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An Efficient Method To Generate Gene Deletion Mutants of the Rapamycin-Producing Bacterium *Streptomyces iranensis* HM 35

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ABSTRACT

***Streptomyces iranensis* HM 35 is an alternative rapamycin producer to *Streptomyces rapamycinicus*. Targeted genetic modification of rapamycin-producing actinomycetes is a powerful tool for the directed production of rapamycin derivatives, and it has also revealed some key features of the molecular biology of rapamycin formation in *S. rapamycinicus*. The approach depends upon efficient conjugational plasmid transfer from *Escherichia coli* to *Streptomyces*, and the failure of this step has frustrated its application to *Streptomyces iranensis* HM 35. Here, by systematically optimizing the process of conjugational plasmid transfer, including screening of various media, and by defining optimal temperatures and concentrations of antibiotics and Ca^{2+} ions in the conjugation media, we have achieved exconjugant formation for each of a series of gene deletions in *S. iranensis* HM 35. Among them were *rapK*, which generates the starter unit for rapamycin biosynthesis, and *hutF*, encoding a histidine catabolizing enzyme. The protocol that we have developed may allow efficient generation of targeted gene knockout mutants of *Streptomyces* species that are genetically difficult to manipulate.**

IMPORTANCE

The developed protocol of conjugational plasmid transfer from *Escherichia coli* to *Streptomyces iranensis* may allow efficient generation of targeted gene knockout mutants of other genetically difficult to manipulate, but valuable, *Streptomyces* species.

Since the discovery of streptomycin in 1943 (1), streptomycetes have been shown to produce thousands of compounds with possibly beneficial features, e.g., antibiotics, immunosuppressants, or anticancer drugs. Actinomycete-derived metabolites comprise over two-thirds of all known antibiotic compounds (2), and recent genome sequencing programs revealed that their biosynthesis potential has been underestimated. In their 8- to 12-Mb genomes, approximately 20 to 30 gene clusters encode the biosynthesis of secondary metabolites (3–5). One of the most important *Streptomyces*-derived compounds is the mechanistic target of rapamycin (mTOR) inhibitor rapamycin (sirolimus), a "billion dollar molecule" and, after cyclosporine, the most widely used immunosuppressant of microbial origin (6). Rapamycin was previously reported as an antifungal antibiotic produced by *Streptomyces hygroscopicus* ATCC 29253 (7), later renamed *Streptomyces rapamycinicus* (8). The rapamycin gene cluster in this strain has been sequenced (9), the biosynthetic pathway has been extensively characterized (10–14), and engineering of the cluster has yielded an impressive range of bioactive modified rapamycins (rapalogs) (15, 16), some in multigram amounts. So far, two other rapamycin-producing species are known: the taxonomically closely related *Streptomyces iranensis* HM 35 (5, 17) and *Actinoplanes* sp. strain N902-109 (18). To elucidate the molecular biology of rapamycin formation in these strains and to further exploit the physiological and pharmacological capability of rapamycin derivatives, genetic manipulation of these alternative producing strains is essential. Until now, lack of a workable conjugation protocol has denied access to additional rapamycin derivatives, as well as to other secondary metabolites of potential interest.

The first method enabling gene cloning in *Streptomyces* was polyethylene glycol-mediated plasmid transformation of protoplasts (19). The procedure required extensive optimization of protoplast formation, regeneration, and transfer, and, thus, numerous strains were only poorly or not at all transformable via protoplasts. The use of electroporation for plasmid DNA transfer into *Streptomyces* (20, 21) enlarged the number of genetically amenable species, but, again, each strain required distinct optimized conditions. An alternative to protoplast transformation is plasmid transfer via conjugation from *Escherichia coli* to *Streptomyces* (22). This approach does not require the recipient to have been extensively characterized genetically. It was further developed to a system that allows not only autonomous replication of the introduced plasmid in the recipient but also its integration via homologous recombination between the cloned DNA and the *Streptomyces* chromosome (23). This system is currently the basis for most *Streptomyces* genetic manipulation, and numerous protocols with further optimized steps exist (see, e.g., reference 24–26). Very recently, the Ca^{2+} ion concentration in the conjugation

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)[a]	Reference or source
Escherichia coli strains		
DH5α	General cloning host	68
BW25113	Strain for propagation of a recombination plasmid	69
ET12567/pUZ8002	Strain for intergeneric conjugation	70
Streptomyces strains		
S. iranensis HM 35	Wild-type strain	17
S. iranensis Δ*hutF*	*S. iranensis hutF*::*aac(3)IV* mutant	This study
S. iranensis Δ*rapK*	*S. iranensis rapK*::*aac(3)IV* mutant	This study
Plasmids		
pIJ790	Helper plasmid, RED/ET recombination plasmid	47
pRSFDuet-1	*E. coli* vector for cloning, Kan^r	Novagen
pOJ260	Suicide vector nonreplicating in *Streptomyces*, Apr^r	23
pRSFDuet_pSG5	Cloning vector, Kan^r, pSG5 replicon	This study
pRSFDuet_pSG5_kan	*E. coli-Streptomyces* shuttle vector, cloning vector, Kan^r, pSG5 replicon, *oriT*	This study
pKOSi	*E. coli-Streptomyces* shuttle vector, cloning vector, Kan^r, pSG5 replicon	This study
pKOSi_hutF	Vector for disruption of *hutF*, based on pKOSi, Kan^r	This study
pKOSi_rapK	Vector for disruption of *rapK*, based on pKOSi, Kan^r	This study
pKOSi_Δ*hutF*	pKOSi_*hutF*::*aac(3)IV* Kan^r Apr^r	This study
pKOSi_Δ*rapK*	pKOSi_*rapK*::*aac(3)IV* Kan^r Apr^r	This study
pTNM	Source of pSG5 for pKOSi	42
pIJ773	Template for amplification of *aac(3)IV* (Apr^r) cassette + *oriT*	47

[a] Kan^r, kanamycin resistance; Apr^r, apramycin resistance.

medium was described as one of the crucial factors that increases the conjugation frequency in *Streptomyces* (27).

Novel rapamycin structures were obtained from *Actinoplanes* sp. N902-109 by addition of enzyme inhibitors, precursor feeding and biotransformation approaches (18). Directed gene disruption in *S. rapamycinicus* was successfully performed by Lomovskaya et al. (28). Using the C$^+$ *attP*-deleted ϕC31 derivative KC515, the rapamycin polyketide synthase-encoding genes were deleted, leading to complete loss of rapamycin formation. KC515-mediated deletions in one or more genes of *rapQONML* generated true rapamycin analogues (11, 29). Later, we applied various strategies to create new rapamycin derivatives ranging from classical strain improvement methods like random mutagenesis via UV irradiation (30), chemical mutagenesis (31), protoplast-related techniques (32), or precursor substitution (33) to overexpression of the putative transcription regulator genes *rapY*, *rapR*, and *rapS* (34). A comprehensive study was published by Kendrew et al. (16), who succeeded in *rapK* deletion in the *S. rapamycinicus* derivative BIOT-3410 by adapting both antibiotic concentrations and media to the conjugation protocol described by Bierman et al. (23).

