INTRODUCTION

Kanamycin, one of the most important subclasses of aminoglycoside antibiotics, has been widely used to treat serious infections caused by gram-positive and gram-negative bacteria by interfering in protein synthesis (Robati, Arab, Ramezani, Langroodi, & Abnous, 2016). The semisynthetic derivatives of kanamycin, such as amikacin and dibekacin. Dibekacin and arbekacin are widely used to solve the issue of resistance effectively (Deguchi, Yokota, Koguchi, & Nakane, 1990; Watanabe, Goi, Hara, & Sugano, 1987). Our group has successfully designed and optimized the synthesis processes of dibekacin and arbekacin from kanamycin B (KB) with good yields (Qiao, Zhang, Zhou, & Li, 2017). KB is generally extracted from the broth of Streptomyces kanamyceticus. However, KB is not the main product, the yield of KB is only...
5%-10% (Ni et al., 2011). The inadequate supply of KB seriously confines the output and leads to the high price of these crucial antibiotics. To solve the problem of KB in short supply, it is urgent and significant to construct engineered strain with the high-yield ability of KB.

Many efforts have been made to make S. kanamyceticus produce more KB relatively. Thapa, Oh, Lee, and Liou (2007) reported the heterologous expression of the kanamycin biosynthetic gene cluster (pSKC2) in Streptomyces venezuelae YJ003, but the isolated compound from the transformant was verified to be KA rather than KB. Ni, Li, Yang, and Huang (2011) demonstrated that genes aprD3 from the transformant was verified to be KA rather than KB. YJ003, but the isolated compound (pSKC2) in Streptomyces venezuelae heterologous expression of the kanamycin biosynthetic gene cluster relatively KB. Thapa, Oh, Lee, and Liou (2007) reported the construct engineered strain with the high-yield ability of KB. To solve the problem of KB in short supply, it is urgent and significant to finess the output and leads to the high price of these crucial antibiotics.

5%–10% (Ni et al., 2011). The inadequate supply of KB seriously confines the output and leads to the high price of these crucial antibiotics. Mycelia are used as recipient rather than traditional spores and there are no same reports in S. kanamyceticus. In our experiments, we found that the conjugation frequency of mycelium was higher than that of spores. The highest conjugation frequency of mycelium was 7.9-fold higher than that of spores.

### 2 | MATERIALS AND METHODS

#### 2.1 | Microorganisms, plasmids, media, and culture conditions

The microorganisms and plasmids used in this study are listed in Table 1. The Mannitol-Soy-agar (MS) medium and seed medium were used for conjugal transfer and selection of S. kanamyceticus mutant. Genomic DNA was isolated from wild-type S. kanamyceticus, which was also used as the host. Both S. kanamyceticus and its mutants were cultivated in 250-ml shaken flasks, with seed medium contained 15 g soluble starch, 4.0 g yeast extract, 0.5 g K2HPO4, and 0.5 g MgSO4 in 1.0 L tap water. After incubation at 28°C for 30 hr, the fermentation medium was inoculated with 3 ml (10% [vol/vol]) seed culture and incubated for 7 days. The fermentation culture medium contained 35 g soyal bean, 30 g maltose, 25 g soluble starch, 8 g Sodium nitrate, and 0.1 g ZnSO4 in 1.0 L tap water. Appropriate antibiotics were added to the media when needed at the following concentrations: apramycin, 35–50 μg/ml; nalidixic acid, 50 μg/ml; kanamycin, 25 μg/ml, and chloramphenicol, 25–50 μg/ml.

#### 2.2 | DNA manipulations

Isolation of the genomic and plasmid DNA, DNA ligations, and other DNA manipulations was performed according to the standard

| Strains or plasmids | Description | Source or reference |
|---------------------|-------------|---------------------|
| Streptomyces kanamyceticus | Wild-type, kanamycin producer | This laboratory |
| ΔkanJ mutant | Mutant of wild-type S. kanamyceticus with a deletion of kanJ | This work |
| Escherichia coli DH5α | Cloning host | Hanahan (1983) |
| E. coli ET12567(pUZ8002) | Cloning host (methyltransfer defective), used for conjugal transfer of DNA from E. coli to Streptomyces | Macneil, Gewain, Ruby, and Dezeny (1992) |
| pSA74 | Vector with chloramphenicol resistant gene; CmR | This laboratory |
| pSET152 | Streptomyces integration vector; ApR, B | Flett, Mersinias, and Smith (1997) |
| pSQ202 | pSET152-derivative plasmid with a deletion of pC31 int | This work |
| pSQ202-J | Recombinant plasmid for kanJ disruption | This work |

