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Identification of Genes Putatively Involved in the Biosynthesis of Antitubercular Peptide in *Streptomyces ribosidificus* NRRL B-11466

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Authors' contributions

Author KMA designed the study, performed the sequence analysis, wrote the protocol, and first draft of the manuscript. Author NMO performed the practical experiments of phenotypic detection. Authors TSE and AAA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To determine the potential antitubercular activity of *Streptomyces ribosidificus* NRRL B-11466 both on genotypic and phenotypic levels.
Methodology: Standard methods and software programs were used for nucleotide/protein sequence analysis and phenotypic detection of antitubercular activity.
Results: Analysis of the submitted DNA segment (accession code = AJ744850) harbouring the ribostamycin biosynthetic gene cluster showed that the respective gene cluster was flanked in the upstream region by three open reading frames (ORFs), encoding putative type II thioesterase (SribL03.14c) and two nonribosomal peptide synthases (SribL03.14c and SribL03.14c). These ORFs were of high amino acid similarities (about 80%) to those located in the viomycin and related antibiotic biosynthetic gene clusters. A DNA segment harbouring three ORFs, putatively involved in

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capreomycidine biosynthesis was submitted into the GenBank database under the accession code HQ327309. Comparative analysis of the respective DNA segment with viomycin and related antibiotic biosynthetic gene clusters showed: firstly, location of the respective DNA segment in the neighbourhood and upstream to the ribostamycine biosynthetic gene cluster; secondly, conservation of six ORFs: SriC (putative L-arginine hydroxylase); SriD (putative L-capreomycidine synthase), SriE (putative permease) located on our submitted DNA fragment; and SribL03.14c, SribL03.15c, SribL03.16c located on the DNA fragment harboring ribostamycin biosynthetic gene cluster, among the tested biosynthetic gene clusters. Phenotypically, *S. ribosodificus* inhibited growth of *Mycobacterium smegmatis* ATCC 19420 and *Mycobacterium phlei* ATCC 11758.

Conclusion: *Streptomyces ribosidificus* NRRL B-11466 produces antimycobacterial agents and this was confirmed genotypically via detection of 6 ORFs with high amino acid similarities (about 80%) to those located in the viomycin and related antibiotic biosynthetic gene clusters as well as phenotypically by determining its inhibitory activity against *Mycobacterium smegmatis* ATCC 19420. This is the first report about identification of genes putatively involved capreomycidine biosynthesis in *Streptomyces ribosidificus* NRRL B-11466**.**

Keywords: Tuberactinomycins; capreomycidine biosynthesis; viomycin; Streptomyces ribosidificus NRRL B-11466.

1. INTRODUCTION

Tuberactinomycin family of peptide antibiotics, (tuberactinomycins; TUBs) is active against *Mycobacterium tuberculosis* infections and is particularly used for the treatment of multidrug-resistant tuberculosis, methicillin-resistant *Staphylococcus aureus* (MRSA) strains and vancomycin-resistant enterococci (VRE) [1,2]. They are peptide antibiotics characterized by the presence of capreomycidine, a nonproteinogenic amino acid with a 6-membered cyclic guanidine side chain that is biosynthesized and condensed with other amino acids via a nonribosomal peptide synthase mechanism to form various TUBs [3]. TUBs include antibiotics such as viomycin, tuberctinomycins, streptothricin and capreomycins that are produced by different *Streptomyces* strains [4-8]. The antibiotic viomycin (tuberactinomycin B), the well-studied antibiotic contain unusual amino acids such as L-capreomycidine, 2,3-diaminopropionate, β-ureidodehydroalanine, and β-lysine [3,8]. The complete biosynthetic pathway of these antibiotics still not biochemically identified, however it was anticipated that they are synthesized via a nonribosomal peptide synthase (NRPS) mechanism [8,9]. The full biosynthetic gene cluster of viomycin antibiotic from *Streptomyces* strain ATCC 11861 was completely isolated and analyzed [3,8]. The unusual nonproteinogenic amino acids were anticipated to be synthesized from α- amino acids in the cell such as 2,3-diaminopropionate from L-serine and L-ornithine. 2,3-diaminopropionate would be further modified to form, β-ureidodehydroalanine, L-capreomycidine from L-arginine, and β-lysine from L-lysine [8]. These amino acids would be condensed to produce these antibiotics via nonribosomal peptide synthases (NRPSs) whose respective genes were also located with the identified biosynthetic gene clusters.

