Biotechnology Reports 34 (2022) e00706

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Evaluation of a new antimicrobial agent production (RSMM C3) by using metagenomics approaches from Egyptian marine biota

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ARTICLE INFO

Keywords:
Metagenomics
DNA
Antimicrobial agents
Cloning
Characterization

ABSTRACT

Diseases and epidemics in the current days need new types of antibiotics in order to be able to eliminate them. The goal of this research is to use metagenomics to identify isolated utilitarian gene (s) as antimicrobial specialists. Collection of diverse locations from sea sediment samples from Alexandria and extraction of total DNA, restriction enzyme fragmentation, cloning into pUC19 vector, and expression of the isolated gene(s) in *E. coli* DH5α were all part of the process. Characterization of Antimicrobial agent was done for the best clone for antimicrobial agent's production to detect efficiency, optimum pH, thermal stability, pH stability, effect of different compounds on antimicrobial activity, and residual activity of product after preservation in room temperature. Amino acid sequence of RSMM C3 gene (1250 bp) was 72% identity with *Herbaspirillum* sp. The ideal temperature level of the RSMM C3 antimicrobial agent production was 36 °C. The antimicrobial agent RSMM C3′s stability was stable at -20 °Celsius for up to two months without thawing. The antibacterial agent RSMM C3 was stable at 4 °C for 14 days without loss in activity. The ideal pH level of the RSMM C3 antimicrobial agent was 6. Remain activity was gradually decreased at pH 5, 6, 6.5 and 7 (86.1, 96.9, 97.2 and 94.9%, respectively). On the other hand, residual activity was (92 and 84%) at (pH 7.5 and 8) for 8 days. The tested antimicrobial RSMM C3 was stable against 1 mM of different compounds (DMSO, Glycerol, NaCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $FeSO_4$, $MnSO_4$ and $CuSO_4$). The research provides for the Metagenomics technique that has the ability for the production of novel antimicrobial agents produced by clone RSMM C3 which has a wide spectrum activity towards different microorganisms comparing to other antibiotics as Ampicillin and Tetracycline.

1. Introduction

Antimicrobial drug discovery has been slowed down in recent years, owing in part to a low rate of drug approval by regulatory organisations [1]. Antimicrobial resistance (AMR) severely restricts infective epidemic treatment options. The global attributable mortality and morbidity rates of drug-resistant infections are estimated to be in the hundreds of millions of people with associated healthcare expenditures in the millions of dollars [2]. Most antibiotics and many other medical compounds were isolated from microorganisms, which resulted in remarkable improvements in human health in the second half of the twentieth century [3]. However, typical culture methods used to isolate natural products from microorganisms result in the rediscovery of known antibiotics (99 percent). As a result, new approaches to drug discovery are required [4]. Using microbial metagenomics, many new antimicrobials and other medicinal chemicals have been discovered, thus far [5]. Metagenomics is a technique for studying the DNA which is extracted from ambient samples without the need to separate and culture microbial communities [6]. Since its inception at the turn of the twenty-first century, metagenomics has attracted the interest of the global scientific community in fields as diverse as health, biotechnology, agriculture, and genetics [7]. Functional metagenomics screenings can be used to uncover new antimicrobial agents by screening microbial populations for antimicrobial activity against indicators or therapeutically relevant pathogens [8]. Other novel bioactive compounds discovered through metagenomics include antitumorigenic compounds like paulomycin G, antioxidants like saccharomonopyrones, cytotoxins like antimycin, anti-inflammatory compounds like somalimycin, heart tissue growth modulating compounds like the bonnevillamides, and compounds like xiamenmycin with antifibrotic properties [5].

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https://doi.org/10.1016/j.btre.2022.e00706
Received 17 November 2021; Received in revised form 22 January 2022; Accepted 24 January 2022
Available online 25 January 2022

Metagenomics can potentially be used to diagnose and to track pathogens [9].

Metagenomic techniques have recently uncovered a class of calcium-dependant antibiotics known as malacidins. These drugs exhibit antimicrobial efficacy against gram-positive bacteria that are multidrug-resistant [10]. The discovery of divamides that has an activity against human immune virus infections are another major accomplishment in the field of metagenomics in medication discovery. Bacteria with an endosymbiotic interaction with marine tunicates produce these chemicals [11]. In general, there are encouraging prospects for antibiotic research, but business structures must be altered if scientific breakthroughs are to be translated into clinically approved treatments [12]. Metagenomics, genomics, combinatorial biosynthesis, and synthetic biology techniques are used for identifying novel natural compounds from microbial marine [13]. Over 30 medicines derived from marine microorganisms are now being tested in clinical or preclinical investigations to treat several varieties of cancers [14]. Individual clones can also be screened for a certain phenotypic trait which is done by identifying a specific phenotypic characteristic [15]. Growth inhibition studies of a suitable tester organism employing soft agar overlays over the clone colonies or a micro titre plate assay utilising supernatant extracts from the clone cultures may be used to detect metagenomic clones harbouring antimicrobial activity [16]. Amongst the benefits of metagenomics applications is the finding of novel bioactive, such as antimicrobials that are active against microbes of concern in both food and medicinal settings [17].

This study aims to assess the genetically antimicrobial metabolites activity isolated from marine sources against a variety of microbial isolates, as well as, the effect of interaction between an active product of clone metabolite and (Ampicillin and Tetracycline) as an alternative to antibiotics in the fight against microorganisms.

2. Materials and methods

2.1. Obtaining and preparing samples

The marine sediments samples were taken from a specific location of the Mediterranean Sea in Alexandria, Egypt, transferred to the study centre, and then inspected in a cold room.

