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Conjugal Transferring of Resistance Gene *ptr* for Improvement of Pristinamycin-Producing *Streptomyces pristinaespiralis*

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Abstract Improving pristinamycin production from *Streptomyces pristinaespiralis* was performed by introducing the resistance gene *ptr* followed by selection for enhanced tolerance to pristinamycin and fermentation test. To transfer *ptr* into *S. pristinaespiralis*, an effective method was established for the first time by using the intergeneric conjugation of DNA from *Escherichia coli* to *S. pristinaespiralis*. The procedure was optimized with heat treatment, spore concentration, optimum medium used in conjugation, concentration of $MgCl_2$, etc. With the optimized conditions, the conjugation frequency was up to 1.36×10^{-3} exconjugants per recipient. The procedure was used to transfer the *ptr* gene into *S. pristinaespiralis*, resulting in 146 exconjugants. These exconjugants were screened on the pristinamycin-resistant plates, and then the fermentation test subsequently. Finally, two strains (SPR1 and SPR2) were obtained with a high yield of 0.11 and 0.15 g/l, respectively, which is about six to eight times more than that of wild-strain ATCC25486. The subculture experiments indicated that the hereditary character of the high-producing *S. pristinaespiralis* SPR1 and SPR2 was stable. Our work suggests that introducing resistance gene *ptr* into *S. pristinaespiralis* could be the way to improve the production of pristinamycin through the enhancement of antibiotic tolerance.

Keywords Strain improvement · *ptr* · Intergeneric conjugation · Pristinamycin · *Streptomyces pristinaespiralis*

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Introduction

Pristinamycin, a member of the streptogramin family of antibiotics, consists of two different groups of ring-like structures [1]. It has a strong activity against methicillin-, penicillin-, and vancomycin-resistant bacteria and exhibits a prolonged postantibiotic effect [2, 3]. Pristinamycin was produced by *Streptomyces pristinaespiralis* [4–7]. In the pristinamycin fermentation with *S. pristinaespiralis*, both mycelium growth and pristinamycin biosynthesis were inhibited by the pristinamycin itself [8].

The multidrug resistance gene *ptr* is interspersed and seems to be organized as a single large cluster [9], conferring resistance to a number of compounds including two structurally dissimilar compounds (pristinamycin I, PI; pristinamycin II, PII), the natural pristinamycin mixture and rifampicin [10]. It has been suggested that introducing drug resistance genes could improve the tolerance of bacteria to antibiotic as well as enhance production of the secondary metabolite in *Streptomyces* [11–15]. For example, transferring the wild-type *relC* gene on a low-copy-number vector has restored the ability of the recombinant in actinorhodin production [14]. Introduction of the resistance gene *ptr* to *S. pristinaespiralis* might enhance pristinamycin resistance in the recombinant strain as well, while little work has been done on this. In this paper, we have tried to transfer the resistance gene *ptr* to *S. pristinaespiralis* and study the effect on its pristinamycin production.

However, due to the strong host restriction modification system, there were no existing efficient methods to transform *Streptomyces* [16]. In general, protoplast and electroporation techniques have been used for *Streptomyces* transformation, but these have low efficiency and limited application [17]. Instead of these methods, there was considerable interest in the use of intergeneric conjugation as a means of transferring single-stranded DNA [18, 19]. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* was firstly reported in 1989 [20]. It is simple and does not rely on the development of procedures for protoplasts formation and regeneration; the restriction barriers may be bypassed or severely reduced by the transfer of single-stranded DNA [21, 22]. In addition, a variety of versatile *oriT* vectors are available and considerably modified according to various needs [23]. This technology was widely applied to many other strains of *Streptomyces* [18, 21, 24]. However, the optimal procedures for different *Streptomyces* strains vary. Therefore, in order to begin a molecular genetic study of *S. pristinaespiralis*, we have previously established an efficient procedure for its transformation to transfer the *ptr* gene into the strain.