However, until now, a standardized routine protocol for targeted genetic modification of either of the alternative rapamycin producers *Streptomyces iranensis* and *Actinoplanes* sp. N902-109 has not been available. To gain better access to the genetic manipulation of these interesting species, we systematically optimized available conjugation protocols with special focus on *S. iranensis* HM 35. Here, we established an effective method of targeted gene deletion in *S. iranensis* HM 35 through intergeneric conjugation. The modified protocol may prove useful for other strains of *Streptomyces* and allied genera that have so far proved intractable.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α and *E. coli* BW25113/pIJ790 were used for cloning. The nonmethylating *E. coli* ET12567 carrying the RK2 derivative pUZ8002 (35) was used as the donor in intergeneric conjugation. Cultivation of all *E. coli* strains was performed as described in Gust et al. (36) and Sambrook et al. (37). *S. iranensis* HM 35 was cultured on oatmeal agar plates (38) for sporulation. Potential exconjugants were isolated from MMAM agar plates (16). DNA manipulation and cloning were carried out according to standard protocols (37). *S. rapamycinicus* ATCC 29253, *S. iranensis* HM 35, and Δ*rapK* mutant strains for the rapamycin production were precultivated in seed medium (soluble starch [10 g/liter], soy peptone [6 g/liter], yeast extract [6 g/liter], Casamino Acids [1.5 g/liter], magnesium sulfate [0.5 g/liter], dipotassium phosphate [1 g/liter]; pH 7.0). The main cultivation for rapamycin production was done in YMM medium (yeast extract [5 g/liter], malt extract [5 g/liter], maltose [5 g/liter]; pH 6.0). Concentrations of antibiotics were 50 μg/ml kanamycin, 50 μg/ml apramycin, 25 μg/ml chloramphenicol, and 25 μg/ml nalidixic acid.

Generation of plasmid pKOSi. In the first step, a plasmid that carried the temperature-sensitive replicon pSG5 (39, 40) and the origin of transfer *oriT* (41) was generated. For this purpose, the 2.86-kb SnaBI/AvrII DNA fragment containing the pSG5 replicon was excised from pTNM (42) and ligated with the 3.5-kb XmnI/AvrII fragment excised from pRSFDuet-1 to generate pRSF_pSG5. Next, the 1.2-kb EcoRV/SpeI DNA fragment containing *oriT* was excised from pOJ260 and cloned into plasmid pRSF_pSG5 using the XbaI and EcoRV restriction sites, leading to pRSF_pSG5_kan.

In a second step, a pRSF_pSG5_kan derivative plasmid that allowed mutant generation via the λ Red-mediated recombination system was constructed (36). To this end, the origin of transfer *oriT* was removed from pRSF_pSG5_kan using the restriction sites BspHI and BglII. The resulting 4.3-kb DNA fragment was ligated with a 0.9-kb PCR fragment containing the pRSF1030 replicon (43) and was amplified from pRSF_pSG5_kan with the primers oTN115 (GTTATTGTCTCATGAGCGGATACATATTTG), containing a BspHI restriction site (underlined), and oTN116 (TCCGTCAGATCTATAATGACCCCGAAGCAGGG), containing a BglII restriction site (underlined). Ligation of both fragments led to pKOSi (Fig. 1).

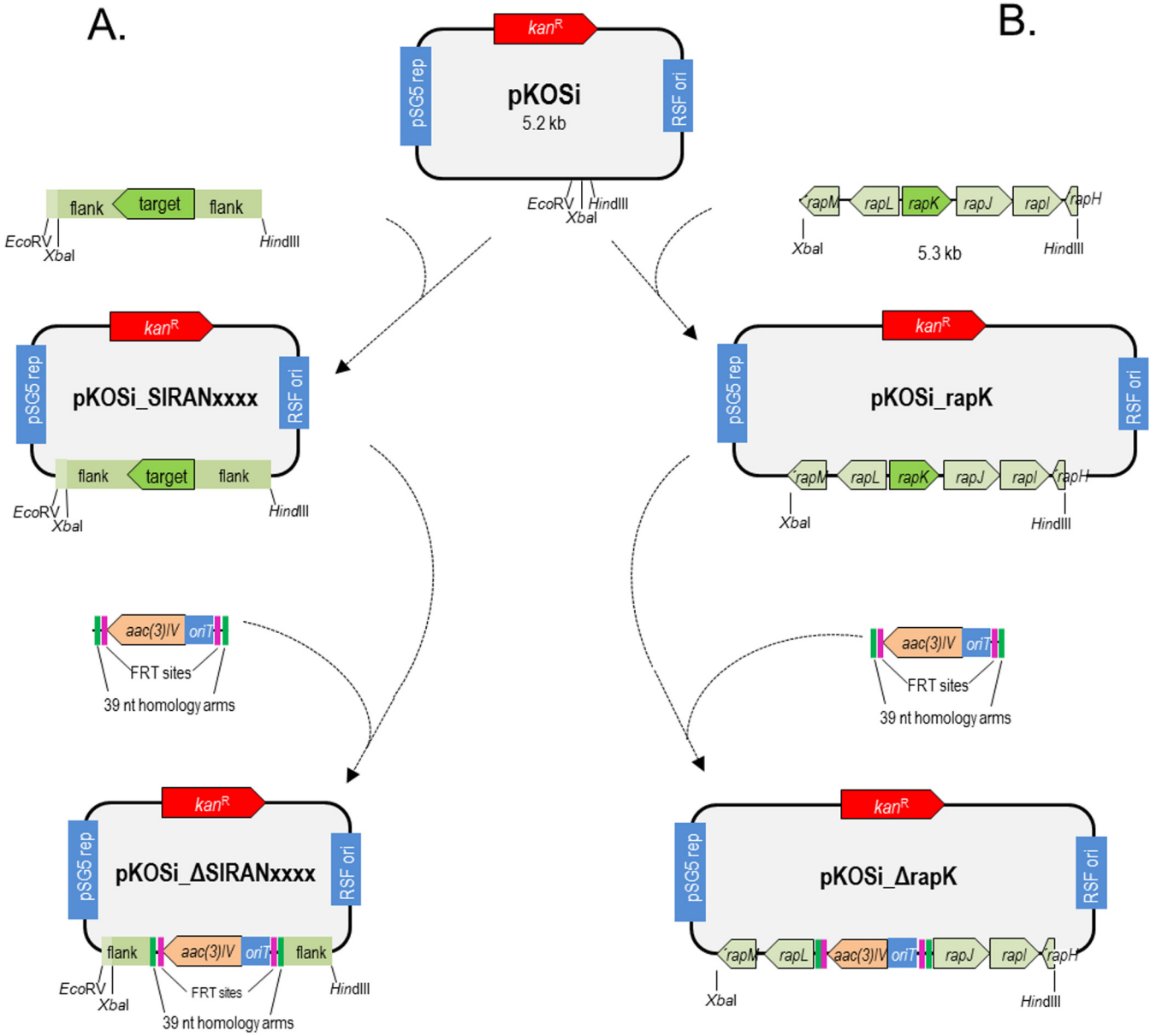


FIG 1 Generation of deletion plasmids. (A) General procedure. (B) Construction of the deletion vector pKOSi_Δ*rapK* for *rapK* as an example. Other deletion plasmids were constructed accordingly. Green, 39-nt homology extensions; pink, FRT sites.

