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Phylogenic Analysis of NRPS and PKS Genes Associated with Antagonistic *Micromonospora* Rc5 and *Streptomyces* Ru87 Isolates

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NAA, ST, AA and EMHW designed the study and wrote the protocol. Authors DHA and CB managed the lab work of the study. Author AA managed the paper organization. Authors DHA and AA wrote the first draft of the manuscript. Authors DHA and AA managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To invistigate phylogenic analysis of NRPS and PKS genes. To discover uncommon and novel types of actinomycetes isolated from Egyptian soils.
Study Design: Fifty rare actinomycetes were isolated from Egyptian soils and tested for antimicrobial activities. Phylogenic analysis of NRPS and PKS Genes was invistigated with unique isolates. Fosmid genomic libraries were constructed and integrated with NRPS and PKS genes.
Methodoloy: Isolation was conducted using humic acid vitamin agar media and starch casein agar media. They were tested against some food and blood borne pathogens. Screening studies of NRPS and PKS genes were conduected via NRPS/PKS PCR assays, and phylogenetic analysis, and fosmid libraries with positive hits of NRPS and PKS genes as a preliminary step for manipulation of these genes.
Results: Forty rare actinomycetes showed significant antimicrobial activities. *Micromonospora* Rc5

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and *Streptomyces* Ru87 isolates reported the highest antimicrobial activities and then revealed positive hits of NPRS and PKS genes. Fosmid genomic libraries were successfully constructed and integrated with NRPS and PKS genes. Adenylation domain of NRPS gene of *Micromonospora* sp. Rc5 showed high similarity (97%) to *Micromonospora haikouensis*. Similar domain of *Streptomyces* sp. Ru87 found identical (100% boost strap) to *Streptomyces viridochromogenes, Streptomyces ribosidificus* and *Streptomyces hygroscopicus.* Whereas, NJ phylogenetic tree of PKS gene fragments of both Rc5 and Ru87 isolates were highly distinctive with bootstrap values (70%, 69%, respectively).

Conclusion: These unique isolates harbour phylogenetically divergent PKS gene cluster that is responsible for distinct biochemical pathways of novel antibiotics. To our knowledge, this is first attempt to tackle phylogenic analysis of NRPS and PKS genes and discover uncommon and novel types of actinomycetes isolated from Egyptian soils. These novel isolates may contain unexpected genes, and subsequently, offers a significant contribution for alternative novel antimicrobial compounds in drug discovery.

Keywords: Antibiotic resistance; actinomycetes; foodborne pathogens; fosmid library; 16s rRNA gene sequencing; NRPS and PKS.

1. INTRODUCTION

Antimicrobial-resistant pathogens has been a major worldwide public health threat causing morbidity, mortality and expanding medicinal services costs [1-5]. Numerous antibiotics against multi-resistant pathogens were reported but with few accomplishments of alternative medications [6]. Inevitably, antagonistic microbes remains the great source for novel bioactive compounds [7-9]. Actinomycetes generate extensive compounds with a variety of biological activities [6,10]. Rare actinomycetes is known as a great potential source of antibiotic production [11-13]. Uncommon and novel types of actinomycetes may contain unexpected genes that are phylogenetically far off related strains, and subsequently, generate alternative novel antimicrobial agents [14].

The biosynthesis mechanism of Nonribosomal peptide (NRPS) and polyketide (PKS) is composed of multiple enzymyes and multiple domains megasynthases that are responsible for the biosynthesis of non-ribosomal peptides (NRPs) and polyketides (PKs) metabolites [15-20]. Many essential and valuable antibiotics are successfully generated by NRPS and PKS gene clusters [21-23] such as antibiotics, antiparasitic agents, antifungals, anticancer drugs, toxins and immunosuppressants [24-26]. Biologically active isolates with positive NRPS and PKS hits and belongs to *Streptomyces, Micromonospora, Saccharothrix, Streptosporangium* and *Cellulomonas* genera are more likely to show higher antimicrobial activity with more diverse structural types [27-29].

NRPS are formed of several domains with a basic structure of an adenylation (A) domain, condensation (C) domain and peptidepeptidyl carrier protein (PCP). PKS modules consist of an acyltranferase (AT) domain, ketosynthase (KS) domain and acyl carrier protein (ACP) with preserved nature [30,17,19,20,31]. The biosynthesis of both PKs and NRPs depends upon using a stepwise, assembly line-type mechanism of modular organization, where each module contains a set of catalytic activities that are responsible for catalysing a single elongation cycle and consequent processing of the intermediates. And therefore, the composition, number, and serial arrangement of elongation modules found within a definite PKS or NRPS biosynthetic system often command the size and chemical structure of the natural compounds produced [32-34]. Phylogenetically distinct domains that is replaced in the modular organization of NRPS and PKS clusters, lead to a different functional complex capable of producing new bioactive molecules [35,36]. Specific degenerate primers were successfully used to amplify NPRS and PKS aiming to select potent strains with a distinct biosynthetic potential [37,38,20]. Fosmid libraries were successfully constructed [39,40] in order to catch and express numerous functional genes [31,20]. Moreover, fosmid libraries assess the diversity of biosynthetic gene clusters and help in finding several new bioactive compounds [41,42].

In this manuscript, we have conducted screening studies of NRPS and PKS genes via NRPS/PKS PCR assays, phylogenetic analysis as an indicator for the novelty of rare actinomycetes strains, and fosmid libraries with positive hits of NRPS and PKS genes as a preliminary step for manipulation of these genes.

2. MATERIALS AND METHODS

2.1 Microorganism and Their Maintainance

In the present study, 50 rare actinomycete isolates were selectively isolated from different Egyptian governorates (Giza, Qualubiya, Alexandria, Assuit and Sinai) using humic acid vitamin agar media [43] and starch casein agar media [44]. Rare actinomycetes isolates were identified via morphological, chemotaxonomy and biochemical methods [44]. These isolates were tested against some food and blood borne pathogens (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumonia* CCM 4415, *Streptococcus mutans* ATCC 25175, *Escherichia coli* O157:H7 ATCC 51659 and *Salmonella enterica* ATCC 25566) provided from Ain shams Specialized Hospital and the Microbial Resources Center (MIRCEN) at the Faculty of Agriculture, Ain shams university, Cairo, Egypt. The Isolates used in this study belonged to several actinomycetes genera such as *Streptomyces*, *Micromonospora, Actinomadura*, *Actinoplanes* and *Kribbella*. Rare actinomycete isolates were cultivated as described [45] with some modifications as follow: A starter spore suspension was performed for each isolate using a loopful of spores cultivated into 35ml starch casein broth for 6 days at 30℃ with shaking at 150 RPM. Then, 1 ml of each isolate spore suspension (3.5 x 10^5CFU/ml), was cultivated again into 35 ml of both starch casein broth media and soya bean meal broth for 8 days at 30℃ with shaking at 150 RPM. Spore suspensions concentration was measured by using spectrophotometer at 600nm and total plate colony count. *Micromonospora* Rc5 and *Streptomyces* Ru87 showed great inhibition activities against above Gram positive and Gram-negative pathogens, and thereafter selected for our study [20]. Food and blood borne pathogens such as (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumonia* CCM 4415, *Streptococcus mutans* ATCC 25175, *Escherichia coli* O157:H7 ATCC 51659 and *Salmonella enterica* ATCC 25566) were used.

2.2 Agar Well Diffusion Method

Agar well diffusion method was conducted with cell free supernatants obtained by centrifugation at (1000 xg) against bacterial pathogens as a preliminary screening for their antimicrobial activities. Actinomycetes spore suspensions were prepared as stated above. Cell-free supernatant (250 µl) was added in each well within nutrient agar petridishes containing 0.5 Mcfarland (1.5 X10^8 CFU/mL) of homogenized tested bacterial spores [46]. Petridishes were then incubated for 24 hrs at 37°C. Results were recorded by measuring the inhibition zone area [47]. All tests and experiments were made in duplicates. The highest antimicrobial activity was obtained with Rc5 and Ru87 isolates, and therefore were selected for this study. Both isolates were previously invistigated via morphological, chemotaxonomy and 16S rRNA gene sequencing and identified as *Micromonospora* sp. Rc5 and *Streptomyces* sp**.** Ru87 [48,49,50].