Materials and Methods

Strains and Plasmids

The wild-strain *S. pristinaespiralis* ATCC25486 was used as the ancestral strain and was cryopreserved in 40% (*v*/*v*) glycerol at −80°C. Its production of pristinamycin in shaking flask was 0.02 g/l. *E. coli* DH5α was used as the cloning host. The methylation-deficient *E. coli* ET12567 (*dam 13::Tn9*, *dcm-6*, *hsdM*), containing pUZ8002 that is a RK2 derivative with a defective *oriT* (*aph*), was used as the donor in intergeneric conjugations [21]. The pMD19-T vector (Promega, USA) was used to ligate and express the resistance gene *ptr* in DH5α; the site-specific integration vector, pSET152 (accession no. AJ414670), containing the 760-bp *oriT* fragment from the plasmid RK2, the φC31 integration function, and the ColEI replication origin of *E. coli*, as well as an apramycin resistance gene, for selection [23]. This plasmid does not contain the replicative functions

of the *Streptomyces* plasmid and thus can be maintained in recipient strains only in the chromosomally integrated state.

Media and Culture Conditions

The slant and plate, seed, and production medium and its cultural conditions for *S. pristinaespiralis* were as described previously [25]. The media for intergeneric conjugation were as those of Kieser et al. [23]. *E. coli* strains were grown in Luria broth (LB) medium [26].

Conjugation Method

A culture of the donor *E. coli* ET12567 (pUZ8002) containing pSET152 was grown in the presence of 50 mg/l apramycin, 25 mg/l chloramphenicol, and 50 mg/l kanamycin (Sigma, USA) to an OD_{600} of 0.5–0.6. To remove the antibiotics, the cells were washed twice with an equal volume of LB medium and finally resuspended in 0.1 volume of LB medium. *S. pristinaespiralis* ATCC25486 spores were resuspended with 0.5 ml 2× YT broth (10 g/l yeast extract, 16 g/l tryptone, and 5 g/l NaCl) after heat treatment; 0.5 ml of *E. coli* donor cells (1.25×10^8 per milliliter) was added to the resuspended spores (10^4–10^9 per milliliter) and the mixtures were spread on MS plates containing 0–50 mM $MgCl_2$. The plates were incubated for 16–20 h at 28°C and then overlaid with 1 ml water containing 0.5 mg nalidixic acid and 0.5 mg apramycin (Sigma, USA). The plates were incubated again for 3–5 days at 28°C and the exconjugants were obtained. All the exconjugants were then transferred onto the slant medium with apramycin to reinsure the integration of pSET152. Each conjugation experiment was repeated three times.

Construction of Recombinant Plasmids

A 1.8-kb *ptr* DNA fragment was amplified by the optimized polymerase chain reaction (PCR) technique on MyCycler thermal cycler (Bio-Rad, USA) with *Taq* DNA enzyme (Takara, China). The primer sequences were 5′-CG*TCTAGA*GCGGGACGAGTTCCGA GAT-3′ and 5′-CG*GAATTC*CGCCCGCCTCCGATGCTAT-3′ with restriction enzyme cutting sites of *Xba*I and *Eco*RI (italicized). It was ligated into pMD19-T vector by T4 ligase (Promega, USA). The new recombinant plasmid (pMD-*ptr*) was then transferred into DH5α strains. Similarly, recombinant pMD-*ptr* plasmid was digested with *Xba*I and *Eco*RI restriction enzymes (Promega, USA) and subcloned into the corresponding MCS of pSET152 to get pSET-*ptr* (Fig. 1).

Other DNA Methods

Standard genetic techniques with *E. coli* and in vitro DNA manipulations were as described [26]. Isolation of plasmids and genomic DNA from *S. pristinaespiralis* was performed as described previously [23].

After transferring the plasmids into *S. pristinaespiralis* ATCC25486, the exconjugants were selected on the plates [25] with apramycin and the plasmid was isolated by the MiniBEST Plasmid Purification Kit (Takara, China). For the integrated function to be placed only on the ϕC31 integration site, the apramycin gene (*apr*) sequence was not changed. A 749-bp fragment was amplified by PCR technique with the primers 5′-AATACGAATGGCGAAAAGC-3′ and 5′-CATCGCATTCTTCGCATC-3′ to examine the *apr* sequence, demonstrating the presence of the pSET152 vector.