Generation of plasmids for homologous recombination with the *Streptomyces* chromosome (deletion plasmids). To inactivate genes in *S. iranensis*, the corresponding gene sequences together with their 2-kb flanking regions were amplified from *S. iranensis* genomic DNA using the Phusion Flash high-fidelity PCR master mix (Thermo Fisher Scientific, Darmstadt, Germany). PCR products were individually cloned into pKOSi (Fig. 1) between the XbaI/EcoRV or XbaI/HindIII restriction sites to create pKOSi_SIRANxxxx vectors. Next, the apramycin resistance-conferring gene *aac(3)IV* was amplified from pIJ773 with 39-nucleotide (nt)-homology arms followed by λ Red-mediated PCR targeting, as described by Gust et al. (36), to yield the final deletion plasmids, which begin with "pKOSi_ΔSIRANxxxx," where xxxx indicates the annotated gene numbers according to Horn et al. (5) (Fig. 1A). Primers used in these cloning steps are detailed in Table 2.

Intergeneric conjugation. Deletion plasmids (pKOSi_ΔSIRANxxxx) (Fig. 1A) were electroporated into the methylation-deficient *E. coli* strain ET12567/pUZ8002. For this purpose, 800 μl of an overnight culture (2× TY medium supplemented with apramycin, kanamycin, and chloramphenicol) was used to inoculate 10 ml 2× tryptone-yeast extract (TY) medium. It was incubated at 30°C with 250 rpm to an A_{595} of ~0.5. Antibiotics were removed by washing the cells twice with 1 ml of antibiotic-free TY medium at 4,000 rpm. Washed cells were resuspended in 250 μl of TY medium.

S. iranensis spores were harvested from 14-day-old oatmeal-agar plates with 20% (vol/vol) glycerol, washed once with TY medium, and resuspended in 1 ml of TY medium before the spores were counted. Per conjugation, 1×10^8 spores/ml were utilized and heat shocked for 10 min at 50°C to induce germination. Then, 250 μl of *E. coli* ET12567/pUZ8002 cells was added, carefully mixed, and immediately plated onto either mannitol soya flour (MS) (containing 10 mM $MgCl_2$) (24) or R6 agar (44) plates. To optimize the conjugation protocol for *S. iranensis* HM 35, different modifications were tested and combined to achieve higher effi-

TABLE 2 Primers used for generation of deletion plasmids

Inactivated gene	Primer (sequence) used for amplification of the gene + flanking sequences	Primer (sequence) used for *aac(3)IV* amplification[a]
hutF	oTN203 (CAAATTAAGCTTTTCGCCTTCCCCGGTTTCGT; HindIII restriction site is underlined)	oTN207 (TGTGGCCGCACTCGCGGAGGTACTGGAGGGCCTGGCGTG**ATTCCGGGGATCCGTCGACC**)
	oTN204 (TATTATTCTAGACCACCG TCAGCGAGTTGGCC; XbaI restriction site is underlined)	oTN208 (TGCTCTCCTTCGTCGGCCGTGCCGGTAGCGGCCGGGTCA**TGTAGGCTGGAGCTGCTTC**)
rapK	oTN247 (GACTGTAAGCTTCACCTCACGAACGTCTTC; HindIII restriction site is underlined)	oTN253 (TTCTTCCTGGTACGCGCCAGGCAACGGCGGGGAAGGGTG**ATTCCGGGGATCCGTCGACC**)
	oTN252 (GAGTCCTCTAGAGATAGGTTTCCATCGGCAG; XbaI restriction site is underlined)	oTN254 (GCTCATCGGTGGTCCTTCCGGCGGGGTCAACTCGGCGGGGTCA**TGTAGGCTGGAGCTGCTTC**)

[a] The priming sites of the *aac(3)IV* disruption cassette are in boldface.

ciency. These modifications are further illustrated in Results. Conjugation efficiency was calculated as the ratio of the number of exconjugants per recipient cell, because we used fewer recipient cells (1×10^8 cells/ml) than donor cells (5×10^8 cells/ml) (45).

Evaluation of exconjugants by Southern blotting. *S. iranensis* exconjugants were cultured in tryptone soy broth for 4 days at 28°C. Genomic DNA was extracted using the NucleoSpin microbial DNA kit (Macherey & Nagel) followed by Southern blotting using an 800- to 1,000-bp-long nonradioactively labeled DNA probe, as previously described (46). *S. iranensis* HM 35 genomic DNA was used as the control.

Rapamycin production. *S. rapamycinicus* ATCC 29253 was precultured in seed medium for 48 h at 28°C, and *S. iranensis* strains were precultured in seed medium for 72 h at 28°C. Subsequently 1/10 of the preculture was cultured in YMM medium for 120 h at 28°C. Bacterial biomass was pelleted by centrifugation, and the pellets were extracted excessively with ethyl acetate. The extracts were filtered to remove the biomass, dehydrated with anhydrous sodium sulfate, filtered again, and reduced to dryness with a rotary evaporator (Laborota 4000 efficient; Heidolph Instruments, Schwabach, Germany). The crude extracts were reconstituted in 1 ml methanol and filtered through a 0.2-μm-pore-size polytetrafluoroethylene filter (Carl Roth, Karlsruhe, Germany). The samples were loaded onto an ultrahigh-performance liquid chromatography (LC)–mass spectrometry system consisting of an UltiMate 3000 binary rapid-separation liquid chromatograph with photodiode array detector (Thermo Fischer Scientific, Dreieich, Germany) and an LTQ XL linear ion trap mass spectrometer (Thermo Fischer Scientific, Dreieich, Germany) equipped with an electrospray ion source. The extracts (injection volume, 10 μl) were analyzed on a 150- by 4.6-mm Accucore reversed-phase (RP)-mass spectrometry column with a particle size of 2.6 μm (Thermo Fischer Scientific, Dreieich, Germany) at a flow rate of 1 ml/min with the following gradient: 0.1% (vol/vol) HCOOH-MeCN/0.1% (vol/vol) HCOOH-H_2O 0/100, which was increased to 60/35 in 30 s and then to 100/0 in 1.5 min, held at 100/0 for 4 min, and reversed to 0/100 in 30 s, with detection at 190 to 400 nm. Rapamycin powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol (final concentration, 100 μg/ml) and used as a standard.

