

Research Article

Targeted Gene Disruption of the Cyclo (L-Phe, L-Pro) Biosynthetic Pathway in *Streptomyces* sp. US24 Strain

Samiha Sioud,¹ Ines Karray-Rebai,¹ Hedi Aouissaoui,¹ Bertrand Aigle,² 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 K, 3038 Sfax, Tunisia

²Laboratory of Genetics and Microbiology, UMR/INRA 1128, IFR 110, Faculty of Sciences and Techniques,
University Henri Poincaré, 54506 Vandoeuvre-lès-Nancy, France

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We have previously isolated a new actinomycete strain from Tunisian soil called *Streptomyces* sp. US24, and have shown that it produces two bioactive molecules including a Cyclo (L-Phe, L-Pro) diketopiperazine (DKP). To identify the structural genes responsible for the synthesis of this DKP derivative, a PCR amplification (696 bp) was carried out using the *Streptomyces* sp. US24 genomic DNA as template and two degenerate oligonucleotides designed by analogy with genes encoding peptide synthetases (NRPS). The detection of DKP derivative biosynthetic pathway of the *Streptomyces* sp. US24 strain was then achieved by gene disruption via homologous recombination using a suicide vector derived from the conjugative plasmid pSET152 and containing the PCR product. Chromatography analysis, biological tests and spectroscopic studies of supernatant cultures of the wild-type *Streptomyces* sp. US24 strain and three mutants obtained by this gene targeting disruption approach showed that the amplified DNA fragment is required for Cyclo (L-Phe, L-Pro) biosynthesis in *Streptomyces* sp. US24 strain. This DKP derivative seems to be produced either directly via a nonribosomal pathway or as a side product in the course of nonribosomal synthesis of a longer peptide.

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1. INTRODUCTION

Peptides are involved in a wide number of physiologic and biochemical processes, including metabolism, pain, reproduction, and the immune response. While many naturally occurring peptides are linear compounds, nature also provides us with a wide variety of monocyclic and polycyclic peptides and proteins of all different shapes and sizes [1]. Among them, diketopiperazines (DKP), cyclic dipeptides, are the smallest head-to-tail cyclic peptides and form a very attractive family because of their many potential uses. In fact, useful biological properties have been demonstrated for some of them such as antibacterial, fungicidal, herbicidal, antiviral, immunosuppressor, and antitumour activities [2, 3]. Although the number of newly isolated naturally occurring DKPs has increased during the last few years, the biosynthetic pathways of these molecules remain largely unexplored. Generally, DKP derivatives seem to be produced by three different ways. The first one is nonribosomal pathway and product formation takes place under the catalytic control of a large multimodular enzyme complex, termed NRP syn-

thetase (NRPS). Molecular characterization of NRPS genes has revealed a modular organisation [4] in which each module within this biosynthetic assembly line is responsible for the recognition, activation, and incorporation of a certain amino acid into the nascent peptide product. Such catalytic unit (module) is composed of functionally specific and independent domains, each of them responsible for catalyzing one single reaction. Much of the attention in the field of NRPSs is attracted by the potential to obtain new peptide products with novel biological activities by directed engineering of the biosynthetic genes. In fact, the modular organisation of NRPSs lends itself to biocombinatorial approaches, such as domain and module swapping, as well as fusion and truncation of existing catalytic units [5].

DKP derivatives can be also obtained as a side product in the course of nonribosomal synthesis. It is the case of the Cyclo (Phe-Pro) formation during the nonribosomal synthesis of tyrocidine A and gramicidin S in certain strains of *Bacillus brevis*. Tyrocidine A, (Phe-Pro-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu-)_{cyc}, is produced at the onset of the stationary phase of growth of *Bacillus brevis* ATCC 8185

strain via a nonribosomal pathway and possesses antibiotic activities against several gram positive bacteria [6]. Gramicidin S, a cyclic decapeptide consisting of two identical pentapeptides (Phe-Pro-Val-Orn-Leu)₂, is produced via a nonribosomal pathway by *Bacillus brevis* ATCC 9999 and presents antibacterial activities [7].

The third DKP biosynthesis way has been described from *Streptomyces noursei*. However, very little is known about the DKP derivatives from *Streptomyces* species and their biosynthetic pathways. The only isolated and studied DKP biosynthetic gene cluster from this bacterium genus is that of albonoursin of the *Streptomyces noursei* [8]. It has been reported by these authors that the biosynthesis of the albonoursin diketopiperazine is independent of nonribosomal peptide synthetases.

We have previously reported the isolation from Tunisian soil of a new actinomycete strain called *Streptomyces* sp. US24 producing divers biological activities, the purification, and the structure elucidation of two active molecules from this bacterium [9, 10]. The first one is the 3-indolethanol and the second active molecule is a DKP derivative, the Cyclo (L-Phe, L-Pro) diketopiperazine. The aim of this work was to detect the biosynthetic pathway of this DKP derivative. Our strategy is based on gene disruption approach by homologous recombination via a suicide vector derived from pSET152 [11] containing a 696 bp DNA fragment amplified by PCR using the *Streptomyces* sp. US24 genomic DNA as a template. The detection of the biosynthetic pathway of the Cyclo (L-Phe, L-Pro) diketopiperazine (DKP) derivative from the *Streptomyces* sp. US24 strain will permit the study and the comprehension of the natural biosynthesis of diketopiperazines from *Streptomyces* genus and consequently the production of hybrid DKP molecules having interesting biological and pharmacological activities.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and primers

Bacterial strains, plasmids, and primers used in this work are listed in Table 1.

