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Research Article
Diversity and Antibacterial Potential of Siderophore Producing Bacteria Isolated from Marine Fish Species

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Abstract

Background and Objective: Siderophores are essential in the extremely iron-reduced environment of the intestine where probiotic bacteria effectively compete for iron. The diversity and antibacterial potential of siderophore producing bacteria isolated from marine fish species from the bight of Bonny at Bonny Island, Nigeria were investigated. **Materials and Methods:** Bacteria were isolated from intestine of 10 marine fish species: Sleeper (*Eleotridae bostrychus*), Smooth grouper (*Dermatolepsis striolata*), Angel fish (*Monodactylus argenteus*), Tilapia (*Tilapia guiniensis*), Silver catfish (*Chrysichthys auratus*), Snapper (*Lutjanus gorensis*), Grunt (*Pomadaysis jubilenie*), Barracuda (*Kudoa sphyraeni*), Mud skipper (*Periophthalmus papillo*) and Mullet (*Mugil cephalus*) using spread plate technique. Isolates were screened for siderophore production using Chrome Azurol Sulphonate (CAS) assay and percent siderophore units were determined. Siderophore producers were screened for antibacterial activity against 5 marine pathogens (*Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio paraheamolyticus*) using agar well diffusion assay. The active strains were identified using molecular method. **Results:** Seventy four bacterial strains belonging to 13 genera (*Pseudomonas*, *Staphylococcus*, *Salmonella*, *Shigella*, *Vibrio*, *Bacillus*, *Klebsiella*, *Escherichia*, *Aeromonas*, *Proteus*, *Providencia*, *Morganella* and *Enterobacter*) were isolated from the samples at different frequencies with *Staphylococcus* and *Vibrio* predominating. Seven fish species harboured at least one siderophore producer. All siderophore producing isolates inhibited at least one pathogen. Six marine fish species that harboured 10 bacterial isolates belonging to 7 genera which produced siderophore with strong antibacterial potential are Angel fish: *Pseudomonas aeruginosa* strain ET05, *Aeromonas* sp. Strain JDMASP8, *Morganella morganii* strain HX08027 and *Vibrio fluvialis* strain FSP561/08; Sleeper fish: *Vibrio fluvialis* strain ATCC33809 and *Morganella morganii* strain FDAARGOS 172; Smooth grouper: *Enterobacter aerogenes* A244438.1; Silver fish: *Proteus* sp. SBP10; Snapper: *Providencia stuartii* strain S2S and Grunt fish: *Aeromonas caviae* strain AH07. **Conclusion:** These organisms and their siderophores can be characterized for use as siderophore genic probiotics for iron nutrition and biocontrol in marine aquaculture.

Key words: Marine bacteria, fish species, siderophore production, antibacterial activity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diverse bacteria are capable of producing siderophore which is a biological molecule[1]. Siderophore is the strongest soluble Fe^{3+} binding agent with extensive application in diverse fields[2-4]. Membrane receptor molecules which are encoded by 5 genes in operon transport siderophore-free iron complex into the cell. When enough iron has been moved into the cell, operon is then turned off. Iron is implicated in vital life processes[5] and in marine microbial diversity regulation[6]. Iron nutrition is a problem for microbes and siderophore production is the most appropriate help for iron assimilation under aerobic environment[7]. Hence, siderophores are essential in the extremely iron-reduced environment of the intestine where probiotic bacteria effectively compete for iron[8].

According to Balcazar *et al.*[9] the utilization of beneficial bacteria or probiotics that control pathogens via many mechanisms, is more and more seen as a substitute to antibiotic treatment owing to increase in strains that are resistant to antibiotic. Verschuere *et al.*[8] affirmed that antibacterial potential of bacteria is as a result of a number of factors that incorporate the siderophores. Consequently, a constant exploration for intestinal microflora with antimicrobial action and siderophore producing capability is essential.

Information on the diversity and antibacterial activity of siderophore producing fish intestinal bacteria from Nigerian coast is not available. Therefore, in this study, the diversity of bacterial isolates from the intestine of coastal marine fishes capable of siderophore production as well as antibacterial ability of the isolates were examined for additional information, for the first time, to the ecology of coastal marine fish intestinal microbiota in the Niger Delta.

MATERIALS AND METHODS

Sample collection: The following fish samples; Sleeper (*Eleotridae bostrychus*), Smooth grouper (*Dermatolepsis striolata*), Angelfish (*Monodactylus argenteus*), Tilapia (*Tilapia guinensis*), Silver catfish (*Chrysichthys auratus*), Snapper (*Lutjanus gorensis*), Grunt (*Pomadaysis jubilenie*), Barracuda (*Kudoa sphyraeni*), Mud-skipper (*Periophthalmus papillo*) and Mullet (*Mugil cephalus*) were collected with the aid of local fisherman from the bight of Bonny, in Bonny Island, Nigeria. They were transported to the laboratory for analysis in plastic container containing habitat water.