Moreover, conversion of (2S)-arginine to (2S,3R)-capreomycidine by VioC and VioD from the viomycin biosynthetic pathway of *Streptomyces sp*. strain ATCC11861 was biochemically analyzed [10]. TUBs also target the catalytic RNAs involved in viral replication [11,12]. Interestingly, some members of TUBs family are listed in the World Health

Organization's model drug list 2002. Recently, it was investigated that tuberactinomycins inhibit translocation on 70S ribosome by stabilizing the tRNA in the A site in the pretranslocation state [13] which is adjacent to the binding sites for the some 2-deoxystreptamine aminocyclitol aminoglycoside antibiotics (2DOS-ACAGA) such as paromomycin and hygromycin B [13]. *Streptomyces ribosidificus* NRRL B-11466 is a producer of ribostamycin, a 2DOS-ACAGA. The ribostamycin biosynthetic gene cluster was completely sequenced and analysed [14]. Analysis of the submitted DNA segment harbouring the ribostamycin biosynthetic gene cluster showed the presence of three ORFs with a very good amino acid identities (about 80%) to those located in the viomycin biosynthetic gene cluster of *S. vinaceus*. These three ORFs were putative type II thioesterase and two NRPSs, however their exact biosynthetic roles in *S. ribosidificus* were not yet known. Whether a full viomycin-related biosynthetic gene cluster is located in *S. ribosidificus* has to be explored. Moreover, isolation, sequencing and annotation of three genes putatively involved in capreomycidine biosynthesis from *Streptomyces ribosidificus* NRRL B-11466 were carried out and submitted into the GenBank database under accession code HQ327309 [15]. Therefore, in this work, comparative analysis of ORFs located on the DNA segment (HQ327309) with the viomycin and capreomycin biosynthetic gene clusters was carried out. Also, preliminary antitubercle inhibitory activity of *S. ribosodificus* was tested against *Mycobacterium smegmatis* ATCC 19420 and *Mycobacterium phlei* ATCC 11758 standard strains.

2. MATERIAL AND METHODS

2.1 Bacterial strains, culture media

Streptomyces ribosidificus NRRL B-11466 (ribostamycin producer) was cultured on tryptic soy broth (TSB) [16,17] or on M65 composed of glucose 4.0 g, yeast extract 4.0 g, malt extract 10.0 g, agar 12.0 g, distilled water ad. 1000.0 ml, pH adjusted to 7.2 (DSMZ, Braunschweig, Germany) at 28°C. *Mycobacterium phlei* ATCC 11758 and *Mycobacterium smegmatis* ATCC 19420 were cultured onto nutrient agar and incubated for 48 hrs at 28°C.

2.2 Testing the Preliminary Antitubercular Inhibitory Activity of *S. ribosidificus*

S. ribosidificus NRRL B-11466 was inoculated into 25 ml TSB and incubated at 28°C for 48 hrs at 160 rpm. About 1 ml from the obtained growth was used for surface inoculation of either tryptic soy agar plate (TSB) or M65 agar plate. The surface inoculated plates were incubated at 28°C for 5 days. From Each plate, agar plug was obtained using a sterile cork borer and added on a surface of inoculated nutrient agar plates (10^5 CFU/ml) with standard testing strains (*Mycobaterium phlei* ATCC 11758 and *Mycobacterium smegmatis* ATCC 19420, a local clinical isolate of *Staphylococcus aureus*). The plates were incubated at 28°C for 24 hrs and the resulted inhibition zones were measured in mm.