2.2. Chemicals

All of the chemical reagents employed in the studies were analytical in nature quality (from Sigma Aldrich, Milan, Italy).

2.3. Cloning and transformation of antimicrobial agents gene

2.3.1. Total DNA preparation

The genomic DNA used in this study was obtained using a modified approach of a particular kit for soil DNA isolation (QIAGEN). Concentration of DNA (1231 ng/μl) and purity (1.81) were measured using Nano drop.

2.3.2. DNA restriction digestion

In a 20 μl reaction volume, *Total* DNA was digested, followed by 2 μl of enzyme buffer and 1–2 units of restriction enzyme. Restriction enzyme digestions were carried out using the reaction conditions recommended by the manufacturers for each enzyme (Fermentas). Various restriction enzymes were employed (*EcoRI, Bam*HI, *HindIII, Xba1* and *PvuI*).

2.3.3. DNA ligation

A ligation to 20 μl reaction length, T4 DNA ligase buffer, T4-DNA ligase protein unit, and vector (4:1) was performed at that moment (Processed DNA). At 16 °C, the reaction was carried out overnight. pUC19 was made up of the screening and expressing vector.

2.3.4. Competent cells preparation and transformation

E.coli was made as follows: 100 ml LB was inoculated with 1 ml overnight culture of *E. Coli* DH5α alpha and incubated at 37°C with shaking (200 rpm) until OD_{600nm}(0.6–0.7), according to Sambrook et al. [18]. The flask was chilled with ice and was divided into 50 ml portions for alkalination. The cells were collected by centrifugation at 4000 rpm and were resuspended in TSS solution. The cell suspension was dispersed in a sterile Eppendorf tube (200 μl aliquots) that had been promptly frozen at −80 °C. On ice, frozen aliquots of the competent cells were allowed to thaw. DNA (ligation blend) was injected into the tube, which was then placed on ice for 20 min. The tube was heated and shocked at 42 °C for 60 s, then 800 μl LB medium was added and the tube was incubated at 37 °C for one hour with shaking. Vials (200 μl) were strewn across LB plates containing XGal and IPTG.

2.3.5. DNA transformation

DNA and pUC19 were digested with the same restriction enzyme before ligation. After transformation, the 270 clones (78 clones from digested DNA using *Bam HI*, 82 clones from digested DNA using *HindIII*, 99 clones from digested DNA using *EcoRI* and 11 clones from digested DNA using SalI) were presented as white clones. Blue/white screening to detect positive clone (carrying gene) which presented as white clone where the blue clones represented negative clones (vector free of gene).

2.3.6. Preparation of plasmid-DNA from transforment clones

The GEBRI kit had directed mini-plasmid extraction kit as follows: overnight culture cells (1.5 ml) were centrifuged at 7000 rpm and were suspended in 300 μl of solution I which was then lysed by inserting 300 μl of solution II with gentle shaking. Solution III (300 μl) was used to precipitate the protein and the genomic DNA, followed by centrifugation at 13,000 rpm for 10 min. At that point, the plasmid DNA supernatant was accelerated with isopropanol and was resuspended in 300 μl of water with 70 percent (v/v) ethanol, followed by centrifugation at 10,000 rpm for 10 min, then using precipitated DNA after air dry.

2.3.7. Screening and assay for antimicrobial activity

The crude extract's antibacterial activity was tested against bacterial isolates (gram negative, gram positive and fungi). Briefly, 100 μl suspension of bacteria (100 CFU/ml) was put into Muller Hinton Agar plates, as well as, 50 μl of fungi were injected onto the potato dextrose agar (PDA) agar plates. 50 μl of both crude extracts of cell clone lysate were put into dried paper discs (8 mm). On the same plates, a control antibiotic of ampicillin and tetracycline paper disc (30 g/ml) was used. The inhibitory zone diameter (mm) was measured after the filled plates were incubated at 35 °C for the duration of 24 h. The experiment was executed three times.

Pure clones were evaluated for antibacterial properties in nutrient agar (NA) (IPTG) inoculated with *E. coli* DH5α for qualitative assessment of antimicrobial compounds. The occurrence of a halo zone across the colonies indicated the production of antimicrobial compounds. The most effective clone was discovered using agar disc diffusion. The disc diffusion (zone of inhibition) technique was likely the most often employed due to its simplicity and its low cost. It was just using small portion of the test. The method entailed preparing a petri plate with agar medium and stretching bacteria at a known concentration across the

medium. A pore was created within the agar using a cork poorer, 50 µl of culture clone that was poured in each well, and it was incubated at 37 °C for 24 h. The disk's inhibition zone was determined and was compared to the zones of the other discs.

Using the cell extract against microorganisms and measuring OD_{600}nm following a 24 h incubation period, a quantitative evaluation of antibacterial activity for positive isolates was done. This was performed by inoculating 50 ml of production LB (ampicillin & IPTG) medium into a 250 ml Erlenmeyer flask with 1 ml suspension from freshly manufactured slant (18 hrs) of each tested isolate. The infected flasks at 37 °C were incubated for 24 h using a reciprocal shaker (200 rpm). Centrifugation at 5000 rpm for 15 min was accustomed to separate the cells. Phosphate buffer had been used to wash the cells and had been sonicated in 1 ml of phosphate buffer pH 7. The bacterial culture was sonicated, then was centrifuged for 5 min at 5000 rpm, with the supernatant transferred to a separate tube to be utilised as antimicrobial agents.

2.4. Evaluation of resistance prevalence of the selected bacteria

All the tested bacteria were subjected to antibiotic resistance using the disc diffusion method according to the modified Kirby-Bauer. Disc Diffusion method was compared to the product of the most efficient RSMM clones (C3, C5, C9, C10 and C13).

