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Isolation and characterization of marine bacteria from East Coast of India: functional screening for salt stress tolerance

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ABSTRACT

Soil salinization has become a severe constraint for crop production world-wide which necessitated development or induced enhancement of salt stress tolerance in plant life to sustain production in saline lands. Recognition and prospecting of valuable stress tolerant genes from natural microbial resources of saline habitat is obscure to date. Therefore, the investigation was towards isolation and characterization of marine salt stress tolerant microbes along the East coast of India for revelation of effective salt stress tolerant genes. Salt stress tolerance was assessed from 98 bacterial isolates obtained from 28 water and soil samples. Among them, 35 isolates which failed to grow beyond 4% salt were discarded and remainder 63 isolates were selected for further functional analysis and only seven isolates recorded ≥8% NaCl stress tolerance. Phylogeny revealed that four isolates belong to Firmicutes and three isolates were members of Proteobacteria. Ribosomal Database Project Release-11 and SILVA SSU database based genotyping and taxonomic identity analysis confirmed that the higher (20%) salt stress tolerant bacteria were *Staphylococcus* sp., *Enterococcus* sp., *Enterobacter* sp. and *Proteus* sp. To investigate candidate, as well as, novel salt stress tolerant genes, the seven bacterial isolates would provide new horizon to focus on the recent developments of salinity stress tolerance. In addition, the findings evidently point out the diversity of salt stress tolerant marine bacteria in coastal Odisha and West Bengal, India.

1. Introduction

Abiotic stresses pose great menace to agriculture and the environment. Abiotic stress akin to high salinity causes considerable reduction of crop production in peninsular South-East Asia, predominantly in India (Behera et al., 2014). Salinity results in hyperosmotic condition, severely disarrays metabolism, causes ion imbalances in plant cells and thereby ensues oxidative damage (Munns and Tester, 2008). In India, 8 million ha out of 329 million ha area and globally on 77 million ha (5%) of 1.5 billion ha farming land is afflicted by salinity (Sheng et al., 2008). Salinization is one of the chief causes for loss of cultivable land which directly adds to the challenge to sustain global food supply. For elevation of the agricultural yield to meet food requirement in future, it is crucial to look up remedial measures for agriculture and aquaculture under natural stresses like salinity. Sustenance or raise of yield of staple food is an utter constraint for mankind. Hence, it is crucial to generate salt stress tolerant plants for cultivation in saline lands (Zhu, 2000; Ueda et al., 2002; Wang et al., 2003). Prokaryotes can survive in broad diversity of ecological stresses, like extremes of temperature, pressure, salinity, pH, radiation etc (Cava et al., 2009; Nath and Bharathi, 2011). Halophilic and halotolerant bacteria inhabit in broad base of salty habitats and the moderately halotolerant bacteria are more distributed than the extreme halotolerant microbes (Baati et al., 2010). Competence of the bacteria to respond and sustain in fluctuations of external osmolarity is noteworthy function for endurance and propagation in diverse ecological niches (Sleator and Hill, 2002) acquired during evolution through expression of large scale of salt stress tolerant genes from bacteria. Isolation and classification of salt stress tolerant microbes from oceanic environment has realistic significance for biotechnological prospective through production of functional biomolecules for instance exopolysaccharides, hydrolytic enzymes, osmolytes etc (Behera et al., 2014). Diverse kind of halophilic and halotolerant microbes have been recognized from broad

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range of aquatic environment (Caton et al., 2004; Grant, 2004; Jiang et al., 2006; Tsiamis et al., 2008; Xiang et al., 2008) and prospecting of the salt stress tolerant genes from these resources, hereafter, is important (Jenkins et al., 1991). Therefore, isolation of bacteria and functional screening intended for salt stress tolerant property, characterization and molecular classification of the marine salt stress tolerant microbes are indispensable to augment agricultural production. Species specific variability regions of the 16S rRNA gene sequence analysis are more supportive for classification of phylogenetic identity in conformity to the phenotypic profiles for recognition of microbes (Mignard and Flandrois, 2006) over conventional technique used for halophilic microbial systematics (Kushner, 1993). Therefore, the reason of the study was to isolate and distinguish the most effective novel salt stress tolerant microorganisms from sediment and water samples of marine ecosystems along the East coast of India for prospecting osmotic stress tolerance of crop plants.

2. Materials and methods

2.1. Sample collection

The water samples (28 nos.) were collected from three dissimilar spots all across the East coast of India viz. Haldia (22°0′59.28″N 88°4′7.88″E), Digha (21°36′9.94″N 87°27′43.93″E) in West Bengal and Puri (19°47′31.15″N 85°49′3.41″E) in Odisha (Fig. 1). Five water samples (500 ml) were collected in sterile water bottles at 5 m apart from each location and each of 0.5 m, 1.0 m and 1.5 m water depth, mixed and the composite samples were instantaneously moved to the laboratory for further isolation and characterization (Chatterjee et al., 2010; Behera et al., 2014). The salinity of the water at Puri, Digha and Haldia was tested and found to be 34 ppt, 35ppt and 33 ppt, respectively.