2.2. Media and culture conditions

E. coli strains were grown in Luria broth (LB) medium. Ampicillin (50 µg mL⁻¹), apramycin (50 µg mL⁻¹), chloramphenicol (25 µg mL⁻¹), nalidixic acid and kanamycin (50 µg mL⁻¹) were added to growth media when required and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 µg mL⁻¹) when appropriate [12]. Transformation of *E. coli* DH5α with pGEM-T derivatives was carried out according to the manufacturer's instructions (Promega, Madison, USA).

Streptomyces sp. US24 strain was grown in Tryptone soya broth medium (TSB: 30 g tryptic soy broth plus 5 g Yeast extract per 1000 mL distilled water) for the preparation of genomic DNA [13] and on R2YE [14] plates for the preparation of spore stocks. TSB supplemented with 30 µg of apramycin mL⁻¹ was used to grow exconjugants.

AS1 medium [18]: 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, and cultures were grown at 37°C.

For biological activity production, *Streptomyces* sp. US24 strain and the corresponding exconjugants were grown in TSB medium, supplemented at 1% (w/v) with starch and 0.1% (v/v) of a trace element solution: (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).

For antibacterial activities determination, indicator microorganism *Micrococcus luteus* LB14110 was grown overnight in LB medium at 30°C, then diluted 1:100 in LB medium and incubated for 5 hours under constant agitation of 200 rpm at the appropriate temperature.

2.3. Intergeneric conjugation

Intergeneric conjugation between *E. coli* and *Streptomyces* sp. US24 strain was performed as described previously by Flett et al. [19], with some 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 *Streptomyces* sp. US24 spore suspension store at –20°C were used as recipients. Spores (eq10⁸) were washed in 2x Yeast extract Tryptone medium [20], resuspended in 500 µL of 2x 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 pellet was resuspended in the residual liquid and the undiluted mixture was plated on AS1 plates containing 10 mmol MgCl₂ and incubated at 37°C for 18 hours. The plates were then overlaid each with 1 mL of water containing 500 µg of nalidixic acid and 1 mg of apramycin, incubated further for 5 days at 37°C. The exconjugants were then counted. Cultures prepared from some arbitrary chosen clones were used for further studies.

2.4. DNA isolation and manipulation

Total DNA preparation was carried out from *Streptomyces* strains (sp. US24 and the corresponding exconjugants) according to Hopwood et al. [21]. Small-scale plasmid preparations from *E. coli* were performed as described by Sambrook et al. [12].

Digestion with restriction endonucleases, separation of DNA fragments by agarose gel electrophoresis, dephosphorylation with alkaline calf intestinal phosphatase, ligation of DNA fragments and transformation were done according to Sambrook et al. [12] for *E. coli* and Hopwood et al. [21] for *Streptomyces*.

For Southern blot manipulations [22], DNA was transferred to Hybond N membranes (Amersham, Buckinghamshire, UK). The hybridization conditions and subsequent

TABLE 1: Bacterial strains, plasmids, and primers used in this work.

Strain, plasmid, and primer	Description	Reference or Source
Strains		
<i>Streptomyces</i> sp. US24	Cyclo (L-Phe, L-Pro) diketopiperazine producer, used in this work as the source of chromosomal DNA to amplify the 696 pb DNA fragment as recipient for intergeneric conjugation and targeted gene disruption	[10]
<i>E. coli</i> DH5 α	General cloning host (F ⁻ ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>deoR thi-1 susE</i> 44 λ ⁻ <i>gyrA96 relA1</i>)	[15]
<i>E. coli</i> ET12567	methylation-defective (<i>dam-13</i> :: Tn9 <i>dcm-6 hsdM</i> Cmr) used for conjugation between <i>E. coli</i> and <i>Streptomyces</i>	[16]
<i>Micrococcus luteus</i>	Indicator microorganism	LB 14110
Plasmids		
pSET152	Integrative vector carrying ϕ C31 integrase gene and ϕ C31 <i>attP</i> site, <i>oriT</i> and an apramycin resistance gene for selection in <i>Streptomyces</i> and <i>E. coli</i>	[11]
pUZ8002	RK2 derivative with a mutation in <i>oriT</i> .	[17]
pGEM-T Easy vector	TA cloning vector Amp ^R (PCR products cloning)	Promega
pSS1	Derivative of pGEM-T Easy vector carrying a 696 pb DNA fragment involved in the biosynthetic pathway of Cyclo (L-Phe, L-Pro) DKP of the US24 strain	This work
pSS2	Suicide vector derivative of the pSET152	This work
pSS3	pSS2 carrying the <i>EcoRI</i> DNA fragment insert from pSS1	This work
Primers		
[P1/P2]	Primers used for the amplification of the 696 pb DNA fragment	This work
P1	5'ATCTACAC(G/C)AGCGGGACGAC(G/C)GGC3'	
P2	5'(G/C)AGGTCGCC(G/C)GTGCGGTACAT3'	

