



HAL
open science

Integrative Gene Cloning and Expression System for Streptomyces sp. US 24 and Streptomyces sp. TN 58 Bioactive Molecule Producing Strains

Samiha Sioud, Bertrand Aigle, Ines Karray-Rebai, Slim Smaoui, Samir Bejar,
Lotfi Mellouli

► **To cite this version:**

Samiha Sioud, Bertrand Aigle, Ines Karray-Rebai, Slim Smaoui, Samir Bejar, et al.. Integrative Gene Cloning and Expression System for Streptomyces sp. US 24 and Streptomyces sp. TN 58 Bioactive Molecule Producing Strains. Journal of Biomedicine and Biotechnology, 2009, 2009 (Article ID 464986), pp.464986. 10.1155/2009/464986 . hal-01659292

HAL Id: hal-01659292

<https://hal.univ-lorraine.fr/hal-01659292>

Submitted on 8 Dec 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Research Article

Integrative Gene Cloning and Expression System for *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 Bioactive Molecule Producing Strains

Samiha Sioud,¹ Bertrand Aigle,² Ines Karray-Rebai,¹ Slim Smaoui,¹ Samir Bejar,¹ and Lotfi Mellouli¹

¹Laboratory of Prokaryotic Enzymes and Metabolites, Centre of Biotechnology of Sfax, Road of Sidi Mansour Km 6, P. O. Box 1177, 3018 Sfax, Tunisia

²Laboratory of Genetic and Microbiology, UMR INRA 1128, IFR 110, Faculty of Sciences and Techniques, University Henri Poincaré, Vandoeuvre-lès-Nancy, 54 003 Nancy Cedex, France

Correspondence should be addressed to Lotfi Mellouli, lotfi.mallouli@cbs.rnrt.tn

Received 2 December 2008; Accepted 26 March 2009

Recommended by Isaac K. O. Cann

Streptomyces sp. US 24 and *Streptomyces* sp. TN 58, two strains producing interesting bioactive molecules, were successfully transformed using *E. coli* ET12567 (pUZ8002), as a conjugal donor, carrying the integrative plasmid pSET152. For the *Streptomyces* sp. US 24 strain, two copies of this plasmid were tandemly integrated in the chromosome, whereas for *Streptomyces* sp. TN 58, the integration was in single copy at the *attB* site. Plasmid pSET152 was inherited every time for all analysed *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 exconjugants under nonselective conditions. The growth, morphological differentiation, and active molecules production of all studied pSET152 integrated exconjugants were identical to those of wild type strains. Consequently, conjugal transfer using pSET152 integration system is a suitable means of genes transfer and expression for both studied strains. To validate the above gene transfer system, the glucose isomerase gene (*xylA*) from *Streptomyces* sp. SK was expressed in strain *Streptomyces* sp. TN 58. Obtained results indicated that heterologous glucose isomerase could be expressed and folded effectively. Glucose isomerase activity of the constructed TN 58 recombinant strain is of about eighteenfold higher than that of the *Streptomyces* sp. SK strain. Such results are certainly of importance due to the potential use of improved strains in biotechnological process for the production of high-fructose syrup from starch.

Copyright © 2009 Samiha Sioud et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

1. Introduction

At the end of the last century, in addition to the conventional screening of naturally occurring bioactive compounds, a strategy was developed to construct desired novel hybrid antibiotics by combinatorial biosynthesis, by specifically combining genes from various biosynthetic pathways originating from different antibiotics producing organisms. In *Streptomyces* species, the most important producers of bioactive molecules, genetic approaches to improve secondary metabolite production are generally hampered by various phenomena such as restriction barriers, absence of an efficient gene transfer systems, and lack of suitable cloning vectors. Recently, in order to circumvent these problems, there has been considerable interest in the use of intergeneric

conjugation as an efficient means of gene transfer and expression in *Streptomyces* species. This technique allows the construction and the manipulation of recombinant plasmids in *E. coli* and their subsequent transfer to a recipient of interest. The initial protocol for intergeneric transfer of plasmids from *E. coli* to *Streptomyces* species was developed by Mazodier et al. [1]. Since then, this method has been successfully applied to several *Streptomyces* species [2–4] via methylation-deficient *E. coli* strains, such as strain ET12567 [5], as a DNA donor to avoid methylated DNA restriction systems of actinomycetes [6]. Several cloning vectors that could be transferred from *E. coli* to *Streptomyces* species by conjugation method have been constructed [7, 8]. Among them, pSET152 is a nonreplicative plasmid in *Streptomyces* that carries the *attP* site and integrase

gene of ϕ C31 phage and consequently can integrate into the chromosomal *attB* site of the bacteriophage ϕ C31. Like λ integrases, ϕ C31 integrase catalyses the site-specific recombination between the chromosomal *attB* site and an *attP* site to form *attL* and *attR*. However, unlike those of λ integrases, the sites are both very small, with *attP* and *attB* being just 39 and 34 pb in size, respectively [9]. The DNA sequences of *attB* were characterized from various *Streptomyces* species, and the analysis of these sequences revealed the presence of high conserved positions. In this genus of bacteria, the presence of pseudo-*attB* sites (50% – 70.3% nt identity to *attB* sites) has also been reported, but with an integration frequency 300 times lower than that with the corresponding *attB* site [10]. However, the absence of this *attB* site from some actinomycete strains, such as *Saccharopolyspora erythraea*, has also been reported [11].

We have previously reported the isolation from Tunisian soil of two new actinomycete strains called *Streptomyces* sp. US 24 [12] and *Streptomyces* sp. TN 58 [13], producing diverse interesting biological activities, and described the purification and structure elucidation of two and five active molecules from the *Streptomyces* sp. US 24 [14] and the *Streptomyces* sp. TN 58 [15], respectively. The active molecules produced by *Streptomyces* sp. US 24 are the 3-indolethanol and a diketopiperazine (DKP) derivative, the Cyclo (L-Phe, L-Pro) diketopiperazine. DKP active molecules form a very important family because of their many potential uses as antibacterial, fungicidal, herbicidal, antiviral, immune-suppressor, and antitumor agents, and therefore they are very attractive for the production of novel hybrid active compounds by combinatorial biosynthesis.