L-Histidine degradation assay. Tripartite petri dishes were prepared containing 8 ml of ISP9 agar (38) per third. To test histidine degradation, the medium was supplemented with (i) 1% (wt/vol) sucrose and 1% (wt/vol) ammonium sulfate, (ii) 1% (wt/vol) sucrose and 25 mM L-histidine, (iii) 25 mM L-histidine and 1% (wt/vol) ammonium sulfate, and (iv) 25 mM L-histidine. Each third of the tripartite petri dish was point inoculated with 5×10^5 spores of the *S. iranensis* Δ*hutF* mutant or the *S. iranensis* HM 35 wild-type strain. For the Δ*hutF* mutant strains, apramycin was added to the medium. The plates were incubated for 14 days at 28°C. The use of L-histidine as the sole carbon and/or nitrogen source was taken as an indication for L-histidine degradation.

RESULTS

To efficiently and reproducibly generate gene deletion mutants of the rapamycin-producing actinomycete *S. iranensis* HM 35, we optimized the procedure for conjugal plasmid transfer from the nonmethylating *E. coli* strain ET12567/pUZ8002 into *S. iranensis* HM 35. A schematic overview of the process is given in Fig. 2. The starting point was the generation of a plasmid that carried a chosen target gene sequence surrounded by flanking regions of approximately 2 kb each. This so-called deletion plasmid of the pKOSi_SIRANxxxx type (Fig. 1A) was introduced into *E. coli* BW25113/pIJ790. The helper plasmid pIJ790 carried the phage λ Red recombinase under the control of an inducible promoter (47) and the homologous recombination machinery genes *bet* and *exo* as well as *gam*, responsible for inhibiting host RecBCD exonuclease V. Gene rearrangement as a prerequisite for subsequent deletion was achieved by homologous recombination, with the

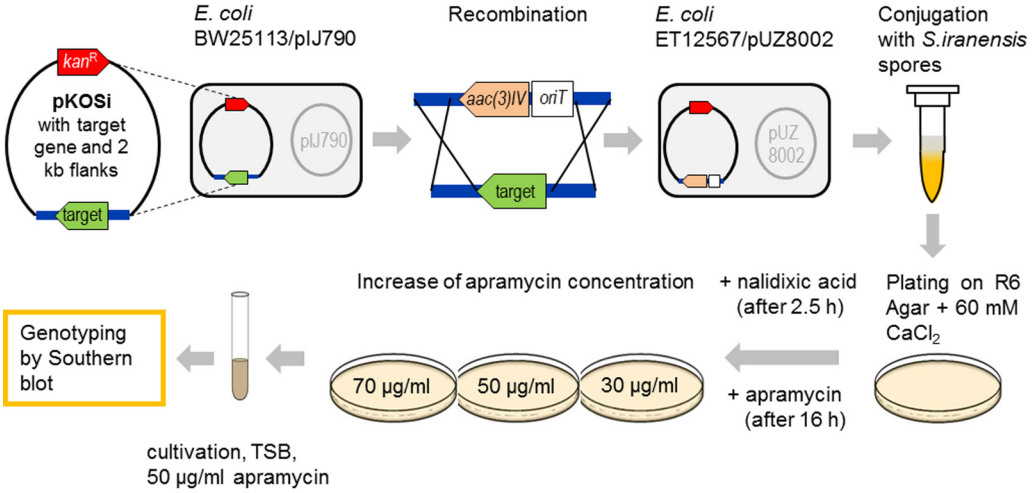


FIG 2 Overview of the generation of *S. iranensis* gene deletion mutants via intergeneric conjugation. A PCR product consisting of a target gene and its 2-kb upstream and 2-kb downstream flanking regions was introduced into pKOSi (Fig. 1). The resulting pKOSi_SIRANxxxx plasmids were cloned via the shuttle host *E. coli* DH5α into *E. coli* BW25113/pIJ790, where gene rearrangement by homologous recombination with a disruption cassette occurred. The disruption cassette carried the apramycin resistance gene *aac(3)IV* and the origin of transfer gene *oriT*. Recombined plasmids were transferred into the nonmethylating *E. coli* strain ET12567/pUZ8002 for conjugation with *S. iranensis* HM 35. The conjugation mixture was plated on R6 agar containing 60 mM $CaCl_2$. The growth of *E. coli* was inhibited by nalidixic acid. Obtained exconjugants were stepwise adapted to increased apramycin concentrations before genotyping by Southern blotting was performed to confirm successful genetic modification. TSB, tryptic soy broth.

disruption cassette carrying the apramycin resistance gene *aac(3)IV* as a selectable marker and the origin of transfer *oriT* (36). Recombined pKOSi derivatives (pKOSi_ΔSIRANxxxx) (Fig. 1A) were then transferred into the nonmethylating *E. coli* strain ET12567/pUZ8002 for conjugation with *S. iranensis* HM 35. The conjugation itself and the steps summarized below were identified as the most critical processes, requiring careful optimization.

Optimization of the conjugation protocol for *S. iranensis* HM 35. Because none of the known conjugation protocols (e.g., see reference 23) provided any exconjugants of the rapamycin producer *S. iranensis* HM 35, a systematic analysis and optimization of protocols were required. The final protocol summarizing all modifications and their consequences regarding conjugation efficiency in comparison to the starting version (23) is presented in Fig. 3. The first modification concerned the incubation time of the conjugation agar plates before they were overlaid with nalidixic acid. Overlaying with 0.5 mg of nalidixic acid was carried out after 2.5 h instead of the 16 h of incubation time at 37°C, followed by an overnight incubation at 37°C to support the loss of the pKOSi derivative. Release of the recombined plasmid was mediated by the temperature-sensitive replicon pSG5, which is functional only at temperatures below 34°C. Therefore, an early temperature shift to 37°C inhibited its replication, thereby promoting plasmid loss. Altogether, this provided an appropriate delivery system for homologous recombination between the plasmid-based sequence and the bacterial chromosome. The time scale for incubation with apramycin remained unchanged; i.e., agar plates were overlaid with 1 mg apramycin (50 mg/ml) after 16 h. Nevertheless, this modification step alone did not lead to exconjugant formation. Hence, the amounts of nalidixic acid and apramycin were decreased in the agar plates after conjugation had occurred at 37°C. Nalidixic acid (0.75 μg) and apramycin (only 1.5 μg) were added to select for growth of *E. coli* ET12567/pUZ8002. Again, none of these amendments improved conjugation frequency. Thus, in addition to identifying the described changes, we tested several media for their suitability for plating of the conjugation mixture of *E. coli* cells and *Streptomyces* spores in place of MS medium, as described in reference 23. Replacement of the MS medium with R6 agar containing 48 mM $CaCl_2$ (a medium suitable for killing *E. coli* [16, 48]) gave stable exconjugants from each postconjugation plate.