2.5. Antimicrobial effect determination of RSMM clones (C3, C5, C9, C10 and C13)

Antimicrobial studies of RSMM clones (C3, C5, C9, C10 and C13) were detected using Escherichia coli (gram negative bacteria) and *Bacillus* and *Staphylococcus* (gram positive bacteria), as well as *Candida albican* and *Asperigillus niger* (fungi). A volume ratio of 1: 1000 was used to dilute the bacterial solution. The sterilised RSMM clones (C3, C5, C9, C10 and C13) (150 µl) were added to the 5 mL bacterial solution and were incubated at 200 rpm for 24 h at 37 °C. At 600 nm, the OD value was measured. In addition, the product of RSMM clones (C3, C5, C9, C10 and C13) was used against fungi using potato dextrose broth that was incubated at 200 rpm for 5 days at 30 °C.

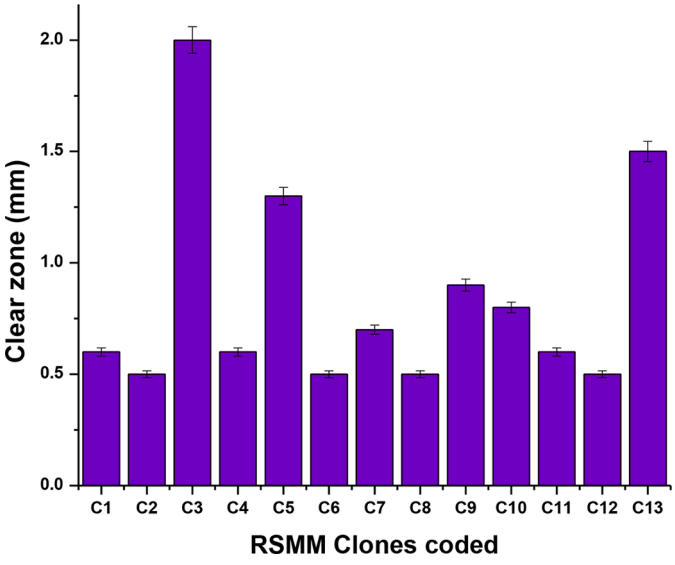

Fig. 1. Screening of antimicrobial agents produced by clones coded RSMM C (1–13).

2.6. Minimum inhibitory concentrations (MIC)

The MIC values of RSMM antimicrobial agents were obtained in a 96-well plate using a micro dilution technique. A cell suspension of bacterial isolates (100 CFU/ml) was inoculated into Muller Hinton broth medium, and 200 µl of the infected medium was dispersed in each well. The studied substances (RSMM) were put through two-fold serial dilution procedure.

2.7. Similarity of sequences

The BLAST tool (www. http://tcoffee.crg.cat) acquired for the PCR product using (M13 F: AGGCCCTGCACCTGAAG, M13 R: TCAGCGCCTGGTACC, **[19]**) was used for comparison with other sequence in the database. Sequencing was done by Bio-vision Company using the Sanger method of DNA Sequencing. Multialignment and molecular analysis of RSMM C3 was applied by different program Sequence Manipulation Suite (https://www.bioinformatics.org/sms2) and EMBOSS Programs (https://www.ebi.ac.uk/Tools/emboss)

2.8. Effects of culture time on growth characteristics and antimicrobial RSMM C3 activities

Clone RSMM C3 was inoculated into LB liquid medium (containing Ampicillin/IPTG) at 37 °C for 50 h, stirring at 200 rpm. The target's absorptivity suspension was measured with a UV spectrophotometer at 600 nm to create a growth curve from the fermentation broth every 3 h, and the cells were centrifuged at 5000 rpm before being sonicated to assess antimicrobial agent synthesis.

2.9. Characterization of antimicrobial agent RSMM C3

2.9.1. Determination of the ideal pH level for production

The ideal pH level of the antibiotic was determined by the inoculation of the RSMM C3 clone at LB broth medium with different pH (5, 5.5, 6, 6.5, 7, 7.5 and 8). Each one was individually used, and after sterilization, IPTG and Ampicillin were added, then, inoculated with RSMM C3. After incubation at 37 °C for 24 h, RSMM C3 antimicrobial agent was tested.

2.9.2. Determination of the ideal pH level of the antimicrobial activity

The ideal pH level of the antibiotic was determined by sonicating the cultured clone with 1 ml of the tested buffer over pH range of 5–9. Citric

Table 1
Remain growth% from *E. coli* DH5*α* after 24 hrs incubation at 37 °C using different concentration of antimicrobial agents produced by clones (RSMM C $_{1–13}$).

Clone ID	Remain growth% from *E. coli* DH5*α* after 24 hrs incubation at 37 °C					Restriction enzymes used in DNA digestion
	10 µl	20 µl	30 µl	40 µl	50 µl	
RSMM C_1	100	89	77	66	54	*EcoRI*
RSMM C_2	100	88	69	52	34	*EcoRI*
RSMM C_3	29	0	0	0	0	*EcoRI*
RSMM C_4	86	72	58	44	30	*EcoRI*
RSMM C_5	73	46	19	0	0	*Bam HI*
RSMM C_6	89	76	63	49	35	*Bam HI*
RSMM C_7	92	83	74	65	66	*HindIII*
RSMM C_8	91	82	73	65	56	*HindIII*
RSMM C_9	64	29	0	0	0	*HindIII*
RSMM C_{10}	79	58	37	16	0	*HindIII*
RSMM C_{11}	98	84	59	34	7	*SalI*
RSMM C_{12}	83	66	50	33	16	*SalI*
RSMM C_{13}	59	17	0	0	0	*SalI*

Table 2
Antibiotic sensitivity of the selected bacterial isolates using stander antibiotic disc comparing with antimicrobial product produced by clones (3, 5, 9, 10 and 13).