Among the five active molecules characterised from the *Streptomyces* sp. TN 58, two belong to the rhamnopyranoside family: the 1-O-(2-Aminobenzoyl)- α -L-rhamnopyranoside and the 4-Hydroxybenzoyl α -L-rhamnopyranoside. These two active compounds possess inhibitory activity towards 3 α -hydroxysteroid dehydrogenase (3 α -HSD), a useful target for anti-inflammatory and antiphlogistic drugs. It should be noted that until now, the *Streptomyces* sp. TN 58 is the only described strain which produces these two molecules directly without any supplement addition into the culture media [15]. Identification and expression of genes involved in biosynthetic pathways of these molecules constitute a real opportunity for the comprehension of their natural biosynthesis and subsequently the production of hybrid molecules. The aim of this work was thus to succeed in carrying out intergeneric conjugation between *E. coli* ET12567 and *Streptomyces* sp. US 24 and TN 58 strains, to analyse the integration sites of the integrative vector pSET152 into the chromosome of these two *Streptomyces* strains, to study whether the site-specific integration of pSET152 affects essential functions such as the production of active molecules in these two studied strains, and to verify the expression of a heterologous gene in *Streptomyces* sp. TN 58 strain using this system.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids. *E. coli* strain ET12567 (pUZ8002) [2, 5], used as the donor in intergeneric conjugation, is a methylation-defective strain (*dam-13::* Tn9 *dcm-6 hsdM* Cmr). *E. coli* strain DH5 α [16] ($F^- \phi 80 \Delta lacZ \Delta M15 \Delta(lacZYA-argF)$ U169 *endA1 recA1 hsdR17* (r^-_k, m^+_k) *deoR thi-1 susE44 \lambda^- gyrA96 relA1*) and *E. coli* HB101 ($F^- hsdS2 recA13 ara^- 14 pro A2 lacY1 galk2 rps L20 xyl^- 5 mtl sup E44$) were used as host strain, and *E. coli* ATCC 8739 and *Micrococcus luteus* LB 14110 were used as indicator microorganisms for the antibacterial activity assays.

The two *Streptomyces* strains US 24 [12] and TN 58 [13] were used as recipient for intergeneric conjugation. *Streptomyces* SK strain is a thermostable glucose isomerase producer [17]. CBS4 strain was the glucose isomerase deficient *Streptomyces violaceoniger* in which the *xylA* gene of the *Streptomyces* SK strain, under the control of the *ermE*-up constitutive promoter, was integrated in its chromosome via the integrative vector pTS55 [18].

Plasmid pUZ8002 is a derivative of RK2 with a mutation in *oriT* [19]. pSET152 is an integrative plasmid carrying ϕ C31 *int* and *att* functions and an apramycin resistance gene for selection in *Streptomyces* and *E. coli* [2]. pGEM-T Easy vector is a TA cloning vector Amp^R (Promega) used for cloning of the PCR products.

2.2. Media and Culture Conditions. *E. coli* strains were grown in Luria broth (LB) medium. Ampicillin (50 μ g mL⁻¹), chloramphenicol (25 μ g mL⁻¹), nalidixic acid, and kanamycin (50 μ g mL⁻¹) were added to growth media when required and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (40 μ g mL⁻¹) when appropriate [20]. Transformation of *E. coli* DH5 α or HB101 with pGEM-T pSET152 derivatives was carried out according to the manufacturer's instructions (Promega).

Streptomyces sp. US 24 and TN 58 strains were grown in Tryptone soya broth (TSB: 30 g tryptic soy broth plus 5 g yeast extract per 1000 mL distilled water) medium for the preparation of genomic DNA [21] and in R2YE [22] plates for the preparation of spore stocks. TSB supplemented with 30 μ g of apramycin mL⁻¹ was used to grow exconjugants for the preparation of genomic DNA and to maintain them.

AS1 medium [23] (1 g Yeast extract, 5 g Soluble starch, 0.2 g L-alanine, 0.2 g L-arginine, 0.5 g L-asparagine, 2.5 g NaCl, 10 g Na₂SO₄ and 20 g agar, pH adjusted at 7.5 with KOH) was used for conjugation experiments.

For bioactive molecules production, *Streptomyces* sp. US 24 strain and the corresponding exconjugants were grown in TSB medium, supplemented at 1% (w/v) with starch and with a trace element solution: (7.5 mL L⁻¹ final; 0.4 g L⁻¹ ZnCl₂; 2 g L⁻¹ FeSO₄·7H₂O; 0.065 g L⁻¹ H₃BO₃ and 0.135 g L⁻¹ MoNa₂O₄·2H₂O). *Streptomyces* TN 58 strain and corresponding exconjugants were grown in TSB medium, supplemented with glycerol at 1% (w/v) and with potassium at 1 mmol L⁻¹. For antibacterial activity determination, indicator microorganisms were grown overnight in LB medium at 30°C for *M. luteus* LB14110 and at 37°C for *E. coli* ATCC 8739, then diluted 1 : 100 in LB medium and

incubated for 5 hours under constant agitation at 200 rpm at the appropriate temperature.

For growth study of the two bacteria: *Streptomyces* TN 58/*xylA* and CBS4, spores at 10^7 mL⁻¹ of each strain were used to inoculate 1000 mL Erlenmeyer flasks with four indents, containing 200 mL of minimum liquid medium ($[\text{NH}_4]_2\text{SO}_4$ 2.5 g L⁻¹, MgSO_4 0.3 g L⁻¹, CaCl_2 0.2 g L⁻¹, KH_2PO_4 1 g L⁻¹, yeast extract 1 g L⁻¹ and 1 mL of trace element solution) in presence of xylose as solely carbon source at 10 g L⁻¹. Biomass of the two studied *Streptomyces* strains was determined at different growth times by measurement by weighing to constant dry weight after drying at 105°C.

2.3. Intergeneric Conjugation. Intergeneric conjugation between *E. coli* and *Streptomyces* US 24 and TN 58 strains was performed as described previously by Flett et al., [24] with minor modifications. *E. coli* ET12567 (pUZ8002/pSET152) was grown to an absorbance of 0.4–0.6 at 600 nm. The cells were pelleted by centrifugation, washed twice in an equal volume of LB, pelleted again, and finally resuspended in 1/10 volume of LB. Aliquots of the two studied *Streptomyces* strain spores suspension stored at -20°C were used as recipients. Spores (eq10⁸) were washed in 2 × yeast extract tryptone medium [25], resuspended in 500 μL of 2 × yeast extract tryptone medium, and incubated at 50°C for 10 minutes to induce germination. Donor cells (500 μL approximately 10⁸ cells) were added to the treated spores, the mixture was pelleted by centrifugation, and finally the pellets were resuspended in the residual liquid, and the undiluted mixtures were plated. The mating mixtures were spread on AS1 plates containing 10 mM MgCl_2 and incubated for 18 hours at 37°C for the *Streptomyces* US 24 and at 30°C for the *Streptomyces* TN 58 strain. The plates were overlaid each with 1 mL of water containing 500 μg of nalidixic acid and 1 mg of apramycin, incubated further for 5 days at the appropriate temperature, and the exconjugants were counted. Cultures prepared from some clones were used for further studies. Control experiments were performed as described above but without the addition of *E. coli* donor cells. The viable count of the donor culture was determined by spreading the cells on LB agar plates supplemented with appropriate antibiotics.