2.3 Nucleotide Accession Code

The nucleotide sequence reported in this study was submitted in the NCBI GenBank database under the accession code HQ327309. The DNA fragment submitted to the NCBI GenBank harboured three ORFs namely SriC (putative L-arginine hydroxylase); SriD (putative L-capreomycidine synthase), SriE (putative permease), This DNA fragment was obtained via DNA sequencing of various PCR products obtained using various heterologous and homologous primers and chromosomal DNA of *S. ribosidificus* as a template. The

obtained DNA sequence files were assembled into one final contig which was submitted into the NCBI GenBank under accession code HQ327309 [15]

2.4 Computer-assisted Analysis of DNA sequences

The programs used for computer-assisted analysis of nucleotide and protein sequences were Staden Package [18], FramePlot [19], Online analysis tools (http://molbiol-tools.ca/), ClustalW2 [20]. Structure of proteins and conserved domain analysis were conducted using Basic Local Alignment Search Tool (NCBI) http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi.

3. RESULTS

3.1 Comparative Analysis of the Submitted DNA Segment (HQ327309) with Various Viomycin-capreomycin Biosynthetic Gene Clusters

As shown in Fig. 1, a total of six ORFs (SriC, SriD, SriE located on our submitted DNA fragment; NCBI GenBank accession code = HQ327309; and SribL03.16, SribL03.15, SribL03.14 located on the DNA fragment harboring ribostamycin biosynthetic gene cluster, NCBI GenBank accession code = AJ744850) were highly conserved among the viomycin and capreomycin biosynthetic gene clusters. It was also obvious that the DNA segment (HQ327309) harboring the putative capreomycidine biosynthetic ORFs (SriC, putative L-arginine hydroxylase and SriD, putative L-capreomycidine synthase) was highly conserved (80% similarities). However, there was a gap between the respective DNA segment (HQ327309) and the ribostamycin biosynthetic gene cluster (accession code = AJ744850).

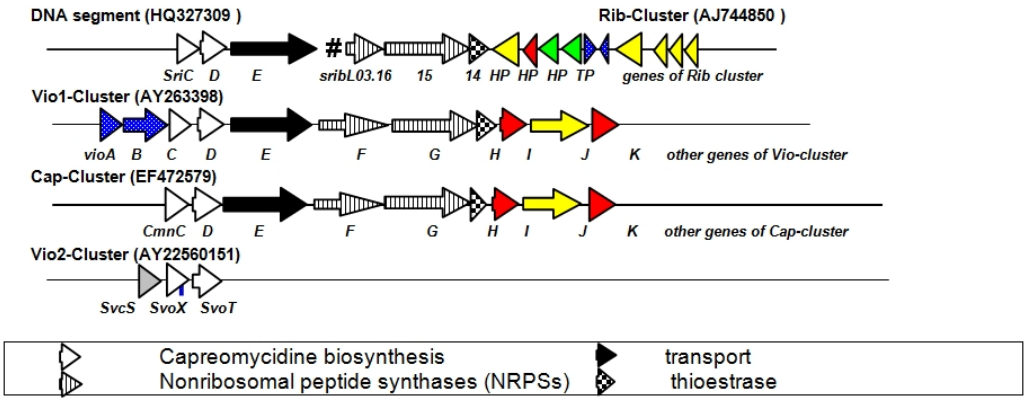

Fig. 1. Comparative analysis of the submitted DNA segment (HQ327309) with various viomycin-biosynthetic gene clusters.

Rib-Cluster = ribostamycin biosynthetic gene cluster, accession code, AJ44850; DNA segment (HQ327309)= DNA segment harbouring putative capreomycidine biosynthetic genes; Vio1-Cluster (AY263398) = Viomycin biosynthetic gene cluster, accession code AY263398; Cap-cluster (EF472579) = capreomycin biosynthetic gene cluster, accession code EF472579; Vio2-Cluster (AY22560151) = Viomycin biosynthetic gene cluster, accession code AY22560151.

