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RESEARCH PAPER

Conjugation of φBT1-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 4×10^{-5} CFU per recipient cell. This integrative vector, based on the φBT1 *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.

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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

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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 DH5α[12] (F$^-$ φ80 d*lacZ*ΔM15Δ (*lacZYA-argF*) U169 *endA*1 *recA*1*hsd*R17 (r^-_k, m^+_k) *deoR thi*-1 *susE*44 λ^- *gyrA*96 *rel*A1) 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 *int-attP* locus 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 medium[16] at 30°C with shaking at 200 rpm for the extraction of genomic DNA and the analysis of growth and rifamycin SV production. YEME medium[17] and modified YEME medium (MYM, YEME supplemented with 5 mM $MgCl_2$, 5 mM $CaCl_2$, 10 g glycine l^{-1}, 6 g KNO_3 l^{-1}, and 220 g sucrose l^{-1}) were used to grow *A. mediterranei* U32 recipient cells[11] 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 $MgCl_2$ and 10 mM $CaCl_2$ was used in the conjugation experiments.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Ref. or source
E. coli DH5α	F- *recA lacZ*ΔM15	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; Apra^r Cm^r	Lab stock

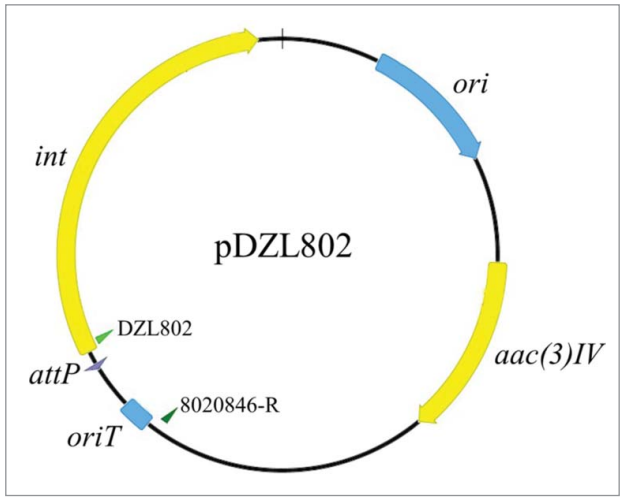

Figure 1. Integrative vector pDZL802 for use in *Amycolatopsis* species. *ori*, pBR322 origin, replicon from pUC18; *aac(3)IV*, apramycin resistance gene; *oriT*, the *oriT* of plasmid RP4; *attP-int*, attachment site and integrase of the *Streptomyces* temperate phage φBT1; DZL802 and 8020846-R, primers used for integration site determination.

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 OD_{600} 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 × 10^6 cells) were added to 500 μl of 2×YT broth and heat shocked at 45°C for 10 min. Donor cells (approximately 10^9 cells) were mixed with recipient cells, and the bacteria were pelleted by centrifugation. The pellets were resuspended, and the mixtures were placed onto 1 cm^2

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 *Apa*I for 3 h, ligated with T4 ligase overnight after deactivating *Apa*I, 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.

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 10^9:4 × 10^5, 10^9:2 × 10^6, and 10^9:2 × 10^7. MS medium containing 10 mM, 50 mM, or 100 mM $CaCl_2$ 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 $CaCl_2$, and 10 mM $MgCl_2$. The proper $CaCl_2$ concentration was especially crucial for the conjugal transfer of pDZL802 from *E. coli* to *A. mediterranei* U32.

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′-CTCCTGCTCCGCGTCCTCCT-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.

Stable integration of pDZL802 does not affect the mycelial growth and antibiotic production of A. mediterranei *U32*

To examine the stability of the integrative plasmid pDZL802 in the absence of apramycin, exconjugants were grown for two successive rounds in 50 ml of liquid Bennet medium for 48 h in the absence of selection. Then, the cultures were diluted and spread onto Bennet plates, and 100 colonies were streaked onto Bennet plates with and without apramycin. As shown in Fig. 3A, all colonies grew normally in the presence and absence of apramycin. This result indicated that pDZL802 was stably integrated in these 100 colonies.