2.2. Functional screening salt stress tolerant bacteria

Cultivable bacterial strains were isolated and enumerated (Brown, 2007). Isolation of bacteria was carried out with Tryptic soya agar (TSA) (HiMedia Pvt Ltd, Mumbai) adopting serial dilution technique and incubated overnight at 37 ± 2 °C for 2–3 days in a BOD incubator. Each colony of the plates were added to 500 ml of liquid TSB medium and overnight incubation was done at 37 ± 2 °C for confluent growth (5–9 x 10^7cells/ml). Broth cultures were plated on TSA media plates and salt tolerance was determined by adding 1.5, 5, 8, 9, 10, 15, 20 and 25% (w/v) NaCl in TSB medium. Optimal growth was examined (Caton et al., 2004).

2.3. Taxonomic studies and growth in selective media

Culture, morphophysiology and biochemical characters of the microbial isolates were studied following standard methods (Ventosa et al., 1998). The isolates were checked for colony characters, motility, shape/size of the organism, and Gram and endospore staining. Biochemical tests, hydrolysis to carbohydrates and its metabolism as well as tolerance to NaCl were performed. Colony characters of isolates were observed on HiCrome *Bacillus* agar, rapid HiEnterococci agar, rapid HiColiform agar, *Enterococcus* confirmatory agar, Kligler iron agar, Chapman stone agar, Pikovskaya's agar, KG agar, Pseudomonas isolation agar, Hifluoro Pseudomonas agar base phenylalanine agar, triple sugar-iron agar, coagulase mannitol agar and HiCrome staph agar base for characterization of the bacteria (HiMedia Pvt Ltd, Mumbai).

2.4. Scanning electron microscopy (SEM)

The salt stress tolerant bacterial isolates were studied for morphological properties through scanning electron microscopy using Hitachi S-

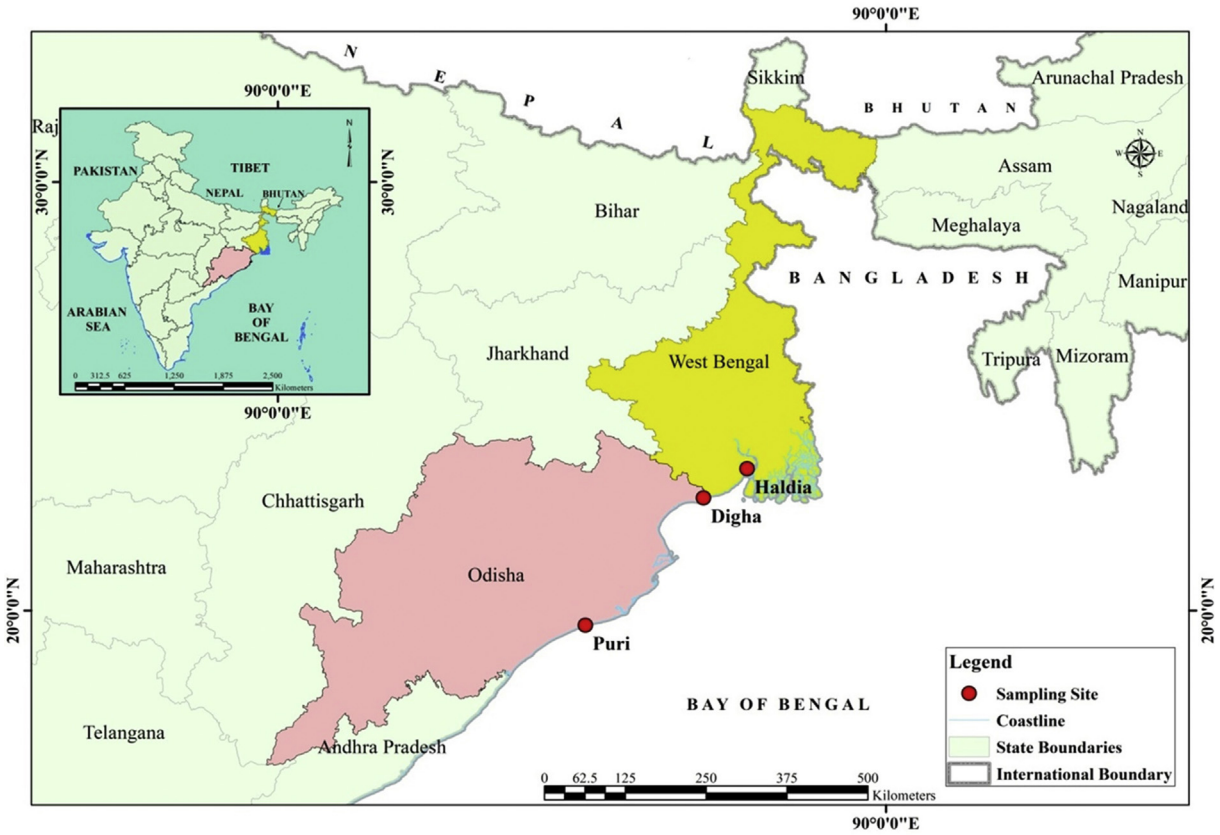

Fig. 1. The map illustrates the diverse spots of sample collection spot across West Bengal and Odisha coastal areas of India. The distinct circles symbolize the specific spots; the latitude and longitude of the places are. mentioned within the parenthses.

530 as per standard protocol (Maji et al., 2012).

2.5. Antibiotic sensitivity test

Reaction of the organisms to diverse standard antibiotic discs with effective concentrations (NCLSS standard level) as mentioned in Table 5 were taken for studying the antibiotic sensitivity test following Brown (2007). The antibiotic discs were placed over Nutrient Agar plates. A clear zone around the discs was accepted as inhibition of growth i.e. sensitivity reaction.