Source of marine pathogenic bacteria: Marine pathogenic bacteria (*Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio parahaemolyticus*) were obtained from culture collection in Environmental Microbiology Division, University of Port Harcourt, Port Harcourt, Nigeria.

Bacterial isolation: Healthy live fish samples were killed by hitting hard object on the head, they were cleaned externally with ethanol and their gastrointestinal tracts dissected under sterile condition. Then, 1 g of intestinal contents were homogenized in separate 9 mL of sterile normal saline from which 10 fold serial dilution of each of the sample was carried out using sterile normal saline. Then, 0.1 mL portions of dilutions 10^{-3} and 10^{-4} of the samples were plated onto already prepared sterile plates of *Salmonella shigella* agar, Thiosulphate citrate bile salt agar, mannitol salt agar and Pseudomonas cetrimide agar. Also 10^{-5} and 10^{-6} dilutions of the samples were inoculated onto sterile nutrient agar plates and incubated at 37°C for 24 -48 h. Colonies on the culture plates were counted, macroscopically characterized and purified by streaking onto fresh nutrient agar plates. The purified isolates were identified to generic level using their characteristics in terms of morphology, physiology and biochemistry with reference to Bergey's Manual of Determinative Bacteriology[10].

Siderophore production and quantification: The amount of siderophore produced was determined using Chrome Azurol Sulphonate (CAS) agar and CAS assay solutions in cell-free culture supernatant[11]. CAS agar plates, prepared according to the method described by Chau *et al.*[12], were inoculated with a loopful of 24 h culture of the isolates by streaking the surface. The plates were incubated at 37°C for 48 h and observed for a change in the medium from blue to orange, indicating siderophore production. Iron deficient defined medium (Minimal medium 9), prepared according to the method described by Chau *et al.*[12], were inoculated with pure culture of bacterial isolates. The flasks were incubated at 30°C for 24 h. Cultures were then centrifuged at 6,000 rpm, 4°C for 15 min and then filtered with 0.2 µm pore size filters to obtain cell free culture supernatants. One millilitre of each cell-free culture supernatant was mixed with equal volume of CAS assay solution and 20 µL of shuttle solution (0.2 M 5-sulfosalicylic acid) was added to the resultant mixture and allowed to reach equilibrium. The absorbance of the mixture at 630 nm was measured using spectrophotometer. The minimal medium served as a blank while CAS assay

solution plus minimal medium plus shuttle served as a reference (r). The quantity of siderophore produced was then calculated:

$$\text{Siderophore units (\%)} = \frac{Ar}{As} \times 100$$

where, Ar is the reference absorbance and As is the sample absorbance[12].

Determination of the antimicrobial activity of the siderophore producing isolates: All the isolates capable of siderophore production were screened for antagonistic activity against selected marine pathogens such as *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio paraheamolyticus* using agar well diffusion assay. Wells measuring 6 mm in diameter were made in plates of nutrient agar seeded with 0.1 mL of 24 h old broth culture of the pathogens. Also 0.1 mL of 24 h old broth culture of the isolates capable of producing siderophore was used to fill the wells. The plates were incubated at 30°C for 24 h. The diameters of the clear zones surrounding the wells were measured and recorded.

Molecular identification of isolates capable of siderophore production: Extraction of genomic DNA of the isolates was done using Zymo Research extraction kit. The DNA concentration and purity were checked in order to know if further dilutions are necessary during PCR using Nano-drop machine. The sub unit 16S rRNA gene amplification was carried out using polymerase chain reaction (PCR) using universal forward primer 27F-5- AGAGTTTGATCCTGGCT CAG -3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′. The product was resolved on a 1% agarose gel at 120 V for 15 min and visualized on a UV trans illuminator. Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequence generated were visualized using bioinformatics algorithms such as chromas lite for base calling, Bio edit was used for sequence editing before performing Basic Local Alignment Search Tool (BLAST) using NCBI data base. Similar sequences were downloaded and aligned with Cluster X and phylogenetic tree was drawn with MEGA 6 software[13]. The evolutionary history was inferred using the Neighbour-joining method[14]. The bootstrap consensus tree inferred from 500 replicates[15] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes and Cantor[16] method.

Statistical analysis: The result obtained from bacterial enumeration of the coastal marine samples was compared by one-way analysis of variance (one-way ANOVA) and multiple range test to find the differences between the values means at 5% (0.5) significant level. The data were analyzed using IBM SPSS statistics version 20.0 (Gailly and Adler, US).