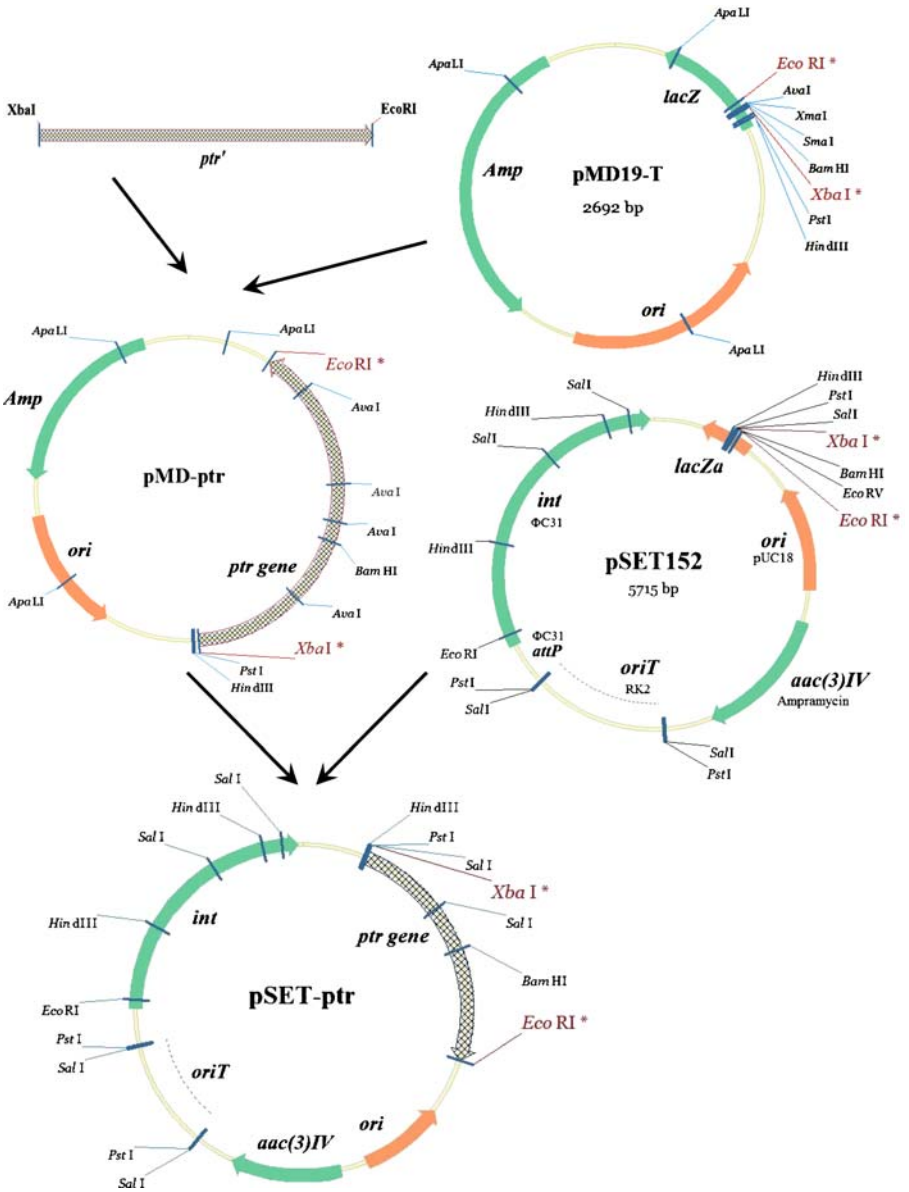

Fig. 1 Schematic representation of the recombinant plasmids. The resulting transformants were selected with apramycin. The restriction sites for the *Eco*RI and *Xba*I enzymes on plasmids are shown with an *asterisk*

Pristinamycin Extraction and Analysis

To determine the concentration of pristinamycin, one volume of fermentation broth sample was directly mixed with two volumes of acetone for 60 min to extract pristinamycin. Subsequently, the mixture was centrifuged at 2,000×*g* for 10 min and then filtered through a 0.45-μm filter to gain the supernatant. Finally, pristinamycin was determined by high-

performance liquid chromatography (HPLC) with a 4.6×250-mm Hypersil ODS C_{18} column. For HPLC determination, the acetonitrile/water mixture (45:55, *v*/*v*) was used as the mobile phase at 1.2 ml·min^{-1} and the eluate was monitored at 206 nm. Commercial Pyostacine from Rhone-Poulenc Rorer (Montrouge, France) was used as a reference standard.

