in A. mediterranei U32**

Exconjugants were streaked from MS plates onto Bennet plates, with and without apramycin, and incubated at 30°C for 3–4 d. Genomic DNA was extracted and digested with ApaI for 3 h, ligated with T4 ligase overnight after deactivating ApaI, and transformed into E. coli DH5α. Transformants on LB plates containing apramycin were inoculated into 3 ml of LB medium and incubated overnight. Rescued plasmids were extracted and sequenced using the primer DZL802-R (5'-CTCAGGGACGTCGGGAGCGA-3'). Plasmid integration sites and orientation were determined via a BLASTN analysis of the sequenced nucleotides.

**Identification of the ϕBT1 attB site in A. mediterranei**

The ϕBT1 attB site of the exconjugants was analyzed by plasmid rescue and sequencing with primer DZL802-R. Linearized pDZL802 integrated into the attachment site within AMED_0846, between nucleotides 903090 and 903091, in all six exconjugants (Fig. 2A). Two other primers, 8020846-F (5' - CTCCTGCTCCGGGCCTCTCCT-3'), located in the U32 genome adjacent to AMED_0846, and 8020846-R (5'-GGCTGCCCTTCCTGGTTGGC-3'), located in pDZL802, were used to further check the exconjugants by PCR. All PCR reactions were positive, yielding a 963-bp fragment as expected (Fig. 2B). Thus, we concluded that there was a single copy integration of pDZL802 into AMED_0846 in the A. mediterranei U32 genome.

**Growth and antibacterial activity assay of exconjugants**

Mycelia of A. mediterranei U32 and exconjugants were grown on Bennet plates at 30°C for 5 d and inoculated into 50 ml of Bennet medium. After incubation at 30°C for 48 h, 2.5 ml of the seed culture was inoculated into another 50 ml of Bennet medium every 8 h. Biomass was determined by measuring the wet weight of the mycelia at different growth times. Rifamycin SV production was determined according to the method of Pasqualucci et al."18"

**Results**

**Conjugal transfer system for pDZL802 in A. mediterranei U32**

The plasmid pDZL802, an integrative vector based on the ϕBT1 int-attP locus, was transferred successfully from ET12567 (pUZ8002) into A. mediterranei U32. The recipient mycelia were prepared in liquid modified YEME medium according to Ding et al."11" and heat shocked at 40°C, 45°C, or 50°C for 10 min. The ratio of donor cells to recipient mycelia was optimized for ratios of 10^5:4 × 10^3, 10^5:2 × 10^5, and 10^5:2 × 10^7. MS medium containing 10 mM, 50 mM, or 100 mM CaCl2 was tested. Finally, a frequency of approximately 4 × 10^-5 for exconjugants was achieved under the following conditions: heat shock of recipient mycelia at 45°C for 10 min, a 10^9:2 × 10^6 ratio of donor cells to recipient mycelia, 10 mM CaCl2, and 10 mM MgCl2. The proper CaCl2 concentration was especially crucial for the conjugal transfer of pDZL802 from E. coli to A. mediterranei U32.
Identification of the &B1 attB sites in *Amycolatopsis mediterranei* U32. (A) Integrative pDZL802 was rescued along with the flanking genomic sequence at the insertion sites; the sequencing results from six colonies using primer DZL802-R indicated that the insertion sites were located between nucleotides 903090 and 903091. (B) Another 20 colonies were verified by PCR using two specific primers adjacent to the insertion site. Lane 21, pDZL802 used as template; Lane 22, genomic DNA of wild-type U32 used as template; Lane 23, no template. All PCR products were of the same length, which implies that there is only one insertion site for pDZL802 in *A. mediterranei* U32. (C) Homologs of AMED_0846 in the genus *Amycolatopsis* were selected, and the amino acid and DNA sequence alignments both show a high degree of conservation. GenBank accession nos. of amino acid sequences: *A. alba* (WP_026467335), *A. benzoatilytica* (WP_027928077), *A. balhimycina* (WP_020643361), *A. decaplanina* (WP_007032049), *A. halophila* (ETAS66274), *A. japonica* (AIG79870), *A. mediterranei* (WP_013222759), *A. methanolica* (WP_026153784), *A. orientalis* (CP003410), *A. rifamycinica* (KDN18277), *A. sp ATCC 39116* (WP_027936416), and *A. vancoresmycina* (WP_003064197). GenBank accession nos. of DNA sequences: *A. alba* (NZ_KB913032), *A. benzoatilytica* (NZ_KB912942), *A. balhimycina* (NZ_KB913037), *A. decaplanina* (NZ_AOHO01000058), *A. halophila* (AZAK1000001), *A. japonica* (CP008953), *A. mediterranei* (CP002000), *A. methanolica* (NZ_AQU01000001), *A. orientalis* (CP003410), *A. rifamycinica* (JMQI01000064), *A. sp ATCC 39116* (NZ_AFVY03000047), and *A. vancoresmycina* (NZ_AOUU01000073). Two nucleotide CT core regions of the attB site are indicated by arrows.
Discussion

Thus far, several methods of transferring DNA into Amycolatopsis, such as protoplast transformation and electroporation, have been developed.9 Conjugation experiments are easier to conduct than electroporation, as they do not require an expensive electroporation apparatus.

The replicable E. coli-Amycolatopsis shuttle-cloning vector pSETRL1 was constructed using the pA387 replicon, and it was successfully transformed into A. mediterranei DSM 40773 and A. orientalis NBRC 12806 by conjugation and electroporation.20 Here, we established a conjugal transfer system for A. mediterranei U32 based on the integrative plasmid pDZL802. Integration of this single-copy plasmid was stable in the absence of selective pressure, which is a distinct advantage compared with the replicable vectors used in genetic studies and industrial strain improvement. Additionally, mycelial growth and rifamycin production were not affected by the integration of pDZL802.

Baltz predicted that a ‘BT1 attB site of A. mediterranei was located in a highly conserved homologous gene encoding an integral membrane protein.21 We experimentally determined that the attB site is located in the AMED_0846 gene, which encodes an integral membrane protein, in agreement with the prediction of Baltz. A BLASTP survey showed that homologs of AMED_0846 are observed not only in Amycolatopsis species (Fig. 2C) but also in several Streptomyces and Saccharopolyspora species. The lowest amino acid sequence homology of these species is approximately 60%. Apparently, the attB site of ‘BT1 is distributed broadly in Amycolatopsis species, making pDZL802 a versatile integrative vector for genetic studies.

According to the above results, a simple and efficient conjugal system for the transfer of pDZL802 from ET12567 (pUZ8002) to A. mediterranei U32 mycelia was developed for the first time. The integration of pDZL802 was stable, and it did not affect mycelial growth or rifamycin production. This conjugation and vector system may also be a powerful tool for strain improvement, heterologous protein expression, and synthetic biology experiments in various other Amycolatopsis species.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Authors’ contributions

Guoping Zhao, Xiaoming Ding and Chen Li conceived and designed the experiments. Chen Li and Li Zhou collected the phenotypic and genotypic data. Chen Li and Ying Wang

Figure 3. Integration of pDZL802 into AMED_0846 has no influence on mycelial growth or antibiotic production. (A) A stability assessment of integration was conducted via three rounds of growth in Bennet liquid medium without selection. One hundred colonies were streaked onto Bennet plates, with and without apramycin, and incubated at 30°C for 4–5 d. (B) The growth curve and rifamycin production of U32/pDZL802 was in accordance with those of the wild-type strain U32.

affect mycelial growth (Fig. 3B) or rifamycin production (Fig. 3C).
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