2.3 DNA Extraction of Selected Actinomycete Strains

Extraction of genomic DNA of *Micromonospora* Rc5 and *Streptomyces* Ru87 was conducted using promega Wizard® Genomic DNA Purification Kit as follows: one milliliter of each actinomycete spore suspension was aseptically added to 35 ml of sterile starch casein broth in 50 ml Erlenmeyer flasks. The flasks were incubated at 30°C in shaking incubator (Spectronics, U.S.A) with an agitation rate (150 RPM) for 7-days. One milliliter of spore suspension was added to 1.5 ml microcentrifuge tube. Cells were pelleted by centrifugation at 13,000 xg for 2 mins and supernatant was discarded. Cells were resuspended thoroughly in a mixture of 480µl of 50 mM EDTA and 120 µl of Lysozyme. Incubation of the samples were conducted at 37°C for 60 min followed by centrifugation for 2 min at 13,000 xg. The supernatant was discarded and 600 µl of Nuclei Lysis Solution was added followed by incubation at 80°C for 5 min followed by an immediate cooling down to 37°C. Three microliters of (4 mg/ml) RNase Solution was added to the lysate. The eppendorf tubes were then inverted 5 times to allow complete mixing followed by incubated at 37°C for 60 min. An aliquot of 200 µl of protein precipitation solution was added to the RNase-treated cell lysate and vigorously vortexed at high speed for 20 sec. The mixture was incubated in ice for 5 min and then centrifuged at 13,000× g for 3 min. The supernatant containing the target DNA was transferred to a clean 0.5 ml eppendorf tube containing 600 µl of isopropanol. Eppendorf tubes were gently inverted until the thread-like strands of DNA formed a visible mass. Samples were centrifuged

at 13,000 × g for 2 min and supernatant was poured off carefully followed by draining the tubes on a clean absorbent paper. An amount of 600 µl of 70% ethanol was added to each eppendorf and tubes were then inverted several times in order to wash the DNA pellet followed by centrifugation at 13,000 × g for 2 min. Careful aspiration of the ethanol was performed, and then tubes were poured on a clean absorbent paper to allow the pellet to air-dry for 15 min. One hundred microliters of DNA rehydration solution was added to each tube and incubated at 65°C for 1 hr. DNA concentration was determined using a Nanodrop spectrophotometer (ND- 2. 1000, Nanodrop Technologies) and stored at -20°C.

2.4 NRPS and PKS PCR Assays

Polymerase chain reaction was successfully used for the identification of PKS and NRPS clusters. Several primers were selected for this study (Table 1).

PCR Reaction mixes consisted of 12.5µl PCR Master Mix (Promega, Madison, WI, USA), 1.25µl DMSO , 2µl of **(**0.8µM) of each primer, 1 µl of (0.1 µM) DNA template and 6.25 µl of double distilled water in 25µl total volume**.** Genomic DNA (10–20 ng) served as template for all PCR reactions. The amplifications of these primers were performed using different PCR programs as follows: A3F and A7R (5 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 59°C, 90 sec at 72°C and a final step of 5 min at 72°C) as described [30]; NRPS_F/R (5 min at 95°C followed by 40 cycles of 30 sec at 95°C, 45 sec at 63°C and 90 sec at 72°C followed by a final extension step for 10 min at 72°C) as described [20]; PKS_F/R (5 min at 95°C followed by 40 cycles of 30 sec at 95°C, 45 sec at 61°C and 90 sec at 72°C followed by a final extension step for 10 min at 72°C) as illustrated [20]; 5LL and 4UU (5 min denaturation at 95°C, followed by 40 cycles of 95°C for 30 sec, 59.2°C for 45 sec, and 72°C for 1.30 min, and then 10 min at 72°C) according to a modified protocol [39]; KSMA-F and KSMB-R (5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 59.1°C and 2 min at 72°C followed by a 5-min extension step at 72°C) as described [51]; 540F and 1100R (5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C and 2 min at 72°C followed by a 5-min extension step at 72°C) as described [52]**;** degKS2F and degKS2R (5 min at 94°C followed by 40 cycles of 40 sec at 94°C, 40 sec at 55°C, 75 sec at 72°C and a final step of 5 min at 72°C) [53]; ADEdom5 and ADEdom3 (5 min at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 60°C and 1.5 min at 72°C, followed by a final extension of 10 min at 72°C) [37]. An aliquot of 5µl of each PCR product and 5 µl of standard 1Kb ladder (Fermentus) were subjected to 1% agarose gel electrophoresis in TAE buffer for 30 min at 90 V. The gel was stained with 50mg/ml of ethidium bromide and then digital images were obtained for the DNA bands of expected size using UV transilluminator (Bio-Rad Laboratories, Hercules, CA).

2.5 Purification and Gene Sequencing of PCR Products

Amplified DNA of NRPS and PKS gene fragments were purified using QIAquick PCR Purification Kit (QIAGEN; Venlo, Netherlands) according to the manufacturer instructions as follows: Fifty microliters of each PCR sample was added to 125 µl PB Buffer in a clean eppendorf. Ten microliters of 3 M sodium acetate (pH 5.0) was added gradually until the mixture turned yellow. The samples were then added in a QIAquick columns, centrifuged for 60 sec at 17, 900 xg and the flow was discarded. QIAquick columns were washed with 0.75 ml BE Buffer and centrifuged for 60 sec at 17, 900 xg. To elute the DNA, each QIAquick column was placed in a clean 1.5 ml microcentrifuge tube, then 50 µl of BE Buffer (10 mM Tris·Cl, pH 8.5) was added carefully to the center of the QIAquick membrane and the column was centrifuged for 60 sec at 17, 900 xg. The purified samples were kept in deep freezer at -20°C and become ready for sequencing. Purified PCR products of both NRPS and PKS gene fragments were sequenced [54] at the Genome service in Warwick University, Coventry, United Kingdom (GATC Biotech AG, Cologne, Germany).

2.6 Sequence Analysis and Phylogenetic Tree Construction

All nucleotide sequences were deposited in the gene bank. ORFfinder Server (https://www.ncbi.nlm.nih.gov/orffinder/) and ExPASy Proteomics Server (http://web.expasy.org) [55], followed by Reverse translation at sequence manipulation site (http://www.bioinformatics.org/sms2/rev_trans.html) were used to predict open reading frames in each NRPS and PKS gene fragment sequences of selected strains [56]. Each open reading frame was blasted against the amino

Table 1. Primes used for the amplification of NRPS and PKS genes

Primers	Sequence (5'_'3)	Gene	fragment (bp)	Reference
A3F	5'- GCSTACSYSATSTACACSTCSGG-3'	NRPS	700–800	**(Ayuso-Sacido & Genilloud, 2004)**
A7R	5'SASGTCVCCSGT SCGGTAS-3'	NRPS	700–800	**(Ayuso-Sacido & Genilloud, 2004)**
NRPS F	5'-CGCGCGCATGTACTGGACNGGNGAYYT-3'	NRPS	480	**(Amos et al., 2015)**
NRPS R	5'-GGAGTGGCCGCCCARNYBRAARAA-3'	NRPS	480	**(Amos et al., 2015)**
PKS F	5'-GGCAACGCCTACCACATGCANGGNYT-3'	PKS	350	**(Amos et al., 2015)**
PKS R	5'-GGTCCGCGGGACGTARTCNARRTC-3'	PKS	350	**(Amos et al., 2015)**
540F	5_-GGITGCACSTCIGGIMTSGAC- 3	PKS II	554	**(Wawrik et al., 2005)**
1100R	5_ CCGATSGCICCSAGIGAGTG-3	PKS II	554	**(Wawrik et al., 2005)**
5LL	5'-GGRTCNCCIARYTGIGTICCIGTICCRTGIGC-3'	PKS	~ 600	**(Parsley et al., 2011)**
4UU	5'-MGIGARGCIYTICARATGGAYCCICARCARMG-3'	PKS	~ 600	**(Parsley et al., 2011)**
KSMA-F	5′-TS GCS ATG GAC CCS CAG CAG-3′	PKS I	~ 700	**(Izumikawa et al., 2003)**
KSMB-R	5′-CC SGT SCC GTG SGC CTC SAC-3′	PKS I	~ 700	**(Izumikawa et al., 2003)**
degKS2F	5'- GCIATGGAYCCICARCARMGIVT-3'	NRPS	~ 600	**(Ayuso-Sacido & Genilloud, 2004)**
degKS2R	5'-GTICCIGTICCRTGISCYTCIAC-3'	NRPS	~ 600	**(Ayuso-Sacido & Genilloud, 2004)**
ADEdom5	5′-ACS GGC NNN CCS AAG GGC GT-3′	NRPS	450	**(Busti et al., 2006)**
ADEdom3	5′-CTC SGT SGG SCC GTA-3′	NRPS	450	**(Busti et al.,2006)**

acid sequences accessible in NCBI database using BlastP and BlastN. Phylogenetic trees were constructed using multiple sequence alignment of amino acid sequences using the CLUSTAL W program [57] and Neighbour-Joining method within the Mega 7 program [58].

2.7 Construction of Fosmid Genomic Libraries

Creation of fosmid genomic libraries were performed for Rc5 and Ru87 isolates according to a modified protocol [20,59] using Copy-control™ Fosmid Library phage packaging system (Epicentre, Madison, WI, USA) as follows:

2. 7.1 Isolation of high molecular weight DNA

High molecular weight DNA was extracted from both strains using promega genomic DNA extraction kit as previously stated. Purified DNA was sheared to approximately 35-40 kb fragments by pipetting 30 times. Then, Low Melting Point agarose gel electrophoresis 1% was prepared. An amount of 10 µl (100 ng/ µl) fosmid control DNA and 10 µl of (140 ng/ µl) DNA sample of both strains were added into each lane. The gel was adjusted at 30-35V overnight. After the run was completed, the gel was stained with ethidium bromide (50mg/ml) and visualized. As the migration of high molecular weight DNA of *Micromonospora* Rc5 and *Streptomyces* Ru87 was similar in size to that of fosmid Control DNA (35-40 kb)Then, end-repair reaction was done for the insert DNA to generate blunt-ended, 5'-phosphorylated DNA. All reagents were thawed, thoroughly mixed and placed on ice.