Results

Parameters Affecting the Efficiency of Conjugal Transfer

To determine the appropriate temperature for spore germination, the heat tolerance of *S. pristinaespiralis* ATCC25486 spores was assessed. As shown in Fig. 2, a significant loss in spore viability was observed at 55°C. While spores were treated at 45°C for 15 min or 50°C for 10 min, the viability reached almost the same high value (around 150%), suggesting that either longer incubation or higher temperature is harmful to spore germination.

Spore sensitivity to heat was reflected in pSET152 transfer efficiency. The highest number of exconjugants (7.5×10^{-5} per recipient) was obtained when using a 50°C heat shock for 10 min, resulting in fivefold to tenfold higher levels than those obtained at 45°C for 15 min. Few exconjugants were obtained when spores were not subjected to the heat treatment.

Because the selection of an appropriate medium can greatly influence the successful conjugal transfer of actinomycetes [18, 21, 27], five representative media (R_2YE, YEME, ISP4, slant medium [SM], and MS) were tested for their effect on conjugation efficiency. Among them, YEME and R_2YE are usually applied in the growth and regeneration of protoplast for *Streptomyces* species; MS and ISP4 are the conjugation media commonly used for *Actinomyces*; and SM is the suitable medium for the growth and spore formation of *S. pristinaespiralis*. As shown in Table 1, ISP4 and MS were suitable for the conjugation of

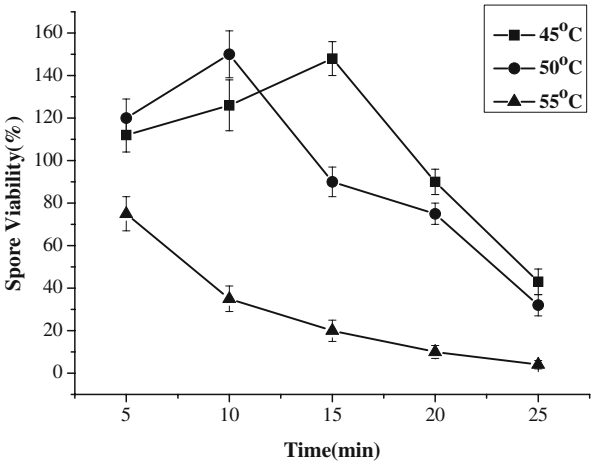

Fig. 2 The effect of temperature on the viability of *S. pristinaespiralis* ATCC25486 spores. Spores (10^3 per milliliter) in 2× YT medium were incubated for 5–25 min at the temperatures indicated. Viability was determined by counting colonies on MS medium after incubation at 28°C for 7 days. The data are the average of three independent experiments and are expressed as a percentage of the colony count obtained with no heat treatment (room temperature, 25°C) as the control

Table 1 Effects of media on transconjugation efficiency.

Medium[a]	Transconjugation frequency[b]
MS	7.2×10^{-5}
ISP4	8.7×10^{-6}
R_2YE	0
YEME	0
SM	0

E. coli ET 12567 (pUZ8002) (1.25×10^{7} per milliliter) and spores (10^{6} per milliliter) were used. Exconjugants were counted on each medium after incubation at 28°C for 7 days

[a] Each medium (see the "Materials and Methods" section) contained 10 mM $MgCl_2$

[b] Values represent the average frequencies from three independent experiment

S. pristinaespiralis ATCC25486; specifically, of the two media, since the MS medium gave 8.3-fold higher conjugation efficiency and better spore formation than ISP4 medium, the MS medium was then selected for the subsequent experiments.

An appropriate ratio of the number of donor cells and recipient spores becomes important in the case of *Streptomyces* species. Because the usual number of recipient spores for conjugation of *Streptomyces* is 10^{7}–10^{9} per milliliter, 10^{4}–10^{9} number of spores per milliliter was tested (the number of *E. coli* donor cells was 1.25×10^{7} per milliliter). As shown in Table 2, when the number of recipient spores was 10^{5}, the transconjugation frequency reached the highest of 4.5×10^{-4}.