RESULTS

Bacterial enumeration and isolation: The bacterial loads of marine fish intestinal contents are presented in Fig. 1. The highest aerobic heterotrophic bacterial count was encountered in Mud skipper while Grunt gave the lowest counts of all the bacterial groups enumerated. Table 1 shows the frequencies of occurrence of different bacterial genera associated with the fish species. A total of 74 bacterial species were identified. Examination of the distribution of the isolated bacterial genera showed that the highest diversity was associated with Mud skipper while the lowest diversity was found in Grunt. The bacteria isolated from the samples were predominantly Gram-negative. Bacteria of the genera *Pseudomonas*, *Staphylococcus*, *Salmonella*, *Shigella*, *Vibrio*, *Bacillus*, *Klebsiella*, *Escherichia*, *Aeromonas*, *Proteus*, *Providencia*, *Morganella* and *Enterobacter* were isolated from the samples at different frequencies with *Staphylococcus* and *Vibrio* predominating.

Siderophore production: Bacterial isolates capable of siderophore production and the siderophore units produced are shown in Table 2. Angel fish (*Monodactylus argenteus*)

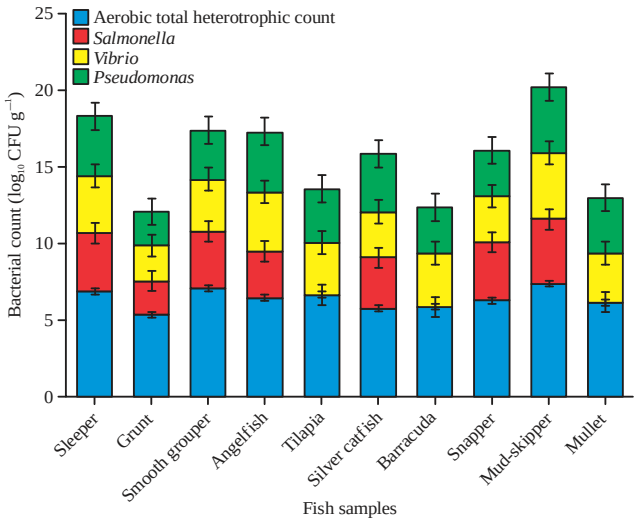

Fig. 1: Bacteria loads of marine fish intestinal samples

Table 1: Frequency of isolation of bacterial genera

Genus	Sleeper	Grunt	Smooth grouper	Angelfish	Tilapia	Silver catfish	Snapper	Barracuda	Mud skipper	Mullet
Pseudomonas	1 (11.1)	0	1 (10)	1 (16.7)	1 (16.7)	1 (16.7)	1 (16.7)	1 (16.7)	1 (7.1)	1 (14.3)
Staphylococcus	2 (22.1)	1 (25)	2 (20)	2 (33.3)	1 (16.7)	1 (16.7)	1 (16.7)	2 (33.3)	1 (7.1)	1 (14.3)
Salmonella	1 (11.1)	1 (25)	1 (10)	1 (16.7)	0	1 (16.7)	0	1 (16.7)	1 (7.1)	0
Shigella	0	0	1 (10)	0	0	0	1 (16.7)	0	1 (7.1)	0
Vibrio	1 (11.1)	1 (25)	2 (20)	1 (16.7)	2 (33.3)	1 (16.7)	1 (16.7)	1 (16.7)	2 (14.3)	2(28.6)
Bacillus	0	0	1 (10)	0	0	0	0	0	2 (14.3)	0
Klebsiella	1 (11.1)	0	0	0	0	0	0	0	0	1 (14.3)
Escherichia	1 (11.1)	0	0	0	1 (16.7)	1 (16.7)	0	0	1 (7.1)	0
Aeromonas	0	0	1 (10)	0	0	0	0	0	1 (7.1)	1 (7.1)
Proteus	0	1 (25)	0	0.0	0	1 (16.7)	0	0	1 (7.1)	0
Providencia	1 (11.1)	0	0	0	1 (16.7)	0	0	1 (16.7)	1 (7.1)	1 (14.3)
Morganella	0	0	0	1 (16.7)	0	0	1 (16.7)	0	1 (7.1)	0
Enterobacter	0	0	1 (10)	0	0	0	1 (16.7)	0	1 (7.1)	0
Total number of genera	8 (61.5)	4 (30.8)	8 (61.5)	5 (38.5)	5 (38.5)	6 (46.2)	6 (46.2)	5 (38.5)	12 (92.3)	6 (46.2)
Total isolates	9 (100)	4 (100)	10 (100)	9 (100)	6 (100)	6 (100)	6 (100)	6 (100)	14 (100)	7 (100)