2.7.2 Preparation of desired size of end repaired DNA

Insert DNA was converted to an end repaired DNA which is a blunt-ended, 5'-phosphorylated DNA. An aliquot of 8 µl 10x End-Repair Buffer, 8 µl 2.5 mM dNTP Mix , 8 µl 10 mM ATP , 20 µl sheared insert DNA (0.5 µg/µl), 4 µl End-Repair Enzyme Mix and 12 µl sterile water were mixed in a 0.5 ml Eppendorf. The reaction was incubated at 37**°**C for 45 min. Another incubation was conducted at 70**°**C for 10 min to stop the end- repair reaction. Purification step of the end-repaired DNA was performed by using centrifugal-filter tubes with ultra-cell membrane 100 kDa (Amicon-Ultra-0.5 mL) as a modification instead of using low melting point agarose gel electrophoresis purification [59]. Purification using centrifugal filter tubes prevents wasting of DNA as well as recovering a higher yield. A volume of 80 µl of end-repaired DNA was added to ultra-cell membrane column of centrifugal-filter tubes and centrifuged at 14000 xg for 20min. The flow in the collection tube was discarded and 10 µl was left in the column. An amount of 500 µl of sterile water was added and centrifuged again at 14000 xg for 20min. The flow was discarded and 10 µl were left. The column was inverted up and down, centrifuged at 16000 xg for 30 sec and then the collection tube was replaced by a new one. An aliquot of 10 µl sterile water was added to the column, inverted up and down and centrifuged at 16000 xg for 30 sec. Membrane columns were discarded. Purified DNA samples were kept in the new collection tube and stored at 4**°**C [59].

2.7.3 Ligation into pCC1FOS vector and MaxPlax Lambda Packaging

Ligation reaction was performed to stick the blunt-ended DNA to the Cloning-Ready Copy Control fosmid pCC1FOS. The following reagents were combined: 1 µl 10X Fast-Link Ligation Buffer, 1 µl 10 mM ATP, 1 µl CopyControl pCC1FOS Vector (0.5 µg/µl), 3 µl concentrated insert DNA(0.25 µg), 1 µl Fast-Link DNA Ligase and 3 µl sterile water. Ligation reaction was incubated at 28**°**C for 4 hours, followed by incubation at 70**°**C for 10 min to inactivate the ligase enzyme. Then, packaging of the ligated DNA into MaxPlax Lambda Packaging extracts was performed. In a clean 0.5 ml Eppendorf tube, an amount of 10 µl of the ligation reaction was added to 25 µl of the thawed MaxPlax Lambda Packaging extracts and held on ice. The solutions were mixed by pipetting several times. Packaging reactions were incubated at 30**°**C for 2 hours. Another aliquot of 25 µl of MaxPlax Lambda Packaging Extract was added to the ligation reaction and incubated extra 2 hours at 30**°**C. At the end of the second incubation, 940 µl phage dilution buffer (PDB) and 25 µl of chloroform were added to the packaging reaction, mixed gently and stored at 4**°**C.

2.7.4 Transformation in *E. coli* cells

A volume of 10 µl previously prepared diluted phage particles was added to 100 µl of prepared EPI300-T1R *E.coli* cells (overnight culture obtained from 0.5 ml EPI300-T1R *E. coli* inoculated in 50 ml of LB broth containing 10 mM MgSO4 and 0.2% Maltose and shaked at 37**°**C

to an $O.D^{600}$ of 0.8-1.0. The reaction was incubated at 37°C for 1 hour and then the infected bacteria was spreaded on LB plates containing chloramphenicol (12.5 mg/ml) and incubated at 37°C overnight to select the CopyControl fosmid clones. Fosmid clones of interest were picked and inoculated in 96-well cell culture plates (Becton Dickinson Labware). Each well contained 150 µl LB medium, 12.5 µg/ml chloramphenicol and 1X CopyControl Fosmid Autoinduction Solution (Epicentre). Genomic library plates were taped to prevent desiccation, incubated at 37°C for 24 hrs and then stored at 4°C and -80°C for further assays.

2.8 NRPS and PKS PCR Screening of Actinomycetes Genomic Libraries

2.8.1 Fosmid DNA extraction from *E.coli* clones

A volume of 150 µl LB broth containing 12.5 mg/ml chloramphenicol was added in each well of 96 well culture plate (Becton Dickinson Labware) using a multichannel pipette. An aliquot of 15 µl of each clone of transformed *E. coli* cells was inoculated in each well. Plates were taped and Incubated at 37°C shaking incubator (150 RPM) for 24 hrs. Using a multichannel pipette, 10 µl from each of 96 wells was transferred into a sterile reservoir and then transferred into a 500 µl eppendorf tube and centrifuged at 13,000 xg for 5 min. The supernatant was discarded and the *E. coli* pellet containing fosmids were extracted using the GeneJET plasmid miniprepkit as per manufacturer's instructions as follows: Fosmid extraction was carried by adding 250 µL of suspension solution, 250 µL lysis solution and 350 µL neutralization solution into a 500 µl eppendorf tube containing pelleted *E. coli* cells and centrifuged for 5 min at 13,000 xg. The supernatant was transferred to the Thermo Scientific GeneJET Spin Column and centrifuged for 1 min. Spin Column was washed twice with 500 µL wash solution and centrifuged for 60 sec at 13,000 xg. The flow through was discarded and centrifuged for 1 min at 13,000 xg. The column containing purified fosmid DNA was transferred into a 1.5 ml microcentrifuge tube and 50 µL of elution buffer was added to the column, incubated 2 min at 28°C and centrifuged for 2 min at 13,000 xg. The flow-through was collected for further PCR assay.

2.8.2 NRPS and PKS PCR assay

A PCR reaction was performed with 1 µl of the purified DNA template aiming to check genes belonging to the NRPS and PKS cluster in *E.coli* clones. Positive PCR results were obtained form each individual well to identify the transformant *E. coli* cells containing the fosmid of interest. A PCR assay using (5LL/4UU, 540F/1100R and ADEdom3/5) primer set was used for screening both libraries. Additional primers (A3F/A7R and degKS2F/R) were also used to screen *Streptomyces* Ru87 genomic library. All PCR thermocycling conditions were conducted as previously described. *S.coelicolor* was used as a positive control in all reactions as it contains both NRPS and PKS genes clusters.

2.8.3 Bioinformatics analysis of fosmid-derived open reading frames (ORFs)

Amplified DNA of NRPS and PKS gene fragments recovered from five selected fosmid *E. coli* clones were purified using QIAquick PCR Purification Kit (QIAGEN; Venlo, Netherlands) according to the manufacturer instructions as previously mentioned and sequenced [54] at the Genome service in Warwick University, Coventry, United Kingdom (GATC Biotech AG, Cologne, Germany). NRPS and PKS PCR screening assays recovered positive *E. coli* fosmid clones harbouring either NRPS or PKS genes. Amplified DNA of NRPS and PKS gene fragments recovered from five selected fosmid *E. coli* clones were purified and sequenced. DNA sequences were analyzed using ORFfinder server ((https://www.ncbi.nlm.nih.gov/orffinder/) or ExPASy Proteomics Server (http://web.expasy.org) [55], followed by conversion into coding DNA sequences using reverse translate tool [56] at Sequence Manipulation site (http://www.bioinformatics.org/sms2/rev_trans.html). The prediction of gene function was accomplished by comparing each ORF DNA sequence against the GenBank database using the BLASTN and BLASTX algorithm.DNA sequences were analyzed in order to confirm the integration of NRPS and PKS genes in the *E. coli* clones. Multiple sequences were aligned using the CLUSTAL W program [57] against corresponding aminoacid sequences. The tree was constructed using the neighbour-joining method and the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 [58]. The numbers besides the branches indicate the percentage bootstrap value of 1000 replicates. The scale bar indicates nucleotide sequence dissimilarity.

3. RESULTS

3.1 Antimicrobial Potential of Rare Actinomycete Isolates

Thirty two rare actinomycetes (64% of total isolates) found to inhibit one or more pathogenic bacteria in soya bean meal broth while twenty nine (58%) showed antimicrobial activities on starch casein broth medium . Antimicrobial activities varied against tested microorganisms grown in soya bean broth as follows: twenty seven isolates (84%) with *Pseudomonas aeruginosa* ATCC 10145*,* nine isolates (28%) against *Escherichia coli* ATCC 51659 and seven isolates (21%) for each of *Staphylococcus aureus* ATCC 6538 and *Salmonella enterica* ATCC 25566). Only Four isolates (12%) have shown antimicrobial activities against *Streptococcus mutans* 25175, and 3 isolates (9%) were active against *Klebsiella pneumonia* CCM 4415 . The antimicrobial activity of rare actinomycetes grown on starch casein broth recorded the following results: 23 (79%) against *Pseudomonas aeruginosa* ATCC 10145*,* followed by 8 (27%) with *Staphylococcus aureus* ATCC 6538 and 7 (24%) against *Klebsiella pneumonia* CCM 441. In addition, Four isolates (13%) were active against *Streptococcus mutans* 25175 and 3 isolates (1%) showed activity against *Salmonella enterica* ATCC 25566. No antimicrobial activity was recorded against *Escherichia coli* ATCC 51659. Our findings confirmed that Forty rare actinomycetes showed significant antimicrobial activities against some food and blood borne pathogens. Among which, *Micromonospora* Rc5 and *Streptomyces* Ru87 reported the highest spectrum of antimicrobial activities against both Gram positive and Gram negative tested pathogens. Rc5 and Ru87 isolates were previously identified as *Micromonospora* Rc5 and *Streptomyces* Ru87 via morphological, chemotaxonomy and 16S rRNA gene sequencing [48, 49,50]..