Generally, 10 mM $MgCl_2$ has been added to the transconjugation media to increase the efficiency of conjugation [23], although there is no clear data available regarding the function and optimal concentration of $MgCl_2$. Thus, the optimal concentration of magnesium in MS medium was determined (Table 3). In the presence of higher concentrations of $MgCl_2$, transconjugation efficiency was further increased. However, at concentrations of 40 mM or higher, both growth and spore formation were severely inhibited. Spore formation occurred at 3–5 days in the presence of 10–30 mM $MgCl_2$, but at least 10 days was required in the presence of 40 mM $MgCl_2$, making 30 mM $MgCl_2$ the practically optimal concentration for transconjugation of ATCC25486.

Finally, the transconjugation frequency was up to 1.36×10^{-3} per recipient for *S. pristinaespiralis* under the optimal condition. The PCR experiments confirmed that the

Table 2 Effects of the number of recipient spores on transconjugation efficiency.

Number of recipient spores (per milliliter)	Transconjugation frequency[a]
10^{9}	+
10^{8}	2.15×10^{-6}
10^{7}	1.12×10^{-5}
10^{6}	8.9×10^{-5}
10^{5}	4.5×10^{-4}
10^{4}	0.6×10^{-4}

E. coli ET12567 (pUZ8002) (1.25×10^{7} per milliliter) and pores (10^{4}–10^{9} per milliliter) were used. Exconjugants were counted on each medium after incubation at 28°C for 7 days

+ too much colonies growing on plates to count

[a] Values represent the average frequencies from three independent experiments

Table 3 Effects of $MgCl_2$ concentration in MS medium on transconjugation efficiency.

$MgCl_2$ (mM)[a]	Transconjugation frequency[b]
0	2.5×10^{-4}
5	3.6×10^{-4}
10	5.1×10^{-4}
15	7.8×10^{-4}
20	9.2×10^{-4}
25	1.19×10^{-3}
30	1.36×10^{-3}
40	1.68×10^{-3}
50	2.15×10^{-3}

[a] The 4.1-mM $MgSO_4$ in MS was replaced by an equivalent amount of $(NH_4)_2SO_4$

[b] Data are the average of three independent experiments

plasmid vector pSET152 was integrated in *S. pristinaespiralis* (not shown) and was stable after ten successive subcultivations, indicating that this intergeneric conjugation strategy can be widely applied for industry application in the future.

Cloning of *ptr* Gene

Because of the relatively high guanine–cytosine content in *Streptomyces* genomes (≥70%), the routine method of PCR should be altered to obtain the *ptr* gene. Here, three solutions were added to optimize the PCR reaction: dimethyl sulfoxide (DMSO), deionized formamide, and glycerol. As shown in Fig. 3, six PCR systems with different kinds or quantities of additives were studied. The *ptr* gene could not been obtained with any single additive here and could sometimes be amplified with treatment 4 or 5, although with weak specificity, low quantity, and poor repeatability. On the contrary, treatment 6 would produce an ideal targeting DNA with high specificity and quantity. Therefore, the optimized PCR condition with designated additives would effectively amplify the gene, facilitating the *Streptomyces* gene cloning, which may widely apply to the rapid amplification strategy for other target genes with high guanine–cytosine content.

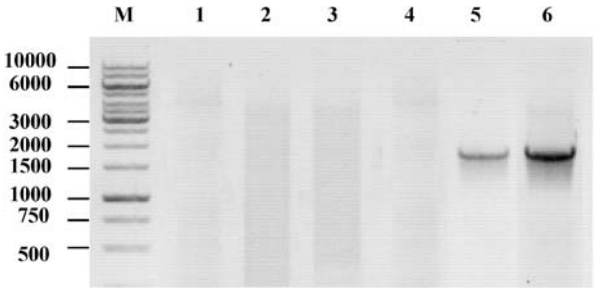

Fig. 3 Optimization of the PCR system. The solutions added in the PCR system: *lane 1* DMSO (5%), *lane 2* DMSO (2%), *lane 3* glycerol (10%), *lane 4* DMSO (0.5%), deionized formamide (2%), and glycerol (3%), *lane 5* DMSO(1%), deionized formamide (1%), and glycerol (3%), *lane 6* DMSO (2%), deionized formamide (1%), and glycerol (1%)

The recombinant plasmid carrying the *ptr* gene was transferred into *S. pristinaespiralis* by the conjugal transfer system established above, resulting in 146 exconjugants of *S. pristinaespiralis* by apramycin resistance screening.