2.4. DNA Isolation and Manipulation. Total DNA preparation was carried out from *Streptomyces* strains according to [21]. Small-scale plasmid preparations from *E. coli* were performed as described by [20].

Digestion with restriction endonucleases, and separation of DNA fragments by agarose gel electrophoresis, alkaline calf intestinal phosphatase, ligation of DNA fragments, and transformation were done according to [20] for *E. coli* and [21] for *Streptomyces*.

For blot manipulations [26], DNA was transferred to Hybond N nylon membranes (Amersham). About 1 μg and 50 ng were loaded for the digested genomic DNA and plasmids DNA, respectively. The hybridization conditions and subsequent detection were in accordance with the manufacturer's instructions. ³²P-labeled probes were prepared using the Random prime labelling system (Amersham).

PCR amplification of the *attB* sites of the two studied *Streptomyces* strains was performed using the two primers ATTB1 and ATTB2 previously described by [10]. Approximately 200 ng genomic template DNA was used with 100 pmol of each primer per 50 μL reaction volume. To improve the denaturation of the DNA, 5% (v/v) DMSO was added to the reaction mixture. Amplifications were performed in a Gene Amp^R PCR System 2700 (Applied Biosystems) using 1U Pfu DNA polymerase (Stratagene) and the recommended buffer system according to the following amplification profile: 94°C (5 minutes) followed by 45 cycles of denaturation at 94°C (30 seconds), annealing at 60°C (1 minute) and extension at 72°C (1 minute). The PCR reaction mix was analysed by agarose gel electrophoresis, and the products having the expected sizes were purified then cloned into pGEM-T Easy vector.

Nucleotide sequences were determined on both strands using the dideoxy chain-termination method [27]. Reactions were performed with a thermo sequenase cycle sequencing kit (Amersham) and specific primers. Homology search was performed using Blast Search algorithm [28].

2.5. Extraction of Active Compounds and Biological Assay of Antimicrobial Activities. For the extraction of the active molecules from the *Streptomyces* sp. US 24 and TN 58 strains and their corresponding exconjugants (four exconjugants randomly chosen for each strain), spores at 10^7 mL⁻¹ were used to inoculate 500 mL Erlenmeyer flasks with four indents containing 100 mL of culture medium. After incubation for 24 hours in an orbital incubator with shaking at 250 rpm at the appropriate temperature, the preculture was used to inoculate (5% v/v) a total volume of 1000 mL culture medium having the same composition of the preculture. After three days incubation at 37°C or 30°C for *Streptomyces* US 24 and TN 58 strains, respectively, and their corresponding exconjugants, in an orbital incubator with shaking at 250 rpm, the culture broths were filtered to separate mycelium and supernatant. Each supernatant was extracted twice with an equal volume of ethyl acetate and then evaporated on a Rotavapor (Laborata 4000), and obtained crude extract was dissolved in 1 mL ethyl acetate and used for antibacterial activities as follows: a paper disk was impregnated with 80 μL of the corresponding sample and then laid on the surface of an agar plates containing 3 mL of top agar inseeded by 40 μL of a 5-hour old culture of *M. luteus* LB 14110 or *E. coli* ATCC 8739. After 2 hours at 4°C, plates containing *M. luteus* were incubated at 30°C and those inoculated with *E. coli* at 37°C, all for overnight. Plates were examined for evidence of antibacterial activities represented by a zone of inhibition of growth of the corresponding indicator micro-organisms around the paper disk. Thin layer chromatography (TLC) was performed on silica gel plates SiO_2 (Merck). Visualization of active compounds was realised with anisaldehyde vapour and Ehrlich's reagent.

2.6. Expression of the *xylA* Gene in *Streptomyces* sp. TN 58. The *Bgl*III fragment carrying the insert *xylA* gene of *Streptomyces* sp. SK placed under the control of the *ermE*-up constitutive promoter [29] from pMM6 [18] was cloned

into the *Streptomyces* integrative vector pSET152 linearised by *Bam*HI, leading to the pSS6 vector construct. This plasmid was firstly obtained within *E. coli* HB101 strain and then transferred to *E. coli* ET12567/pUZ8002 for the construction of the *Streptomyces* TN 58/*xylA* strains by conjugal transfer. Exconjugants were selected on the basis of apramycin resistance.

Preparation of the cell-free lysate of the *Streptomyces* SK, TN 58, TN 58/pSET152, TN 58/pSS6, and CBS4 (used as positive control) strains was achieved as follows. The cells were grown in TSB medium for 48 hours and were harvested by centrifugation at 8000 rpm for 10 minutes, and the pellets were suspended in TE-buffer with 10 mM MgCl₂ and 1 mM CoCl₂. After incubation for an hour on ice in the presence of 5 mgmL⁻¹ lysozyme, 100 µgmL⁻¹ PMSF, and 1 µgmL⁻¹ Pepstatin A, cells were disrupted by sonication at 4°C for 6 minutes (pulsations of 3 seconds, amplify 90) using a vibra cell Sonicator (Fisher-Bioblock Scientific), and debris were removed by centrifugation at 20 000 rpm for 30 minutes.

Glucose isomerase activity was determined using fructose as substrate by quantitatively measuring the glucose production. The activity was assayed in a reaction mixture containing the enzyme (100 µL of an appropriate diluted purified or crude extract) with 10 mM MgCl₂, 1 mM CoCl₂ and 15% fructose, in a volume of 400 µL. In standard condition, assays were incubated for 30 minutes at 80°C, and the reaction was stopped by cooling the tubes on ice. The amount of glucose generated was determined by glucose-oxidase (GOD-PAP) enzyme system, and A₆₅₀ was measured after 40 minutes at room temperature. One unit of glucose isomerase activity is defined as the amount of enzyme needed to produce 1 µmol of product per minute under the assay conditions. Protein concentration was determined by Bradford's method [30] using the Bovine Serum Albumin as standard.

3. Results and Discussion

3.1. Conjugal Transfer of pSET152 and ϕ C31-Directed, Site-Specific Recombination in *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 Strains. The plasmid pSET152 was mobilized from *E. coli* ET12567 (pUZ8002) into *Streptomyces* sp. US 24 and TN 58 strains. Exconjugants were obtained at a frequency of approximately 5×10^{-5} and 3×10^{-4} per recipient spore of *Streptomyces* sp. US 24 and TN 58 strains, respectively, which showed a high frequency of exconjugants. Indeed, Nikodinovic et al., [31] consider that the transformation efficiency (5×10^{-5} exconjugants recipient⁻¹) of the *Streptomyces nodosus* by conjugal transfer of DNA from *E. coli* was a high transformation frequency.