Pathogenic bacterial ID	Antibiotic sensitivity (IZ mm)										
	Levofloxacin (LE)	Amikacin (A/K)	Ceftazidime (CAZ)	Cefoperazone (CFP)	Ampampicilin +Sulbactam (A/S)	Cefepime (CPM)	Colistin (CT)	Azetreonum (ATM)	Penicillin (P)	Meropeneme (MEM)	Clindamycin (DA)
E.coli	1	1.5	-	-	-	-	-	1.2	-	-	-
Enterococcus spp	-	-	-	-	-	-	-	-	-	1.3	-
Bacillus spp	-	-	-	-	-	-	-	-	0.8	2.5	-
Proteous spp	-	1.2	-	-	-	-	-	-	0.9	2.5	1.4
Salmonella spp	-	-	0.8	-	0.8	0.8	1.2	-	-	-	-
Shigella spp.	-	-	-	-	-	-	1.6	-	0.8	3.5	2
Staphylococcus aureus	-	-	-	-	-	-	1.4	-	1.1	-	-
Staphylococcus spp	1.3	1.5	-	1.4	0.9	-	1.2	-	-	-	-

acid, phosphate, tris and borat at 50 mM buffer strength were utilised in this test. After incubating 50 ml of cell suspension with *E. coli* DH5α at 37 °C for 24 h using the crock borer method, actives were determined.

2.9.3. pH stability of RSMM C3

The pH stability was determined by incubating an antimicrobial agent (RSMM C3) at pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5 (100 ml of antimicrobial /100 of buffer) for 2 h, then, inoculating 150 µl of treated product into a 5 ml LB cultivated with *E. coli* DH5α in liquid medium at 200 rpm and 37 °C. The residual activity was estimated by comparing the OD value of untreated RSMM C3 with buffers to a control of untreated RSMM C3 with buffers.

2.9.4. Determination of the ideal temperature level for production

The ideal temperature level of the RSMM C3 antimicrobial agent was determined by inoculation of RSMM C3 clone at LB broth medium at different temperature (32, 34, 36, 37, 38 and 40 °C), each one was individual, and after sterilization, IPTG and Ampicillin were added, then, inoculated with RSMM3.

2.9.5. Thermal stability

Thermodynamic stability of the antimicrobial agent (RSMM C3) was examined by both incubating it at room temperature and at 4 °C for 14 days and incubating at 35–50 °C for up to 2 h. The stability of the antimicrobial agent was also tested by gradually thawing for up to 10 days at different intervals time through measuring the remaining activity.

2.9.6. Residual activity of the RSMM C3 product after preservation in room temperature

In a separate Eppendorf tube, 150 µl of sterile treated RSMM C3 product was incubated for 7 day under aseptic condition. Sterilization of RSMM C3 product was done by using syringe bacterial filter. Residual activity of the RSMM C3 after preservation in room temperature was determined by inoculating the 150 µl of treated RSMM C3 product into the 5 ml LB cultivated with *E. coli* DH5α.

2.9.7. Effect of certain compounds on antimicrobial RSMM C3 activity

The tested antimicrobial agent RSMM C3 was tested against 1 mM of different compounds (DMSO, Glycerol, NaCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $FeSO_4$, $MnSO_4$ and $CuSO_4$). The RSMM C3 products tested independently with the tested agent for 30 min (1/1 volum); the residual efficiency was calculated by inoculating 150 µl of treated RSMM C3 product into the 5 ml LB cultivated with *E. coli* DH5α in liquid medium under 200 rpm and 37 °C, residual activity were calculated by determination of OD_{600nm} against control of untreated RSMM C3 with the compounds under test individually.

3. Results and discussion

The choice of isolation from marine waters, especially from the Mediterranean waters of the Egyptian territorial waters, as a result of the presence of many researches indicating that marine waters are full of resources that can be exploited to produce materials that are useful in many fields. The following references provide some instances of marine-derived microbes: terpenoids [20], alkaloids [21], peptides [22] polyketides [23] and hybrids [24, 25]. The choice of metagenomic technology due to the inability to isolate organisms present in many environments from which new materials can be produced. Large-scale direct sequencing of metagenomic DNA can provide a more thorough understanding of microbial communities [26].

3.1. Screening of antimicrobial agents

13 pure clones were positive for antibacterial activity in NA (IPTG) inoculated with *E. coli* DH5α for qualitative antimicrobial agent assessment. The presence of a halo zone around the 13 colonies showed that antimicrobial agents were being produced. The diffusion of the agar plate was used to test the positive clones. The inhibition zone around the disc was computed and was compared to Ampicillin and tetracycline inhibition zones. RSMM C were used to code the positive clone (1–13). Most antimicrobial compounds were inhibited in a zone around clones coded RSMM C3, RSMM C5, RSMM C9, RSMM C10 and RSMM C13 (2, 1.3, 0.9, 0.8 and 1.5 mm, respectively) (Fig. 1).

Antimicrobial activity was quantitatively measured for the 13 positive clones. The quantitative determination of an effective concentration of RSMM C (1–13) was accomplished by utilising different concentrations to inhibit *E. coli* DH5α cultivated in LB medium for 24 h at 37 °C, with growth inhibition measured using a spectrophotometer. Remain growth% from *E. coli* DH5α after 24 hrs incubation at 37 °C using different concentration of antimicrobial agents produced by clones (RSMM C 1–13) were presented in (Table 1) to confirm the most producer clones for further study. A wide range of microbial characteristics were characterised using the sequencing method [27, 28]. The gene for indirubin (chemical substance produced primarily as a result of bacterial metabolism) was discovered by Kumar et al. [29] and Nazir [30] from soil metagenome with antibacterial activity as well as antimicrobial activity due to indirubin pigment isolated from soil DNA [31].

