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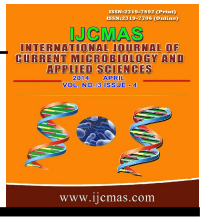

Original Research Article

Antagonistic activity of *Lysinibacillus fusiformis* n 139 strain isolated from marine fish *Triacanthus strigilifer* and genome sequence

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A B S T R A C T

Lysinibacillus is a gram positive, mesophilic rod shaped bacterium. Under harsh conditions, *Lysinibacillus* can form dormant endospores that are resistant to heat, chemicals and ultraviolet light. These spores may remain viable for a longer time. *Lysinibacillus* is an important organism to study because it can be used as an insecticidal toxin that control mosquito growth. Above reason, we preliminary screening of antibacterial activity against antibiotic resistant, eye pathogens and Urinary tract infections pathogens. Five endophytic bacteria were isolated from marine fish of *Triacanthus strigilifer* and isolated for its antibacterial compound production against human pathogens. The screening was carried out by agar well diffusion method and screening of supernatant for antagonistic activity. Among the five isolates, strain-3 was more activity against all clinical isolates. Genome sequencing of these organisms is useful because it increases the knowledge of the *bacilli* and also offers insight for future improvement of important biological control agents. Hence the present study also attempted that, the strain 3 with more antagonistic activity was identified as *Lysinibacillus fusiformis* by 16S rRNA gene sequencing. Further study is needed to find out compound purification from active strains.

Keywords

Antibiotic resistance pathogens; bioactive compounds; gene sequencing

Introduction

The marine environment is a prolific resource for the isolation of less exploited microorganisms. In recent years microorganisms have become important in the study of novel microbial products exhibiting antimicrobial, antiviral, and antitumor as well as anticoagulant and cardio active properties. These active compounds may serve as model systems in the discovery of new drugs. Many organisms had developed complex adaptive and self-protecting mechanisms to survive, often associated with the production of structurally unique bioactive compounds. Many such compounds have been extracted from various marine organisms such as bacteria, cyanobacteria, seaweeds, sponges, cnidarians, tunicates,

soft corals, bryozoans, molluscs, echinoderms, fish and sea snakes. Marine bacteria produce broad-spectrum classical antibiotics and a variety of toxins such as tetradotoxins, saxitoxin, cigualoxins and brevetoxins which are useful in neuro physiological and neuro pharmacological studies. Production of antimicrobial compound seems to be a general phenomenon for most bacteria. An antimicrobial is a substance that kills or inhibits the growth of microbes such as bacteria, fungi or viruses. The discovery of antibiotics has revolutionized the world of medicine. The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse objective compounds (Natham et al., 2004).

Lysinibacillus is a Gram-positive, rod-shaped, and round-spore-forming bacterial genus in the family *Bacillaceae*. Organisms in this genus were previously regarded as members of the genus *Bacillus*, but the taxonomic status of these microorganisms, i.e., rRNA group 2 of the genus *Bacillus*, was changed to the genus *Lysinibacillus* in 2007 (Ahmed et al., 2007). Compared with *Bacillus*, *Lysinibacillus* contains lysine and aspartate in the cell wall peptidoglycan as diagnostic amino acids, in contrast to *meso*-diaminopimelic acid in the genus *Bacillus* (Miwa et al., 2009). *Lysinibacillus* is commonly found in soil (Ahmed et al., 2007) and has been isolated from plant tissues (Melnick et al., 2011), from fermented plant seed products (Parkouda, 2010), and even from puffer fish liver specimens (Wang et al., 2010). As an insecticidal microorganism, the genome of *Lysinibacillus sphaericus* strain C3-41 was the first strain in the genus *Lysinibacillus* (Hu,2008).

Environmental isolates of the genus *Lysinibacillus* are potential biological control agents for diseases that affect cacao (Melnick, 2011). The genome sequence of this *Lysinibacillus* strain will facilitate the investigation of its beneficial properties in pharmaceutical industry. By considering the scope of marine bacteria and the less exploited nature of marine microorganisms, in the present study a screening was made to isolate and identify the bacteria using sequencing of 16S rRNA gene for the production of secondary metabolites against human pathogens.