The integrative plasmid pSET152 cannot replicate in *Streptomyces*; so stable exconjugants should be obtained only if pSET152 has been integrated into the chromosome of studied strain. In order to identify the copy number of pSET152 in the recipient *Streptomyces* sp. US24 and *Streptomyces* sp. TN 58, eight arbitrary chosen exconjugants were studied for each strain. Chromosomal DNAs were extracted, digested by *Bam*HI and probed with the whole linearised vector pSET152 in a blot analysis. Since pSET152

had a single *Bam*HI site, DNA from exconjugants was expected to show hybridisation of two fragments to probe in the case of single copy integration.

For *Streptomyces* sp. US24 exconjugants, obtained hybridisation patterns were identical and showed the presence of three bands of about 5.7, 6.2, and 7.8 Kb (Figure 1(a)). The same probe did not hybridise to any DNA fragment of the wild type genomic DNA. This result implied that the plasmid was presumably integrated at the same locus in all corresponding exconjugants. However, among the three obtained bands, the one of 5.7 kb corresponds exactly to the size of the linearized vector pSET152. This fact can be explained via the tandem integration of two copies of the pSET152 vector in the chromosome of *Streptomyces* sp. US24 strain (Figure 1(b)). The presence of tandemly repeated copies of pSET152 vector in the *attB* site can be explained by the property of pSET152 to integrate at *attR* or *attL* sites via *attP/attR* or *attP/attL* recombination. Normally, the integration of pSET152 vector will be via *attB/attP* recombination. So, in certain cases, we can assist to the multiple independent recombination events, and pSET152 can integrate at *attL* or *attR* sites via *attP/attL* or *attP/attR*. As was hypothesised by Combes et al. [10], if this event occurs, this reaction must be specific to the *attL* and *attR* sites as tandem repeats of pSET152 in the pseudo-*attB* sites were not generally observed. Alternatively, the generation of tandem repeats of pSET152 in the *attB* may be a consequence of the rate of integration. If the rate of recombination into the *attB* site is rapid, integration occurs early during the mating period, and when the hyphae containing integrated pSET152 receives a further copy of pSET152, this last one may, generally, integrate by homologous recombination to generate a tandem repeat.

Concerning *Streptomyces* TN 58, two identical hybridisation signals were obtained for all eight analysed exconjugants corresponding to two DNA fragments of about 6.8 and 8 Kb (Figure 2(a)). The same probe did not hybridise to any DNA fragment of untransformed *Streptomyces* sp. TN 58 genomic DNA. This result implied that a single copy of the pSET152 plasmid was integrated at the same locus in all the exconjugants in strain *Streptomyces* sp. TN 58. We can suggest that the chromosome of this strain contains a single functional *attB* site for vectors that integrate site specifically using the bacteriophage ϕ C31 *att/int* system (Figure 2(b)). All together, our data strongly suggest the presence of an efficiently recognised *attB* site in the chromosome of the two studied *Streptomyces* strains.

3.2. Identification of the Φ C31 *attB* Sites. Integration of pSET152 into the *Streptomyces* sp. US 24 and TN 58 strains chromosomes implies the existence of one *attB* site recognised by the Φ C31 integrase. Taking advantage of the two PCR primers (ATTB1 and ATTB2) described by Combes et al., [10], we recovered a unique PCR product at the expected size of approximately 0.3 Kb from the chromosomal DNAs of the two studied strains. These fragments were cloned into the pGEM-T Easy vector yielding pSS4 and pSS5 plasmids for US 24 and TN 58 strains, respectively. The insert of three randomly chosen clones for pSS4 and

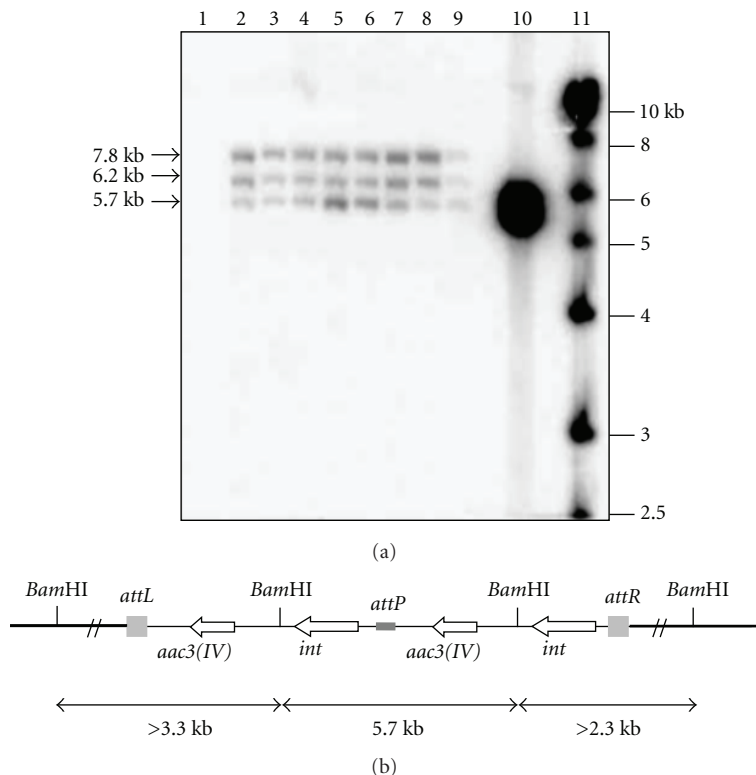


FIGURE 1: (a) Southern blot of *Streptomyces* sp. US24 *Bam*HI-digested total DNA hybridised with 32 P-labeled pSET152 plasmid. Lane 1, untransformed wild type *Streptomyces* sp. US24; lanes 2–9, the eight studied exconjugants; lane 10, *Bam*HI-digested pSET152 plasmid DNA (5.7 Kb); lane 11, the 1 kb ladder used as DNA marker. (b) Schematic representation of tandemly insertion of the pSET152 into the *Streptomyces* sp. US24 *attB* site. Chromosomal DNA hybridising is represented by thin lines and pSET152 by thick line. The integrase gene (*int*), and the apramycin resistance gene [*aac3(IV)*] from pSET152 *attP*, *attL*, and *attR* sites are also shown.

pSS5 plasmids was sequenced. All three nucleotide sequences were identical for each plasmid insert. The two inserts possess a size of 292 bp and 293 bp for US 24 and TN 58 strains, respectively, and a high nucleotide identity of 86% (Figure 3). These two amplified DNA fragments encode a homolog of the *Streptomyces coelicolor* A3(2) *Sco3798* ORF with a nucleotide identity of 92% and 86% for *Streptomyces* sp. US 24 and *Streptomyces* TN 58 strains, respectively. This ORF is a putative chromosome condensation protein, with sequence similarity to a mammalian-encoded protein, pirin (an identified nuclear protein that interacts with Bcl-3 and nuclear factor I), in which the *attB* site of Φ C31 lies in various *Streptomyces* species [32].