The exconjugants obtained were patched onto MMAM agar plates with increased apramycin concentrations. Afterward, a PCR was carried out to distinguish between mutants with single or double crossovers (see Fig. S1 in the supplemental material). Only the genotype of the double-crossover mutants was examined by Southern blotting. The conjugation frequency achieved was still relatively low, in the range of 1.6×10^{-7} (Fig. 4). Based on the study of reference 27, we increased the $CaCl_2$ concentration in the R6 agar plates to 60 mM, which led to a conjugation frequency of 3.1×10^{-7}. We further improved the conjugation frequency to 6.8×10^{-7} by inserting a 6-h germination time step at 28°C for the heat-shocked spores (27) prior to mixing them with the *E. coli* ET12567/pUZ8002 cells (Fig. 3). In summary, an efficient *S. iranensis* gene deletion strategy was achieved by applying all of the above modifications in a single experiment.

Verification of the conjugation protocol by deleting various genes. To exemplify the final protocol, 10 *S. iranensis* genes were chosen for deletion: *SIRAN1006*, *SIRAN1022*, *azlA*, *SIRAN1968*, *SIRAN4334*, *SIRAN4988*, *hutF*, *rapK*, *SIRAN8167*, and *SIRAN8652*. In each case, exconjugants for knockouts of each target gene were obtained. The successful deletion of the indicated genes in each of the 10 generated *S. iranensis* deletion mutants was confirmed by Southern blotting, as shown for *hutF* and *rapK* (Fig. 4).

According to *anti*biotics and *s*econdary-*m*etabolite *a*nalysis *sh*ell (antiSMASH) (49, 50), the genes *SIRAN1006*, *SIRAN1022*, and *azlA* are members of a polyketide synthase (PKS) gene cluster, whereas *SIRAN8652* belongs to a terpene synthase-containing cluster. *SIRAN1986* represents a glycoside hydrolase and *SIRAN4334* a monooxygenase. The gene *SIRAN4988* codes

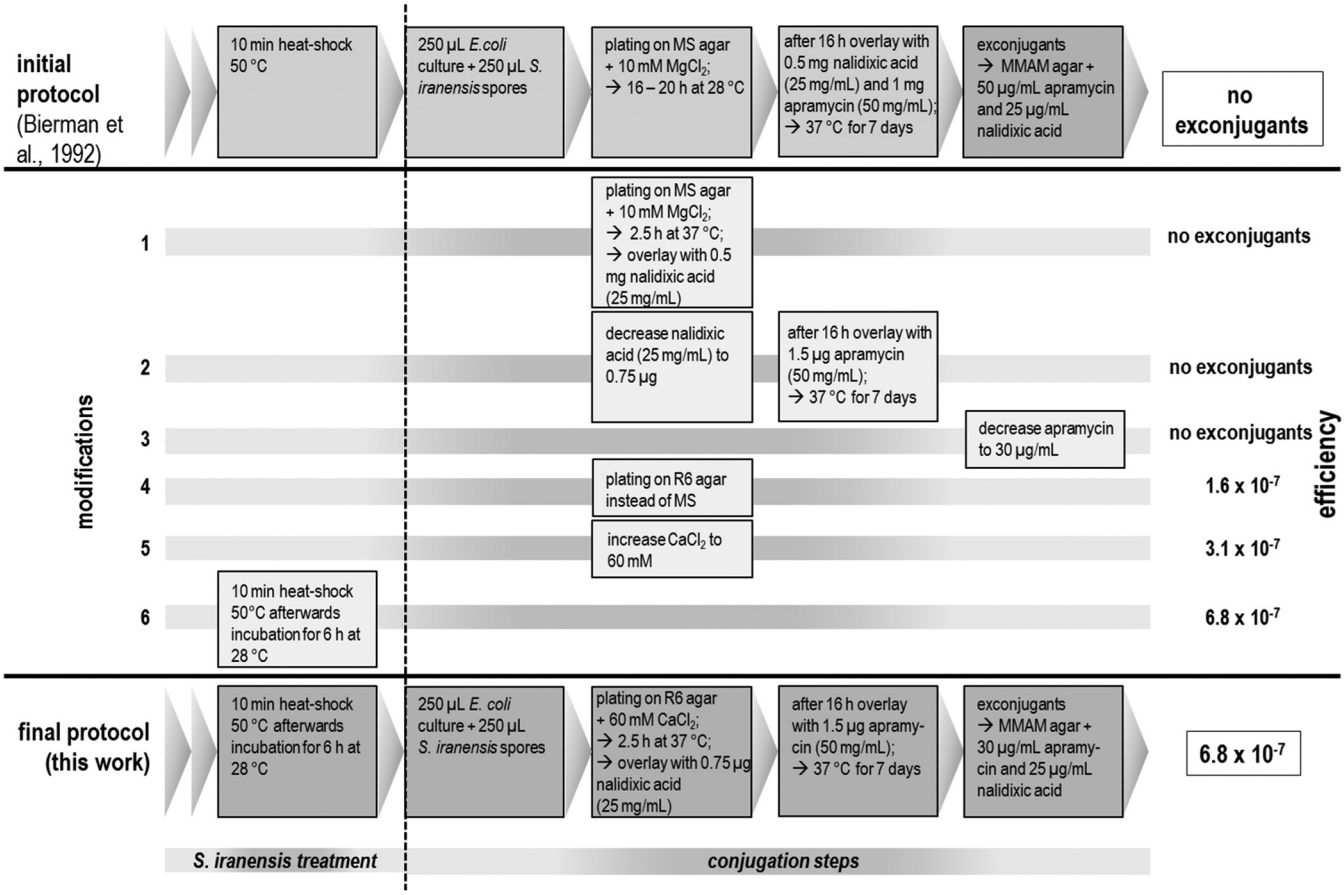


FIG 3 Development of an efficient conjugation protocol for the rapamycin producer *S. iranensis* HM 35. Stepwise modification of the basic protocol (23) led to increased conjugation efficiency and allowed reproducible generation of gene deletion mutants of *S. iranensis* HM 35. The highest conjugation efficiency (6.8×10^{-7} cells ml^{-1}) was achieved when all modifications were applied in a single experiment.