Screening for the Ideal Recombinants

There were two steps to select the strains with higher pristinamycin production. The wild strain was not able to grow on the agar plates containing more than 10 μg/ml pristinamycin, and the introduction of the resistance gene *ptr* would reinforce the tolerance of the recombinant to pristinamycin. These exconjugants were primarily screened on the plate medium with 15 μg/ml pristinamycin. After 5–7 days, 96 pristinamycin-resistant exconjugants were obtained, which might be the recombinants producing more pristinamycin. Then, these pristinamycin-resistant exconjugants were screened for high-yield strains by fermentation test and measuring the pristinamycin yield. Firstly, the potential strains were all screened by one single shake-flask fermentation test, and then the secondary screening was accomplished in the triplicate shake-flask fermentation test (Table 4).

As a result of one single shake-flask fermentation test, compared to the 0.02 g/l production of wild-strain ATCC25486, most exconjugants had an improved yield at various levels, 53.1% exconjugants had a slight increase of production between 0.02 and 0.04 g/l and 38.5% exconjugants could produce two to five times pristinamycin vs. wild-strain ATCC25486. Particularly, two high-yield strains, SPR1 and SPR2, with sixfold to eightfold increase were obtained, and their yields were 0.11 and 0.15 g/l, respectively. This is a dramatic improvement of antibiotic yield by introduction of self-resistance gene.

The hereditary stability of recombinants SPR1 and SPR2 was tested by a colonial subculture method. As shown in Fig. 4, the yields were not changed too much among ten generations, which meant that the genetic stability of recombinants SPR1 and SPR2 was very good (Fig. 5).

Discussion

In order to transfer the target gene into *Streptomyces*, an efficient transfer system should be established first. For *S. pristinaespiralis* ATCC25486, some attempts have been taken for protoplast formation and regeneration before. Although it could be successful to generate protoplast of strain ATCC25486, transformation using the PEG-induced method always failed, which suggested that strain ATCC25486 had the restriction barrier. Instead, as a means of transferring single-stranded DNA, the intergeneric conjugation method is proved to evade the restriction barrier effectively. The procedure is simple, and the required vectors can be made relatively easily in *E. coli* [23]. The high-copy-number vectors such as pIJ702 plasmids were not applied in this study because it was shown to be structurally unstable and

Table 4 The pristinamycin yields of 96 highly resistant exconjugants.

Highly resistant exconjugants	The improved yield times vs. wild-strain ATCC25486					
	0–1	1–2	2–3	3–4	4–5	>5
Number	6	51	21	13	3	2
Frequency (%)	6.3	53.1	21.9	13.5	3.1	2.2

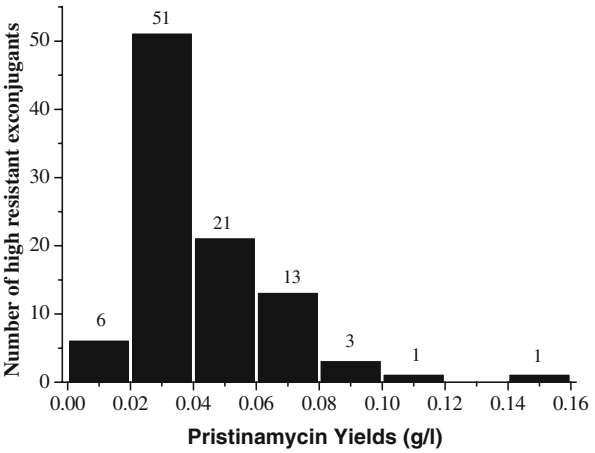

Fig. 4 The pristinamycin yields distribution of 96 high-resistance exconjugants

recombinant pIJ702 plasmids frequently showed deletions of cloned inserts [28]. Moreover, the expression of multiple copies of cloned genes leads to unexpected variations in secondary metabolite production [16]. Therefore, one of the best ways to stably clone a single copy of a gene is to integrate the gene of interest in the chromosome itself. The integrated vector pSET152 was chosen herein, and it was verified that the pSET152 vector could be transferred to *S. pristinaespiralis* successfully and was stable after ten successive generations. Furthermore, exconjugants were obtained at a frequency of 1.36×10^{-3} per recipient for *S. pristinaespiralis* with almost 18-fold improvement of the initial value, which provided a powerful tool for genetic modification of *S. pristinaespiralis*.