For *Streptomyces* species, all characterised Φ C31 *attB* sites showed high nucleotide sequence similarity varying from 82.4% to 100%. However, the presence of pseudo-*attB* sites (50% – 70.3% nt identity to *attB* sites) into which integration occurred at a frequency 300 times lower than into the corresponding *attB* site [10] has also been reported in this genus of bacteria. The multiple-sequence alignment of *attB* sites of *Streptomyces* sp. US 24 and TN 58 with several other *Streptomyces* species is shown in Figure 4. This alignment indicates that these sequences are conserved, and some positions in the minimal site can tolerate nucleotide changes. Kuhstoss and Rao [33] reported, by comparing the *S. ambifaciens attB* site with *attP*, that the core sequence

(i.e., the region at which the crossover occurs) is 5' TTTG. Combes et al., [10] had shortened this sequence to two nucleotides 5' TT which was present in the *attB* site of both studied strains *Streptomyces* sp. US 24 and TN 58.

3.3. Site-Specific Integration Does Not Affect the Essential Functions and Active Molecule Production of *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 Strains. Despite their wide use and clear advantages, it has been reported that integration of the integrative vectors into the ϕ C31 *attB* site can cause detrimental effects on antibiotic production in some strains [34]. In fact, it should be considered that the decrease in antibiotics production for many *Streptomyces* strains carrying integrated site specific plasmids such as *S. fradiae* producer of tylosin, *S. kanamyceticus* producer of kanamycin and others has been reported [35]. Therefore, while integrative vectors could be used for the development of stable recombinant producers of antibiotics, their possible negative effect on the level of antibiotic production should be considered. Thus, in order to determine whether the site-specific integration of the plasmid pSET152 affected relevant functions of *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 strains, we have followed the growth of both strain and their corresponding exconjugants carrying plasmid pSET152. All exconjugants grew well in the minimal

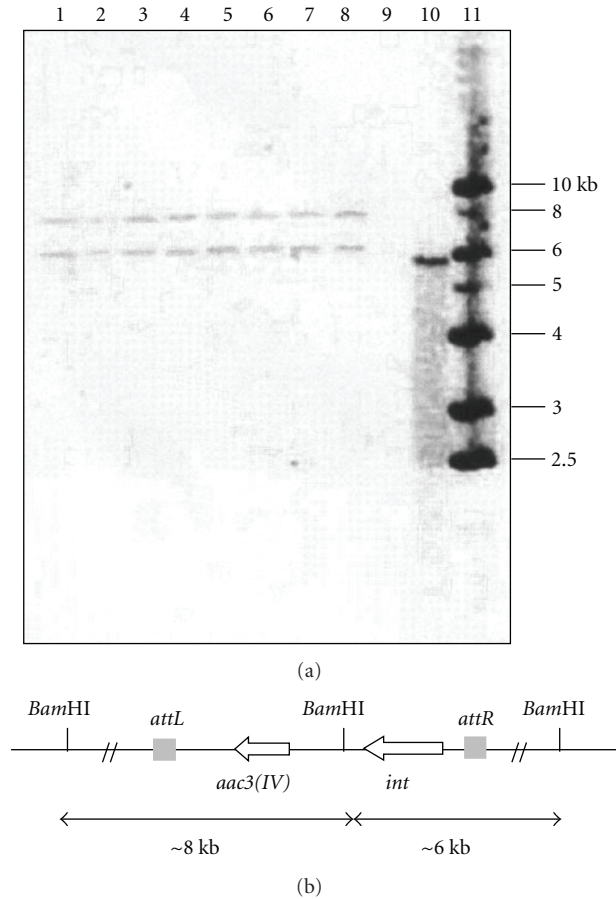


FIGURE 2: (a) Southern blot of *Streptomyces* sp. TN 58 *Bam*HI-digested total DNA hybridised with ³²P-labeled pSET152 plasmid. Lanes 1–8, the eight studied exconjugants; lane 9, untransformed wild type *Streptomyces* sp. TN 58; lane 10, *Bam*HI-digested pSET152 plasmid DNA (5.7 Kb); lane 11, the 1 kb ladder used as DNA marker. (b) Schematic representation of the integration of plasmid pSET152 in the chromosome of *Streptomyces* sp. TN 58 by site-specific recombination.

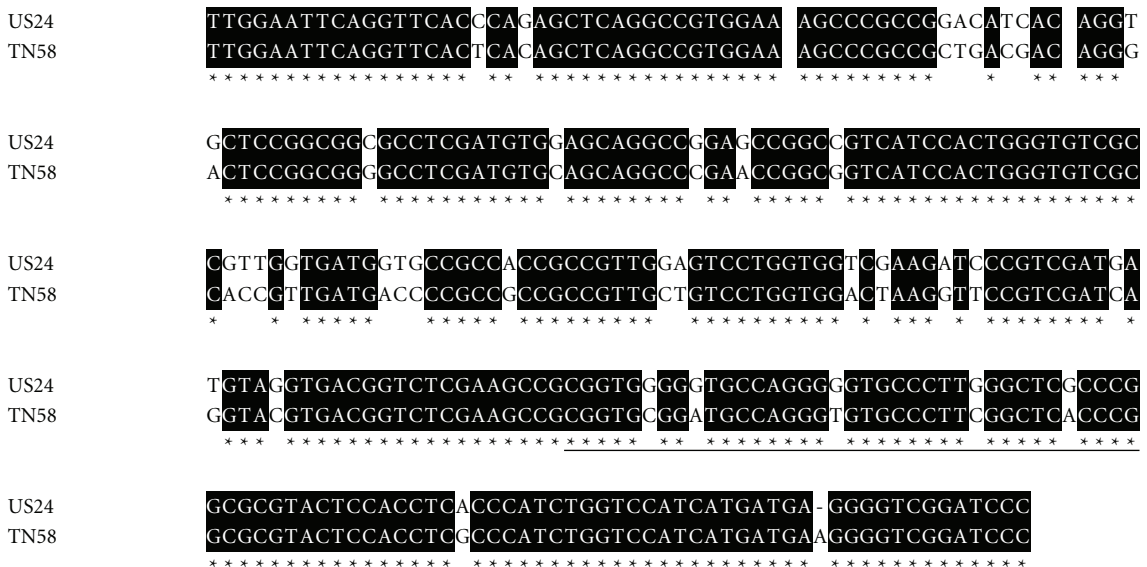


FIGURE 3: Alignment of the *attB* region of *Streptomyces* sp. US 24 and TN 58 strains. Solid boxes, identical sequences, underlined sequence, *attB* site.