3.2 Alignment of Sric (putative L-arginine hydroxylase) and Homologous Proteins

As shown in Fig. 2, SriC showed more than 80% similarities in the amino acid sequences with VioC (L-arginine hydroxylase; accession code, AAO66427) from *S. vinaceus* (viomycin producer;) and CmnC (L-arginine hydroxylase; accession code, ABR67746) from *Saccharothrix* sunsp. *capreolus* (capromycin producer). The catalytic sites were also conserved.

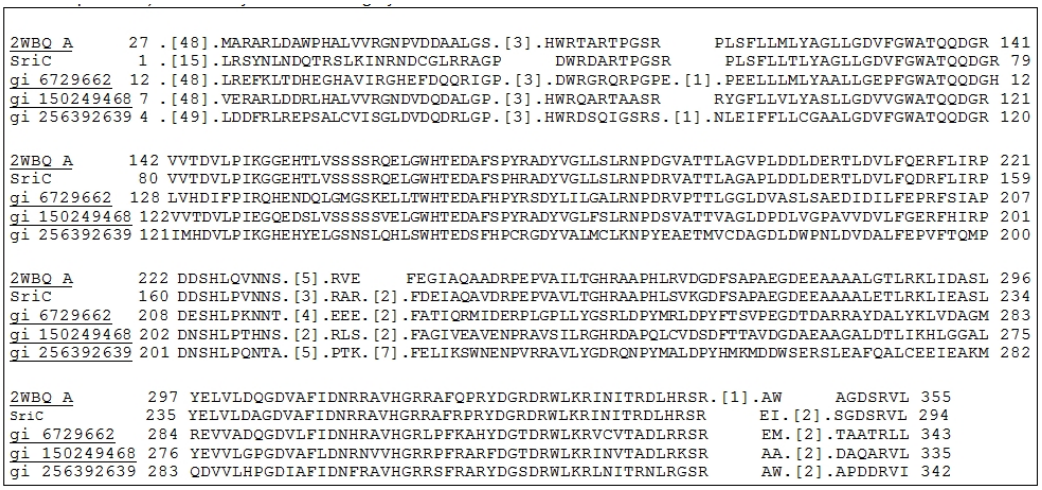

Fig. 2. Multiple amino acid sequence Alignment of L-arginine hydroxylase of *Streptomyces ribosidificus* NRRL B-11466 (SriC; ADR02786) and its homologous. *The numbers indicate the position within the corresponding proteins: 2WBQ_A = Chain A, crystal structure of VioC in complex with (2s,3s)-hydroxyarginine, accession code (AC) = 2WBQ_A; gi 6729662 = putative oxygenase of Streptomyces rochei, AC= CAB67713; gi 150249468 = CmnC of Saccharothrix mutabilis subsp. Capreolus, AC= ABR67746; gi 256392639 = hypothetical protein Caci_3456 of Catenulispora acidiphila DSM 44928, AC= YP_003114203.*

3.3 Alignment of SriD (putative L-carpreomycidine synthase) and Homologous Proteins

As shown in Fig. 3. SriD showed about 80% similarities in the amino acid sequences with VioD (L-carpreomycidine synthase; accession code. AAO66428) from *S. vinaceus* (viomycin producer;) and CmnD (L-arginine hydroxylase; accession code, ABR67747) from *Saccharothrix* subsp. *capreolus* (capromycin producer). Multiple amino acid sequence alignment also showed conservation of the amino acid residue lysine (K; position 231 within SriD) that would be necessary for the catalytic activities of the respective proteins via forming an internal aldimine bond (Schiff base linkage) with pyridoxal -phosphate (PLP).