2.6. Extraction of DNA, PCR amplification and 16S rRNA gene sequencing

DNA isolation, PCR amplification of 16S rRNA gene and sequencing of the rRNA fragment DNA was prepared from seven bacterial isolates by sarkosyl method (Maniatis et al., 1982). DNA samples were treated with RNase A. The concentrations of DNA were measured by optical assessment of 1% agarose gels stained with ethidium bromide in addition to spectrophotometric assessment. The 16S rRNA gene PCR amplification was executed by 16S rRNA gene specific primers by GeneAmp PCR system 9700 thermal cycler (ABI, Foster City, USA). For 16S rRNA gene amplification, forward primer [8F (5′–3′) AGAGTTTGATCCTGG CTCAG] and reverse primer [1492R (5′–3′) ACGGCTA CCTTGTTACGACTT] were used (Edwards et al., 1989). The protocol for PCR amplification was: primary heating for 2 min at 95 °C, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, extension for 1 min 30 s at 72 °C, followed by a final extension step for 10 min at 72 °C. The final PCR reaction mixture (50 μl) was as follows: 50 ng of isolated genomic DNA, 1X PCR buffer with 1.5 mM $MgCl_2$, 30 mM each dNTP (Sigma, USA), 10 pmol each primer (Sigma, USA) and 1 U Taq DNA polymerase (Sigma, USA). PCR amplified gene products were resolved on agarose gel (1.8%). DNA fragments of 1500 bp were cut out from the agarose gel and the fragments were purified with a QIAquick PCR purification kit (QIAgen Inc., USA) following manufacturer's protocol. Amplified PCR fragments of the 16S rRNA genes were sequenced in ABI 3730xl genetic analyzer using universal primers. The sequencing was carried out by Big Dye by means of the terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol.

2.7. Blast search and phylogenetic study

Seven partial gene sequences of 16S rRNA were BLAST searched with NCBI database (Altschul et al., 1990). The taxonomic hierarchies of the test sequences were obtained establishing the nearest neighbours based on shared words (%) between test and query sequences using SEQ MATCH tool (RDP Release-11). The alignment and pintail quality, alignment identity and ambiguity were checked using SILVA SSU database. The SILVA SSU analysis of the sequences illustrated no ambiguity in the sequences. The analysis further explained almost 100% alignment identity (99.85–100%) between the target and reference database sequences. Phylogenetic analyses of partial gene sequences were executed by Clustal X (v 1.83) (Thompson et al., 1994). The sequences were combined with developmentally interconnected halotolerant microbial species using NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) (Larkin et al., 2007). All gaps i.e. indels obtained by the input sequence alignment were considered as missing data during the phylogenetic estimation. Neighbour joining algorithm (Hillis and Bull, 1993) was used to calculate the evolutionary distances through 1000 bootstraps, including Kimura-2 parameter substitution model (Kimura, 1980). MEGA 6.0 (Tamura et al., 2011) was used to create and visualise the phylogenetic tree.

2.8. Accession numbers

The accession numbers of seven bacterial isolates obtained after submitting the sequences to GenBank were: KP117091 (P-TSB-70), KP117092 (P-TSB-72), KP117093 (P-TSB-75), KP117094 (D-TSB-84), KP117095 (P-TSB-78), KP117096 (D-TSB-86) and KP117097 (H-TSB-70).

3. Results

3.1. Isolation and characterization of salt stress tolerant marine bacteria

98 bacteria were isolated from 28 water samples. All 98 isolates could grow at pH 7.3 ± 0.2 and 35 isolates endured only up to 4% NaCl, but 63 isolates tolerated beyond 4% salt which were further tested thoroughly and seven isolates (D-TSB-84, D-TSB-86 H-TSB-70, P-TSB-70, P-TSB-72, P-TSB-75 and P-TSB-78) were found extreme (8–20%) NaCl stress tolerant (Fig. 2) and further investigations were carried out with the seven bacteria. Out of seven osmotolerants, three isolates (P-TSB- 70, P-TSB-72 and P-TSB-75) tolerated 20% NaCl stress and four isolates (D-TSB-84, P-TSB-78, D-TSB-86 and H-TSB-70) tolerated 9% NaCl stress (Table 1).

3.2. Cultural and morphological characterization

The detailed culture, morphology and colony characters were not identical and detailed characters of the seven bacteria on selective medium are shown in Supplementary Tables 1 and S2. The P-TSB-70, P-TSB-72 and D-TSB-84 colonies were golden brown, non-motile, entire and convex; the cells were spherical, non-spore former, non-motile occurred in singles, pairs and irregular clusters arranged in irregular or tetrad shapes. The P-TSB-75 and H-TSB-70 formed tanned and motile colonies, and the bacteria were rod shaped. P-TSB-78 colonies were yellow and motile; cells were cocci in clusters, short chains, diplococci and single cocci. D-TSB-86 formed tanned colonies and the bacteria were motile and straight rods. The colonies of the isolates on different selective media also differed viz. colour, acidic reaction, gas/H_2S production etc (Supplementary Table S1).