Previous research has shown that some insertions may have a negative effect on the production of antibiotics[19]; thus, the growth and rifamycin production of wild-type *A. mediterranei* U32 and the exconjugants were examined. The integration of pDZL802 in *AMED_0846* was stable and did not

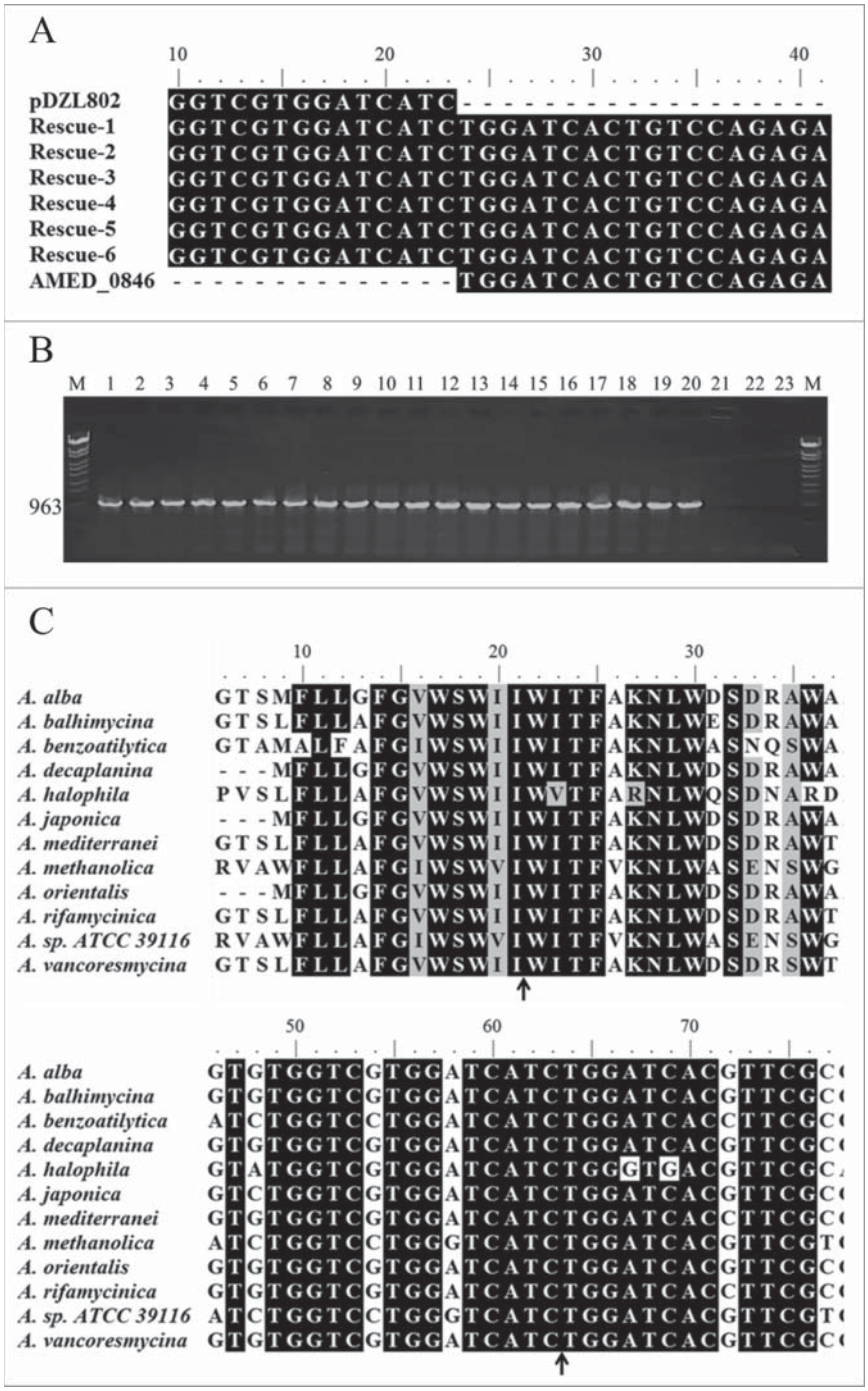

Figure 2. Identification of the φBT1 *attB* sites in *Amycolatopsis mediterranei* U32. (A) Integrative pDZL802 was rescued along with the flanking genomic sequence at the insertion sites; the sequencing results from six colonies using primer DZL802-R indicated that the insertion sites were located between 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* (**ETA66274**), *A. japonica* (**AIG79870**), *A. mediterranei* (**WP_013222759**), *A. methanolica* (**WP_026153784**), *A. orientalis* (**CP003410**), *A. rifamycinica* (**KDN18277**), *A.* sp ATCC 39116 (**WP_027936416**), *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* (**AZAK01000001**), *A. japonica* (**CP008953**), *A. mediterranei* (**CP002000**), *A. methanolica* (**NZ_AQUL01000001**), *A. orientalis* (**CP003410**), *A. rifamycinica* (**JMQI01000064**), *A.