Table 2 (*continued*)

Antibiotic sensitivity (IZ mm)													
Tetracycline (TE)	Chloramphenicol (C)	Metronidazole (MTZ)	Rifampine (RA)	Streptomycine (S)	Ofloxacin (OFX)	Doxycycline (DO)	Nitrofurantion (F)	Trimethoprim-sulfamethoxaz (STX)	RSMM C_3	RSMM C_5	RSMM C_9	RSMM C_{10}	RSMM C_{13}
-	-	-	-	-	-	1.2	-	1.2	1.8	0.9	0.8	0.7	1.7
1.4	-	-	-	0.8		1	0.8	1	1.8	0.6	0.8	0.8	1.3
-	1.3	-	-	1.9	2		1.3	2.2	2	0.8	0.7	0.9	1.6
0.7	1.5	-	2	1.5	0.9	1	1.5	-	1.4	0.8	0.7	0.7	0.9
-	-	-	-	-	-	-	-	-	1.5	0.8	0.8	-	0.8
2.2	1.2	-	2.3	2.2	2	2.7	1.5	1.9	1.4	0.9	1.2	0.7	1
-	1.1	-	-	-	-	-	-	-	1.3	1	1.1	-	0.9
-	-	-	-	-	-	-	-	-	1.4	0.8	0.9	0.7	1.1

3.2. Evaluation of resistance prevalence of the selected bacteria

All the tested bacteria (*E. coli, Enterococcus spp, Bacillus spp, Proteous spp, Salmonella spp, Shigella spp* and *Staphylococcus spp*) were subjected to antibiotic resistance using the disc diffusion method. Disc diffusion of stander antibiotic discs were compared to the product of the most efficient RSMM clones (C3, C5, C9, C10 and C13). Antimicrobial products produced by clones coded (RSMM C3 and RSMM C13) were the most effective clones (**Table 2**).

3.3. Antimicrobial effect and MIC determination for RSMM C3 antimicrobial agent

The effective concentration of antimicrobial agents by RSMM clones (3, 5, 9, 10 and 13) were quantitatively determined by using different concentrations of (RSMM clones) to inhibit bacteria (*Bacillus subtlis, E. coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*) cultured in LB medium for 24 h at 37 °C and (*Candida albican & Asperigillus niger*) cultured in potato dextrose liquid broth for 6 days at 30 °C, with growth inhibition evaluated using a spectrophotometer (600_{nm}) (**Table 3**).

3.4. Sequence similarity

Molecular characterization of the active genetic materials for the active clones (1–13) was tested using universal primer of the vector (M13: F & R). As illustrated in **Fig. 2**, there were variation in molecular weight of PCR product from different clones. According to MIC and data of antimicrobial activity. RSMM C3 Clone was selected for analysis of nucleotide sequences. The PCR product molecular weight of RSMM C3 clone was 1250 bp. The sequence retrieval system Swiss Prothtt p://hcuge.ch/srs5/ and Multialigment were used to align several sequences for clone RSMM C3. Amino acid sequence similarity with others presented in data base were illustrated in **Table 4**. Phylogenetic relation of RSMM C3 with genes available in GenBank database was illustrated in **Fig. 3**. The dendogram was generated by the neighbour-joining method using MEGA7.0 Software. Amino acid sequence of RSMM C3 gene was 72% identity with *Herbaspirillum* spp. Donia et al. **[32]** highlighted the concept of bacteria producing antibiotics to minimise competition with the creation of lactocillin in the genital symbiotic organisms *Lactobacillus gasseri* **[32]** and **[33]** underscored the evolution of lugdinin by *Staphylococcus lugdunensis* in the nasopharynx to inhibit methicillin-resistant Staphylococcus aureus (MRSA). *Herbaspirillum* sp. had been found in maize, rice, beans, bananas, sugar cane, pineapple, and other plants' roots and stems **[34, 35]** as well as, groundwater **[36]** and drinking water distribution systems **[37]**.

3.5. Effects of culture time on growth characteristics and antimicrobial RSMM C3 activities

Clone RSMM C3 was inoculated into LB liquid medium (containing Ampicillin/IPTG) and incubated at 37 °C for 50 h with 200 rpm stirring. At 3 h intervals, samples were taken. **Fig. 4** depicted the target RSMM C3 clone growth curve and antimicrobial production curve. The target RSMM C3 clone developed significantly, reaching a stable stage after 24 h, as illustrated in Fig. 4. RSMM C3 antimicrobial agent production grew fast in the first 30 h, then stable till 50 h, with the best enzyme production period being 24 h.

The production process of antibiotics is affected by external factors and environmental conditions affecting growth. pH, temperature and growth period were important factors affecting the production process

Table 3
Minimum inhibitory concentration (MIC) of the antibiotic against the tested microorganisms.

Tested microorganisms			MIC (µl/ml) of clone				
Name	Type	Gram reaction	RSMM C_3	RSMM C_5	RSMM C_9	RSMM C_{10}	RSMM C_{13}
Bacillus subtlis	Bacteria	+	20	50	30	50	40
E. coli	Bacteria	–	20	40	30	50	30
Pseudomonas aeruginosa	Bacteria	–	30	40	40	50	40
Staphylococcus aureus	Bacteria	+	30	50	40	50	40
Candida albican	Yeast	–	50	80	90	70	60
Asperigillus niger	Fungi	–	50	80	70	100	80

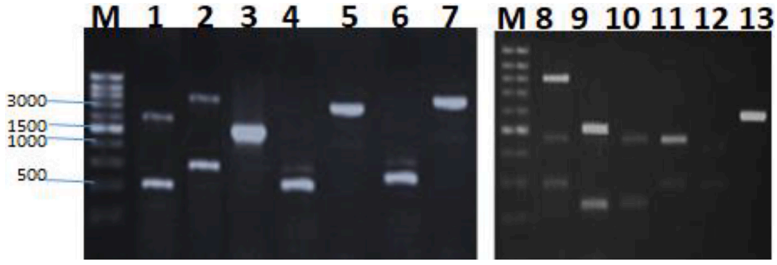

Fig. 2. M13 PCR products using plasmid of clones coded RSMM C (1–13).