3.3. Physiological and biochemical characterization

Physiological as well as biochemical characters of the salt tolerant bacteria are specified in Table 2. The isolates were urease, catalase

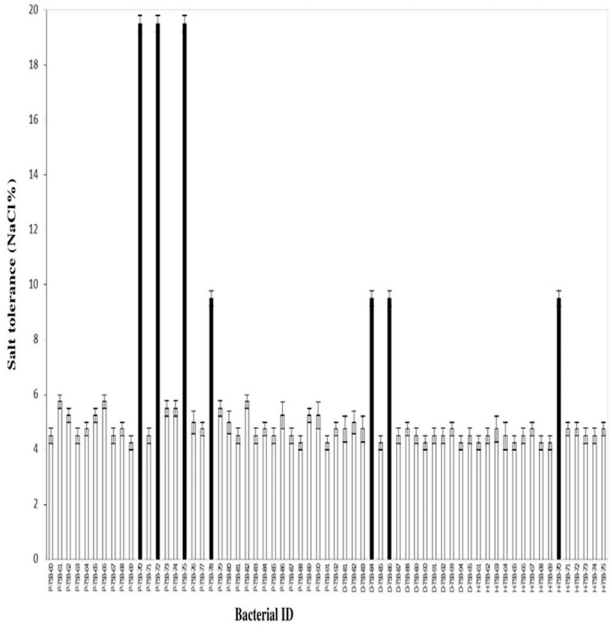

Fig. 2. Salt stress tolerance (%) of 63 bacterial isolates sampled from East coast of India.

Table 1
NaCl tolerance of all bacteria.

Isolate No.	Location	Isolation source	Isolation medium	Max. salt (NaCl) conc. (%)
P-TSB-60, P-TSB-61, P-TSB-62, P-TSB-63, P-TSB-64, P-TSB-65, P-TSB-66, P-TSB-67, P-TSB-68, P-TSB-69, P-TSB-71, P-TSB-73, P-TSB-74, P-TSB-76, P-TSB-77, P-TSB-78, P-TSB-79, P-TSB-80, P-TSB-81, P-TSB-82, P-TSB-83, P-TSB-84, P-TSB-85, P-TSB-86, P-TSB-87, P-TSB-88, P-TSB-89, P-TSB-90, P-TSB-91 (n = 29)	Puri, Odisha	Water	Tryptone soya agar	8
P-TSB-78 (n = 1)	Puri, Odisha	Water	Tryptone soya agar	9
P-TSB-70, P-TSB-72, P-TSB-75 (n=3)	Puri, Odisha	Water	Tryptone soya agar	20
D-TSB-81,D-TSB-82, D-TSB-83, D-TSB-85, D-TSB-87, D-TSB-88, D-TSB-89, D-TSB-90, D-TSB-91, D-TSB-92, D-TSB-93, D-TSB-94, D-TSB-95 (n = 13)	Digha, W.B.	Water	Tryptone soya agar	8
D-TSB-84, D-TSB-86 (n = 2)	Digha, W.B.	Water	Tryptone soya agar	9
H-TSB-61, H-TSB-62, H-TSB-63, H-TSB-64, H-TSB-65, H-TSB-66, H-TSB-67, H-TSB-68, H-TSB-69, H-TSB-71, H-TSB-72, H-TSB-73, H-TSB-74, H-TSB-75 (n = 14)	Haldia, W.B.	Water	Tryptone soya agar	8
H-TSB-70 (n = 1)	Haldia, W.B.	Water	Tryptone soya agar	9

Positive growth (+), negative growth (-), W.B. (West Bengal).
Seven bacteria (boldface) out of all the isolates in the table which tolerated above 8% were used in this present study.

Table 2
Physiological and biochemical properties of the seven bacterial isolates.

Tests	Bacterial isolates						
	D-TSB-84	D-TSB-86	H-TSB-70	P-TSB-72	P-TSB-75	P-TSB-78	P-TSB-70
Catalase	+	+	+	-	+	+	+
Indole production	-	-	-	-	-	-	-
Methyl red test	+	-	+	+	+	+	+
Vogues Proskauer test	-	+	-	-	-	-	-
Nitrate reduction	+	-	-	+	-	+	+
Urease production	+	+	+	+	+	+	+
Citrate utilization	-	-	+	-	+	-	-
Oxidase	-	+	+	-	+	-	-
Coagulase	-	-	-	-	-	-	-
Blood haemolysis	-	-	-	-	-	-	-
Lecithinase	-	-	-	-	-	-	-

Positive result (+), negative result (-).

(except P-TSB-72) and methyl red (except D-TSB-86) positive, but negative for coagulase, blood haemolysis, indole production and lecithinase tests. Exo-enzyme production (amylase, protease, lipase, chitinase) by the seven bacteria is shown in Table 3. All isolates fermented glucose, sucrose, arabinose, mannose, raffinose, lactose, dextrose, trehalose, glycogen and aesculin; produce acid without gas but none fermented salicin except for *Enterococcus* sp (P-TSB-78) (Table 4). Under scanning (Supplementary Figure S1), the organisms also showed spherical (P-TSB-70, P-TSB-72, D-TSB-84), rod (P-TSB-75), cocci (P-TSB-78) and straight rod (D-TSB-86).