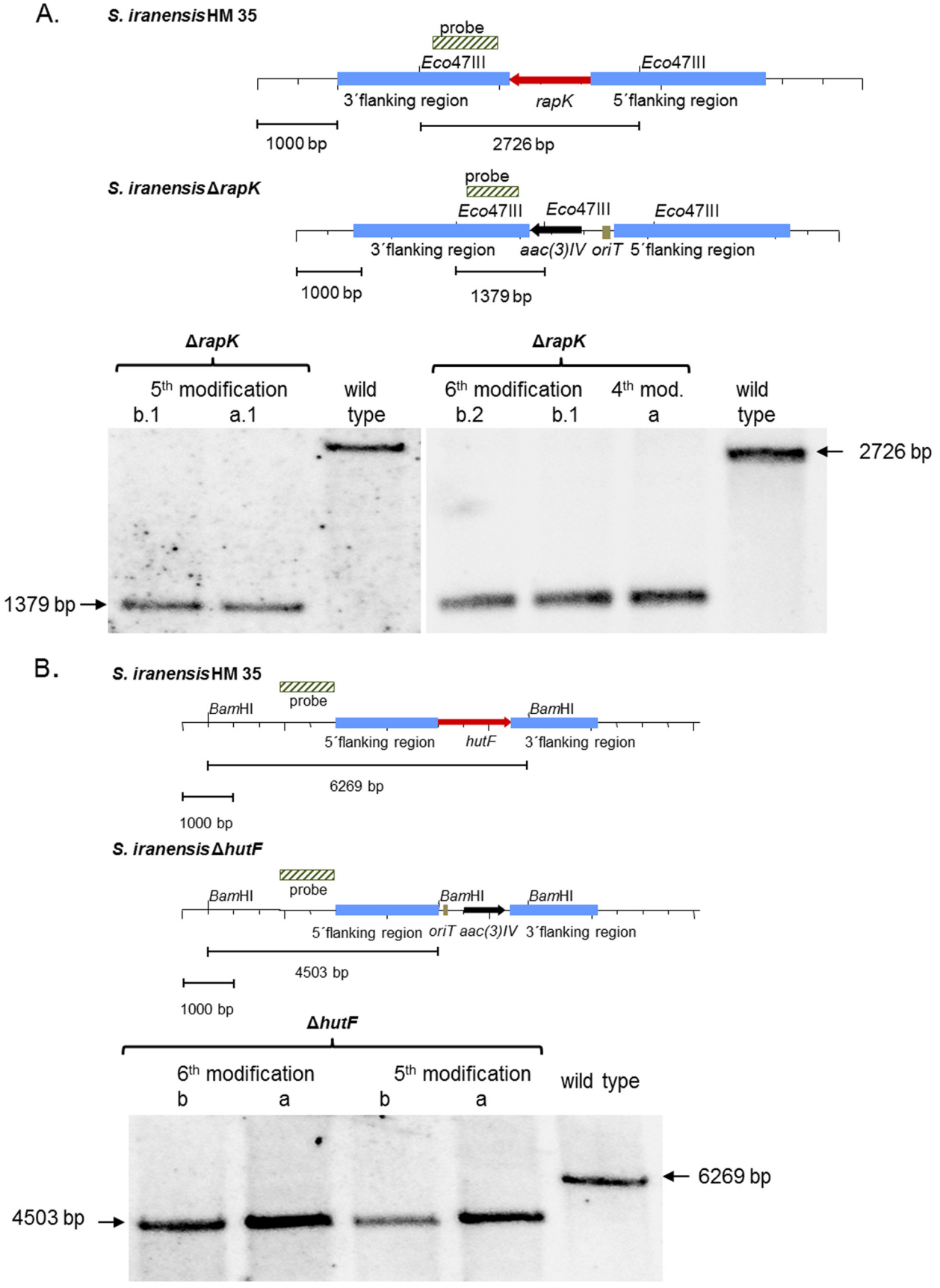
for a putative serine/threonine phosphatase. *hutF* is annotated as formiminoglutamate deiminase, an enzyme involved in histidine catabolism (51, 52), whereas *SIRAN8167* encodes a transmembrane efflux protein. Finally, we deleted *rapK*, which is part of the rapamycin biosynthesis gene cluster. The chorismatase RapK generates 4,5-dihydrocyclohex-1-ene-carboxylic acid (DHCHC) from chorismate. DHCHC serves as starter unit for the type I PKS encoded by *rapA* for rapamycin biosynthesis (16, 53). Two mutant strains, the Δ*rapK* and Δ*hutF* mutants, were further characterized.

Characterization of the Δ*rapK* and Δ*hutF* deletion mutants. Like *S. rapamycinicus*, *S. iranensis* is able to produce the immunosuppressant rapamycin (5) (see Fig. S2 in the supplemental material). Consequently, a Δ*rapK* mutant should be incapable of rapamycin biosynthesis. As shown in Fig. 5, liquid chromatography-mass spectrometry analysis confirmed that the *rapK* deletion strain lost its ability to produce rapamycin. This result is in agreement with data obtained for the Δ*rapK* mutant of *S. rapamycinicus* (16, 53).

The gene *hutF* is annotated as formiminoglutamate deiminase, an enzyme involved in formation of *N*-formyl-L-glutamic acid from *N*-formininoglutamic acid. The former compound is further metabolized into L-glutamic acid that can be transferred via α-ketoglutaric acid into the citric acid cycle. In this process, two molecules of NH_4^+ are released. Therefore, growth of *S. iranensis* on L-histidine as the sole carbon and/or nitrogen source is possible. The Δ*hutF* mutants were expected to lack the ability to degrade L-histidine. Both Δ*hutF* mutants and the wild type were grown on ISP9 agar containing L-histidine as the sole carbon and/or nitrogen source to test L-histidine degradation. Figure 6 shows the growth of colonies of these mutants. On control agar, no difference between the mutant and the wild-type strain was observed. In contrast to the wild type, the mutant strain did not germinate when agar plates with L-histidine as the sole carbon and/or nitrogen source were used. This phenotype was indicative of the deletion of *hutF*. These successful gene deletions demonstrated the efficiency of the modified conjugation procedure for generation of gene knockouts in *S. iranensis*.