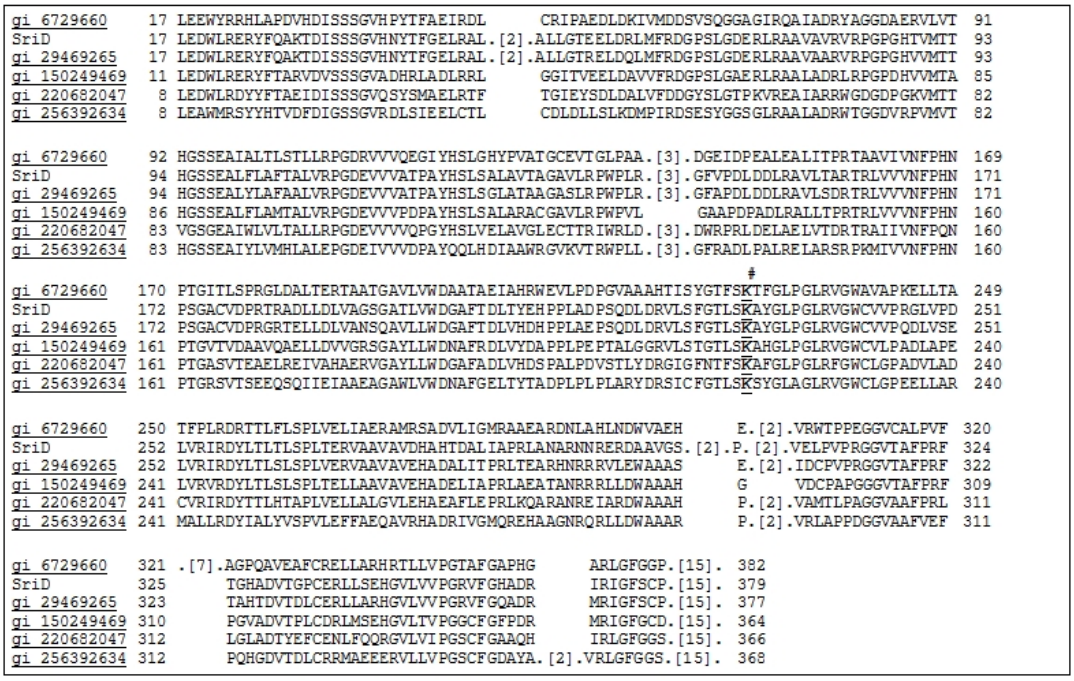

Fig. 3. Multiple amino acid sequence Alignment of L-capreomycidine synthase of *Streptomyces ribosidificus* NRRL B-11466 (SriD; ADR02787) and its homologous.
The numbers indicate the position within the corresponding proteins:; gi 6729660 = putative amino-transferase of Streptomyces rochei, accession code (AC)= CAB67711; gi 29469265= putative L-capreomycidine of Streptomyces vinaceus (VioD), AC= AAO66428; gi 150249469= L-capreomycidine synthase (CmnD) of Saccharothrix mutabilis subsp. capreolus, AC= ABR67747 ; gi 220682047= putative L-capreomycidine synthase of Catenulispora yoronensis, AC= ACL80152; gi 256392634 = putative L-capreomycidine synthase of Catenulispora acidiphila DSM 44928, AC = YP_003114198. # = conservation of lysine amino acid (K) required for catalytic activity.

3.4 Testing the Preliminary Antitubercular Activity of *S. ribosidificus*

Results revealed that *S. ribosidificus* NRRL B-11466 was sporulated upon incubation on M65 agar while was not sporulated on TSB agar when using similar conditions of inoculation and incubations (5 days at 28°C). As shown in figures 4,5,6, the M65 agar plug of sporulated *S .ribosidificus* showed large inhibition zones (22 mm; 25mm, 27 mm) with all of the tested strains .The TSB agar plug of the non-sporulated *S. ribosidificus* showed only very weak inhibition zone (10 mm) with the local clinical isolate of *Staphyloccocus aureus* and showed no inhibition with both of the tested *Mycobacteria*.