3.2 PCR Screening of PKS and NRPS Gene Fragments

Digital images of agarose gel captured by UV transilluminator (Bio-Rad Laboratories, Hercules, CA) confirmed the successful amplification of the predicted sizes (350-700 bp) of NRPS and PKS gene fragments for both *Micromonospora* Rc5 and *Streptomyces* Ru87 (Figs. 1,2,3,4,5). Adenylation domains of NRPS clusters were detected in *Micromonospora* sp. Rc5 using primer sets (Ade dom3/5 F/R, A3F/A7R and NRPS F/R) and generated adenylation domain sequences similar to *Micromonospora carbonaceae* with an identity (95%) and *Micromonospora siamensis* strain DSM 45097 with (80%) blast identity (Table 2). PKS gene fragment screening by using (540-1100 F/R and 5U/4L F/R) primers detected gene fragments related to PKS clusters in *Micromonospora haikouensis* and *Micromonospora carbonaceae* with a blast identity (97%) and (87%), respectively. While, fragments recovered by (KSMA/B, PKS F/R and degk2 F/R) primer sets showed blast identity (82%, 81% and 74%) to *Micromonospora* sp. YIM*, Micromonospora* sp. SAUK6030 and *Micromonospora chalcea* (Table, 2)*.* In case of *Streptomyces* sp. Ru87, Blastn analysis of NRPS gene fragments detected by (A3A7 F/R and NRPS F/R) primer sets showed nucleotide identity (75% and 68%) to *Streptomyces venezuelae* and *Streptomyces* strain MM14, respectively (Table, 2). While, PKS gene fragments detected by using (540-1100 F/R and PKS F/R) primer sets showed (83%) identity to *Streptomyces marokonensis* and identity (87%) to *Streptomyces eurocidicus* (Table 2). All sequenced amplicons can be viewed under GenBank accession numbers (MF928516, MF928517, MF928518, MF928519, MF928520, MF928521, MF928522, MF928523, MF928524, MF928525, MF928526, MF928527).

3.3 Phylogenetic Analysis of NRPS Genes

NRPS gene fragments of our identified strains were converted into amino acid sequences (open reading frames) using open reading frame identifiers. Each open reading frame was blasted against the amino acid sequences accessible in NCBI database via Blastn and Blastp. Similar Comparative sequences were used for phylogenetic tree consruction. NJ phylogenetic tree showed that (adenylation domain) NRPS gene fragment of *Micromonospora* sp. Rc5 is grouped in the same clade with different species of genus *Micromonospora*. In addition to, it shared a high bootstrap value (99%) with NRPS gene fragment of *Micromonospora carbonaceae* and *Micromonospora haikouensis* (Fig.6). NJ phylogenetic tree showed that NRPS gene sequence of *Streptomyces* sp. Ru87 is gathered with other related sequences in *Streptomyces* genus including *Streptomyces ribosidificus, Streptomyces hygroscopicus* and *Streptomyces viridochromogenes* with (54%) boot strap value. However, NRPS gene fragment of *Streptomyces* sp. Ru87 was separated in a distinct branch (Fig. 7).

Table 2. Blast identity of NRPS and PKS gene fragments of *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87. NRPS and PKS PCR screening assays were conducted using different primer sets. NRPS and PKS Positive hits were sequenced and Nucleotide sequence identity was recorded using blastp and blastn algorithm

Primers	Strain	Accession No.	Blast ID	gene Annotation	Closest match accession No.	E value	% Identity	Search tool	Open Reading frame identifer
Ade dom3-5-F/R	*Micromonospora* sp. Rc5	MF928516	*Micromonospora carbonacea*	adenylation domain (NRPS)	WP_043963323.1	4e-70	95%	blastp	ORFfinder
540F/1100R	*Micromonospora* sp. Rc5	MF928519	*Micromonospora haikouensis*	PKS ketosynthase	SCE93413.1	1e-42	97%	blastp	ORFfinder
KSMA/B F/R	*Micromonospora* sp. Rc5	MF928520	*Micromonospora* sp. YIM	Polyketide synthase I	KT359257.1	1e-106	82%	blastn	Expasy server
A3F/A7R	*Micromonospora* sp. Rc5	MF928517	*Micromonospora carbonacea*	adenylation domain(NRPS)	WP 043963323.1	4e-70	95%	blastp	ORFfinder
5U/4L-F/R	*Micromonospora* sp. Rc5	MF928522	*Micromonospora carbonacea*	TypeI Polyketide synthase	WP 043967820.1	7e-50	87%	blastp	ORFfinder
PKS F/R	*Micromonospora* sp. Rc5	MF928521	*Micromonospora* sp. SAUK6030	Type II polyketide synthase gene	GQ118939.1	2e-72	81%	blastn	Expasy server
NRPS F/R	*Micromonospora* sp. Rc5	MF928518	*Micromonospora siamensis* strain DSM 45097	adenylation domain(NRPS)	LT607751.1	6e-21	80%	blastn	Expasy server
degk2F/R	*Micromonospora* sp. Rc5	MF928523	*Micromonospora chalcea*	tetrocarcin polyketide synthase	EU443633.1	2e-92	74%	blastn	Expasy server
540-1100 F/R	*Streptomyces* sp. Ru87	MF928526	*Streptomyces marokonensis*	Type II polyketide synthase	AGO59072.1	3e-39	83%	blastp	ORFfinder
A3F/A7 R	*Streptomyces* sp. Ru87	MF928524	*Streptomyces venezuelae*	NRPS	CP018074.1	2e-111	75%	blastn	Expasy server
PKS F/R	*Streptomyces* sp. Ru87	MF928527	*Streptomyces eurocidicus*	Ketosynthase gene(PKS)	ADW44002.1	1e-20	87%	blastp	ORFfinder
NRPS F/R	*Streptomyces* sp. Ru87	MF928525	*Streptomyces* strain *MM14*	NRPS	KX708215.1	8e-26	68%	blastn	Expasy server

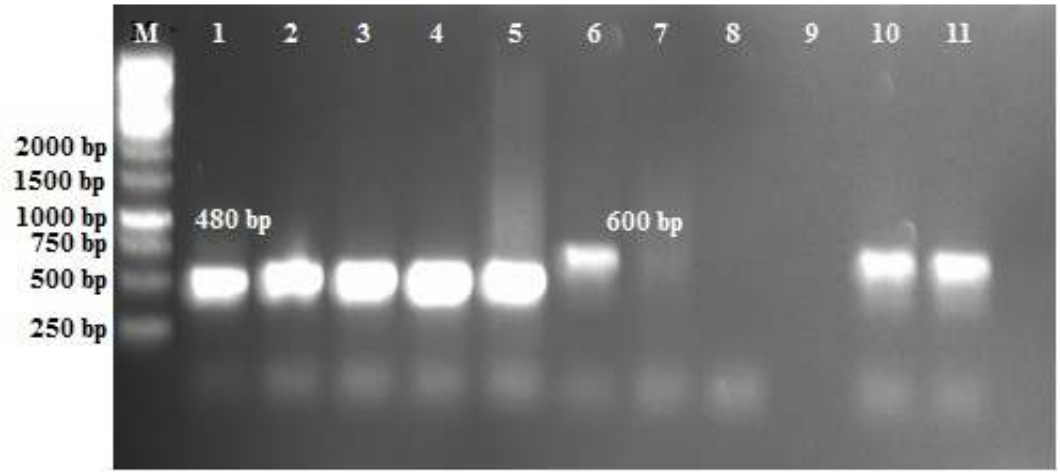

Fig. 1. PCR positive hits using (NRPS F/R) NRPS PCR assay and (degk2 F/R) PKS PCR assay
PCR amplicons were subjected to electrophoresis in 1% agarose gels with 50mg/ml of ethidium bromide. Electrophoresis running buffer (0.2MTris base, 0.1Msodium acetate, and 0.01 M Na2EDTA, pH 7.8) for 30 min at 90 V. Digital images were obtained using a UV transilluminator (Bio-Rad Laboratories, Hercules, CA). Lane M: 1 KB ladder, NRPS Positive hits by NRPS F/R primers, Lane 1: control, Lane 2,3: PCR product (480 bp) of Micromonospora sp. Rc5 , Lane4,5, : PCR product of (480 bp) Streptomyces sp. Ru87. PKS Positive hits by degk2 F/R primers. Lane 6: control, Lane 7, 8, 9: Negative PKS gene detection results in case of Streptomyces sp. Ru87, Lane 10, 11: PCR product (600bp) of Micromonospora sp. Rc5.