Materials and Methods

Collection of marine fish

Marine fish were collected from Palk Strait for isolation of endophytic bacteria for screening of human pathogens. The intestine of fish was sampled aseptically for bacteriological examination and antibacterial assay.

Isolation of endophytic bacteria from collected marine fish

Isolation of endophytic bacteria from the *Triacanthus strigilifer* intestine by adding 10 mL of Phosphate Buffered Saline (PBS) to 2 g of target organ. The tissue extract was prepared by blending the tissue for 1 min with homogenizer in ice bath. Tissue residue and fat were filtered off and subjected to serial dilutions (10−1 and 10−2) using PBS. 100 µL of each diluted sample inoculated to the Zoberl marine agar (Himedia, Mumbai, India) using streak plate method. The plates were maintained at 30 °C for 24 hours to allow bacterial growth. Each discrete colony was further subcultured for several times to ensure that it was a pure culture before

subjected to the antagonistic activity and bacterial identification. The marine bacterial isolates designated as Strain 1 to 5 and isolated strains were stored at 4°C for antagonistic study and identification.

Biochemical Test of Isolates

The endophytic bacteria isolates were initially evaluated by biochemical tests i.e. indole test, methyl red test, voges-proskauer test, citrate utilization test, nitrate reduction test, urease test, hydrogen sulphilde test, catalase test, oxidase test, motility test, sugar fermentation test, gram staining and shape were screened for the identification of isolated bacteria.

Production and screening of bioactive compounds from isolates against human pathogens

The overnight grown cultures of endophytic bacteria from marine fish grown in Nutrient broth were centrifuged at 4000 rpm for 10 minutes and supernatant was collected. The overnight cultures of the Five antibiotic resistant pathogens (ABR) *viz., Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus epidermis, Pseudomonas aeruginosa* and *Streptococcus pneumonia,* Five urinary tract infection bacterial pathogens *viz., Pseudomonas* sp, *Enterobacter* sp, *Escherichia coli, Klebsiella pneumoniae* and *Proteus* sp., and Ten eye pathogens namely *viz., E. coil*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Staphylococcus epidermis*, *Streptococcus pneumoniae*, *Klebsiella, Pneumoniae*, *Streptococcus vividins*, *Proteus* sp. and *Acinitobacter* in Nutrient broth were uniformly swabbed on the sterile Muller Hindon agar plates using sterile cotton swabs. Five wells of 6mm size were made with sterile cork borer on the seeded plates. Around 100µl of the supernatant collected was added to each well aseptically. The plates were incubated without inverting for 24 hours at 37^0C and the zone of inhibition was noted and recorded.

Genomic DNA extraction, Cloning and sequencing of 16S rRNA gene of isolates

The most potent isolated bacterial strain was grown in 2ml Zobell Marine Broth (HiMedia, India) overnight at 27^o C. The culture was centrifuge at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 37°C. To this tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K from 20 mg/ml were added and incubated at 550C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water. The amplified product (1,500-bp) was purified using GFX ™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/AcloneTM PCR Product Cloning Kit #K1214, MBI Fermentas). Full-length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea). The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST (Altschul, 1997).

Results and Discussion

The most potent isolates were identified as *Lysinibacillus fusiformis* based on the

results of 16 S rRNA sequencing. The Based on nucleotide homology and phylogenetic analysis the microbe was detected as *Lysinibacillus fusiformis* and the nearest species was found to be *Lysinibacillus boronitolerans.* More than 1470 bp of the 16 S rRNA genes of the strains was sequenced (Fig. 1). Analysis of the 16 SrRNA sequences confirmed the strain was found to be most similar to *Lysinibacillus fusiformis* (Accession Number: KF193525). Screening of antibacterial activity of 5 isolates was carried out against different clinical strains. Inhibitory activity against atleast one clinical isolate was detected for 5 isolates (Table 1).