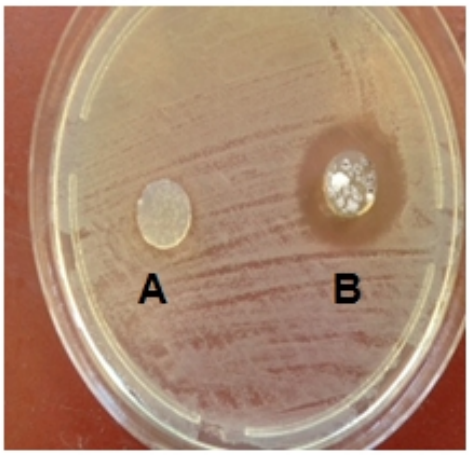

Fig. 4. Growth inhibition of *Mycobacterium smegmatis* ATCC 19420
Using :A; TSB agar plug of S .ribosidificus NRRL B-1146 (non-sporulated) B; M65 agar plug of S . ribosidificus NRRL B-11466 (sporulated)

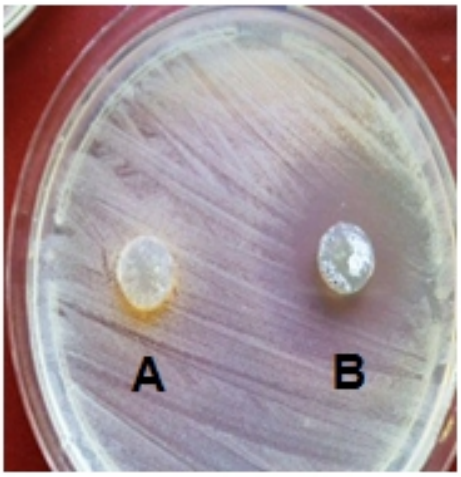

Fig. 5. Growth inhibition of *Mycobacterium phlei* ATCC 11758
Using: A; TSB agar plug of S. ribosidificus NRRL B-11466 (non-sporulated), B; M65 agar plug of S. ribosidificus NRRL B-11466 (sporulated)

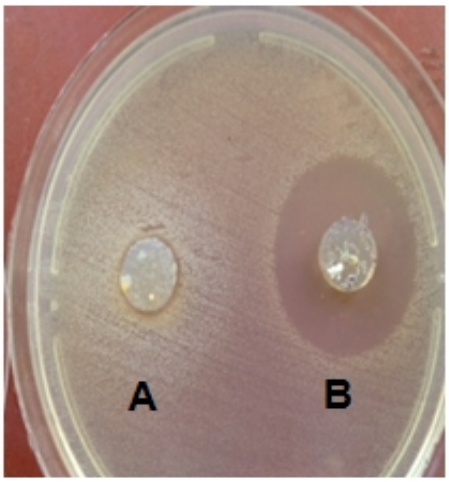

Fig. 6. Growth inhibition of *Staphylococcus aureus* clinical isolate
Using: A; TSB agar plug of S. ribosidificus NRRL B-11466 (non-sporulated), B; M65 agar plug of S. ribosidificus NRRL B-11466 (sporulated)

4. DISCUSSION

Viomycin, tuberctinomycins, streptothricin and capreomycins are major peptide antibiotics of tuberactinomycin family with enormous activity against *Mycobacterium tuberculosis* infections and are of particular importance in the treatment of the most clinically relevant pathogens such as methicilin-resistant *Staphylcoccus aureus* (MRSA) as well as vancomycin-resistant enterococci (VRE) [1,2,8,15]. The biosynthetic gene clusters of these peptide antibiotics were fully isolated and sequenced, however their complete biosynthetic pathways were not biochemically identified [3,7,8]. L-capreomycidine (amino acid with a 6-membered cyclic guanidine side chain) is the most important nonproteinogenic residue in these antibiotics was biochemically identified where VioC (L-arginine hydroxylase) and VioD (L-capreomycidine synthase) gene products were involved [10].