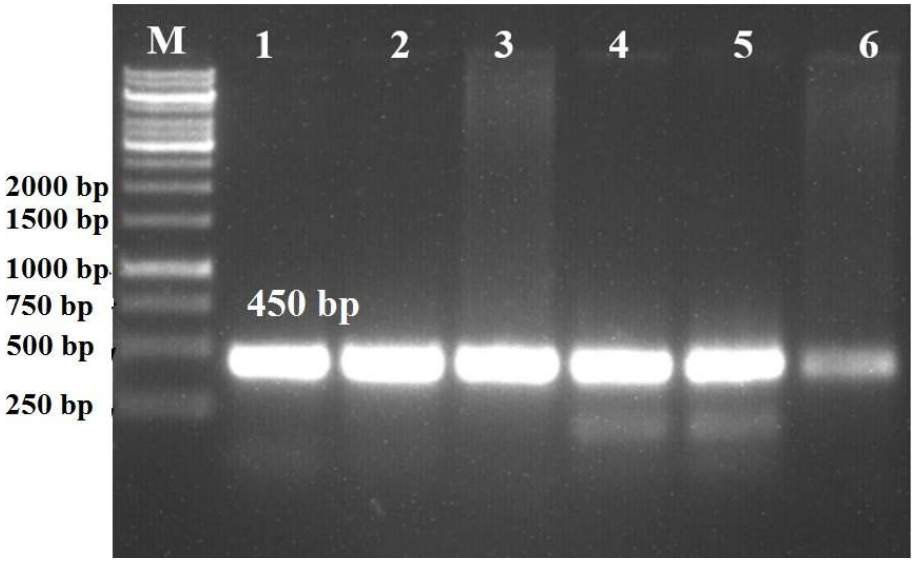

Fig. 2. PCR positive hits using (ADEdom 3/5 F/R) NRPS PCR assay.
PCR amplicons using ADEdom 3/5 F/R primers were subjected to electrophoresis in 1% agarose gels as previously described in Fig.1, Lane M: 1 KB ladder, Lane 1: control, Lane 2-6: PCR product (450 bp) of Micromonospora sp. Rc5.

3.4 Phylogenetic Analysis of PKS Genes

NJ phylogenetic tree showed that PKS gene fragments of *Micromonospora* sp. Rc5 are grouped with *Micromonospora haikouensis* with a boot strap of 70% (Fig.8). NJ phylogenetic tree showed that PKS gene sequence of *Streptomyces* sp. Ru87 is gathered with *Streptomyces yeochonensis* and *Streptomyces flaveus* with (43%) bootstrap value (Fig.9). This is to confirm that *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 strains harbour an exceptional phylogenetically divergent PKS gene cluster and would definitely generate distinctive

biochemical pathways of novel antibiotics. We suggest that Rc5 and Ru87 isolates are among uncommon and novel types of actinomycetes and therefore, may contain unexpected genes, and subsequently, generate alternative novel antimicrobial agents [14].

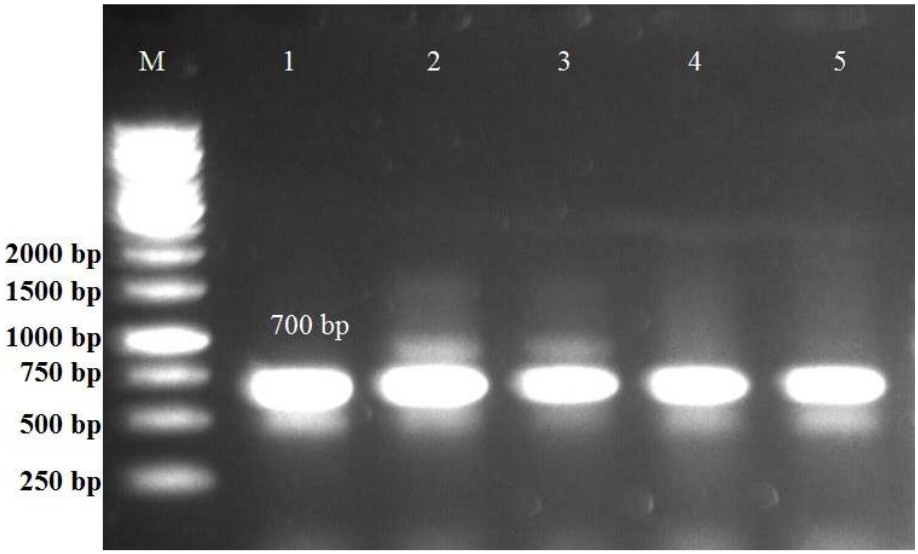

Fig. 3. PCR positive hits using (A3F/A7R) NRPS PCR assay

PCR amplicons using A3F/A7R primers were subjected to electrophoresis in 1% agarose gels as previously described in Fig.1, Lane M: 1 KB ladder, Lane 1: control, Lane 2,3: (700 bp) PCR product of Micromonospora sp. Rc5 , Lane 4,5 : PCR product of Streptomyces sp. Ru87.

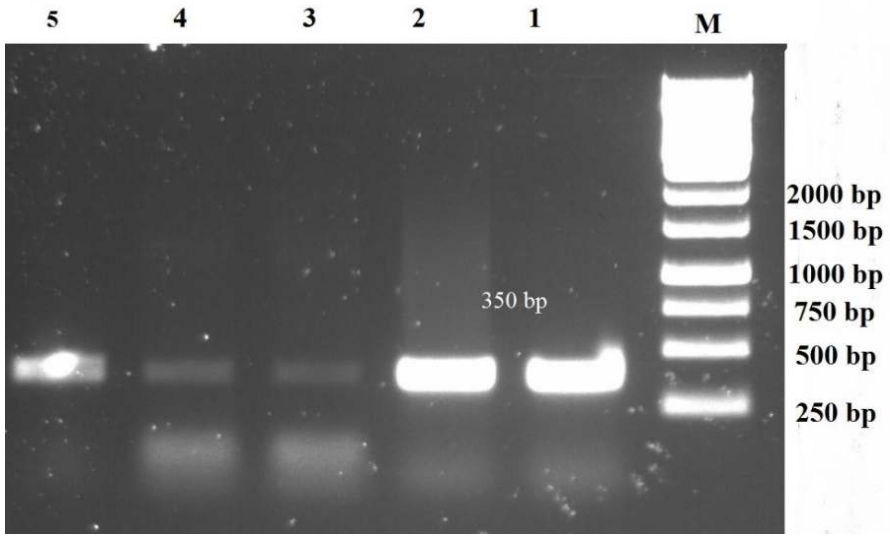

Fig. 4. PCR positive hits using (PKS F/R) PKS PCR assay

PCR amplicons using PKS F/R primers were subjected to electrophoresis in 1% agarose gels as previously described in Fig.1, Lane 5: control, Lane: 1, 2: PCR product (350 bp) of Streptomyces sp. Ru87, Lane 3,4 : PCR product (350 bp) of Micromonospora sp. Rc5.

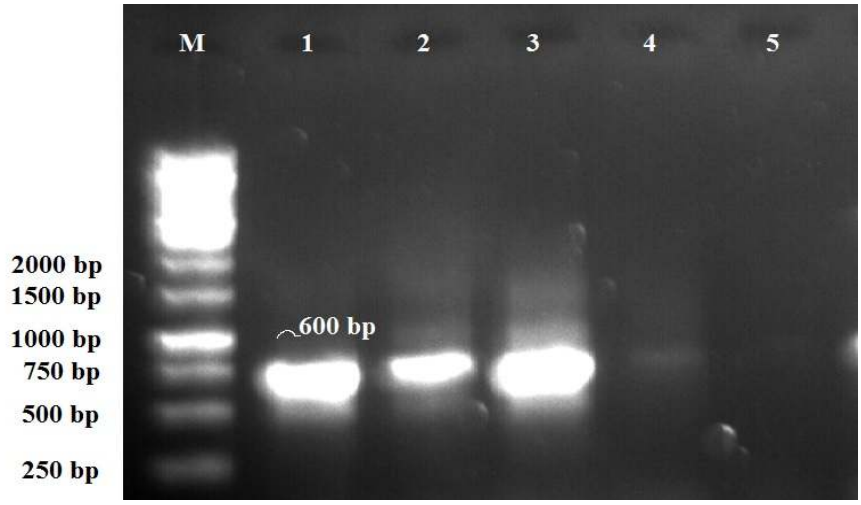

Fig. 5. PCR positive hits using (5LL/4UU F/R) PKS PCR assay

PCR amplicons using 5LL/4UU primers were subjected to electrophoresis in 1% agarose gels as previously described in Fig.1, Lane M: 1 KB ladder, Lane 1: control, Lane 2-4: Positive hits of 600 bp PKS gene of Micromonospora sp. Rc5. Lane 4-5: Negative PKS gene detection results in case of Streptomyces sp. Ru87.