3.4. Antibiotic sensitivity test

All seven bacteria were sensitive to experimental doses of kanamycin, vancomycin, nalidixic acid, ciprofloxacin, tetracycline, erythromycin, levofloxacin, doxycyclin and rifampicin; and resistant to ampicillin, nystatin and amoxicillin (except for D-TSB-84) (Table 5). Besides, four bacteria were resistant to bacitracin except for P-TSB-75, D-TSB-84 and D-TSB-86, and five isolates were sensitive to polymyxin B except for D-TSB-86 and H-TSB-70 and five isolates were sensitive to penicillin G (except P-TSB-70 and D-TSB-84).

3.5. BLAST search and phylogenetic study

The seven extreme osmotolerant bacteria showed similarity between the bacterial morphology and 16S rRNA sequences. Derived from 16S rRNA sequences, bacteria were documented into genus e.g. *Staphylococcus*, *Enterococcus*, *Enterobacter* and *Proteus* (Table 6). The phylogenetic lineages between the 16S rRNA gene sequences is signified through the neighbour-joining tree acquired in the phylogenetic analysis. The 16S rRNA amplification for each of the seven bacteria produced about 1500 bp amplicons and the phylograms were generated with the 16S rRNA gene sequences of very much similar sequence of other microorganisms of NCBI database (Fig. 3). The 16S rRNA sequences of *Staphylococcus* sp. viz. P-TSB-70 (KP117091), P-TSB-72 (KP117092) and D-TSB-84 (KP117094) were 100% similar with the 16S rRNA gene sequence of *Staphylococcus* sp., *Staphylococcus haemolyticus* and *Staphylococcus epidermidis, Proteus* sp. viz. P-TSB-75 (KP117093) and H-TSB-70 (KP117097) branched with the 16S rRNA gene sequence of *Proteus* mirabilis having 100% bootstrap value, *Enterobacter* sp. viz. D-TSB-86 (KP117096) branched with the 16S rRNA gene sequence of *Enterobacter* sp. having 100% bootstrap value and *Enterococcus* sp. viz. P-TSB-78 (KP117095) branched with the 16S rRNA gene sequence of *Enterococcus* sp. available in NCBI database. 16S rRNA sequencing and phylogenetic study reveal 99–100% similarity of the seven isolates collected from Eastern coastal areas to the sequences of GenBank. The 16S rRNA

Table 3
Exo-enzyme production by the seven bacteria.

Test	Bacteria						
	P-TSB-70	P-TSB-72	P-TSB-75	D-TSB-84	P-TSB-78	D-TSB-86	H-TSB-70
Starch Hydrolysis (amylase)	+	+	-	+	-	-	-
Gelatin Hydrolysis (protease)	-	-	-	-	-	-	-
Casein hydrolysis (protease)	-	-	-	-	-	-	-
Fat hydrolysis (lipase)	-	-	-	-	-	-	-
Chitin hydrolysis (chitinase)	-	-	-	-	-	-	-

Table 4
Carbohydrate fermentation properties of the seven bacterial isolates.

Carbon Source (1%)	Bacterial isolates													
	D-TSB-84		D-TSB-86		H-TSB-70		P-TSB-72		P-TSB-75		P-TSB-78		P-TSB-70	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
Glucose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Sucrose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Arabinose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Mannose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Raffinose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Lactose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Dextrose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Trehalose	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Glycogen	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Aesculin	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Salicin	-	-	-	-	-	-	-	-	-	-	+	-	-	-

Positive (+), negative (-).

Table 5
Antibiotic sensitivity tests of the seven bacterial isolates against recommended doses of antibiotics.

Antibiotics	Bacterial isolates						
	P-TSB-70	P-TSB-72	P-TSB-75	D-TSB-84	P-TSB-78	D-TSB-86	H-TSB-70
Ampicillin (10 μg/disc)	R	R	R	R	R	R	R
Kanamycin (30 μg/disc)	S	S	S	S	S	S	S
Vancomycin (30 μg/disc)	S	S	S	S	S	S	S
Nalidixic Acid (30 μg/disc)	S	S	S	S	S	S	S
Ciprofloxacin (30 μg/disc)	S	S	S	S	S	S	S
Nystatin (10 μg/disc)	R	R	R	R	R	R	R
Tetracycline (30 μg/disc)	S	S	S	S	S	S	S
Amoxicillin (10 μg/disc)	R	R	R	S	R	R	R
Erythromycin (10 μg/disc)	S	S	S	S	S	S	S
Bacitracin (0.04U/disc)	R	R	S	S	R	S	R
Polymyxin B (300U/disc)	S	S	S	S	S	R	R
Levofloxacin (5 μg/disc)	S	S	S	S	S	S	S
Doxycyclin (30 μg/disc)	S	S	S	S	S	S	S
Penicillin G (10U/disc)	R	S	S	R	S	S	S
Rifampicin (5 μg/disc)	S	S	S	S	S	S	S

Sensitive (S), Resistant (R).

sequences have brought about definite information about the salt stress tolerance of diversified bacterial population along the East coast of India. Study revealed that, two isolates of *Staphylococcus* and one isolate of *Proteus* grew well in 20% salt stressed medium in contrast to rest of the four bacterial isolates. One isolate of *Staphylococcus*, one isolate of *Proteus*, one isolate of *Enterobacter* and one isolate of *Enterococcus* tolerated upto 9% NaCl stress.