The isolated strains were inhibited against majority of antibiotic resistance pathogens. The isolate strain 3 presented the highest inhibition zone (15mm) against *Streptococcus pneumonia*. Moreover, the isolated endophytic bacteria were suppressing the growth of ophthalmic pathogens. Among the isolated strains, strain 3 revealed the most effective activity against *Pseudomonas aerugninosa* (18 mm) (Table 2). In addition, endophytic isolates were also tested against Urinary tract infection pathogens. The results showed, strain 3 was the highest activity against *Klebsiella sps.* (Table 3). Table 4 shows the biochemical identification test results. The Based on these criteria, strain 3 was selected to 16S rRNA sequence

Marine bacteria producing a pyrrole antibiotic had been isolated and identified by Burkholde *et al.,* (1966). A new hybrid antimicrobial antibiotic, Thiomarinol, was isolated from a marine bacterium, *Alteromonas rava*. Thiomarinol showed excellent *invitro* antimicrobial activity against Gram-positive and Gram-negative bacteria. (Hideyuki Shiozawa et al., 1993). Antagonistic interactions among marine pelagic bacteria were studied by Richard and Farooq (Richard, et al., 2001). Five endopytic isolates of the present study exhibited antibacterial activity against the most chosen clinical pathogens. The highest activity was found against pathogens exhibited by the Strain 3.

The isolated strain 3 showed more antibacterial activity than the other isolates. It was identified by 16S rRNA gene sequencing. Based on nucleotide homology and phylogenetic analysis the Microbe was detected as *Lysinibacillus fusiformis*. The information about the phylogenetic relationship of these microbe with other bacteria were found using combination of NCBI GeneBank and RDP database. The sequence description revealed that the marine isolate strain 3 was phylogenetically related to *Lysinibacillus boronitolerans* strain. Understanding antibiosis at the phylogenetic level may allow a more focused search for antibiotics that are active against a bacterial species or group. Such an understanding may also help to devise strategies for pathogen control in aquatic environment. Current assay for antimicrobial activity are inadequate because some antibiotic producing bacteria may require the presence of an inducer compound produced in the presence of another bacterial species. These findings have important implications for the discovery of novel antimicrobial compounds from marine bacteria and may allow the development of new methods for screening novel

Table.1 Antagonistic activity of isolates from *Triacanthus strigilifer* against antibiotic resistant pathogens

Test organisms	Zone of Inhibition (mm diameter)				
	Isolates				
	1	**2**	**3**	**4**	**5**
Proteus sp.	02	14	04	02	02
Serratia merchen	03	05	06	04	02
E. coli	11	02	03	7	4
Bacillus aerogenosa	4	9	5	4	8
Bacillus megaterium	2	2	3	2	4
Staphylococcus aureus	1	5	12	14	13
Streptococcus viridae	1	4	3	1	6
S. epidermis	2	3	4	4	4
Pseudomonas aeroginosa	1	5	6	4	7
Solmonella typhii	3	4	5	2	9
Streptococcus pneumoniae	2	6	15	4	**8**
Klebsiella pneumoniae	3	1	5	4	1

Table.2 Antagonistic activity of isolates from *Triacanthus strigilifer* against eye pathogens

Test organisms	Zone of Inhibition (mm diameter)				
	Isolates				
	1	**2**	**3**	**4**	**5**
Pseudomonas aerugninosa	12	13	18	14	12
Enterobacter aerugenosa	10	17	12	11	10
Micrococcus luteus	4	6	5	9	4
Staphylococcus epidermis	2	2	1	1	1
Streptococcus pneumoniae	2	4	14	11	12
Klebsiella pneumoniae	2	7	2	4	2
Streptococcus vividins	4	8	6	4	2
Proteus sp.	4	3	2	2	2
Acinitobacter sp.	3	4	2	4	2