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 696 bp DNA fragment, involved in the biosynthesis pathway of Cyclo (L-Phe, L-Pro) DKP of the *Streptomyces* sp. US24 strain, was performed using two degenerate primers (P1, P2) as described in Table 1. Approximately 150 ng genomic DNA was used as template with 100 pmol of each primer per 50 μ L reaction volume. To improve the denaturation of the DNA, 5% (v/v) DMSO was added to the reaction mixture. Amplification was performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster city, USA) using 1U Pfu DNA polymerase (Stratagene, Amsterdam, The Netherlands) 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 (60 seconds) and extension at 72°C (90 seconds). The PCR products were analysed by agarose gel electrophoresis and fragment of the expected size was purified then cloned into pGEM-T Easy vector yielding pSS1 plasmid.

Nucleotide sequences were determined on both strands using the dideoxy chain-termination method [23]. Reactions were performed with a Thermo sequenase cycle sequencing kit (Amersham) and specific primers. Homology search was performed using Blast Search algorithm [24].

2.5. Extraction, purification of active compounds, biological assay of antimicrobial activities, LC/MS and LC/MS/MS analysis

For the purification of the active molecules from the *Streptomyces* sp. US24 strain and three corresponding mutants (obtained after transfer of the pSS3 "see Table 1" into the *Streptomyces* sp. US24 strain), spores at 10⁷ mL⁻¹ were used to inoculate 500 mL Erlenmeyer flasks with four indents, containing 100 mL TSB medium supplemented at 1% (w/v) with starch and 0.1% (v/v) of a trace element solution [9]. After incubation at 37°C for 24 hours in an orbital incubator with shaking at 250 rpm, this preculture was used to inoculate (5% v/v) a total volume of 1 L culture medium having the same composition of the preculture. After three days incubation at 37°C in an orbital incubator with shaking at 250 rpm, each culture broth was filtered to separate mycelium and supernatant. The supernatant was extracted twice with an equal volume of ethyl acetate and then evaporated on a Rotavapor (Laborata 4000). The crude extract, approximately 100 mg for each culture, was dissolved in 500 μ L dichloromethane-methanol 1:1 and fractionated by HPLC. This analysis was performed with an analytical column (C18 column 7 μ m, 4.6 mm inner diameter 25 cm length). The elution was at a flow rate of 1 mL min⁻¹ with a gradient of two solutions A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid). After injection of the sample, the column was eluted

with a linear gradient from 100% buffer A to 50% buffer A and 50% buffer B over the first 40 minutes, followed by a linear gradient to 100% buffer B from 40 to 60 minutes, and then a steady flow of 100% buffer A through 10 minutes [10]. Elution was monitored using a UV detector at 220 nm.

LC/MS and LC/MS/MS analyses were performed using an LC/MSD Trap XCT–Electrospray (Agilent Technologies), operated in positive ionisation mode, equipped with an HPLC Agilent 100 DAD detector (C18 column Zorbax 300 2.1 × 150 mm). After injection of 5 µL of the corresponding sample, the column was eluted with a linear gradient 95% buffer A (water, 0.1% formic acid) and 5% buffer B (acetonitrile, 0.1% formic acid) through 2 minutes, followed by a linear gradient to 80% buffer B and then a steady flow of 20% buffer A, 80% buffer B through 3 minutes. The elution was at a flow rate of 250 µL min⁻¹.

For biological assay of antimicrobial activity, 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 seeded by 40 µL of a 5-hour-old culture of *M. luteus* LB 14110. After 2 hours at 4°C, plates were incubated at 30°C for overnight and then examined for evidence of antimicrobial activities represented by a growth inhibition zone of the indicator micro-organism around the paper disk.

3. RESULTS AND DISCUSSION

3.1. PCR amplification of a *Streptomyces* sp. US24 DNA fragment potentially involved in the Cyclo (L-Phe, L-Pro) DKP derivative biosynthesis

Although the biosynthetic pathways of DKP derivatives remain largely unexplored, in bacteria and fungi these active molecules generally seem to be produced by nonribosomal pathways (NRPS: nonribosomal peptide synthetases). In analogy to type-I polyketide synthases (PKSs), NRPSs exhibit a modular architecture and a module can be further subdivided into different domains, each responsible for a certain biochemical reaction. The adenylation domain (A-domain) controls the entry of the substrates into the peptide as it recognizes and adenylates its cognate substrate. The thiolation domain (T-domain) is referred to as peptidyl carrier protein (PCP), from where it is condensed with the aminoacyl or peptidyl moieties at the neighboring modules. The latter reaction is catalyzed by the condensation domain (C-domain). These three domains, C, A, and T, constitute a minimal elongation module, the basic repetitive unit of multimodular NRPSs. Accordingly, the first module of an NRPS complex lacks a C-domain, whereas the last module usually contains also a termination domain (Te-domain) to release the product. This modular architecture offers a real potentiality to construct new hybrid peptides with novel biological activities by directed engineering of the biosynthetic genes. The aim of this work was to detect the biosynthetic pathway of the Cyclo (L-Phe, L-Pro) active molecule produced by *Streptomyces* sp. US24. For that purpose, two degenerate oligonucleotides (P1 and P2) were prepared, according to the typical *Streptomyces* codon usage, based on the amino acid sequences alignment of two synthetases 1 of the Tyrocidine A and Gram-

