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Research Article

Streptomyces enissocaesilis: A New Anti-MRSA Antibiotic Producer Isolated from Little Rann of Kutch, Gujarat

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ABSTRACT
LK-08, A Haloalkaliphilic actinomycete, was isolated from soil samples which were accumulated from the Little rann of Kutch, Gujarat. Strain has been identified as Streptomyces *enissocaesilis* based on morphological, biochemical and 16SrRNA sequence homology analysis. Maximum antibiotic production was produced in media comprising lactose 6%, peptone 0.3%, NaCl 4% at pH 8.0 for 7 days at 28°C. Partially purified culture broth of a streptomyces isolate, LK-08 exhibited antibacterial activity on MRSA (Methicilin Resistant *Staphylococcus aureus).* Phylogenetic analysis indicated that strain LK-08 is 99% like wise to *Streptomyces enissocaesilis*, produce secondary metabolites. The study owns significance as only few haloalkaliphilic actinomycetes have been explored as an anti-MRSA agent from geological rare habitat, Little rann of Kutch.

KEYWORDS: Antibiotic; Streptomyces; Haloalkaliphilic; MRSA; Little rann of Kutch

INTRODUCTION

The bacteria that are mostly caused trouble in the resistance process are the, so called the ESKAPE pathogens emphasizing their strength to "escape" from ordinary antibacterial treatment [1]. The resistance of MRSA against different antimicrobials is globally increasing at alarming rate and treatment of MRSA infections has become more challenging since it is a grievous concern among health care professionals [2]. MRSA strains have obtained a mobile genetic element called staphylococcal cassette chromosome (SCCmec), carrying mecA encoding a penicillin-binding protein with low affinity to beta-lactam antibiotics. The most concurrent SCCmec kinds found in hospital isolates are I, II and III; also type IV is associated with community-acquired strains [3]. Now, however, nosocomial infections are perennial problem around the world due to increasing bacterial resistance to classical

antimicrobials and searching for new antibacterial mechanisms is of great criticality.

The region under study, little rann of Kutch has scare exploration with microbial biodiversity and its biotechnological potential. Haloalkaliphilic actinomycetes are a kind of extremophiles which are likely to reside in extreme environment and so they are having great research query of microbial physiology from extreme environments. [4]. The present report has focused on the isolation, screening and partial purification of an anti MRSA metabolites from a new Haloalkaliphilic *Streptomyces enissocaesilis* strain LK-08, isolated from the saline desert little rann of Kutch, Western India.

MATERIALS AND METHODS

Isolation of Actinomycetes from soil samples

LK 08 was isolated from Soil collected from little run of Kutch, GPS (lat)23˚C/30min/ 6.8"(long) 71˚C/29min/4.0".The samples were gathered from 5-25 cm depth in sterile plastic bags and transported aseptically to the laboratory. The saline soil (10 g) was incubated at 45˚C with CaCl2 (1 g) for 1 week [5]. This is 0followed by centrifugation of soil suspension for 20 minutes at 2000 rpm and use the supernatant for isolation of microorganism. Isolation and enumeration of actinomycetes were done by serial dilution and spread plate technique [6]. A good selective media, GAA(Glucose Asparagines Agar) was used. Samples were inoculated into glucose asparagines broth supplemented with 50 µg/ml cycloheximide (antifungal antibiotic). All flasks were put on shaker at 100 rpm, 28˚C for 5 to 7 days. After incubation, loopful of sample was streak on to actinomycetes isolation agar and starch casein agar [7]. All plates were incubated at 28˚C for 5 to 7 days. All chosen isolates were screen for their anti-MRSA activity. The most promising isolates were further characterized by the 16S rRNA sequencing technique at chromous biotech, Banglore.

Morphological, physiological, and biochemical characteristics

The haloalkaliphilic actinomycete, LK 08 was characterized with regard to its salt (0–10%, w/v) and pH (6–10) endurance in starch casein agar. The organism was recognized on the basis of morphological features, pigment production, and biochemical characteristics along with carbon utilization tests. The isolates were investigated for cell shape, arrangement and Gram reaction by standard methods given by Cappuccino and Sherman in Microbiology laboratory manual.

Isolation of MRSA strain from hospital using selective medium

The sample collection was carried out from different government hospitals. Isolation of *Staphylococcus aureus* was done by presumptive isolation method using Mannitol-Salt broth followed by the streaking with overnight old culture on same solid media, MSA [8]. Screening and conformation of MRSA was done using selective means MeReSa agar base [9]. and Oxacillin resistance screening agar base (with supplement) [10].

Anti-biogram of all MRSA isolates

Susceptibility of isolates against various antimicrobial agents was determined by the agar disk diffusion method as by Bauer-Kirby [11]. HiMedia**, Dodeca G -V-Plus (DE032)** multi discs was used to observe antibiotic susceptibility patterns.

Antibiotic production by LK 08 isolate in liquid culture

A single colony of LK 08 isolates was inoculated into 50 ml of SCB with 4%NaCl,8 pH containing in 150 ml of Erlenmeyer flask and incubated in the shaker at 100 rpm at 28°C for 24 hours. After 1 day, 10 ml of grown seed culture from SCB transferred in 100 ml of SCB containing in 250 ml of Erlenmeyer flask and incubated in shaker at 100 rpm at 28°C for 7 days.