*aChloramphenicol resistant bApramycin resistant

**TABLE 1** List of plasmids and strains used in this work
protocols. Table 2 summarizes the oligonucleotide primers, which were synthesized at Beijing Genomics Institute (Beijing, China). The polymerase chain reaction (PCR) enzymes were purchased from New England Biolabs (Beverly, MA, USA), while FastDigest enzymes and T4 DNA ligase were from Thermo Fisher Scientific (Waltham, MA, USA). A Gel Extraction Kit (OMEGA Bio-Tek, USA) was used to recover the target DNA fragments from agarose gels. All chemicals used in this work were molecular biology grade and commercially available.

2.3 | Construction of the plasmids

On the basis of pSET152, the plasmid pSQ202 was used to knockout the kanamycin biosynthetic gene (kanJ). A chloramphenicol resistance gene (Cmr) was required because no proper selection marker was available in pSQ202. First, the gene was amplified from pSA74 using the Cmr forward (F) and Cmr reverse (R) primers that had been inserted with SacI and XbaI, respectively. Subsequently, to amplify a 1,020-bp fragment containing the upstream sequence of kanJ (kanJ-U) (GenBank ID: AJ628422.2), we used kanJ-U F and kanJ-U R primers, which had been inserted with HindIII and SacI, respectively. To amplify a 1,073-bp fragment containing the downstream sequence of kanJ (kanJ-D) (GenBank ID: AJ628422.2), we used kanJ-D F and kanJ-D R primers, which had been inserted with XbaI and EcoRI, respectively. All three PCR fragments were digested with their corresponding enzymes and inserted to the HindIII/EcoRI cloning sites of pSQ202 generating plasmid pSQ202-J.

2.4 | Establishment and optimization of a conjugal transfer system

The intergeneric conjugation between E. coli and S. kanamyceticus was carried out as described previously by Mazodier, Petter, and Thompson (1989) with some modifications. The culture of the donor E. coli ET12567 (pSQ202, pUZ8002) containing conjugative plasmid (kanJ-D) (GenBank ID: AJ628422.2), we used kanJ-D F and kanJ-D R primers, which had been inserted with XbaI and EcoRI, respectively. All three PCR fragments were digested with their corresponding enzymes and inserted to the HindIII/EcoRI cloning sites of pSQ202 generating plasmid pSQ202-J.

2.5 | Confirmation of the exconjugants by PCR

The exconjugants genomic DNA was extracted and then was confirmed by two PCR primers. The first pair of primers, Cmr F and Cmr R, was designed to prove that Cmr replaced kanJ; the expected PCR product was 898 bp. The second pair of primers, kanJ F and kanJ R, was used to verify whether the kanJ gene was present in the exconjugants. The amplification product was subjected to sequence analysis, and the result was compared with the sequence in GenBank.

2.6 | Antibiotic isolation and analysis

After the wild-type strain and kanJ mutant were cultured in fermentation medium at 28°C for 7 days, the culture broth was collected. Then, the pH of the culture broth was adjusted to 2 with H2SO4, and the acidified broth was stirred for 30 min and then centrifuged (1,680 g; 15 min). The supernatant was subsequently readjusted to pH 7 using NaOH and then re-centrifuged (1,680 g; 15 min). The supernatant of the culture broth was prepared for bioassays and further separation and purification. Then further purification and product analysis were referred to a novel method that Qiao’s laboratory has developed previously for the direct determination of

| Gene       | Sequence (5’−3’)             | Restriction site |
|------------|------------------------------|------------------|
| kanJ-U F   | CCCAAGCTTCCGTGTACCAGCGGATGATG | HindIII          |
| kanJ-U R   | CGAGCTCTGCTCTTGGAGGTGTCACTTG  | SacI            |
| Cmr F      | CGAGCTCAGTTGATCGGCACGCTAAG    | SacI            |
| Cmr R      | GCTCTAGATTAACGACCCCTGCCCCCTGA | XbaI            |
| kanJ-D F   | TGCTCTAGACGATCGGCAACCCACACCGG | XbaI            |
| kanJ-D R   | CCGAATTCTCGGGGATCGGCAACGAGGG | EcoRI           |
| kanJ F     | TCTCGGCGATCCTTGGACCAGGGCATCGAG|                |
| kanJ R     | GGCCTACGGGGCGGTACCGCTTGGGC    |                |

Note. **Bold**: Restriction sites
KB in the presence of KA in fermentation broth using HPLC-ELSD (Zhang, He, Zhang, & Liu, 2015). The isolated compound was also restored by dissolving the dried precipitates in water, and then, it was analyzed by electrospray ionization–mass spectrometry (ESI-MS).