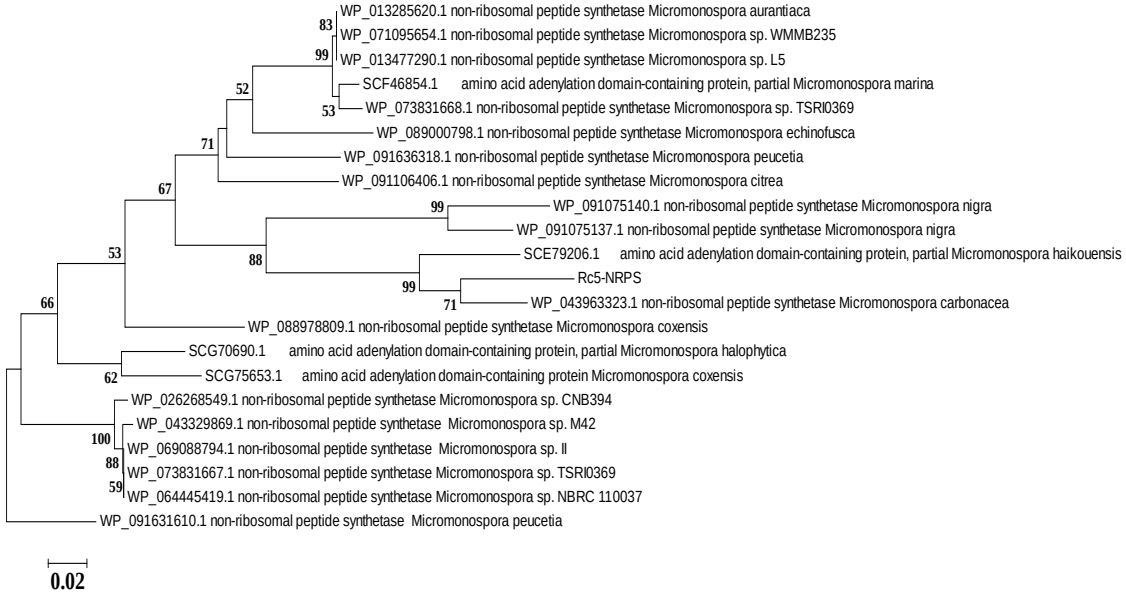

Fig. 6. Phylogenetic tree based on amino acid sequence of NRPS gene fragments of *Micromonospora* sp. Rc5 amplified by primer pair A3/A7 F/R.

Multiple sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) against corresponding aminoacid sequences. The tree was constructed using the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Tamura et al, 2011). The numbers besides the branches indicate the percentage bootstrap value of 1000 replicates. The scale bar indicates nucleotide sequence dissimilarity.

To our knowledge, this is first attempt to tackle phytogenic analysis of NRPS and PKS genes for rare actinomycetes isolated from Egyptian soils. Therefore, offers a significant contribution for novel antimicrobial compounds in drug discovery.

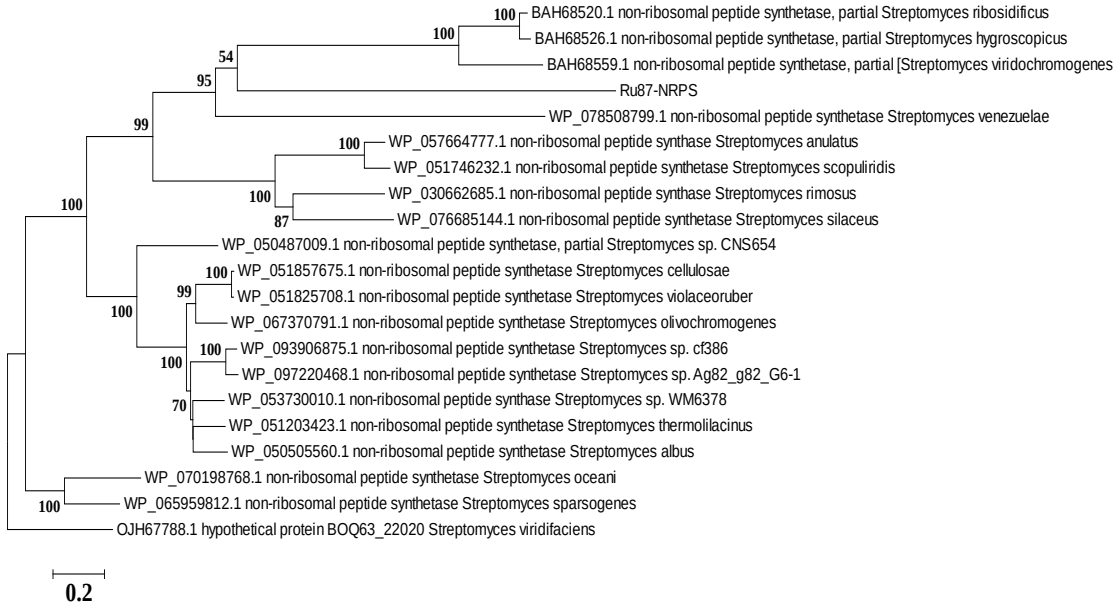

Fig. 7. Phylogenetic tree based on amino acid sequence of NRPS gene fragment of *Streptomyces* sp. Ru87 amplified by by primer pair NRPS F/R.

Multiple sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) against corresponding aminoacid sequences. The tree was constructed using the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Tamura et al, 2011). The numbers besides the branches indicate the percentage bootstrap value of 1000 replicates. The scale bar indicates nucleotide sequence dissimilarity.

3.5 Construction of Rare Actinomycetes Fosmid Libraries

High molecular weight DNA of both *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 were extracted and successfully sheared into approximately 35 Kb according to manufacturer instructions (Fig. 10). DNA ligation and packaging was confirmed via the growth of *E. coli* clones on LB-chloramphenicol plates. Three hundred *E. coli* clones were selected to construct *Micromonospora* sp. Rc5 genomic library. On the other hand, *Streptomyces* sp. Ru87 genomic library consisted of ~500 *E. coli* clones were recovered in 96- well plates. If we assume that the average insert DNA size captured by a fosmid is approximately 35 kb. Therefore, the total library size of *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 were 10.5 Mb and 17.5 Mb, respectively. One genome equivalent = total genome length / average insert size. We concluded that our genomic libraries contain sufficient DNA sequences, which is one genome equivalent in case of *Micromonospora* sp. Rc5 and 2 genome equivalent in case of *Streptomyces* sp. Ru87.This means that the DNA captured in the fosmids of each library gives a full representation of the whole genome of each strain.Our genomic libraries is available upon request for PCR screening for any desired gene sequence or even DNA manipulation for antibiotic production using heterologous hosts, after supervisor's approval.

3.6 PKS and NRPS Screening for Actinomycetes Strains Genomic Libraries

NRPS PCR screening assay allowed the recovery of 10 positive NRPS *E.coli* clones from *Micromonospora* sp. Rc5 genomic library and *23* positive NRPS *E.coli* clones from *Streptomyces* sp.Ru87 library (Figs.11,12). PKS PCR screening assay indicated that only 2 clones were positive for PKS genes recovered from *Micromonospora* sp. Rc5 and 12 were positive for PKS clones from *Streptomyces* sp.Ru87 genomic libraries (Figs.11,12). Those positive hits suggest that NRPS or PKS biosynthetic pathways are existing within the genome. Our results ensures successful library construction of both strains including integrated PKS and/ or NRPS gene fragments in *E.coli* clones. Selected

sequenced clone amplicons can be viewed under GenBank accession numbers (MF928528, MF928529, MF928530, MF928531, MF928532).

3.7 Bioinformatic Analysis DNA Sequences Obtained from *E.coli* Fosmid Clones

Five *E. coli* clones were selected including A4, C8 and C9 from *Micromonospora* sp. Rc5 genomic library, while 2 clones B5 and G5 were recovered from *Streptomyces* sp. Ru87 genomic library. Bioinformatics investigations of sequence data obtained from *E.coli* clones derived from both *Micromonospora* sp Rc5 and *Streptomyces* sp. Ru87 genomic libraries**,** indicated that they contained NRPS and PKS genes with significant homology to NRPS and PKS genes of *Micromonospora* and *Streptomyces* genera, respectively. Our results confirmed the integration of NRPS and PKS within the fosmid clones (Table,3).

4. DISCUSSION

In this study, we have conducted antimicrobial screening for 50 rare actinomycetes isolated from Egyptian soil against some food and blood borne pathogens. Our results confirmed that 40 isolates inhibited the growth of one or more food and blood borne pathogens. This is the first study to target NRPS and PKS genes captured from rare actinomycetes isolated from Egyptian soil using, NRPS and PKS gene sequencing, phylogenetic analysis based on amino acid sequences, and fosmid library construction. Our study confirmed that *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 contained both PKS and NRPS gene clusters. Therefore, they can produce diverse bioactive molecule**s.** Similar studies clarified the presence of NRPS and PKS genes in *Streptomyces* sp**.** against gram positive and gram negative pathogens [30,60,61] recorded the presence of NRPS and PKS genes in antibiotic producing *Micromonospora* genera.