DISCUSSION

Streptomycetes produce an immense number of secondary metabolites, including compounds of medical interest. Among them is the valuable immunosuppressant rapamycin. Methods to genetically manipulate the genome of its alternative producers, e.g., by introducing or deleting selected genes, might open new avenues toward the production of novel rapamycin variants without the need for postfermentative chemical modification. Despite this importance, until this study, there had been no effective protocol for the generation of targeted gene deletions in rapamycin-pro-


A.
S. iranensis HM 35
probe
Eco47III
Eco47III
3´flanking region
rapK
5´flanking region
1000 bp
2726 bp
S. iranensis ΔrapK
probe
Eco47III
Eco47III
Eco47III
3´flanking region
aac(3)IV oriT
5´flanking region
1000 bp
1379 bp
ΔrapK
5th modification
b.1
a.1
wild type
ΔrapK
6th modification
b.2
b.1
4th mod.
a
wild type
2726 bp
1379 bp
B.
S. iranensis HM 35
BamHI
probe
BamHI
5´flanking region
hutF
3´flanking region
6269 bp
1000 bp
S. iranensis ΔhutF
BamHI
probe
BamHI
BamHI
5´flanking region
oriT aac(3)IV
3´flanking region
4503 bp
1000 bp
ΔhutF
6th modification
b
a
5th modification
b
a
wild type
6269 bp
4503 bp

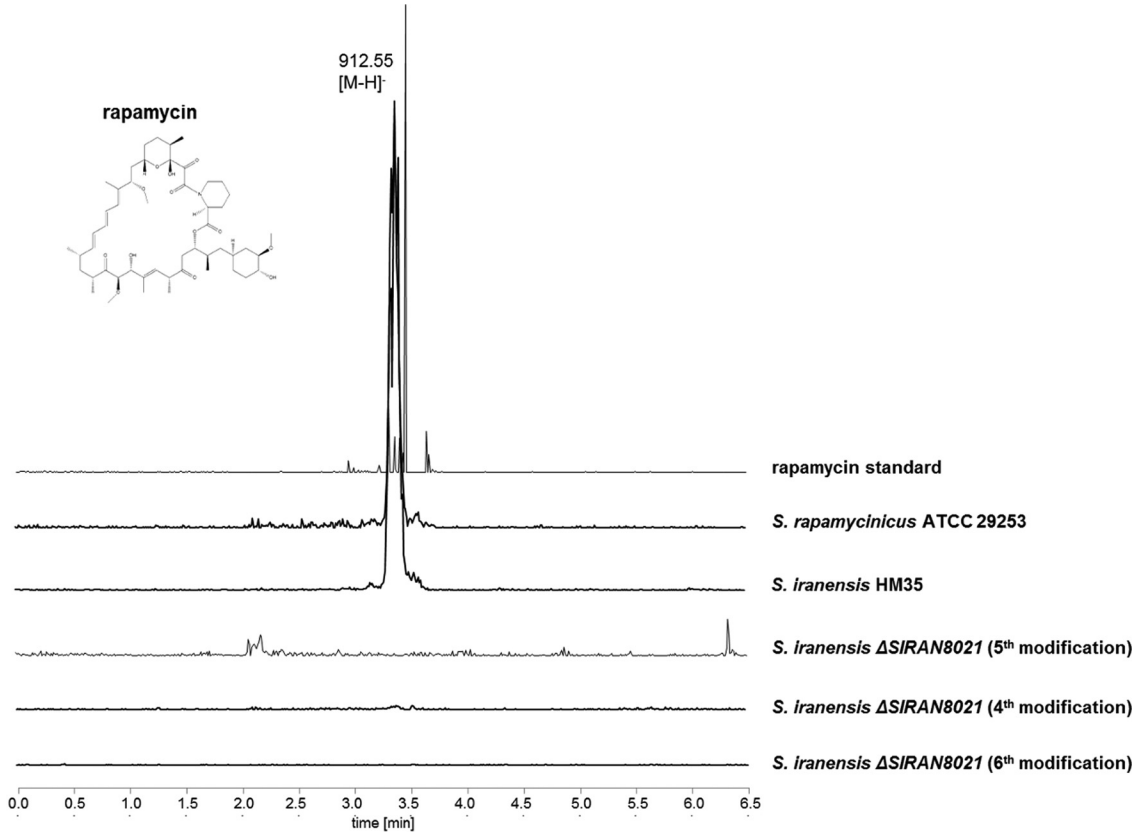


FIG 5 Identification of rapamycin by LC-mass spectrometry. Extracted ion chromatogram (EIC) showing mass-to-charge ratios (*m/z*) of 912 to 914 [M-H], corresponding to rapamycin extracted from biomass. Unlike with the Δ*rapK* mutants obtained by each modification of the transformation protocol, a mass corresponding to rapamycin was detectable only in *S. rapamycinicus* ATCC 29253 and *S. iranensis* HM 35.

ducing *Actinoplanes* sp. N902-109 (18), for *S. iranensis* (17), or even for the established producer *S. rapamycinicus* (8); the working protocol (16) had not been systematically optimized. Two major challenges hinder gene deletions in actinomycetes: (i) the transfer of the vector carrying the foreign DNA into the cell and (ii) the site-specific integration of DNA into the chromosome. Typically, intergeneric conjugation is used as a means of gene transfer into streptomycetes to accomplish the first step (22, 54, 55). Intergeneric conjugation has already been successfully applied to species of various genera, e.g., *Streptomyces*, *Actinomadura*, *Amycolatopsis*, *Arthrobacter*, *Micromonospora*, *Nocardia*, *Rhodococcus*, *Kitasatospora*, and *Saccharopolyspora* (56–60). The advantage of this strategy is that it allows for the design and construction of recombinant plasmids in *E. coli* prior to transfer to the recipient of interest. The second step, the integration of recombinant DNA into the genome, is often mediated by the integration vector pSET152 (23) or derivatives thereof that contain the attachment site (*attP*) and the integrase (*int*) function of the temperate phage ϕC31. pSET152 derivatives undergo site-specific integration into the *attB* sites of the *Streptomyces* chromosome (61). However, even this was challenging with *S. rapamycinicus* and *S. iranensis* and not feasible with the standard conjugation protocol (23). Only by applying the mentioned modifications could pSET152 be reproducibly inserted into both species (data not shown).

While the insertion of pSET152 proves that the introduction of recombinant gene cassettes into a given species is possible, it also means that pSET152 derivatives are not suitable to achieve targeted gene deletions. To address this problem, diverse solutions exist. The basic required step relies on gene rearrangement by homologous recombination, typically between the gene to be deleted and a cassette consisting of a selection marker and an origin of transfer. As already mentioned, the appropriate cassette can easily be generated in *E. coli*. An elegant way to perform such gene rearrangement consists of λ Red-mediated PCR targeting (36). Therefore, we designed a pKOSi-based deletion vector that, besides carrying the temperature-sensitive replicon pSG5 (39, 40), carries a cassette encoding the kanamycin resistance gene *kan* and

FIG 4 Southern blot analysis. (A) *S. iranensis rapK* deletion mutant. The genomic locus was replaced by an apramycin resistance cassette. Genomic DNA was cut with Eco47III. An 801-bp PCR fragment carrying a *rapK* downstream sequence was used as the probe. Δ*rapK* deletion mutants (lanes 1, 2, 4 and 6) were characterized by a band of 1,379 bp; the typical 2,726-bp band of the wild type (lane 3 and 7) had disappeared. (B) *S. iranensis hutF* deletion mutant. The wild-type *S. iranensis* HM 35 strain was replaced at the locus of *hutF* by an apramycin resistance cassette. Genomic DNA was digested with BamHI. A 1,047-bp PCR fragment containing the upstream sequence of *hutF* was used as the probe. In Δ*hutF* deletion mutants (lanes 1 to 4), the characteristic 6,269-bp band of the wild type (lane 5) disappeared. Instead, a band of 4,503 bp represented successful gene replacement.