* sp *ATCC 39116* (**NZ_AFWY03000047**), and *A. vancoresmycina* (**NZ_AOUO01000073**). Two nucleotide CT core regions of the *attB* site are indicated by arrows.

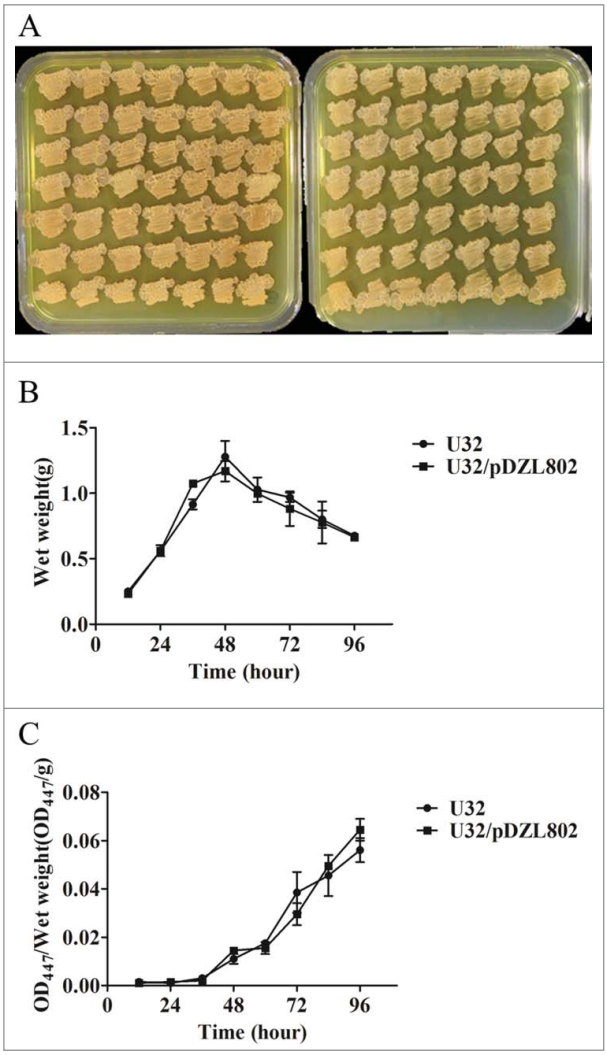

Figure 3. Integration of pDZL802 into *AMED_0846* has no influence on mycelial growth or antibiotic production. (A) A stability assessment of integration was conducted via three rounds of growth in Bennet liquid medium without selection. One hundred colonies were streaked onto Bennet plates, with and 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).

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 φBT1 *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 φBT1 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

performed computational analysis. Guoping Zhao, Xiaoming Ding and Chen Li wrote the paper. All authors read and approved the final manuscript.

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