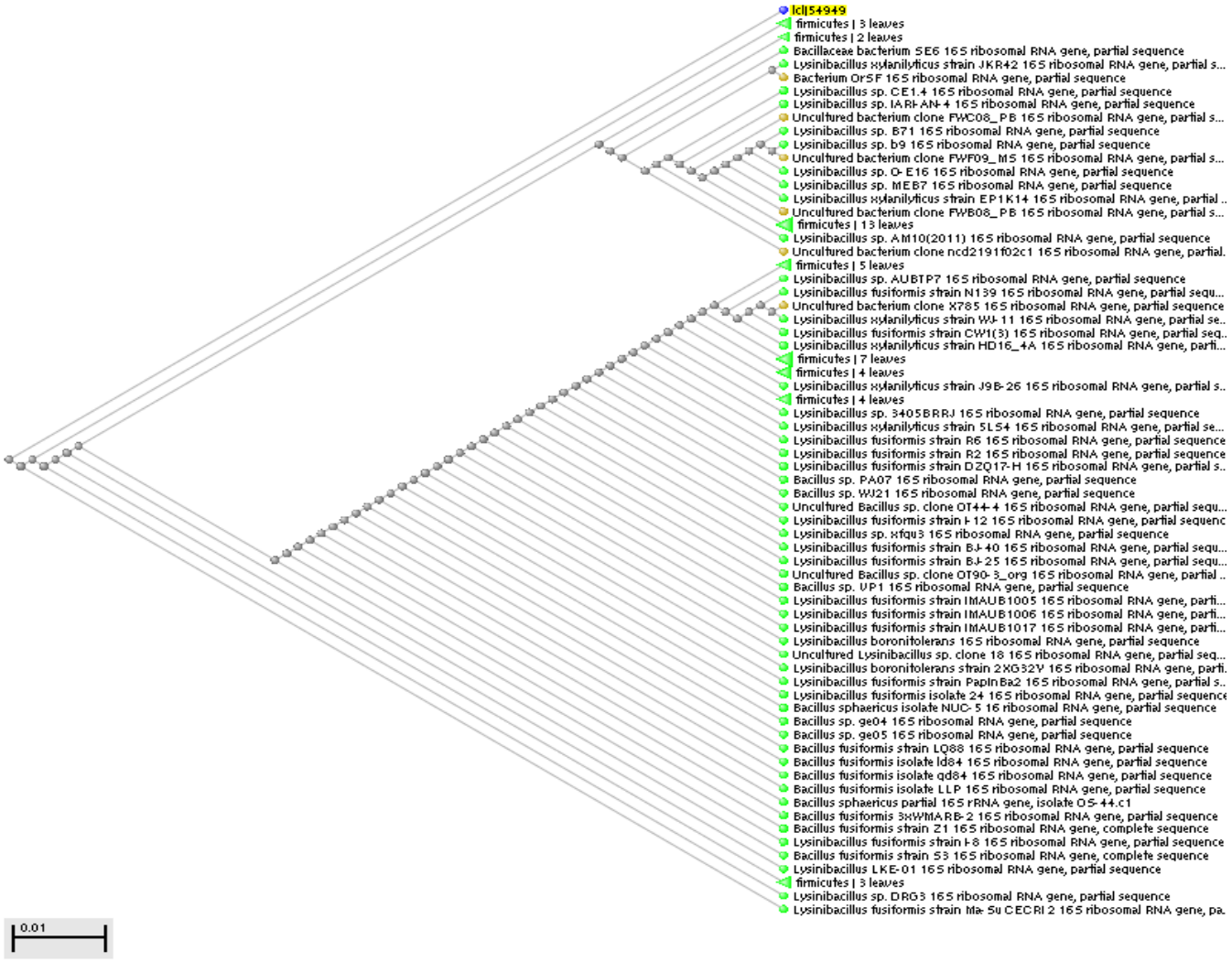
Table.3 Antagonistic activity of isolates from *Triacanthus strigilifer* against Urinary tract infections (UTI) pathogens

Test organisms	Zone of Inhibition (mm diameter)				
	Isolates				
	1	**2**	**3**	**4**	**5**
Pseudomonas sps.	2	4	5	8	2
Enterobacter sps.	11	11	08	14	12
Klebsiella sps.	13	14	22	11	05
E. coli	06	07	08	14	12
Proteus morganii	09	04	05	11	14