4. Discussion

4.1. Isolation of salt stress tolerant bacteria

In the current study, salt stress tolerant bacteria were categorized based on their biochemical and physiological properties as per adjudging Chatterjee et al. (2010) and 16S rRNA gene sequence studies of Behera et al. (2014). Here, seven microbial strains were isolated from three locations in the coastal vicinity of Eastern region of India. The techniques of isolation and selection of the bacteria were subsequently selected for functional screening the salt stress tolerant microbial strains from the initially isolated remainder bacterial community. The 16S rRNA gene phylogeny is the recommended technique for classification and has been broadly used for difference of diverse microorganisms including the diazotrophic microbes (Zehr et al., 2003).

The 98 isolates were not uniformly distributed in 28 water samples of three locations of West Bengal and Odisha, and 35 were not salt tolerant (<2% salt), 56 isolates could grow with <8% salt but 7 organisms endured 8–20% salt (extremophiles) (Table 1). The results proved that all microbes of coastal habitat were not salt tolerant and degree of osmotolerance also vary in the microbial guilds. Similarly, both salt tolerant and sensitive bacteria were reported in saline habitat (Behera et al., 2014), as well as, in non-saline terrestrial habitat harbouring 5–12% salt tolerant bacteria (Das and Dangar, 2008). Prevalence of salt tolerant microbes in general and extremophillic osmotolerant (20% salt) in West Bengal and Odisha indicated that osmotolerant bacteria were generally more in water samples of Odisha. The results conformed to the results of

Table 6
Analyses of the seven partial 16S rRNA gene sequences in RDP Release-11 and SILVA SSU databases.

Isolate Number	Isolate Name	Length of sequence (bp)	RDP-11 (*S_ab* score)	SILVA alignment identity	Phylum	Similarity with nearest strain	Accession Number
P-TSB-70	*Staphylococcus* sp.	1434	1.000	100	Firmicutes	*Staphylococcus epidermidis*	KP117091
P-TSB-72	*Staphylococcus* sp.	1442	1.000	100	Firmicutes	*Staphylococcus haemolyticus*	KP117092
P-TSB-75	*Proteus* sp.	1434	0.997	99.85	Proteobacteria	*Proteus mirabilis*	KP117093
D-TSB-84	*Staphylococcus* sp.	1429	1.000	99.93	Firmicutes	*Staphylococcus epidermidis*	KP117094
P-TSB-78	*Enterococcus* sp.	1449	1.000	100	Firmicutes	*Enterococcus* sp.	KP117095
D-TSB-86	*Enterobacter* sp.	1430	0.996	99.93	Proteobacteria	*Enterobacter* sp.	KP117096
H-TSB-70	*Proteus* sp.	1424	1.000	99.85	Proteobacteria	*Proteus mirabilis*	KP117097