3 | RESULTS

3.1 | Effect of the concentration of MgCl₂ on E. coli and S. kanamyceticus conjugation

MgCl₂ is commonly added to conjugation medium to improve the frequency of conjugation (Choi, Lee, Wang, & Kinoshita, 2004), and different Streptomyces strains correspond to respective optimal MgCl₂ concentration. In our study, conjugation medium MS was further optimized with a supplement of 5, 10, 15, 30, 40 mM of MgCl₂, respectively. The results revealed that the conjugation frequency enhanced with the increasing of MgCl₂ concentration, as depicted in Figure 1. The most effective conjugation appeared in the medium containing 40 mM MgCl₂. However, the formation and growth of spores were markedly inhibited when MgCl₂ concentration was over 30 mM. The results suggested that 15 mM MgCl₂ appeared to be the optimal concentration for conjugation of S. kanamyceticus.

3.2 | Effect of heat-shock on E. coli and S. kanamyceticus conjugation

The heat-shock of the S. kanamyceticus spores was assessed in a temperature range between 45 to 60°C in order to determine the optimal temperature for conjugation. After heat-shock, Streptomyces spores were precultured for 2–3 hr at 37°C to shorten the germination time. Different temperatures were selected to evaluate the effect of heat treatment on the conjugation frequency. As shown in Table 3, the rising temperature affiliated to the conjugation efficiency, and the maximum appeared at 55°C. However, the conjugation frequency rapidly decreased to $0.7 \times 10^{-7}$ when the temperature was 60°C. Based on these results, 55°C was chosen as the best heat-shock condition for the spores and used for all subsequent experiments.

3.3 | Effect of donor-to-recipient ratio on E. coli and S. kanamyceticus conjugation

The ratio of donor-to-recipient cell number was a crucial parameter in the intergeneric conjugation of Streptomyces (Enriquez, Mendes, Anton, & Guerra, 2006). In order to establish an optimal spores recipient number for a given number of E. coli donor, we set up 4 series of matings. Different concentration of S. kanamyceticus spores and mycelia was mixed with E. coli ET12567 (pUZ8002, pSQ202) cells, respectively. The conjugation frequency was calculated and shown in Table 4. For spores recipient, no exconjugant was observed at the donor-to-recipient ratio of 10:1. The number of exconjugant colonies was found to increase with the number of spores recipient. The conjugation frequency increased to $6.7 \times 10^{-6}$ when the ratio was up to 1:100. Subsequently, mycelia were used as recipient instead of spores. The highest conjugation frequency was obtained at the ratio of 1:1, which was 7.9 times higher than that optimized spores. However, few exconjugants were observed when the number of mycelia cells was $10^9$.

3.4 | Effect of overlaying time of antibiotics on E. coli and S. kanamyceticus conjugation

Donor and recipient cells were mixed and spread on plates with incubation for 2–3 hr at 28°C. Subsequently, each plate was overlaid with 1 ml of antibiotic solution (1 ml of sterile water containing 50 μg of nalidixic acid against E. coli and 35 μg of chloramphenicol against...
TABLE 4 The effect of donor-to-recipient ratio on Escherichia coli and Streptomyces kanamycticus conjugation

| S. kanamycticus | Concentration of MgCl₂ (mM) | Temperature of heat-shock (°C) | Donor/recipient | Conjugation frequency |
|----------------|-----------------------------|------------------------------|-----------------|-----------------------|
| Spores         | 15                          | 55                           | 10⁷:10⁶         | —                     |
| Spores         | 15                          | 55                           | 10⁷:10⁷         | 2.0 × 10⁻⁶            |
| Spores         | 15                          | 55                           | 10⁷:10⁸         | 4.2 × 10⁻⁶            |
| Spores         | 15                          | 55                           | 10⁷:10⁹         | 6.7 × 10⁻⁹            |
| Mycelia        | 0                           | —                            | 10⁷:10⁶         | 3.2 × 10⁻⁵            |
| Mycelia        | 0                           | —                            | 10⁷:10⁷         | 5.3 × 10⁻⁵            |
| Mycelia        | 0                           | —                            | 10⁷:10⁸         | 1.3 × 10⁻⁶            |
| Mycelia        | 0                           | —                            | 10⁷:10⁹         | 2.6 × 10⁻⁹            |

Note. Time of heat-shock is 10 min.