icidin S, respectively. For these two decapeptides, Phe and Pro are the residues number one and two, respectively. Three genes (*tycA*, *tycB*, and *tycC*) coding for three proteins of 124, 405, and 724 kDa, respectively, are involved in the biosynthesis of the Tyrocidine A. TycA (synthetase 1) comprises only one module corresponding to the phenylalanine activase (phenylalanine activating domain) and racemase [25]. In the biosynthesis of the Gramicidin S, three open reading frames organised in one transcriptional unit were identified and the corresponding genes are called *grsT*, *grsA*, and *grsB*. The GrsA (Gramicidin S synthetase 1) is an enzyme with a molecular weight of 126.6 kDa which activates and racemize the amino acid phenylalanine as the first step in nonribosomal biosynthesis of the peptide antibiotic gramicidin S [7]. Comparison of the amino acid sequences of GrsA and TycA revealed an extensive degree of homology over 56%. If similar amino acids are also considered, the degree of homology increases to over 70% [26]. This amino acid comparison lets us find two conserved regions IYTS GTTG and MYRTGDL belonging to the adenylation domains of the two synthetases 1 GrsA and TycA. These two conserved regions are part of the known highly conserved core motifs of the adenylation domains of all known NRPSs. Accordingly, two degenerate PCR primers forward P1 and reverse P2 were designed (Table 1) and used in a PCR reaction with the *Streptomyces* sp. US24 genomic DNA as template. A PCR product with an expected size of approximately 700 bp was obtained. After purification, this DNA fragment was cloned in the pGEM-T Easy vector, yielding the pSS1 plasmid. The insert of four randomly chosen clones was sequenced. All obtained nucleotide sequences are identical and showed a typical *Streptomyces* GC content (about 76%). This identity can be explained by the high-stringency PCR conditions and the low degree of degeneration of the two used primers. Frame analysis of the nucleotide sequence (696 bp, accession no. AM282973) revealed the presence of a unique internal open reading frame. The alignment of the corresponding amino acid sequence (aa) with all available protein sequences using the Gapped BLAST and PSI-BLAST program [24] showed as expected important aa identities with different NRPS adenylation domains. This identity reaches 50% and 48% with the adenylation domains of the pristinamycin I (PI) synthetase 2, and actinomycin (ACM) synthetase III, respectively (Figure 1). These two adenylation domains activate the two amino acids L-threonine and L-aminobutyric of the PI produced by *Streptomyces pristinaespiralis*, and the three amino acids proline, N-methylglycine and N-methyl-L-valine of the ACM produced by *Streptomyces chrysomallus*, respectively [27, 28].

3.2. Construction of suicide vector derived from pSET152

Streptomyces sp. US24 was refractory to classical polyethylene glycol transformation and electroporation procedures (data not shown). Therefore, we decided to use another approach involving intergeneric conjugation from *E. coli* into *Streptomyces* [29]. As part of this approach, we have constructed a conjugative suicide vector derived from the integrative conjugative pSET152 vector. This plasmid was

US 24	1	-----YTSGTGEPK
SnbC	1603	VLLTDRATDTAHECRSMAGDADVLVVDLELPTGGGEPLPVVPAHPHQLAYMYTSGSTGNPK
AcmC	3096	LLITDHTTDLDTTTTQFN-PADTPHDGEDP---GNPNHTTHPDDAAAYIMYTSGSTGRPK
Consensus		vlltldrtdw ad ep p hp ayvmYTSGS TG PK
US 24	12	PSPYATATWPPWPPTAASPAVCATGCC LHSPVAFDAATFEVWGPLL TGGCVVVASRPLD
SnbC	1663	GVAITHGDVAALAAADTCWRGTGNQQRVLLHSPYSFDTSQYELWVPLL SGGTVVVAAPPGLD
AcmC	3151	GVIAATHRNIT ALALDPRDPTAHRVLLHSP TAFDASTYEIWVPLLNGNTVVIAP TGDLD
Consensus		g v th a v a l a d w r v l L H S P a F D a s t y E v W v P L L t G g t V V v A P s g d L D
US 24	72	SALLRRLVRDTGLSALWLTAGLFRLLAQDAPDCFTGLRQVWTGGDVVPA AAVRRLAACP
SnbC	1723	TRALERAIIVDGGVTGMWLT SGLFNLLAEESPHCFGTGVRVWVTGGDVVS PAAVARVLAASP
AcmC	3211	VHTYHRVITDQQIT ALWLT SWVFNLLTEQSPETFTTRVRQIWTGGEAVSGATVTR LQQACP
Consensus		s r l r R l i D g l t a l W L T s g l F n L L a e d s P d c F T g v R q v W T G G d v V s a A a V R v l a A c P
US 24	132	GLTVVDGYGPTETTTTFATSSAITDAAITVPDTPVPIGHPLDDMRVHVLDGRLRPVPPGCPGE
SnbC	1783	STMVADGYGPTETTTFATHFMRAPWQOETTVPVPIGTPLDNTTCHVLDLDRPVPVPGVTGE
AcmC	3271	DTTVVDGYGPTETTTFATHFPYPTPYTGSAVVPIGRPMATMHTYVLDDSLQPVAPGVTGE
Consensus		t t V v D G Y G P T E T T T F A T h h l p w t t V P I G h P l d m r h V L D d r L r P V p P G v t G E
US 24	192	LYLAGEGVARGYLGRPGD TAARFLADPACPPGS RMYRTGDL-----
SnbC	1843	LYICGSGLARGYLNRP ELTAERFVADPYAP-GGRMYRTGDLVRLRRDGVLTFLGRADHQV
AcmC	3331	LYLAGSGLARGYLD RPALTAERFVANPYAPG ERMYRTGDLARWNPDDHLEVA GRADHQV
Consensus		LYlAGsGlARGYL R P g l T A e R f v A d P y a p p G R M Y R T G D L r d l f g r a d h q v