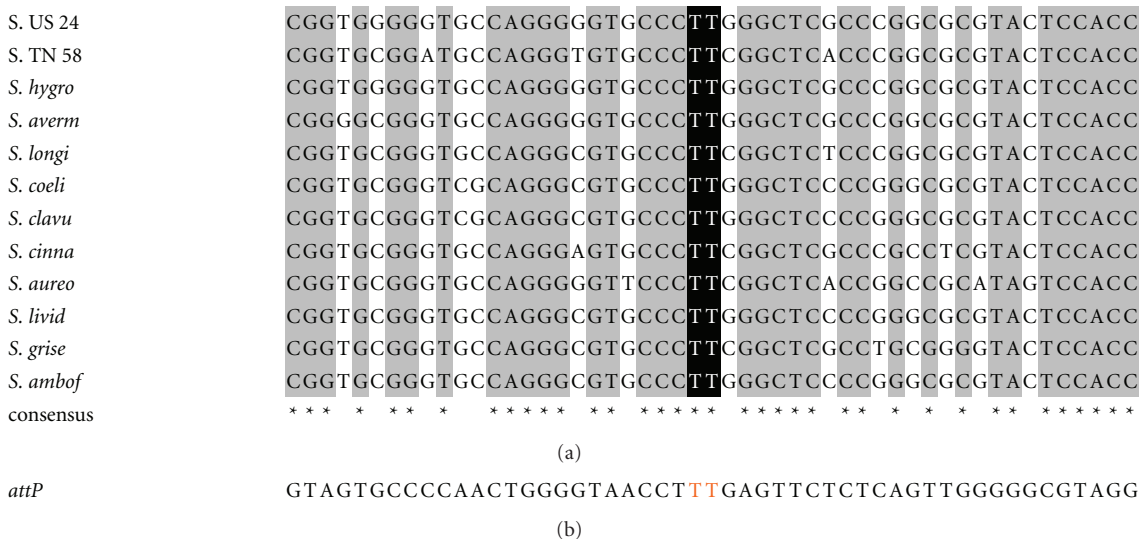


FIGURE 4: Natural variation of Φ C31 *attB* sites in various species of *Streptomyces*. (a) Alignment of *attB* nucleotide sequence of *Streptomyces* sp. US 24 strain (S. US 24) and *Streptomyces* sp. TN 58 (S. TN 58) with *attB* sequences of *S. hygroscopicus* NRRL5491 (*S. hygro*); *S. avermitilis* MA-4680 (*S. aver*); *S. longisporoflavus* 83E6 (*S. longi*); *S. coelicolor* (*S. coel*); *S. clavugerus* (*S. clav*); *S. cinnamomensis* (*S. cinna*); *S. aureofaciens* (*S. aureo*); *S. lividans* 66 TK64 (*S. livid*); *S. griseus* ATCC 12475 (*S. grise*); *S. ambofaciens* (*S. ambof*). Dark boxes, identical sequences; black shading sequence at which crossover occurs (TT) for the natural *attB* and *attP* sites. (b); Sequence of the *attP* site in ϕ C31.

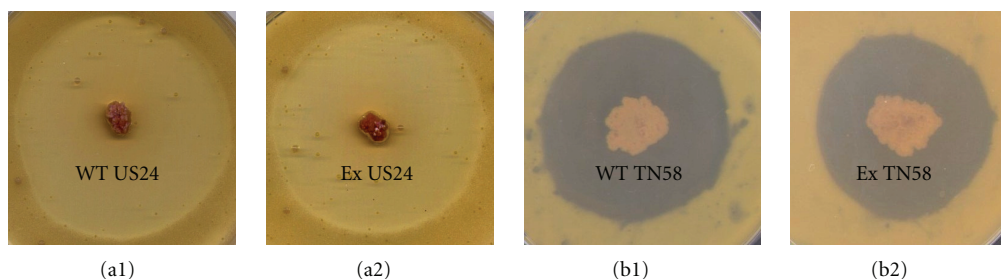


FIGURE 5: Antibacterial activities in solid media against *M. luteus* of the *Streptomyces* sp. US 24 strain wild type “WT US24” (a1) and one corresponding exconjugant US24/pSET152 “Ex US24” (a2) and *Streptomyces* TN 58 strain wild type “WT TN 58” (b1) and one corresponding exconjugant TN 58/pSET152 “Ex TN 58” (b2).

medium, and there was no difference compared to the wild type strains. The presence of pSET152 had no effects on growth and sporulation of the two strains. We have studied the stability of the pSET152 inheritance in exconjugants. This stability was determined as the proportion of colonies that retained resistance to apramycin (at $50 \mu\text{g mL}^{-1}$) after several passages of the exconjugants under nonselective conditions. Plasmid pSET152 was inherited every time for the all studied exconjugants under nonselective conditions for both strains. Meanwhile, antimicrobial activity was determined by observing bacterial growth inhibition of the wild type strains (US 24 and TN 58) and corresponding exconjugants. There was no obvious difference between wild type *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 and their corresponding exconjugants (Figure 5 and Table 1). In addition, the analysis on TLC plates of the ten studied active crude extracts revealed the presence of the expected active molecules in each strain and its corresponding exconjugants. For the *Streptomyces* sp. US 24 strain and its four studied exconjugants, two bands

were easily detected on TLC plates under UV light at 254 nm after spraying with anisaldehyde/sulphuric acid. The first band gives an orange colouration with a retention factor, $R_f = 0.46$ ($\text{CHCl}_3/\text{MeOH}$ 10%) and the second one a violet colouration, $R_f = 0.37$ ($\text{CH}_2\text{Cl}_2/9\%$ MeOH). According to our previously work [14], these two bands correspond to the 3-indolethanol and the Cyclo (L-Phe, L-Pro) dike-topiperazine active molecules, respectively. Concerning the *Streptomyces* sp. TN 58 strain and its studied exconjugants, the five active molecules (M1 to M5) were present in all five crude extracts. M1 belongs to the acetyltryptamine family and gives a colouration orange and violet after anisaldehyde/sulphuric acid and Ehrlich’s reagent visualisation. The R_f of this molecule is 0.5 ($\text{CHCl}_3/5\%$ MeOH). M2, $R_f = 0.57$ ($\text{CH}_2\text{Cl}_2/1\%$ MeOH), and M3, $R_f = 0.47$, ($\text{CHCl}_3/5\%$ MeOH) belong to the thiazole and brevinamide families, respectively. M2 gives a chestnut colouration on spraying with anisaldehyde/sulphuric acid and M3 gives a pinkish colouration after Ehrlich’s reagent visualisation.

TABLE 1: Antibacterial activities in liquid media against *M. luteus* and *E. coli* of the two studied *Streptomyces* strains and their corresponding exconjugants. WT (wild type). Ex (Exconjugant).

Indicator microorganisms	Diameter (mm) of inhibition zones									
	<i>Streptomyces</i> sp. US24					<i>Streptomyces</i> TN 58				
	WT	Ex.24 ¹	Ex.24 ²	Ex.24 ³	Ex.24 ⁴	WT	Ex.58 ¹	Ex.58 ²	Ex.58 ³	Ex.58 ⁴
<i>M. luteus</i>	20	19	20	21	20	17	17	16	17	17
<i>E. coli</i>	21	21	20	21	21	15	15	15	16	16

TABLE 2: Glucose isomerase activity of *Streptomyces* TN 58 wild type (*S.* TN 58), *Streptomyces* TN 58/pSET152 (*S.* TN 58/pSET152), *Streptomyces* sp. SK (*S.* SK), CBS4, and *Streptomyces* TN 58/*xylA* (*S.* TN 58/*xylA*) strains from 48-hour old cultures.