TABLE 5 The effect of concentration and overlaying time of antibiotics on Escherichia coli and Streptomyces kanamycticus conjugation

| S. kanamycticus | Concentration of MgCl₂ (mM) | Donor/recipient | Temperature of heat-shock (°C) | Overlaying time of antibiotics/hr | Conjugation frequency |
|----------------|-----------------------------|-----------------|------------------------------|-----------------------------------|-----------------------|
| Spores         | 15                          | 10⁷:10⁹         | 55                           | 14                                | 2.2 × 10⁻⁹            |
| Spores         | 15                          | 10⁷:10⁹         | 55                           | 16                                | 2.9 × 10⁻⁶            |
| Spores         | 15                          | 10⁷:10⁹         | 55                           | 18                                | 6.1 × 10⁻⁶            |
| Spores         | 15                          | 10⁷:10⁹         | 55                           | 20                                | 6.7 × 10⁻⁶            |
| Spores         | 15                          | 10⁷:10⁹         | 55                           | 22                                | a                     |
| Mycelia        | 0                           | 10⁷:10⁷         | —                            | 8                                 | 1.2 × 10⁻⁹            |
| Mycelia        | 0                           | 10⁷:10⁷         | —                            | 10                                | 5.3 × 10⁻⁵            |
| Mycelia        | 0                           | 10⁷:10⁷         | —                            | 12                                | 6.2 × 10⁻⁵            |
| Mycelia        | 0                           | 10⁷:10⁷         | —                            | 14                                | a                     |

*The antibiotics selection for exconjugants did not work, allowing the growth of both donor and recipient colonies. Time of heat-shock is 10 min.

S. kanamycticus. The antibiotic overlaying time was another factor which may influence the conjugation frequency (Table 5). More exconjugants were obtained with the time prolonged from 16 to 18 hr. To some extent, premature overlaying weakened the growth ability of mycelia on the culture medium. Nevertheless, when the overlaying time of antibiotics was extended to 22 hr, the exconjugants of a single colony could not be observed on the plate and it induced false-positive result of exconjugants. This suggested that the best overlaying time of antibiotics mix-culture plates was 18–20 hr for using S. kanamycticus spore as recipient, but only 10–12 hr for S. kanamycticus mycelia.

3.5 | Construction of recombinant plasmids pSQ202-J

To construct the kanJ disruption plasmid, pSQ202 was used to delete the inner HindIII fragment (0.8 kb) of the ΦC31 int gene. The plasmid was constructed as described in materials and methods. The genetic organization and the restriction endonuclease map are shown in Figure 2.

3.6 | Transformation of pSQ202-J into S. kanamycticus

According to the best conjugal transfer condition mentioned above, E. coli ET12567 (pUZ8002, pSQ202-J) was used as donor to
exconjugants with recipient of S. kanamyceticus mycelia. After the exconjugants appeared, single-crossover exconjugants of S. kanamyceticus were obtained using chloramphenicol as the selective marker. After three rounds of mycelia forming on the seed agar medium without antibiotic selection, chloramphenicol was added into the seed agar medium for double-crossover exconjugants selection. The three double-crossover exconjugants not sensitive to chloramphenicol were isolated to obtain ΔkanJ mutant. Then genomic DNA of exconjugants from ΔkanJ mutant was isolated and analyzed by PCR. As shown in Figure 3, ΔkanJ mutant showed an 898-bp fragment with Cmr F and Cmr R as the primers and does not possess the 825-bp kanJ sequence. The phenotypic characteristics including colony color, growth rates, and mycelium morphology were not altered in comparison with the original strain.