FIGURE 1: Alignment of amino acid sequences of the 696 bp DNA fragment of *Streptomyces* sp. US24 with pristinamycin I synthase 2 (SnbC) of *Streptomyces pristinaespiralis*, and actinomycin synthase III (AcmC) of *Streptomyces chrysomallus*. Consensus sequence (cons) corresponds to the amino acids conserved. The two conserved regions used to design the two degenerate oligonucleotides (P1 and P2) are highlighted.

chosen because it is successfully transferred from *E. coli* ET12567/pUZ8002 into *Streptomyces* sp. US24 strain by intergeneric conjugation, and corresponding directed-site specific recombination has been characterised and analysed (data not shown). To obtain a nonintegrative form of pSET152, the plasmid was partially digested by the restriction endonuclease *Hind*III and a resulting 4.8 kb DNA fragment was purified, in which, a part of the integrase encoding gene of the pSET152 has been deleted. After ligation and transformation of *E. coli* cells, recombinant clones were obtained harbouring the desired pSET152 suicide derivative vector called pSS2.

3.3. Integration by homologous recombination

The 696 bp DNA fragment was purified from pSS1 after its digestion by *Eco*RI and then cloned into pSS2 digested by the same enzyme giving pSS3. This plasmid can be integrated into the chromosome of the *Streptomyces* sp. US24 strain only via homologous recombination. After transfer of the pSS3 plasmid into the *E. coli* ET12567 (pUZ8002) strain, we have realized an intergeneric conjugation between *E. coli* ET12567 (pUZ8002/pSS3) and *Streptomyces* sp. US24 strain. *Streptomyces* sp. US24 Apr^R (apramycin resistant) exconjugants were obtained with a frequency of 10^{-9} . This frequency is a thousand times less comparing to the transconjugation

efficiency (10^{-6}) (data not shown) of the native pSET152 which integrates into the *Streptomyces* sp. US24 chromosome by site-specific recombination between its *attP* and the chromosomal *attB* site. This observation lies well with the previously reported finding illustrating that integration via homologous recombination usually occurs 10^2 – 10^3 times less frequently than *attB/P*. mediated integration. All obtained exconjugants grew well and there are no morphological differences comparing to the wild-type strain. Homologous integration of pSS3 was verified by Southern hybridisation using the 696 bp DNA fragment and the pSS2 as probes (Figure 2(a)). When the 696 bp DNA fragment is used as probe, *Bam*HI digested chromosomal DNA of the wild-type strain *Streptomyces* sp. US24 hybridized with only one 6 kb DNA fragment, whereas with the *Bam*HI digested chromosomal DNA of one analysed exconjugant, two hybridization signals of about 4 and 10 kb were obtained. Using as probe the pSS2 plasmid linearized by *Bam*HI, there is single *Bam*HI site within the multiple cloning site of this vector, one hybridization signal of about 10 kb was obtained for the chromosomal DNA of the analysed mutant strain and no signal for the wild type. Knowing that the 696 bp amplified DNA fragment has no *Bam*HI site, obtained hybridization results are in concordance with a homologous recombination of the 696 bp DNA fragment via a single crossover into the chromosome of the *Streptomyces* sp. US24 strain (Figure 2(b)).