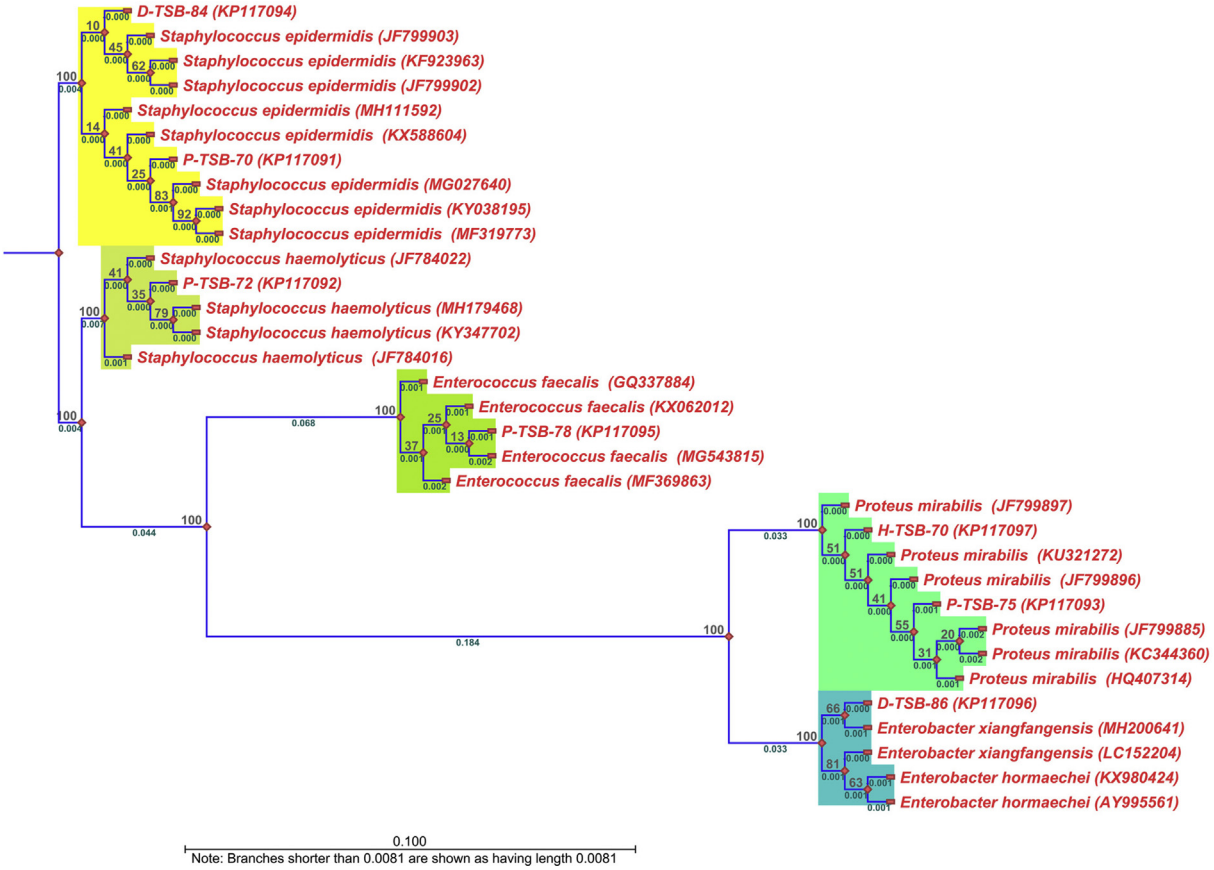

Fig. 3. Phylogenetic tree of salt stress tolerant bacterial isolates by neighbor-joining method based on 16S rRNA partial gene sequences of bacterial isolates from East coast of India. Numbers at nodes represent bootstrap percentages founded on a neighbour-joining analysis of 1000 resampled datasets.

Behera et al. (2014) who reported variation, as well as, more bacterial population in water of coastal samples of Odisha.

Morpho-physio-biochemical characters (Tables 2, 3, and 4) including the colony characters in different media (Supplementary Table S1) of the seven salt tolerant bacteria were not identical which proved that the structure and functions of the bacteria were different. The phenotypic characters (Table 3) were matched with the phenotypic data of standard bacteria for identification following manuals of systematic bacteriology (Smibert and Krieg, 1995; Holt, 1984) and identified the organisms as *Staphylococcus* sp (strain P-TSB-70 and P-TSB-72, D-TSB-84), *Proteus* sp (strain P-TSB-75 and H-TSB-70), *Enterobacter* sp (strain D-TSB-86) and *Enterococcus* sp (strain P-TSB-78). However, minor differences of morpho-physio-biochemical characters of the bacteria of the same species (Tables 2, 3, and 4) are not unusual in the microbial communities (Holt, 1984).

Seven bacterial strains showed varied sensitivity to different antibiotic assays (Table 5). Seven bacterial strains were found to be resistant to ampicillin (10 μg/disc) and nystatin (10 μg/disc), but had diverse sensitiveness to the remaining antibiotics. Mishra et al. (2009) revealed that all isolates were sensitive to various antibiotics and showed that the bacteria had no inherent resistance to numerous sets of antibiotics. Ming-xiang et al. (2012) studied the clinical isolates of *Staphylococcus aureus* for the molecular epidemiological characteristics and antimicrobial resistance in Changsha area and reported that the strain was resistant to erythromycin, ampicillin, penicillin G and clindamycin. However, in the present study, the *Staphylococcus* sp. strain D-TSB-84 showed sensitivity to erythromycin, *Staphylococcus* sp. P-TSB-72 showed sensitivity to both penicillin and erythromycin and *Staphylococcus* sp. P-TSB-70 showed sensitivity to erythromycin. Bouza and Cercenado (2002) reported that *Enterobacter* sp. is intrinsically resistant to ampicillin which supports the present observation of *Enterobacter* sp. strain D-TSB-86. Further, Cernohorska and Chvilova (2011) studied on the resistance to antibiotics and biofilm development of *Proteus mirabilis* isolated from urine and observed that *Proteus mirabilis* strains were resistant to ampicillin. Similarly, Stock (2003) studied the natural antibiotic vulnerability of *Proteus* sp. referring to *P. mirabilis* and *P. penneri* strains and found that both the strains were in nature resistant to penicillin G. He found that *P. penneri* was naturally resistant to amoxicillin. However, in the present study both the strains *viz. Proteus* sp. strain H-TSB-70 and *Proteus* sp. strain P-TSB-75 were found resistant to ampicillin but sensitive to penicillin G. Hollenbeck and Rice (2012) reported that ampicillin resistance of *Enterococcus faecium* which supports the data of *Enterococcus* sp. strain P-TSB-78 was observed in the present study.

4.2. 16S rRNA sequence, distribution and diversity analysis

The 16S rDNA amplification of the seven bacteria produced about 1500 bp amplicons which was used for phylograms generation through NCBI database (Fig. 3). The 16S rRNA sequences of P-TSB-70 (KP117091), P-TSB-72 (KP117092) and D-TSB-84 (KP117094) were 100% similar with the 16S rRNA sequence of *Staphylococcus* sp. of NCBI database (JF799903, JF799902, KF923963, MH111592, KX588604, MG027640, KY038195, MF319773, JF784022, MH179468, KY347702 and KY038195) which were isolated from the various sources (viz. food, tannery effluent, clinical samples, mouse faeces, ice cubes and root of plants). Similarly, the 16S rRNA sequence of P-TSB-75 (KP117093) and H-TSB-70 (KP117097) claded with 100% similarity with the 16S rRNA gene sequence of *Proteus* sp. of NCBI database (JF799897, KU321272, JF799896, JF799885, HQ407314, KC344360) from different sources (viz. fish, midgut of *Musca domestica*) with 100% bootstrap value. The D-TSB-86 (KP117096) clustered 100% similarity with the 16S rRNA gene sequence of Enterobacter sp. of NCBI database (MH200641, LC152204,

KX980424, AY995561) from different sources (viz. honey from honey bee) with 100% bootstrap value. The 16S rDNA sequence of P-TSB-78 (KP117095) branched 100% similarity with the 16S rRNA gene sequence of *Enterococcus* sp. of NCBI database (MF369863, GQ337884, KX062012, MG543815) isolated from different sources (viz. pickle, raw meat) with 100% bootstrap value. As *Staphylocccous, Proteus, Enterobacter and Enterococcus* found in this study was identical in homogeneity to the *Staphylococcus* found in NCBI database, the bacteria isolated from the various sources revealed that the source of bacteria migrated from terrestrial environment to aquatic environment was contributed via anthropogenic resources. The results confirmed the phenotype identity of the organisms (Fig. 3). However, members of each phylotype differed from some phenotypic characters (Fig. 3).