	<i>S.</i> TN 58	<i>S.</i> TN 58/pSET152	<i>S.</i> SK	CBS4	<i>S.</i> TN 58/ <i>xylA</i>
Specific activity U mg ⁻¹	0	0	0.1	1.68	1.81

The two other active molecules (M4 and M5) characterised from the *Streptomyces* sp. TN 58 strain, and belonging to the rhamnopyranosides family, do not have antimicrobial activities, but they possess inhibitory activity towards 3 α -hydroxysteroid dehydrogenase (3 α -HSD). Indeed, the rhamnosylated aromatic compounds were easily detected on TLC plates due to their striking yellow/greenish colouration after visualisation with anisaldehyde vapour. For all five analysed crude extracts, the wild type one (from the *Streptomyces* sp. TN 58 strain) and those from the four corresponding exconjugants, we obtained similar TLC migration profiles and two yellow/greenish bands having a retention factor (CHCl₃/15% MeOH) of 0.22 and 0.33 corresponding to the two molecules belonging to the rhamnopyranoside family. For the five active molecules of the *Streptomyces* sp. TN 58 and its four studied exconjugants, TLC profiles, bands colouration, and retention factors of the different active compounds were in perfect concordance with the results of previously works [15]. These data clearly demonstrated that the morphological differentiation and active molecule production of all studied pSET152 integrated exconjugants were similar to those of wild type *Streptomyces* sp. US 24 and *Streptomyces* sp. TN 58 strains. Consequently, conjugal transfer using *attP/B* site integration can be concluded to be a suitable means of gene transfer and expression for both studied strains.

3.4. Heterologous Expression of *xylA* Gene in *Streptomyces* sp. TN 58 Strain. The establishment of a gene transfer system and the stable integration of cloned genes at the specific loci in the genome of *Streptomyces* sp. TN 58 prompted us to express some heterologous gene in this strain. Xylose isomerase (D-xylose ketol isomerase EC 5.3.1.5) catalyses the reversible isomerisation of D-xylose into D-xylulose. It is also referred to as glucose isomerase (GI) because of its ability to convert D-glucose to d-fructose. This property is widely exploited industrially for the production of high-fructose syrup from starch [36]. Thus, the enhancement of strain productivity is certainly of importance. So we expressed the *xylA* gene cloned from *Streptomyces* sp. SK [17], and we attempted to examine whether this gene could express and fold effectively in strain TN 58. It should be noted that the wild

type *Streptomyces* TN 58 strain cannot assimilate the xylose as solely carbon source. Heterologous *xylA* gene was inserted into pSET152 to yield pSS6 plasmid. Based on intergeneric conjugal transfer, transformation efficiency of strain TN 58 by plasmid pSS6 was achieved at high frequency (10⁻⁴ exconjugants recipient⁻¹). The chromosomal structure of three TN 58/*xylA* strains, arbitrary chosen, was checked by Southern Blot hybridisation using the 800 bp *Pst*I DNA fragment internal to pSET152 as probe, which contain the oriT region. As expected, a unique and identical large *Bam*HI band was obtained for the three TN 58/*xylA* strains chromosomal DNA, whereas wild type strain TN 58 chromosomal DNA does not hybridise to the probe (data not shown). One of the three recombinant strains studied was subcloned five times on a solid media in the absence of apramycin followed by cultivation for several generations in liquid media in the absence of selection pressure. The plating of this culture on solid media, on both with and without selective pressures, showed that 100% of the colonies were apramycin resistant and able to grow in the presence of xylose as solely carbon source.

Determination of the mycelial intracellular glucose isomerase activity of *Streptomyces* TN 58, *Streptomyces* TN 58/pSET152, *Streptomyces* sp. SK, CBS4, and TN 58/*xylA* strains shows that glucose isomerase activity of the TN 58/*xylA* recombinant strain is of about eighteenfold higher than that of the SK strain and comparable to that of CBS4 strain. No activity was detected for the wild type TN 58 strain and the recombinant strain TN 58/pSET152 (Table 2). Study of the growth of the two strains, *Streptomyces* TN 58/*xylA* and CBS4, in minimum liquid media containing xylose as solely carbon source showed that the two strains grew well, and for each time, the resulting biomass was quite similar. It should be noted that glucose isomerase activity is correlated with biomass production, and optimum activity has been obtained after 48 hours of incubation.

According to these results, we can deduce that site-specific recombination directed by pSET152 makes it possible to stably insert heterologous DNA in strain TN 58. This should be very important for a number of cases such as genes expression, genetic complementation, and biosynthetic pathway manipulations of the active molecules of this strain especially those belonging to the rhamnopyranoside family.

Acknowledgments

This work was supported by the Tunisian government (Contract Program CBS-LEMP) and the CMCU project (2006–2008) no. 06/S 0901 “MELLOULI/AIGLE”.