3.7 Effect of kanJ knockout in S. kanamyceticus

Subsequently, the wild-type strain and ΔkanJ mutant were fermented under the same conditions and their products were analyzed. HPLC-ELSD analysis demonstrated that the products of wild-type strain contained both KA and KB, while ΔkanJ mutant did not contain KA. And the main component of ΔkanJ mutant had the same retention time as KB (Figure 4a). The KB yield of wild-type S. kanamyceticus was 46.57 ± 12 mg/L, and the KB yield of ΔkanJ mutant reached to 543.18 ± 42 mg/L. ESI-MS demonstrated that the main component of the fermentation product had a molecular ion peak at 484.5 m/z (Figure 4b). These results confirmed that ΔkanJ mutant mainly produces KB.

4 DISCUSSION

There are different restriction-modification systems in Streptomyces due to the diversity of them (Sadeghi, Soltani, Nekouei, & Jouzani, 2014). Transformation systems for several Streptomyces strains have been developed. Rocha (Rocha, Ruiz-Villafán, & Manzo, 2018) reported a reliable genetic system for Streptomyces peucetius var. caesius, and the optimal conjugation

FIGURE 3 Verification of the recombination strains. PCR analysis with the genomic DNA from Streptomyces kanamyceticus and ΔkanJ mutant. Using primers Cmr F and Cmr R, PCR product from 1. wild-type strain 2. ΔkanJ mutant; using primers kanJ F and kanJ R, PCR product from 3. wild-type strain, 4. ΔkanJ mutant

FIGURE 4 Metabolite analysis of wild-type Streptomyces kanamyceticus and ΔkanJ mutant. (a) HPLC analysis of metabolites. (b) ESI-MS analysis of the product of ΔkanJ mutant
frequency was up to 5 × 10⁻⁴. In Park, Jang, and Hwang (2012) work, a conjugal transfer system for Streptomyces acidiscabies was established. The highest conjugation frequency was 1.4 × 10⁻³. However, no conjugation efficiency of S. Kanamycetificus was reported to our knowledge. A rational genetic operating system of S. kanamyceticus should be established according to its own characteristics. In this work, a conjugal transfer system for the kanamycin producer S. kanamyceticus ATCC12853 was established and optimized successfully.

The results revealed that the donor-to-recipient ratio played a crucial role for affecting conjugation frequency in S. kanamyceticus. Different number of E. coli donor cells was used to explore the optimal one for a given number of spores. However, there were no obvious differences in the consequences and 10⁷ was applied for further experiments. As the recipient cells' number increased, the number of exconjugant colonies increased simultaneously when spores were used in the tested range. For mycelia as recipient, in contrast, the increasing donor cells were not expected to achieve the improvement of conjugation frequency, which was not consistent with the results of spores.

The present work firstly demonstrated that S. kanamyceticus mycelia could be used quite effectively as recipient for intergeneric conjugation instead of spores. In addition, compared to 5–7 days culture time for spores, it was only 2–3 days when mycelia were used as recipient, and no heat-shock operation was required. In particular, the optimal conjugation frequency achieved with S. kanamyceticus mycelia (6.2 × 10⁻⁵) was more satisfactory than that obtained with spores (6.7 × 10⁻⁶). It suggested that mycelia were more appropriate in S. kanamyceticus conjugation experiments.

Generally, several conjugal transfer conditions were screened, and the optimal one was confirmed to transfer the foreign plasmids into S. kanamyceticus. Kanamycin biosynthetic gene (kanJ) was knocked out through derivatives of pSET152, restraining the transformation of metabolites from KB to KA. The ferment result proved that we successfully made KB to be the main product in the fermentation broth. The highest KB yield of ΔkanJ mutant reached 585.33 mg/L, which was 10.7-fold higher than that of the original strain. Thus, the current work provided a strategy to obtain adequate KB as raw materials for synthesis of dibekacin and arbekacin. Importantly, the possibility to create a new engineered strain, by using the similar genetic manipulation, is particularly significant for the future application of other antibiotics production.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTION

Shuman Zhang performed the experiment and edited the final version of the manuscript. Tiansheng Chen performed the experiment. Jia Jia, Liwen Guo, and Huizheng Zhang analyzed data. Chao Li and Renzhong Qiao contributed reagents and analytical tools. Shuman Zhang and Tiansheng Chen contributed equally.

DATA ACCESSIBILITY

The data will be available on request from the corresponding authors.

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