Table.4 Biochemical identification of isolates from *Triacanthus strigilifer*

S.No.	Biochemical tests	Results				
		Strain-1	Strain-2	Strain-3	Strain-4	Strain-5
1.	Indole test	+	-	+	-	-
2.	Methyl red test	-	+	-	+	+
3.	Voges-proskauer test	+	-	+	+	+
4.	Citrate utilization test	+	+	+	-	+
5.	Nitrate reduction test	-	+	-	-	-
6.	Urease test	-	-	+	-	-
7.	Hydrogen sulphide test	+	-	-	-	-
8.	Catalase test	-	-	+	+	+
9.	Oxidase test	+	-	-	+	+
10.	Motility test	+	+	-	+	+
11.	Sugar fermentation test	-	+	+	-	-
12.	Gram staining	G^{+ve}	G^{-ve}	G^{+ve}	G^{-ve}	G^{-ve}
13.	Shape	Rod	Coccii	Rod	Coccii	Coccii

Fig.1 Phylogenetic analysis of *Lysinibacillus fusiformis*



compounds active against multi- drug-resistant bacteria. The present findings highlight the significance of the 16SrRNA gene sequence of *Lysinibacillus fusiformis* strains as potential sources of potent broad spectrum antimicrobial agents. Purification and structural analysis of the active compounds from these strains may prove to be novel. Further structural studies are required to isolate the types of compounds responsible for the antibacterial effects. The study encourages the use of bacterial extracts demonstrated that medicine can be used as effective modern medicine to combat pathogenic microorganisms.

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References

Ahmed I, Yokota A, Yamazoe A, Fujiwara T. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to*Lysinibacillus sphaericus* comb. nov. *Int. J. Syst. Evol. Microbiol.* 2007, 57, 1117–1125.

Altschul SF. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997, 25, 3389-3402.

Burkholder, P. R., M. Robert, Pfister and Frederick, H.L. Production of an antibiotic by a marine bacterium. *Appl. Microbiol*. 1966, 14(4), 649-653.

Hideyuki Shiozawa., Takeshi Kagasaki., Takeshi Kinoshita and Haruyama, H. Thiomarinol, a new hybrid antimicrobial antibiotic produced by a marine bacterium - fermentation, isolation, structure, and antimicrobial activity. *The J. Antibio*. 1993, 251-255.

Hu X, . Complete genome sequence of the mosquitocidal bacterium *Bacillus sphaericus* C3-41 and comparison with those of closely related Bacillus species. *J. Bacteriol*. 2008, 190, 2892–2902.

Melnick RL, Suárez C, Bailey BA, Backman PA. Isolation of endophytic endospore-forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases. *Biol. Control* 2011, 57, 236–245.

Miwa H, Ahmed I, Yokota A, Fujiwara T. . *Lysinibacillus parviboronicapiens* sp. nov., a low-boron-containing bacterium isolated from soil. *Int. J. Syst. Evol. Microbiol*. 2009, 59, 1427–1432.

Natham, A., Magarvey., Jessica, M.Keller., Valerie Bernam., Martin Dworkin and Sherman, D.H. Isolation and characterization of novel marine-derived *Actinomycete* taxa rich in bioactive metabolites. *Appl. Environ. Microbiol.* 2004, 70(12), 7520-7529.

Parkouda C, Microorganisms associated with maari, a baobab seed fermented product. Int. *J. Food Microbiol*. 2010, 142, 292–301.

Richard, A. Long and Farooq Azam, Antagonistic interactions among marine pelagic bacteria. *Appl. Environ. Microbiol*. 2001, 67(11), 4975- 4983.

Wang J, Fan Y, Yao Z. Isolation of a *Lysinibacillus fusiformis* strain with tetrodotoxin-producing ability from puffer fish *Fugu obscurus* and the characterization of this strain. *Toxicon* 2010, 56, 640–643.