References

- [1] P. Mazodier, R. Petter, and C. Thompson, “Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species,” *Journal of Bacteriology*, vol. 171, no. 6, pp. 3583–3585, 1989.
- [2] M. Bierman, R. Logan, K. O’Brien, E. T. Seno, R. N. Rao, and B. E. Schoner, “Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp,” *Gene*, vol. 116, no. 1, pp. 43–49, 1992.
- [3] P. Matsushima and R. H. Baltz, “A gene cloning system for ‘*Streptomyces toyocaensis*,’” *Microbiology*, vol. 142, no. 2, pp. 261–267, 1996.
- [4] R. Fouces, M. Rodríguez, E. Mellado, B. Díez, and J. L. Barredo, “Conjugation and transformation of *Streptomyces* species by tylosin resistance,” *FEMS Microbiology Letters*, vol. 186, no. 2, pp. 319–325, 2000.
- [5] D. J. MacNeil, J. L. Occi, K. M. Gewain, et al., “Complex organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase,” *Gene*, vol. 115, no. 1-2, pp. 119–125, 1992.
- [6] E. Stegmann, S. Pelzer, K. Wilken, and W. Wohlleben, “Development of three different gene cloning systems for genetic investigation of the new species *Amycolatopsis japonicum* MG417-CF17, the ethylenediaminedisuccinic acid producer,” *Journal of Biotechnology*, vol. 92, no. 2, pp. 195–204, 2001.
- [7] T. Voeykova, L. Emelyanova, V. Tabakov, and N. Mkrtumyan, “Transfer of plasmid pTO1 from *Escherichia coli* to various representatives of the order Actinomycetales by intergeneric conjugation,” *FEMS Microbiology Letters*, vol. 162, no. 1, pp. 47–52, 1998.
- [8] R. Fouces, M. Rodríguez, E. Mellado, B. Díez, and J. L. Barredo, “Conjugation and transformation of *Streptomyces* species by tylosin resistance,” *FEMS Microbiology Letters*, vol. 186, no. 2, pp. 319–325, 2000.
- [9] A. C. Groth, E. C. Olivares, B. Thyagarajan, and M. P. Calos, “A phage integrase directs efficient site-specific integration in human cells,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 11, pp. 5995–6000, 2000.
- [10] P. Combes, R. Till, S. Bee, and M. C. M. Smith, “The *Streptomyces* genome contains multiple pseudo-*attB* sites for the ϕ C31-encoded site-specific recombination system,” *Journal of Bacteriology*, vol. 184, no. 20, pp. 5746–5752, 2002.
- [11] M. A. Gregory, R. Till, and M. C. M. Smith, “Integration site for *Streptomyces* phage ϕ BT1 and development of site-specific integrating vectors,” *Journal of Bacteriology*, vol. 185, no. 17, pp. 5320–5323, 2003.
- [12] L. Mellouli, R. Ben Ameer-Mehdi, S. Sioud, M. Salem, and S. Bejar, “Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated *Streptomyces* sp. US24 strain,” *Research in Microbiology*, vol. 154, no. 5, pp. 345–352, 2003.
- [13] L. Mellouli, I. Karray-Rebai, S. Sioud, R. Ben Ameer-Mehdi, B. Naili, and S. Bejar, “Efficient transformation procedure of a newly isolated *Streptomyces* sp. TN58 strain producing antibacterial activities,” *Current Microbiology*, vol. 49, no. 6, pp. 400–406, 2004.
- [14] R. Ben Ameer-Mehdi, L. Mellouli, F. Chabchoub, S. Fotso, and S. Bejar, “Purification and structure elucidation of two biologically active molecules from a new isolated *Streptomyces* sp. US 24 strain,” *Chemistry of Natural Compounds*, vol. 40, no. 5, pp. 510–513, 2004.
- [15] R. Ben Ameer-Mehdi, K. A. Shaaban, I. Karray-Rebai, S. Smaoui, S. Bejar, and L. Mellouli, “Five naturally bioactive molecules including two rhamnopyranoside derivatives isolated from the *Streptomyces* sp. strain TN58,” *Natural Product Research*. In press.
- [16] D. Hanahan, “Studies on transformation of *Escherichia coli* with plasmids,” *Journal of Molecular Biology*, vol. 166, no. 4, pp. 557–580, 1983.
- [17] K. Srih-Belghith and S. Bejar, “A thermostable glucose isomerase having a relatively low optimum pH: study of activity and molecular cloning of the corresponding gene,” *Biotechnology Letters*, vol. 20, no. 6, pp. 553–556, 1998.
- [18] M. Mezghani, M. A. Borgi, R. Kammoun, H. Aouissou, and S. Bejar, “Construction of new stable strain over-expressing the glucose isomerase of the *Streptomyces* sp. SK strain,” *Enzyme and Microbial Technology*, vol. 37, no. 7, pp. 735–738, 2005.
- [19] M. S. B. Paget, L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner, “Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2),” *Journal of Bacteriology*, vol. 181, no. 1, pp. 204–211, 1999.
- [20] J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2nd edition, 1989.
- [21] D. A. Hopwood, M. J. Bibb, K. F. Chater, et al., *Genetic Manipulation of Streptomyces: A Laboratory Manual*, The John Innes Foundation, Norwich, UK, 1985.
- [22] C. J. Thompson, J. M. Ward, and D. A. Hopwood, “DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species,” *Nature*, vol. 286, no. 5772, pp. 525–527, 1980.
- [23] R. H. Baltz, “Genetic recombination by protoplast fusion in *Streptomyces*,” *Developments in Industrial Microbiology*, vol. 21, pp. 43–54, 1980.
- [24] F. Flett, V. Mersinias, and C. P. Smith, “High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes,” *FEMS Microbiology Letters*, vol. 155, no. 2, pp. 223–229, 1997.
- [25] T. Kieser, M. J. Bibb, M. J. Bittner, K. F. Chater, and D. A. Hopwood, *Practical Streptomyces Genetics: A Laboratory Manual*, The John Innes Foundation, Norwich, UK, 2000.
- [26] T. E. Maniatis, E. F. Fritsch, and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA, 1982.
- [27] F. Sanger, S. Nicklen, and A. R. Coulson, “DNA sequencing with chain-terminating inhibitors,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 12, pp. 5463–5467, 1977.
- [28] S. F. Altschul, T. L. Madden, A. A. Schäffer, et al., “Gapped BLAST and PSI-BLAST: a new generation of protein database search programs,” *Nucleic Acids Research*, vol. 25, no. 17, pp. 3389–3402, 1997.
- [29] M. J. Bibb, G. R. Janssen, and J. M. Ward, “Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*,” *Gene*, vol. 38, no. 1–3, pp. 215–226, 1985.
- [30] M. M. Bradford, “A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the

- principle of protein dye binding,” *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.
- [31] J. Nikodinovic, K. D. Barrow, and J.-A. Chuck, “High frequency transformation of the Amphotericin-producing bacterium *Streptomyces nodosus*,” *Journal of Microbiological Methods*, vol. 55, no. 1, pp. 273–277, 2003.
- [32] S.-U. Choi, C.-K. Lee, Y.-I. Hwang, H. Kinoshita, and T. Nihira, “Intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to *Kitasatospora setae*, a bafilomycin B₁ producer,” *Archives of Microbiology*, vol. 181, no. 4, pp. 294–298, 2004.
- [33] S. Kuhstoss and R. N. Rao, “Analysis of the integration function of the streptomycete bacteriophage ϕ C31,” *Journal of Molecular Biology*, vol. 222, no. 4, pp. 897–908, 1991.
- [34] R. H. Baltz, “Genetic manipulation of antibiotic-producing *Streptomyces*,” *Trends in Microbiology*, vol. 6, no. 2, pp. 76–83, 1998.
- [35] A. N. Luzhetskii, B. E. Ostash, and V. A. Fedorenko, “Intergeneric conjugation *Escherichia coli*—*Streptomyces globisporus* 1912 using integrative plasmid pSET152 and its derivatives,” *Russian Journal of Genetics*, vol. 37, no. 10, pp. 1123–1129, 2001.
- [36] S. H. Bhosale, M. B. Rao, and V. V. Deshpande, “Molecular and industrial aspects of glucose isomerase,” *Microbiological Reviews*, vol. 60, no. 2, pp. 280–300, 1996.



Hindawi

Submit your manuscripts at
<http://www.hindawi.com>