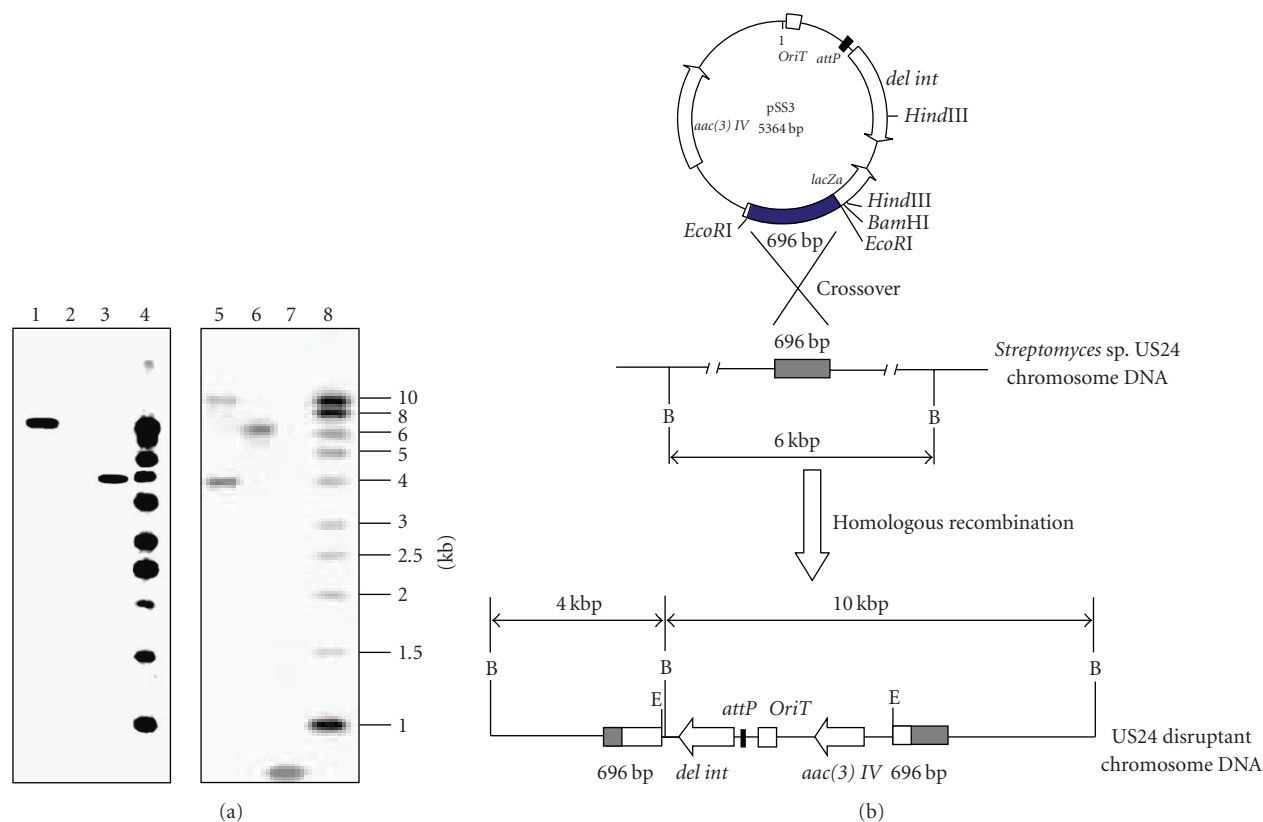


FIGURE 2: (a) Southern blot analysis confirming insertion of pSS3 plasmid into the *Streptomyces sp. US24* chromosome via homologous recombination. Blots were probed with the 696 bp DNA fragment (right panel) or the whole linearized pSS2 (left panel). Lanes 4 and 8, 1 kb ladder used as molecular marker, lanes 2 and 6, wild-type genomic DNA cut with *Bam*HI, lanes 1 and 5, apramycin resistant (*Apr*^R) exconjugant mutant genomic DNA cut with *Bam*HI, and lanes 3 and 7, the used probes linearized pSS2 and 696 bp DNA fragment, respectively. (b) Schematic representation of the integration of plasmid pSS3 in the chromosome of *Streptomyces sp. US24* by homologous recombination. The resident copy of the 696 bp locus involved in the biosynthetic pathway of Cyclo (L-Phe, L-Pro) DKP of the US24 strain (shaded boxes) present in the chromosome of *Streptomyces sp. US24* is shown. *del int*: deletion of a part of the integrase-encoding gene by partial digestion *Hind*III; *aac(3)IV*: apramycin resistance gene; B: restriction site for *Bam*HI; E: restriction site for *Eco*RI.

3.4. The 696 bp amplified DNA fragment required for the Cyclo (L-Phe, L-Pro) DKP derivative biosynthesis

To determine whether the amplified DNA fragment of 696 bp is really involved in Cyclo (L-Phe, L-Pro) biosynthesis, approximately 100 mg of crude extract for each culture, obtained after ethyl acetate extraction from 1 L supernatant culture of the *Streptomyces sp. US24* strain and of three different *Apr*^R exconjugant mutants (MI, MII, and MIII), was dissolved in 500 μ L dichloromethane-methanol 1:1 and fractionated with an analytical HPLC column. For the three analysed exconjugants, obtained chromatograms are identical and present global profiles quite similar to that of the wild type. Six fractions were collected separately between 0 and 50 minutes both for the wild type and one exconjugant (Figure 3). F1 to F6: (F1 “0–13 minutes”; F2 “13–21 minutes”; F3 “21–26 minutes”; F4 “26–31 minutes”; F5 “31–40 minutes,” and F6 “40–50 minutes”). These fractions were concentrated by Rotavapor and then tested for their an-