Table 4
Similarity percentages and accession numbers obtained after comparing the amino acid sequence of the RSMM C3 gene to the submitted sequences in Gene Bank.

Accession number	Description	Max score	Total score	Identity (%)
WP_034336552.1	Herbaspirillum spp.	188	188	72.03
WP_145602679.1	Herbaspirillum huttiense	187	187	72.03
WP_209533287.1	Herbaspirillum spp.	186	186	71.33
WP_008327711.1	Unclassified Herbaspirillum	185	185	70.63
EOA02466.1	Herbaspirillum frisingense	183	183	69.93
WP_039876989.1	Herbaspirillum spp.	182	182	69.93
WP_217507394.1	Herbaspirillum robiniae	179	179	67.13
KAF1042488.1	Herbaspirillum frisingense	176	176	65.73
WP_174525326.1	Herbaspirillum hlorophenolicum	174	174	65.03
WP_061789339.1	Herbaspirillum rubrisubalbicans	167	167	69.23
WP_151634651.1	Noviherbaspirillum aerium	155	155	58.74
AIA14267.1	Uncultured bacterium	153	153	57.34
WP_108439068.1	Glaciimonas spp.	155	155	60.14
WP_211450905.1	Collimonas antrihumi	153	153	57.14
WP_167541843.1	Herbaspirillum rhizosphaerae	150	150	60.00
WP_144966446.1	Herbaspirillum hiltneri	150	150	60.84
WP_194721211.1	Noviherbaspirillum malthae	149	149	57.34
WP_053199601.1	Herbaspirillum hiltneri	149	149	60.14
WP_016832202.1	Herbaspirillum lusitanum	149	149	59.44
WP_084199891.1	Noviherbaspirillum autotrophicum	148	148	59.03
WP_050463333.1	Herbaspirillum autotrophicum	147	147	55.24
WP_175626240.1	Oxalobacteraceae	145	145	54.55
WP_062114450.1	Collimonas pratensis	144	144	55.24
WP_157889211.1	Herminiimonas arsenitoxidans	144	144	54.55
WP_038488168.1	Collimonas arenae	142	142	56.93
WP_061542380.1	Collimonas fungivorans	133	133	55.71
WP_188419591.1	Oxalicibacterium solurbis	130	130	48.95
MBI1772947.1	Burkholderiales bacterium	129	129	51.39

[38]. Antibiotic production was strongly depending on the nature of the medium, according to Messaoudi et al. **[39]**. The finest part of the process was the stationary phase and the best phase for developing antimicrobial agents at various stages of growth **[40]**. For successful and effective fermentation of a specific bioactive molecule and high product yields, the performance of a supporting production medium was critical **[41, 42]**.

3.6. Characterization of antimicrobial agent RSMM C3

3.6.1. Effect of temperature

The ideal temperature level of the RSMM C3 antimicrobial agent production was 36 °C which determined by inoculation of RSMM C3 clone at LB broth medium at different temperature (30, 32, 34, 36, 37, 38 and 40 °C)(Fig. 5A).

RSMM C3′s stability was tested at −20 °Celsius for up to two months without thawing. The antibacterial agent RSMM C3 was stable at 4 °C for 14 day without loss in activity although loss of activity was gradually decrease to 10% after 14 days (Fig. 5B). Remain activity was tested at different temperature (35–50 °C) for up to 2 0 (Fig. 5C) where the antimicrobial agent RSMM C3 was stable at 35 °C for 2 h and the residual activity for (40, 45 and 50 °C) was (97.8, 96.8 and 89.1%, respectively) after incubation for 2 h. The stability of the sample was also tested by gradually weakly thawing it for up to 10 days in an antibacterial agent solution at different time intervals and measuring the remaining activity (Fig. 5D).

3.6.2. Effect of pH

The ideal pH level of the RSMM C3 antimicrobial agent was 6 as presented in Fig. 6(A) which was determined by inoculation of RSMM