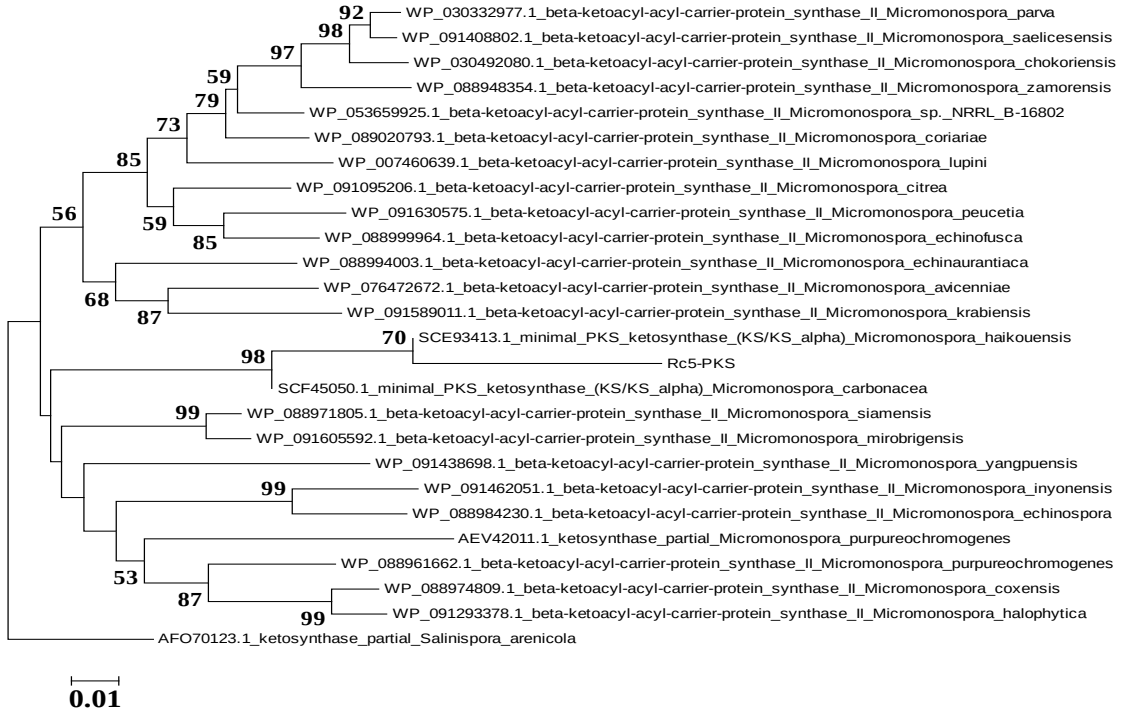

Fig. 8. Phylogenetic tree based on amino acid sequences of PKS gene fragments of *Micromonospora* sp. Rc5 amplified via primer pair degk2F/R.

Multiple sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) against corresponding aminoacid sequences. The tree was constructed using the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Tamura et al, 2011). The numbers besides the branches indicate the percentage bootstrap value of 1000 replicates. The scale bar indicates nucleotide sequence dissimilarity.

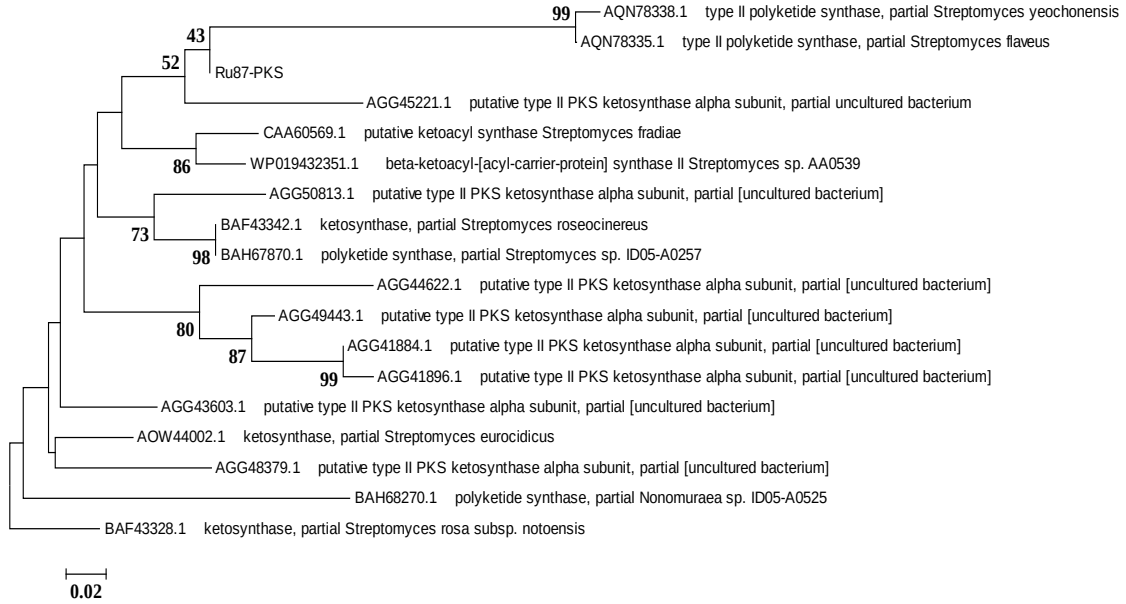

Fig. 9. Phylogenetic tree based on amino acid sequences of PKS gene fragments of *Streptomyces* sp. Ru87 amplified via primer pair540-1100 F/R.

Multiple sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) against aminoacid sequences. The tree was constructed using the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Tamura et al, 2011). The numbers besides the branches indicate the percentage bootstrap value of 1000 replicates. The scale bar indicates nucleotide sequence dissimilarity.

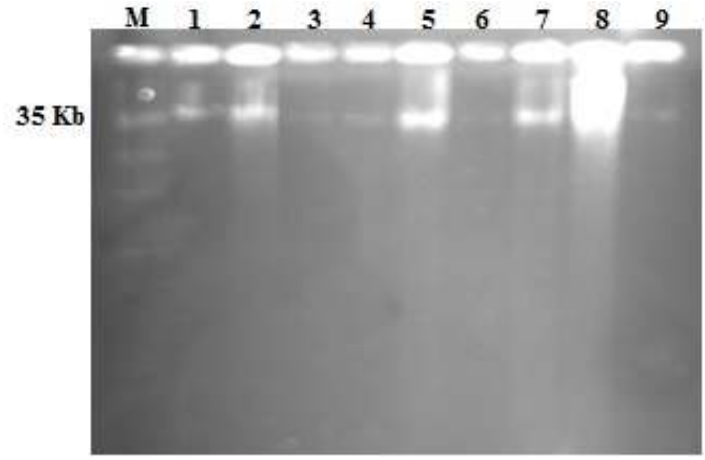

Fig. 10. Sheared genomic DNA of *Streptomyces* sp. Ru87 and *Micromonospora* sp. Rc5.

Purified DNA was sheared to approximately 35-40 kb fragments by pipetting 30 times. Then, Low Melting Point agarose gel electrophoresis 1% was prepared. An amount of 10 µl (100 ng/ µl) fosmid control DNA and 10 µl of (140 ng/ µl) DNA sample of both strains were added into each lane. The gel was adjusted at 30-35V overnight. After the run was completed, the gel was stained with ethidium bromide (50mg/ml) and visualized. Gel electrophoresis of agarose gel was illustrated as follows: The first band pattern is the lambda ladder. Lane 2 indicates the 35 kb of control lambda DNA. Lane 2 -5 shows Streptomyces sp. Ru87 genomic DNA, while lane 5-9 shows Micromonospora sp. Rc5 genomic DNA

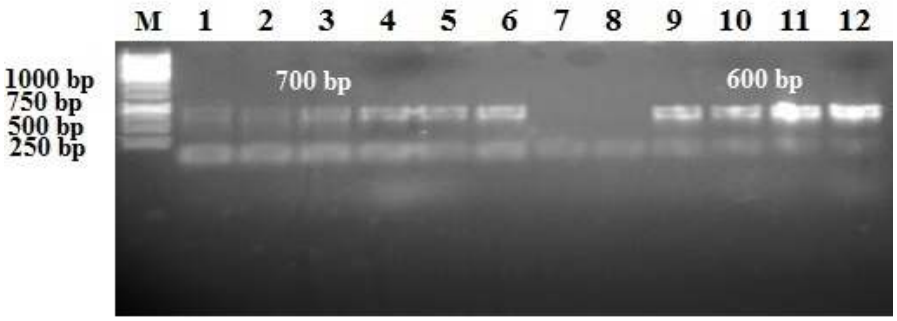

Fig. 11. NRPS and PKS PCR screening of *Micromonospora* sp. Rc5 genomic library.
PCR amplicons were subjected to electrophoresis in 1% agarose gels as previously described in Fig.1, Lane M: 1 KB ladder, Lane 3-6: Positive hits of NRPS (700 bp) genes using A3F/A7R primers, , Lane: 9-12: Duplicates of positive hits of PKS (600 bp) using 5LL/4UU F/R primers

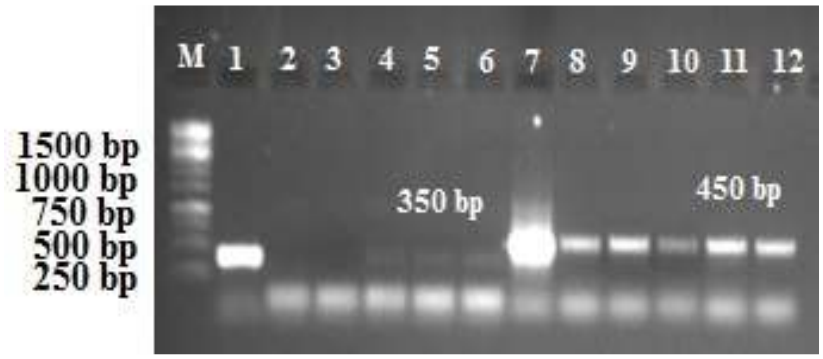

Fig. 12. PKS and NRPS PCR screening of *Streptomyces* sp Ru87 genomic library.
PCR amplicons were subjected to electrophoresis in 1% agarose gels as previously described in Fig.1. Lane M: 1 KB ladder, Lane 1: control, Lane 4-6: Positive hits of 350 bp of PKS genes isolated from E. coli clones of the Streptomyces sp. Ru87 genomic library by PKS F/R primers. While, lane 7: control, Lane 8-12: Positive hits of 450 bp of NRPS genes isolated from E. coli clones of the Streptomyces sp. Ru87 genomic library by ADEdom 3/5 primers.