Heat treatment is used because spore germination is thought to be necessary for an efficient conjugation [20] and because heat may temporarily reduce the activity of restriction enzymes that could attack the incoming DNA [29, 30]. A few reports have claimed that heat treatment was harmful both to spore germination and conjugation frequency [31, 32], but it is still proved effective in this study. While discussing the magnesium concentration in the MS plate, it was observed that the transconjugation

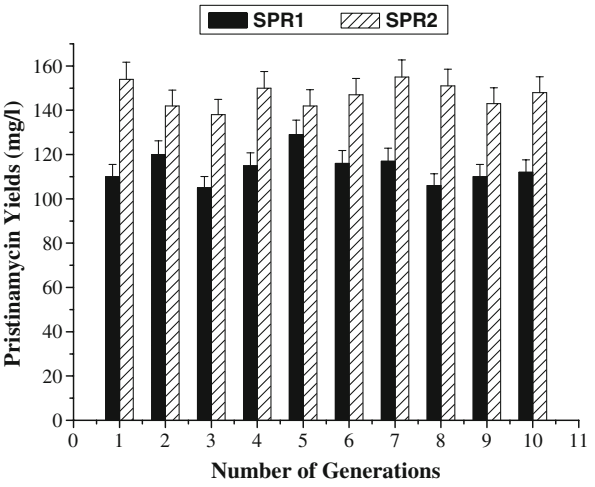

Fig. 5 Production of pristinamycin fermentation by SPR1 and SPR2 strains in ten generations

frequency was increased while the concentration of magnesium was getting higher (Table 3). This fact demonstrates that Mg^{2+} is essential in the conjugative plate, which is also in accord with some views of a previous study [27]. Although the mechanism of magnesium is not very clear at present, it has been reported that the presence of Mg^{2+} is required for some DNA relaxases (the main players in the initiation of conjugative plasmid transfer) [33]. It might function in a similar way with $oriT_{RK2}$ of pSET152 in the conjugation procedure, which introduces a nick at the *oriT* towards the site-specific and strand-specific cleavage.

As is known, in addition to genes involved in antibiotic biosynthesis, *Streptomyces* often carry resistance genes to protect themselves against the antibiotics they produce [34]. Enhancing the resistance ability to antibiotics was applied widely, whereas most of them have been referred to mutation and random selection method, which is a time-consuming and high-cost process. Recently, some strategies have already been applied to eliminate pristinamycin toxicity by the addition of adsorbent resin on fermentation level [8] or by genome shuffling on cell level [25]. However, in addition to biosynthetic gene cluster [35], few studies have focused on the genetic manipulation on *S. pristinaespiralis*. As it was suggested that the level of antibiotic production depended on the copy number of the biosynthetic gene cluster covering the resistance gene, such as *Streptomyces kanamyceticus* [36], we supposed that it would facilitate the efficient production of the recombined strain to the introduction of an additional copy of the *ptr* gene independent of the biosynthetic and resistance gene cluster [9] in the genome of *S. pristinaespiralis*. The *ptr* gene was amplified and transferred into *S. pristinaespiralis* ATCC25486 in this research. After pristinamycin-resistant screening and fermentation test, high-yield strains SPR1 and SPR2 with sixfold to eightfold increase of pristinamycin yield were obtained. In spite of the relatively low pristinamycin production of two high-yield strains, the increasing degree of pristinamycin production was striking, which put forward a very effective technique for further study to improve the strain. The low yield could be attributed to the parent strain with absolutely low production. In further study, it is worthwhile to introduce a strong promoter such as P_{ermE} [37] or take the high-yield strains in a previous study [25] as a start for the next evolution.

In conclusion, a conjugation method between *E. coli* and *S. pristinaespiralis* for efficient gene cloning and manipulation was constructed. By amplifying the resistance gene *ptr* apart from the biosynthesis gene cluster, a dramatic increase in pristinamycin production was noticed in *S. pristinaespiralis*, indicating the usefulness of the genetic approach to improve pristinamycin production by increasing resistance gene copies.

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