Partial Purification of the antibiotic metabolites from LK 08

An equal volume of ethyl acetate was added into the sterile flask containing supernatant aseptically, incubated them in the shaker at 80 rpm at 37°C for 1.5 hours [12]. After 1.5 hours, transfer organic phase into sterile crucible and placed them into the hot air oven at 120° C, after some time interval (1 to 1.5 hours) crystals were observed in the crucible. Dissolve the crystals into 10% DMSO and stored at 4°c.

Anti-MRSA activity of ethyl acetate extract of LK 08

The productions of bioactive metabolites were assayed against MRSA isolate on Muller Hinton agar by agar well diffusion method [13].

16S rRNA sequencing of LK 08

LK 08 strain was sequence at chromous biotech, Bangalore. The 16S rRNA sequence data of sequenced strain was deposited in the Gene bank nucleotide data base for the accession number [14].

RESULTS

It is propounded from literature that the distribution of the most actinomycetes in the alkaline, saline soda lime was same as that observed for submarine region and saline desert Kutch and slightly vivid than that found in soda lakes [15]. The little rann of Kutch has derived scientific attention which in turn has led to the isolation of many new species and genera of actinomycetes and their antimicrobial properties. It has wide range of salinities. Up to the present, only a few species have been isolated from such habitats and only limited metabolites have been featured.

Characterization and identification of LK-08 strain

The actinomycetes strain LK-08 isolated from soil samples of little rann of Kutch, India. It is a Gram-positive filamentous bacterium. The vegetative mycelium showed light yellow color while the aerial mycelium showed light grey color. This strain could grow best at 28˚C. LK-08 was able to grow up to 4% w/v NaCl. LK-08 was able to grow optimally at pH range 7 to 10 with slow growth at neutral pH 7. LK-08 utilized glucose, fructose, maltose, lactose and sucrose as sources of carbon without acid production. Tests for lipid hydrolysis, casein hydrolysis, starch hydrolysis, indole production, ammonia production, citrate utilization showed positive results. Gelatin hydrolysis, nitrate reduction and H2S production showed negative results (Table 1).

Table 1: Morphological, Physiological and Biochemical characteristics of strain *Streptomyces enissocaesilis* (LK-08)

Characteristics	Result
MORPHOLOGICAL	
Aerial mycelium colour	Light gray
Substrate mycelium colour	creamy yellow
PHYSIOLOGICAL	
Optimum temperature for growth	28˚C
pH range for growth	7 to 10
Optimum pH for growth	8
NaCl tolerance	4%
BIOCHEMICAL	
Catalase	-
H2S production	-
Nitrate reduction	-
Ammonia production	+
MR test	+
VP color change/gas	+
Indole production	+
Gelatin hydrolysis	-
Casein hydrolysis	+
Starch hydrolysis	+
Litumus milk test	-

Lipid hydrolysis	+
Citrate utilization	+
Triple sugar iron test	-
Glucose	+
Fructose	+
Maltose	+
Lactose	+
Sucrose	+

[Utilization: +, positive result; -, negative result]

Based on the comparative study of 16S rRNA gene sequence, phylogenetic relationship and according to eztaxon (http://eztaxon-e.ezbiocloud.net/ezt_identify), LK-08 strain was designated as *streptomyces enissocaesilis*. It share 99%sequence similarity with type strain *Streptomyces plicatus* (Figure-1)

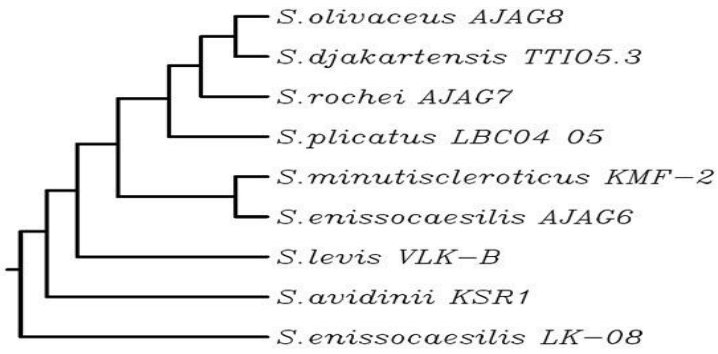

Fig.1: Neighbor-joining tree based on 16SrRNA gene sequence exhibiting relationship between LK-08 and related taxa within the genus *Streptomyces*

Phylogenetic analyses was conducted using UPGMA and tree was constructed. The 16S rRNA gene sequence of strain LK-08 has been deposited in the Gen Bank database under accession number MF773428.Therefore, on the basis of the microscopic, macroscopic, biochemical, Physiological and 16S rRNA gene sequence, the designated isolate LK-08 was found to be of genus streptomyces.