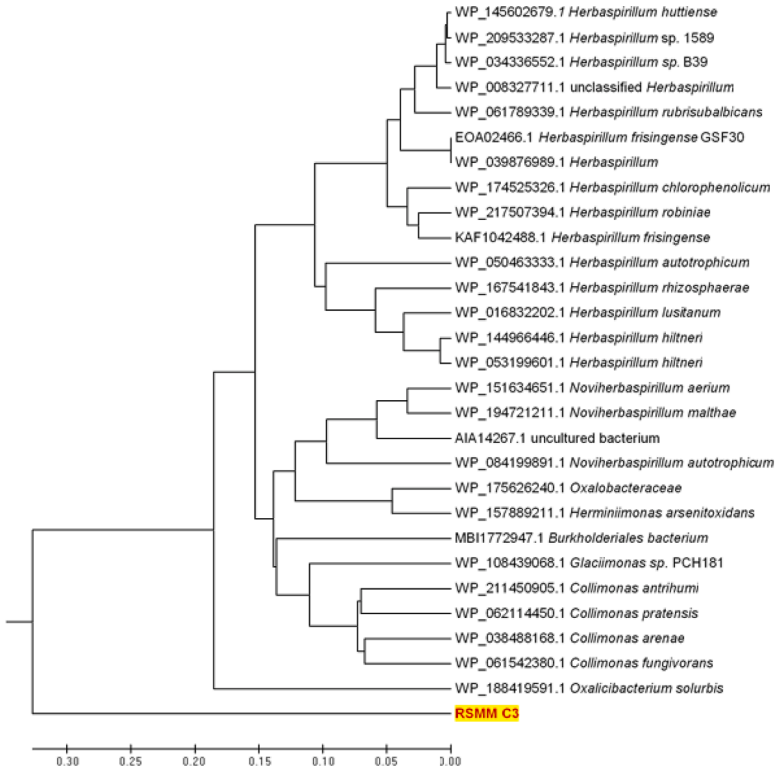

Fig. 3. Phylogenetic relation of RSMM C3 gene with genes available in GenBank database using MEGA 7.0 Software.

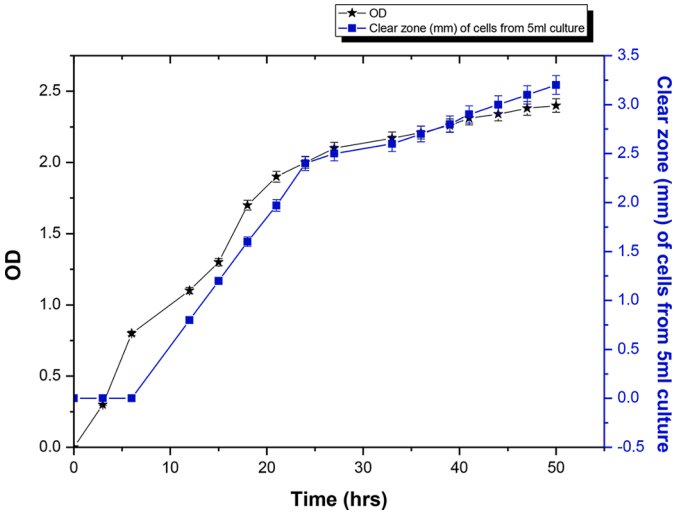

Fig. 4. Growth curve of RSMM C3 clones upon heterologous expression of antimicrobial activity (clear zone) under lacz promoter cultured in LB (Amp &$_{IPTG}$) broth medium along 48 h incubation under shacking at 37 °C.

C3 clone at LB broth medium at different pH (5–8). The optimal pH range of RSMM C3 antimicrobial agent activity was (6–7.5) as illustrated in Fig. 6(B), which was determined by inoculation of RSMM C3 product in LB agar plate inoculated with bacterial culture with different pH. The pH stability was determined by incubation of antimicrobial agent RSMM C3 at pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8 as presented in Fig. 6**(C)**. Remain activity was gradually decreased at pH 5, 6, 6.5 and 7 (86.1, 96.9, 97.2 and 94.9%, respectively). On the other hand, residual activity was (92 and 84%) at (pH 7.5 and 8) for 8 days.

3.6.3. Effect of certain compounds on antimicrobial RSMM C3 activity

The tested antimicrobial RSMM C3 was tested against 1 mM of different compounds (DMSO, Glycerol, NaCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $FeSO_4$, $MnSO_4$ and $CuSO_4$). Its activity was stable at different tested compounds as presented in Fig. 7 when treated for 30 min. The activity of the substances used as antibiotics was affected by some chemicals [43, 44], some did not affect their activity as DMSO **[45]**, Glycerol **[45]**, NaCl **[38]**, $CaCl_2$ **[45]** and $FeSO_4$, and others, had little effect as $MgCl_2$, $ZnCl_2$, $MnSO_4$ and $CuSO_4$. This study has an added value importance in the sense that antibiotics are to reach as being the final product used as medicine.