Analysis of the DNA fragment (NCBI accession code = AJ744850) harbouring the ribostamycin biosynthetic gene cluster showed that the respective gene cluster was flanked in the upstream region by three open reading frames (ORFs), encoding putative a type II thioestrase (SribL03.14c) and two NRPSs (SribL03.14c and SribL03.14c) [14]. These ORFs were of high amino acid similarities (about 80%) to those located in the viomycin and related antibiotic biosynthetic gene clusters [3,8,15]. In order to know, whether a full viomycin-related biosynthetic gene cluster is located in *S. ribosidificus* or not, a previous study was conducted where a series of heterologous and homologous primers were designed and used in PCR to amplify and sequence genes homologous to those in the viomycin and related antibiotic gene clusters [15]. This previous study resulted in a final assembled contig of 3884 bp which was submitted into the NCBI GenBank database under accession code HQ327309. Analysis of the respective DNA segment (contig) using FramePlot program revealed the presence of two complete ORFs (SriC, encode putative L-arginine hydroxylase and SriD, encode putative L-capreomycidine synthase) and another incomplete ORF (SriE, encode permease) [15]

Comparative analysis of the respective DNA segment with the viomycin and capreomycin biosynthetic gene clusters showed: firstly, location of the respective DNA segment in the neighbourhood and upstream to the ribostamycine biosynthetic gene cluster; secondly, conservation of six ORFs: SriC (putative L-arginine hydroxylase); SriD (putative L-capreomycidine synthase), SriE (putative permease), located on our submitted DNA fragment; and SribL03.14c , SribL03.15c, SribL03.16c located on the DNA fragment harboring ribostamycin biosynthetic gene cluster, with high amino acid identities to homologous ORFs (AAP92496.1, AAP92497.1, AAP92498.1) in the viomycin biosynthetic gene cluster [8]. This means that the presence of these genes/ORFs will be correlatated with the nature and structure of metabolic products formed by the respective clusters.

Moreover, amino acid alignment of SriC and SriD with homologous proteins together with their putative tertiary structure gave evidence about their similar catalytic activities. Thomas et al. [8] proved the essential presence of the catalytic residue lysine that forms an internal aldimine bond (Schiff-base linkage) with pyridoxal 5'-phosphate (PLP) [8,21]. This catalytic residue was also conserved in SriD (position 230). VioC and VioD proteins were biochemically analyzed to be involved in conversion of (2S)-arginine to (2S,3R)-capreomycidine [10]. Accordingly, SriC and SriD are anticipated to be involved in the biosynthesis of capreomycidine, the essential nonproteinogenic residue in the tuberactinomycin peptide antibiotics. Furthermore, conservation and arrangement of the 6 conserved ORFs by this way gave clue about presence of a peptide antibiotic biosynthetic gene cluster in a close vicinity to the ribostamycin biosynthetic gene cluster. For further confirmation, *S. ribosodificus* was tested phenotypically for growth inhibition of *Mycobacterium smegmatis* ATCC 19420 and *Mycobacterium phlei* ATCC 11758 standard strains as a preliminary indication of its antitubercular activity. Results showed that *S. ribosidificus* inhibit growth of both *Mycobacterial* standard strains, however the growth inhibition occurred only upon sporulation. This would means that the production of this inhibitory metabolite occurred in the stationary phase of bacterial growth which is the case of all secondary metabolites such as antibiotics. This is the first report about inhibition of *Mycobacterium smegmatis* growth by *Streptomyces ribosidificus* NRRL B-11466 as well as identification of genes putatively involved in the biosynthesis of a new peptide antibiotic of tuberactinomycin family in *Streptomyces ribosidificus*. Therefore, the prospective of this study is to isolate this antibiotic in a pure form, elucidate its chemical structure and confirm its activity against *Mycobacterium tuberculosis* in order to be used in future as antitubercular drug. Also, construction of knock-out mutant of the different genes obtained in this study followed by recording the different phenotypic changes that will occur on the mutant strain.

5. CONCLUSION

Streptomyces ribosidificus NRRL B-11466 inhibited growth *Mycobacterium smegmatis* ATCC 19420 and this inhibition was confirmed genotypically via isolation, sequencing and amino acid analysis of 6 ORFs with high amino acid similarities (about 80%) to those located in the viomycin and related antibiotic biosynthetic gene clusters. These ORFs were anticipated to be involved in the biosynthesis of antitubercular peptide metabolite synthesized via a nonribosomal peptide mechanism.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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