tibacterial activities against *M. luteus*. For the *Streptomyces sp. US24* strain, an inhibitory activity was detected for the two fractions F3 and F5. However, concerning the three exconjugant mutants, only the F5 fraction was active and the corresponding diameter of inhibition zone was comparable to that of F5 fraction of the *Streptomyces sp. US24* strain (Table 2). These microbiological results strongly suggest the presence of the Cyclo (L-Phe, L-Pro) active molecule in the crude extract (fraction F3) only for the *Streptomyces sp. US24* wild-type strain. It should be noted that according to our previous work [10], the active molecule Cyclo (L-Phe, L-Pro) was purified from the culture supernatant of *Streptomyces sp. US24* strain with a retention time of 25 minutes using the same HPLC conditions used in the present work. This retention time was included in the time interval of the fraction F3 (21–26 minutes). The biological activity observed in the fraction F5 for the four crude extracts (that of the wild type and those of the three mutants) is due to the 3-indolethanol molecule. In fact, Mehdi-Ben Ameer et al. [10] have extracted purified and characterized two active

TABLE 2: Antibacterial activities against *M. luteus* of the crude extracts HPLC fractions of the wild-type *Streptomyces* sp. US24 and the three studied exconjugant mutant strains.

Strain	Diameter (mm) of inhibition zones					
	F1	F2	F3	F4	F5	F6
<i>Streptomyces</i> sp. US24	0	0	14	0	10	0
Exconjugant mutants						
MI	0	0	0	0	10	0
MII	0	0	0	0	10	0
MIII	0	0	0	0	10	0

molecules from the supernatant culture of the *Streptomyces* sp. US24 strain: the Cyclo (L-Phe, L-Pro) “M⁺ = 244”, and the 3-indolethanol “M⁺ = 161”. These results are in perfect concordance with those obtained by the determination of the extracted ion chromatograms (EIC), after acetonitrile removing of the four F3 fractions and LC/MS and LC/MS/MS studies. Indeed, for the three exconjugant mutants, we have obtained identical (EIC) which are completely different to that of the wild-type *Streptomyces* sp. US24. Only this later presents two overlapping important peaks between 4.5 and 5.2 minutes (Figure 4(a)). LC/MS analysis of the different products of these two peaks reveals the presence of an ion corresponding to [M+H]⁺ = 244.8 at 4.7 minutes. LC/MS/MS analysis of this ion gives three ion fragments at m/z 216.8, m/z 119.9, and m/z 70.2 (Figure 4(b)). These expected fragments have been already obtained in EI/MS analysis during our previous works concerning the Cyclo(L-Phe, L-Pro) of the *Streptomyces* sp. US24 strain (data not shown). The ion fragments at m/z 120 and m/z 70 correspond to the immonium ion of Phe and Pro, respectively. Otherwise, LC/MS and LC/MS/MS analysis of the four active F5 fractions showed the presence of an ion of [M+H]⁺ = 162 at 7 minutes for the wild type as well as for the three mutants (MI, MII, and MIII) crude extracts. Corresponding ion fragments were identical for all the four F5 fractions [M+H]⁺ = 162 “m/z 118.7 and m/z 118.7”. These results are in concordance with those obtained previously (data not shown) concerning the active molecule 3-indolethanol: “M⁺ = 161”.

Together, all these data (microbiological, chemical, and spectroscopic studies) supported the presence of the active molecule Cyclo (L-Phe, L-Pro) only in the crude extract of the wild-type *Streptomyces* sp. US24, and the active molecule 3-indolethanol in all the four analysed crude extracts (that of *Streptomyces* sp. US24 and those of MI, MII, and MIII exconjugant mutants). Consequently, the amplified DNA fragment of 696 bp from the genomic DNA *Streptomyces* sp. US24 strain is proven to be involved in its Cyclo (L-Phe, L-Pro) biosynthetic pathway.

However, to our knowledge for *Streptomyces* species, the only isolated and studied DKP biosynthetic gene cluster from