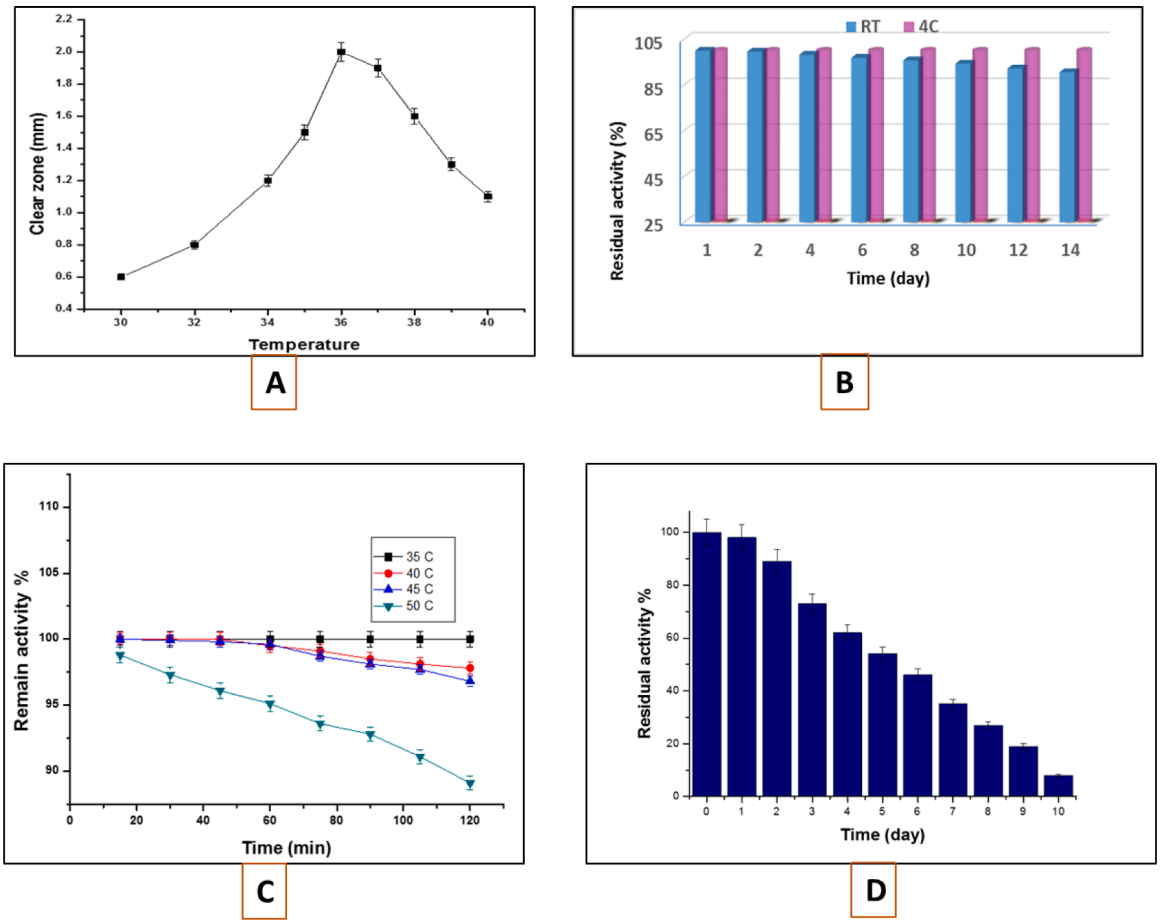

Fig. 5. Effect of temperature. A: The ideal temperature for production of RSMM C3 antimicrobial agents. B: Preservation effect of recombinant antimicrobial RSMM C3 at room temperature and preservation at 4 °C for 14 days. C: Preservation effect of recombinant antimicrobial RSMM C3 at (35, 40, 45 and 50 °C) for 2 h. D: Effect of freezing/thawing on recombinant antimicrobial RSMM C3.

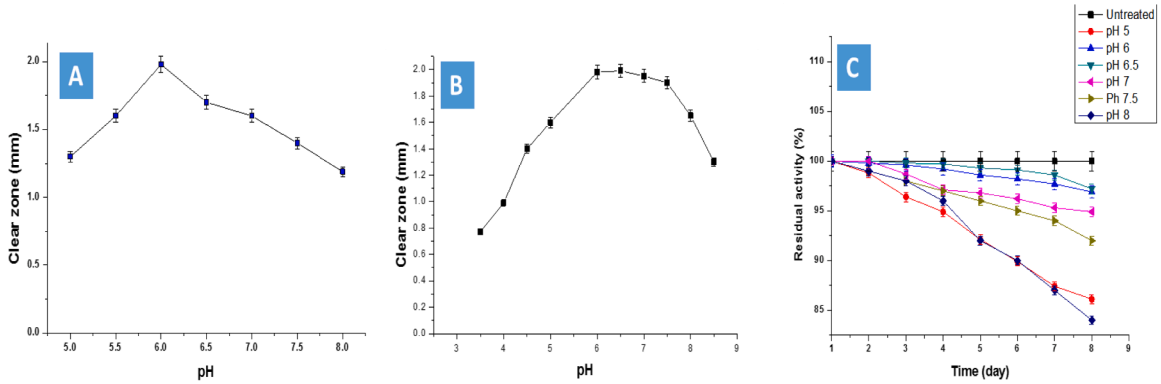

Fig. 6. Effect of pH. A: The ideal pH level for production of RSMM C3 antimicrobial agents. B: Optimum pH for antimicrobial activity of RSMM C3. C: pH stability of antimicrobial RSMM C3 at pH (5, 6, 6.5, 7, 7.5 and 8).

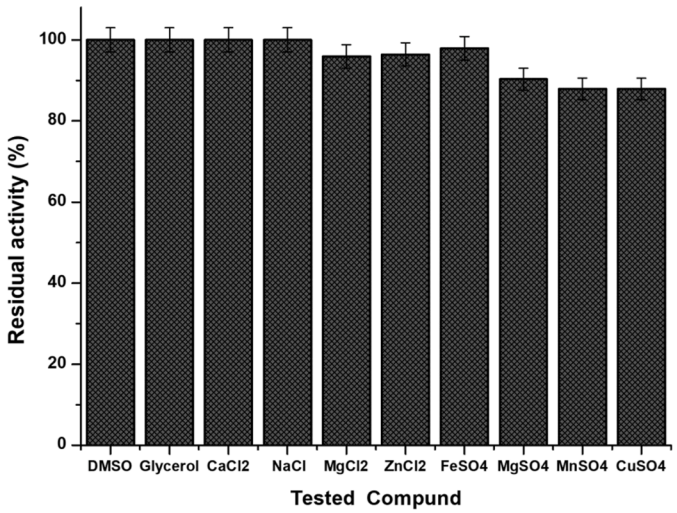

Fig. 7. Effect of certain compounds on antimicrobial RSMM C3 activity.

4. Conclusion

This isolated RSMM C3 antimicrobial agent is thought to be a unique broad spectrum molecule based on chemical and physical characterization. It is made up of novel amino acids that are not found in any of the present antibiotic groups. This agent must be medically evaluated for its impact on human health; if shown to be safe, it could be employed as a human medicine to combat a variety of microbial pathogens. The RSMM C3 antimicrobial agent is a novel antibiotic source for microbes, as well as, a promising bio-source for a variety of active secondary metabolites.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgement

The authors acknowledge City of Scientific Research and Technological Applications, Alexandria, Egypt for support and facilitating this study.

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