Isolation and Antibiogram of MRSA

Strain were selected for the study based on cultural characteristics, growth characteristics of strains on selective media including MeReSa agar base, oxacillin resistance screening agar base. MeReSa agar base contain chromogenic mixture, which is specifically cleaved by *Staphylococcus aureus* to give bluish green colored colonies. High concentration of sodium chloride also supports in restraining the accompanying micro flora. Cefoxitin is recommended to use for selective isolation of MRSA [16]. The medium is made selective for MRSA by the addition of MeReSa Selective Supplement (FD229) & Cefoxitin supplement (FD259) in combination.

All the strains were obtained as Gram positive cocci. The microscopic and macroscopic characteristics were completely akin to the typical colony characters of *Staphylococcus aureus*. All isolates also show coagulase positive. According to Kirby-Bauer zone interpretive standards, *Staphylococcus aureus* at 5µg Methicillin rendered ≤9(inhibition zone diameter(mm)) is known to be Resistant [17]. One strains denote absolutely non- compliance to methicillin. Antibiogram of all MRSA isolate against multi disc is listed in Table-2.

Table 2: Anti Biogram of MRSA isolates [Dodeca G-V-plus (DE032)]

Antibiotic	Concentration	MRSA 1	MRSA 2	MRSA 3
Penicillin G (P)	10 unit	0	0	18
Amoxicillin (AMX)	10 µg	0	0	9
Carbenicillin (CB)	100 µg	10	0	11
Methicillin (MET)	5 µg	18	0	21
Azithromycin (AZM)	15 µg	23	0	15
Clindamycin (CD)	2 µg	23	0	21
Roxithromycin (RO)	15 µg	21	0	18
Lincomycin (L)	2 µg	14	0	18
Vancomycin (VA)	30 µg	17	0	15
Rifampicin (RIF)	5 µg	11	0	13
Teicoplanin (TEI)	30 µg	17	0	19
Linezolid(LZ)	30 µg	21	0	20

Antibiotic production and Extraction from LK-08

The antibiotic production by LK-08 was carried out in starch casein broth (4% NaCl, pH 8). After Fermentation extraction of antibiotic was carried out using ethyl acetate. This extract was bioassay against MRSA-2 isolates (Figure-2).

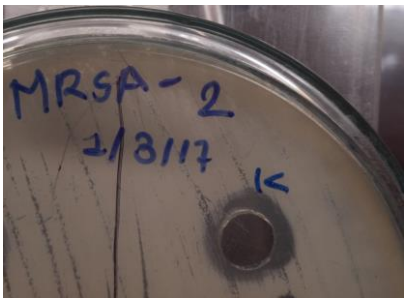

Fig. 2: Bioassay of ethyl acetate extract of LK08 (K) against MRSA -2

The production of antibiotic was begun after 6th day. Bioassay was performed for 6th, 7th and 8th day extract [18]. Best result was obtained on 8th day. It means production started during mid-stationary phase that confirmed the antimicrobial compounds to be secondary metabolites. The extraction of the metabolites was carried out by ethyl acetate on the basis of the maximum solubility and antimicrobial activities.

For instance, a new macrolide structural class with anti-MRSA and anti-VRE activity was reported from *Streptomyces enissocaesilis* that was isolated from the Thar desert, Rajasthan [19]. Our results are also equivalent to that Singh et.al which had reported eleven compounds having same molecular formula like oleandomycin, 2-piperidinone and derivatives of either erythromycin or tylonolide from *streptomyces levis* [20]. Our LK-08 strain also shows 99% similarity to *streptomyces levis.*

DISCUSSION

Currently, resistant among *staphylococcus aureus* has increased against the available antimicrobial compounds. Apart from normal actinomycetes, the haloalkaliphilic actinomycetes from little rann of Kutch are much less explored in this context for their antimicrobial potential. A notorious case is the MRSA, which is resistant not only to methicillin but usually also to aminoglycosides, macrolides, tetracycline, chloramphenicol, and lincosamides. Such strains also do not respond to disinfectants, and MRSA can act as a major source of hospital-acquired infections. So, search of novel natural source for production of antibiotic is significant. New antibiotics are essential to treat microbial pathogens that are turning increasingly

resistant to available treatment [21]. The Saline desert environment is an untapped source of novel actinobacterial diversity and thus of new metabolites. In our search for new anti-MRSA antibiotics from saline desert (little rann of kutch) derived microorganisms, we identified an actinomycete strain LK-08, *Streptomyces enissocaesilis*,(http://eztaxon-e.ezbiocloud.net/ezt_identify) The strain was identified as a Streptomyces sp. based on 16S rRNA gene sequence analysis and shares greatest similarity (99%) with the type strain *Streptomyces levis* having derivatives of either erythromycin or tylonolide. (Figure-4) It shows also similarity with *streptomyces rochei* which carry Antibiotic biosynthetic genes pSLA2-L for lankamycin and lankacidin [22]. This low level of sequence identity implies it may be a new species.

In the current study, it is apparent that a soil bacterium of Streptomyces sp. LK-08 produced extracellular antibiotics, which are found very efficacious against pathogenic MRSA, test microorganisms in vitro. Further investigations are required with a view to determining the structure of active compounds before it assumes the form of a medical treatment. Endeavour's to establish the complete structure elucidations are underway.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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