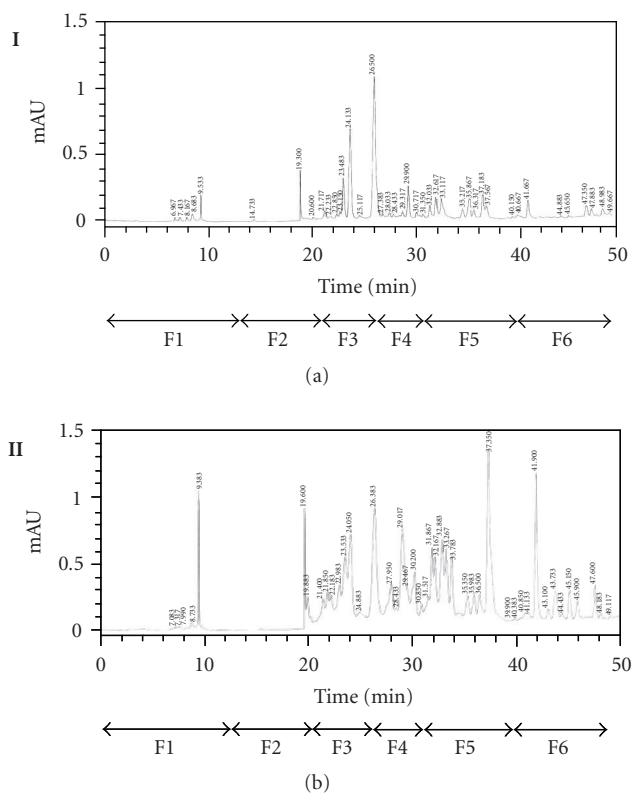


FIGURE 3: HPLC chromatograms in semipreparative conditions of the crude extracts of 1 L supernatant cultures of the wild-type *Streptomyces* sp. US24 strain (I) and one exconjugant mutant (II). Six fractions were collected separately between 0 and 50 minutes both for the wild type and one exconjugant. F1 to F6: (F1 “0–13 minutes,” F2 “13–21 minutes,” F3 “21–26 minutes,” F4 “26–31 minutes,” F5 “31–40 minutes,” and F6 “40–50 minutes”).

this bacterium genus is that of albonoursin of the *Streptomyces noursei* [8]. It has been reported by these authors that the biosynthesis of the albonoursin diketopiperazine is independent of nonribosomal peptide synthetases. Contrarily, in our case, the biosynthesis of the DKP derivative Cyclo (L-Phe, L-Pro) by the *Streptomyces* US24 seems to be a nonribosomal pathway. To this level of work, two hypotheses can be proposed. The first one is to consider the direct production by the *Streptomyces* sp. US24 strain of the Cyclo (L-Phe, L-Pro) via a nonribosomal pathway. The second hypothesis is the obtaining of this active molecule as a side product in the course of nonribosomal synthesis of a longer peptide by the *Streptomyces* sp. US24. In fact, the Phe-Pro dipeptide can be released from the NRPSs by a nonenzyme-catalysed reaction to give the Cyclo (Phe-Pro). This is due to the high proportion of cis conformation in the Phe-Pro dipeptide, which is induced by N-alkylated amino acids such as Pro. Actually, our study is focused on the construction of a cosmid library from the genomic DNA of the *Streptomyces* sp. US24 to identify the complete biosynthetic pathway of this DKP derivative.

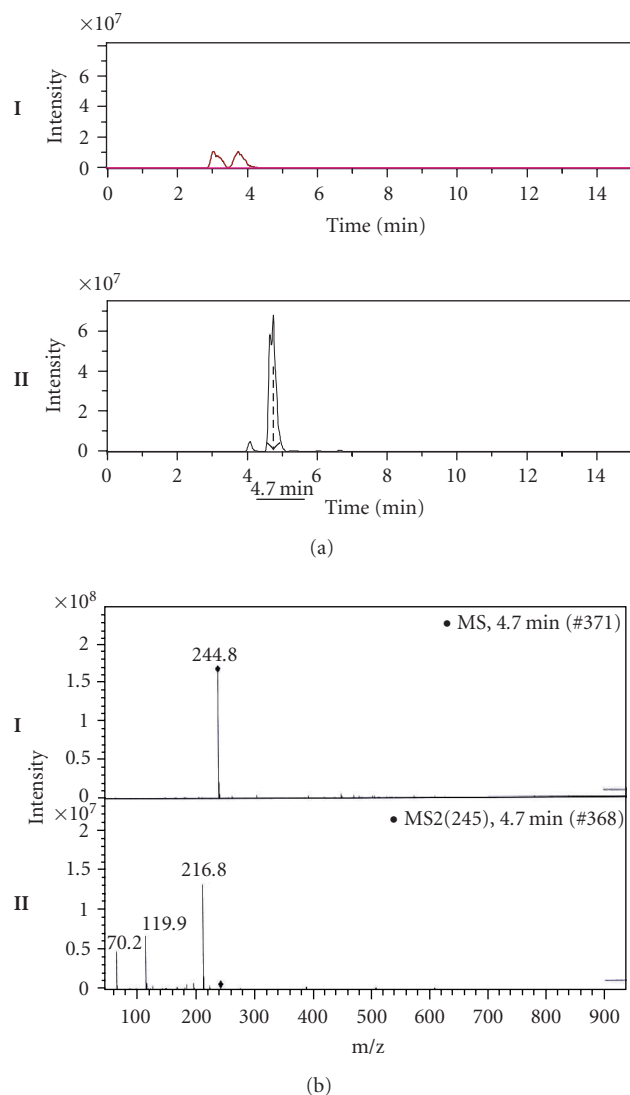


FIGURE 4: (a) Extracted ion chromatograms (EIC) of two fractions F3: one of exconjugant mutant (I) and the second of the wild-type *Streptomyces* sp. US24 strain (II). (b) LC/MS analysis (I) and LC/MS/MS (II) fragments determination of the product of the peak 4.7 minutes (fraction F3 of the wild-type *Streptomyces* sp. US24 strain) corresponding to the ion $[M+H]^+ = 244.8$.

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