Choi et al. (2014) reported that, the genus *Staphylococcus* comprises of 47 type species as well as 24 subspecies with authentically available names. According to Choi et al., (2014), sources for isolation of *Staphylococcus* species comprise of amber (Lambert et al., 1998), insects (Hájek et al.1992), estuarine and marine surface water (Gunn and Colwell, 1983), fermented fish (Tanasupawat et al., 1992), cheese (Vernozy-Rozand et al., 2000), soil, and plant, representing prevalence of the *Staphylococcus* in the normal environment, though, ecology and physiology of non-pathogenic *Staphylococcus* in the usual habitat remained ambiguous. A common feature of *Staphylococus* species is tolerant to high concentration of NaCl (Choi et al., 2014). Several researchers reported numerous *Staphylococcus* including *S. saprophyticus, S. epidermidis* and other several *Staphylococcus* to grow under 10% NaCl (Schleifer and Kloos, 1975). *S. condimentii, S. carnosus* and *S. piscifermentans* can propagate at 15% NaCl (Choi et al., 2014) and *S. agnetis* can endure even upto 19% NaCl (Taponen et al. 2012). *Staphylococcus* sp. are predictable to experience altering ecological surroundings and stresses, including osmotic stress as *Staphylococcus* sp. exists in a variety of environments which includes outside the normal surroundings, food, animal and human host. Consequently, resistance to osmotic stress would execute a significant part for adaptation of *Staphylococcus* below high NaCl state (Choi et al.,2014).

The 16S rDNA phylogenetic analysis identified the isolates P-TSB-70, P-TSB-72 and D-TSB-84 as the phylotype *Staphylococcus*, P-TSB-75 and H-TSB-70 as phylotype *Proteus*, P-TSB-78 isolate as *Enterococcus* and D-TSB-86 isolate as *Enterobacter* (Fig. 3). Thus the phenotyping and phylotyping confirmed genotype identities of the bacteria P-TSB-70, P-TSB-72 and D-TSB-84 as *Staphylococcus* sp., P-TSB-75 and H-TSB-70 as *Proteus* sp., P-TSB-78 isolate as *Enterococcus* sp. and D-TSB-86 isolate as *Enterobacter* sp (Table 6, Fig. 3). However, the observations revealed that occurrence of the extreme salt tolerant genotypes were uneven in different locations, states and countries as well. In similar kind of study, Behera et al. (2014) isolated bacteria along eastern coast of India, genotyped the bacterial isolates and found *Bacillus*, *Vagococcus*, *Pseudomonas*, *Alcaligenes*, *Vibrio* and *Serratia* in addition to our findings. Similarly, *Halorubrum* sp. was found from salty lake on the Qinghai-Tibet Plateau at China and Great Salt Lake (Xu et al., 2007; Jones, 2014). Therefore, occurrences of extremely salt tolerant genotypes are highly diversified in different marine environments. Therefore, from the above evidences it is clearly visible that researchers have gone through the path of isolating salt stress tolerant bacteria from the saline as well as non-saline habitats and identifying the isolates through 16S rRNA gene sequence. Although numerous possibilities exist of getting salt stress tolerant bacteria from the said area due to higher salinity, isolating salt stress tolerant bacteria along the East coastal area has remained with comparatively lesser investigated evidence. Hence, the authors focussed on the marine environment of the East coastal part of India in anticipation to obtain diverse microbial strains having variable stress tolerant properties.

5. Conclusion

Salinity stress is key concern for upsetting production of several food plants (Qados and Amira, 2011) as several crop varieties are extremely procumbent to salinity which affects production (Patel et al., 2018). The indigenous salt tolerant bacteria obtained during the investigation could be exploited for induced salt tolerance of crop plants or production of beneficial metabolites (Hanin et al., 2016). The phenotypic, genetic and proteomic analysis of the salt stress tolerant bacterial isolates of the East coast of India proved that, the resident microbial guilds were variable in different locations which would tolerate high concentration of NaCl. Thus it could be concluded that, the bacterial population of the East coast of India is highly diverse, varies in antibiotic sensitivity and tolerating upto 20% salt stress. These identified salt stress tolerant bacteria probably would be highly useful in prospecting salt stress tolerant genes in future to develop the salt stress tolerant transgenic plant varieties.

Declarations

Author contribution statement

Priyanka Das: Performed the experiments; Wrote the paper.

Soumendranath Chatterjee, Tushar Kanti Dangar: Analyzed and interpreted the data.

Bijay Kumar Behera, Trilochan Mohapatra: Conceived and designed the experiments.

Basanta Kumar Das: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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