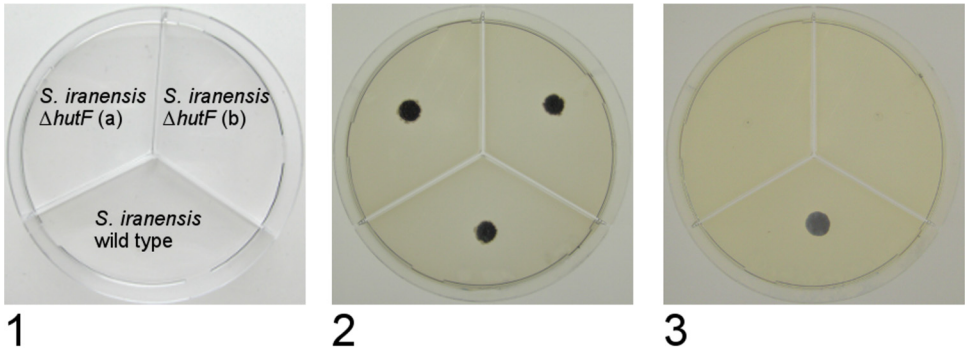


FIG 6 Growth of *S. iranensis* HM 35 and Δ*hutF* mutants on ISP9 agar plates. Plates were inoculated with 5×10^5 spores and incubated for 14 days at 28°C. (1) Inoculation scheme; (2) plate supplemented with 1% (wt/vol) sucrose and 1% (wt/vol) ammonium sulfate (control); (3) plate supplemented with 1% (wt/vol) sucrose and 25 mM histidine. Growth on all other agar plates containing histidine as the sole C or N source was comparable to that on plate 3.

2-kb flanking regions of the gene to be deleted. The apramycin resistance gene *aac(3)IV*, the origin of transfer gene *oriT*, and twin Flp recognition target (FRT) sequences were amplified from pIJ773 containing arms with 39 nt of homology to the gene of interest.

Subsequently, the PCR product was electroporated into *E. coli* BW25113/pIJ790. This strain expresses the genes encoding the λ Red recombination machinery, thus promoting homologous recombination between the genomic region and the homology arms of the PCR product and generating the final plasmid for intergeneric conjugation with *S. iranensis*. The advantage of this method is its rapidity, compared to step-by-step cloning of the resistance cassette, the *oriT* gene, and the FRT sequences. Theoretically, the described procedure provides a promising approach for the ultimate site-specific homologous recombination between this cassette and the bacterial chromosome to generate targeted gene knockouts. However, in practice, it does not work properly for every *Streptomyces* species, and further optimization is often required to successfully conjugate *E. coli* with refractory species (62–65). Various species-dependent parameters which strongly affected the transformation efficiency were identified. Sun et al. (64) found that the ratio of donor to recipient cell numbers was the most influential factor for conjugation into *Streptomyces noursei*. Also, for *Streptomyces diastatochromogenes*, the number of spores and their use instead of mycelia, in combination with a cultivation step at 30°C subsequent to the heat shock, were found to be the most important factors (65). In contrast, Du et al. (63) achieved the highest conjugation efficiencies for *Streptomyces lincolnensis* by using mycelia rather than spores as recipients and by addition of 10.3% sucrose to the SM medium (63). Due to the fact that several streptomycetes degrade methylated DNA, a nonmethylating *E. coli* strain can be preferable as the DNA donor in intergeneric conjugation (55). However, when an effective gene transfer system for *Streptomyces ipomoeae*, the causative agent of soil rot disease of sweet potatoes, was engineered, plasmids could be introduced and maintained with approximately equivalent frequencies from either methyl-proficient or methyl-deficient *E. coli* donors (66).

The parameters that determine successful intergeneric conjugation include the following: (i) the type of cells, (ii) the media used for sporulation and conjugation protocols, (iii) the duration of the incubation steps, and (iv) the temperature of the incubation steps. All of these factors were important when we transferred a basic conjugation protocol into an effective conjugation system for rapamycin-producing *S. iranensis*. Modifications of each of the parameters were found to be required. The protocol developed for deletion mutant generation was applied to *S. iranensis* spores. Screening of media indicated that R6 agar instead of MS agar in the conjugation agar plates is a prerequisite for the growth of exconjugants. We also changed the time of addition and amount of nalidixic acid and apramycin. By significantly decreasing the concentrations of both compounds, an even higher number of transconjugants was obtained. Based on data from Wang and Jin (27), we changed the $CaCl_2$ concentration in the conjugation agar plates and found 60 mM to be optimal. Another important step included 6 h of incubation directly after the heat shock. By combining all of the described modifications in a single experiment, we achieved a conjugation efficiency of 6.8×10^{-7} transconjugants per recipient CFU. This is still low compared to those obtained for *Streptomyces coelicolor* (1.1×10^{-1}) when the methylation-deficient donor *E. coli* ET12567/pUB307 was applied for the first time (55) or compared to those achieved for *S. lincolnensis* mycelia exconjugants (2.8×10^{-5}) when regenerated on solid MS medium containing 10.3% sucrose (63).

Conjugation efficiencies have proved to be highly varied, depending on the *Streptomyces* species and on the optimization steps used. For *S. peucetius* and *Streptomyces* sp. strain C5 conjugation efficiencies of 1.5×10^{-4} per recipient cell or 1.1×10^{-5} exconjugants per recipient spore, respectively, were calculated (67). Phornphisutthimas et al. (62) obtained high efficiencies of conjugation (10^{-2} to 10^{-3}) for *S. rimosus* with a procedure based on heat treatment of the spores at 40°C for 10 min prior to their being mixed with *E. coli* ET12567(pUZ8002/pIJ8600). In that study, tryptic soy agar medium containing 10 mmol/liter $MgCl_2$ was the preferred medium for conjugation. Finally, Sun et al. (64) obtained 8×10^{-3} exconjugants per recipient when spores were heat shocked at 50°C for 10 min, mixed with *E. coli* ET12567/pUZ8002/pSET152 in the ratio of 1:100, plated on 2CMY medium (71) containing 40 mmol/liter $MgCl_2$, and incubated at 30°C for 22 h.

Taken together, the methods and strategies applied to develop and optimize intergeneric conjugation for a given species are as diverse as the species themselves. The present protocol focuses on *S. iranensis*, aiming to generate mutants producing nonnative rapamycin variants, to study the regulation of rapamycin biosynthesis in this species, and also to make the species amenable to the discovery of novel secondary metabolites by genetic engineering.

To our knowledge, the modified protocol is the first successful directed gene deletion protocol for this species.

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