There is an evident that isolates which contain NRPS and PKS genes, have the capability to produce a wide range of bioactive molecules than others that lack those clusters [20,27]. Our study confirmed that *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 contained PKS and NRPS gene clusters. Similar studies clarified the presence of NRPS and PKS genes in antagonistic *Streptomyces* sp. S34 to gram positive and gram negative pathogens [60]. Antimicrobial activity was reported in *Streptomyces* that contain PKS-I, PKS-II and NRPS and isolated from Saudi Arabian soil [61]. The presence of NRPS and PKS genes in antibiotic producing *Streptomyces* and *Micromonospora* genera was also isolated from soil samples in Qinghai-Tibet Plateau[29]. Moreover, the presence of NRPS and PKS-I genes was reported in Avermectin producing *Streptomyces avermitilis* NRRL 8165, Rapamycin producing *Streptomyces venezuelae* ATCC 15439, Methymicin producing *Streptomyces hygroscopicus* NRRL 5491, Spiramycin producing *Streptomyces ambofaciens* ATCC 23877, Rifamycins producing *Micromonospora lacustris* ATCC 21975, Megalomicin producing *Micromonospora megalomicea* subsp. *niera* NRRL 3275 and Rosamicin producing *Micromonospora rosaria* ATCC 29337 [30]. Additionally, it was emphasized that 75% of actinomycetes isolates showed antagonistic activity and contained NRPS and PKS genes [62]. Similarly, it was illustrated that, 50% of actinomycetes strains were active against bacterial and fungal pathogens with positive NRPS and PKS genes [63].

Table 3. Bioinformatics analysis of NRPS and PKS positive hits of *E. coli* fosmid clones

Clone Number	Accession number	Rare actinomycetes Strain	Size	Open reading frame	Closest match	Closest match Accession number	Annotation	% Identity	E value	Search tool	Open Reading Frame Identifer
A4	MF928528	*Micromonospora* sp. Rc5	680 bp	ORF4/5	*Micromonospora chalcea*	ACB37742.1	putative type I polyketide synthase	83%	1e-75	blastp	ORFfinder
C8	MF928529	*Micromonospora* sp. Rc5	401 bp	ORF5	*Micromonospora peucetia*	SCL71897.1	Adenylation Domain (NRPS)	84%	6e-63	blastp	ORFfinder
C9	MF928530	*Micromonospora* sp. Rc5	531 bp	ORF2	*Streptomyces gilvosporeus* strain F607	CP020569.1	Adenylation Domain (NRPS)	84%	0.92	blastn	Expasy server
B5	MF928531	*Streptomyces* sp. Ru87	517 bp	ORF5	*Streptomyces iranensis*	LK022848.1	Adenylation Domain (NRPS)	100%	0.96	blastn	Expasy server
G5	MF928532	*Streptomyces* sp. Ru87	513 bp	ORF 2	*Streptomyces* sp. LC-6-2	KY432814.1	abyssomicin biosynthetic gene cluster (PKS)	91%	3	blastn	Expasy server

NRPS and PKS PCR screening assays recovered positive E.coli fosmid clones harbouring either NRPS or PKS genes. Amplified DNA of NRPS and PKS gene fragments recovered from five selected fosmid E.coli clones were purified and sequenced. DNA sequences were analyzed using ExPASy Proteomics Server (http://web.expasy.org) ***(Walker, 2005)****, followed by conversion into coding DNA sequences using reverse translate tool according to* ***Stothard, 2000*** *at Sequence Manipulation site or by using ORFfinder. The prediction of gene function was accomplished by comparing each ORF DNA sequence against the GenBank database using the BLASTN and BLASTP algorithm.*

In our study, Adenylation domains of NRPS clusters in *Micromonospora* sp. Rc5 indicated that the most similar adenylation domain sequences is related to *Micromonospora carbonaceae* with an identity (95%) and *Micromonospora siamensis* strain DSM 45097 with (80%) blast identity. While, PKS fragments was related to *Micromonospora haikouensis* and *Micromonospora carbonaceae* with a blast identity (97%) and (87%), respectively. In case of *Streptomyces* sp. Ru87, Blastn analysis of NRPS gene showed nucleotide identity (75% and 68%) to *Streptomyces venezuelae* and *Streptomyces* strain MM14, respectively. PKS fragments showed identity (83%) to *Streptomyces marokonensis* and identity (87%) to *Streptomyces eurocidicus.* Lower blast identity results ensure that our strains contained different distinct NRPS and PKS clusters other than comparative sequences on the data base.

NJ phylogenetic tree showed that adenylation domain of NRPS gene of *Micromonospora* sp. Rc5 shared a high bootstrap value (99%) with NRPS gene fragment of *Micromonospora carbonaceae* and *Micromonospora haikouensis*. Moreover, adenylation domain of NRPS gene of *Streptomyces* sp. Ru87 is gathered with related NRPS sequences of *Streptomyces ribosidificus, Streptomyces hygroscopicus, Streptomyces viridochromogenes* and *Streptomyces venezuelae* with (95%) boot strap value. NRPS gene fragment of *Streptomyces* sp. Ru87 was separated in a distinct branch pointing out that our isolate have different ancestor than that of other comparative sequences on the data base. This indicates the possibility of new natural compounds production by *Streptomyces* sp. Ru87.

Furthermore, NJ phylogenetic tree of PKS showed that PKS gene fragments of both *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 were distinct from comparative sequences on the database with low bootstrap value (70%, 43%, respectively). A low boot strap value of phylogenetically divergent gene sequences is a great indication of the production of enzymes encoding for pathways producing structurally novel classes of polyketide bioactive compounds such as tetrocarcins, formicamycins, amphotericin B, Mithramycin, Daunorubicin and doxorubicin [64,65,66].

Fosmid libraries are highly important for evaluating the diversity of biosynthetic gene clusters in microorganisms and detecting numerous new bioactive compounds [41,42]. A fosmid genomic libraries for *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 were successfully constructed. Positive hits of both NRPS and PKS were recovered using NRPS and PKS PCR assays. BLASTN and BLASTP analysis of sequence data obtained from *E.coli* clones derived from both *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 genomic libraries, indicated that they contained NRPS and PKS genes with significant homology to NRPS and PKS genes of *Micromonospora* and *Streptomyces* genera, respectively. Our results confirmed the successful integration of NRPS and PKS within the fosmid clones. We confirmed that genes encoding natural products can be readily captured by using this strategy. Larger libraries with larger inserts and the use of prescreening should greatly enhance the ability to detect novel and useful secondary metabolites [67]. Efficient expression of entire biosynthetic gene clusters is often limited by the chosen host strain. High GC content of actinomycetes genes and differences in regulatory elements of gene expression limits the utilization of classical microorganisms like *E. coli* or *Saccharomyces cerevisiae* as hosts for secondary metabolism gene expression [68]. Similar results clarified the construction of two metagenomic libraries from microbial cells of the marine sponge *Aplysina aerophoba* using an *E. coli-Streptomyces* shuttle cosmid vector, pAY1 and were used for NRPS screening [69].

Furthermore, the construction of fosmid libraries from Cuban and Antarctic soil samples and the recovery of NRPS and PKS in *E.coli* was illustrated [20]. Similar studies showed that the construction of actinomycete fosmid libraries harboring distinct clones with PKS genes [38] and the construction of environmental DNA library with PKS and NRPS genes [64]**.** Moreover, the heterologous production of PKS/NRPS hybrid yersiniabactin was produced by *Yersinia pestis* in a genetically engineered *E. coli* host [70]. This study clarified the ability to manipulate biosynthetic gene clusters in a different host. This modified biosynthetic gene clusters can subsequently be heterologously expressed in a suitable host organism to produce new natural products with potentially improved properties.

5. CONCLUSION

NRPS and PKS phylogenetic analysis demonstrates that our strains possess similar

NRPS genes to the comparative NRPS genes on NCBI data base, while they harbor distinct PKS genes. Our findings suggest that the phylogenic study of both NRPS and PKS clusters for each individual strain is essential and should go parallel for a complete documentation of its metabolites. This will open the door for scientists to explore more about the biochemical pathways and consequently the discovery of novel bioactive molecules. We suggest that *Micromonospora* sp. Rc5 and *Streptomyces* sp. Ru87 has uncommon enzymatic pathways due to phylogenetically distinct PKS genes, and hence unique molecules biosynthesis. Furthermore, the construction of fosmid libraries for our strains along with successful integration of NRPS and PKS genes would be the first step for future metabolic gene manipulation. Our research would contribute in monitoring and controlling the threat of antimicrobial drug resistance and therefore improve the Health care by enhancing drug discovery.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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