Numbers in parentheses represent the percentage frequencies

Table 2: Bacterial isolates capable of siderophore production

Isolate code	Sources	Tentative identity	Siderophore unit (%)
AR1	Angelfish	*Pseudomonas* sp.	21.70
AR2	Angelfish	*Pseudomonas* sp.	5.65
AR3	Smooth grouper	*Enterobacter* sp.	3.96
AR4	Angelfish	*Proteus* sp.	11.44
AR5	Silver catfish	*Proteus* sp.	6.67
AR6	Angelfish	*Vibrio* sp.	6.89
AR7	Snapper	*Providencia* sp.	25.44
AR8	Sleeper	*Vibrio* sp.	19.21
AR10	Grunt	*Aeromonas* sp.	9.75
AR11	Sleeper	*Morganella* sp.	2.42

harboured the highest number of siderophore producing bacteria. The siderophore units produced by the isolates ranged from 2.42-25.44 as detected by the CAS assay method. Seven out of ten fish species studied haboured at least one bacterial species that produced siderophore. Seven out of thirteen genera isolated produced siderophore. The ten isolates capable of siderophore production were Gram-negative rods.

Antibacterial potential of siderophore producing strains: The antibacterial activities of siderophore producing strains against marine pathogens are shown in Table 3. All siderophore producing isolates inhibited at least one marine pathogen with inhibition zones ranging from 16-21 mm.

Molecular identification of siderophore producing isolates: Table 4 shows the ten isolates that were successfully identified using molecular method and their accession numbers as well as gene bank closest relatives. The siderophore producing strains and the fish species that haboured them are as follows-Angel fish: *Pseudomonas aeruginosa* strain ET05, *Aeromonas* sp. Strain JDMASP8, *Morganella morganii* strain HX08027 and *Vibrio fluvialis* strain FSP561/08; Sleeper fish: *Vibrio fluvialis* strain ATCC33809 and *Morganella morganii* strain FDAARGOS 172; Smooth grouper: *Enterobacter aerogenes* A244438.1; Silver fish: *Proteus* sp. SBP10; Snapper: *Providencia stuartii* strain S2S and Grunt fish: *Aeromonas caviae* strain AH07.

DISCUSSION

The abundance and diversity of bacterial populations in the gut contents of marine fish species as reported in Fig. 1 and Table 1, respectively are noteworthy. This is because most of the bacteria found in intestinal content and mucus are opportunistic pathogens[17]. Pond *et al.*[18] proposed that fish digestive tract is a reservoir of many pathogens. The overgrowth of pathogens and the transmission of disease may then break out following a breach of intestinal microflora due to a stressor such as pollution and intensive farming cultivation[19]. Members of the genera Vibrio[17, 20], *Pseudomonas*[21], *Aeromonas*[17,22], *Providencia*[23], *Morganella*[24], *Proteus*[25] and *Enterobacter*[26,27] have previously been detected in the gut of wide variety of fresh and marine fishes. Some strains of these bacteria posses the virulence factors necessary to induce disease[28].

Thirteen fish intestinal bacteria produced siderophore (Table 2) indicating their ability to survive and grow in the iron deficient conditions. Chau *et al.*[12] stated that iron is a limiting bioactive metal in seawater and essential for the growth of marine bacteria. Competition for iron is also a possible mechanism for aquaculture probiotics to control the pathogens. Numerous studies have implicated siderophores as bacteriostatic substances produced by *Pseudomonas* species[29,30] and the inhibitory activity of many *Pseudomonas* strains seemed indeed mediated by siderophores[31,32].

Table 3: Antibacterial activity of siderophore producing bacterial isolates against marine pathogens

			Zone of inhibition (mm)				
Isolate code	Source	Bacterial isolate	A	B	C	D	E
AR1	Angelfish	*Pseudomonas* sp.	-	-	-	21	-
AR2	Angelfish	*Pseudomonas* sp.	-	-	-	17	18
AR3	Smooth grouper	*Enterobacter* sp.	-	-	16	-	-
AR4	Angelfish	*Proteus* sp.	-	-	17	-	-
AR5	Silver catfish	*Proteus* sp.	-	-	-	18	-
AR6	Angelfish	*Vibrio* sp.	-	-	-	-	16
AR7	Snapper	*Providencia* sp.	-	16	-	19	-
AR8	Sleeper	*Vibrio* sp.	-	16	-	-	-
AR10	Grunt	*Aeromonas* sp.	16	-	-	-	-
AR11	Sleeper	*Morganella* sp.	16	-	-	-	-

A: *Escherichia coli*, B: *Salmonella* sp., C: *Staphylococcus aureus*, D: *Vibrio cholerae*, E: *Vibrio parahaemolyticus*

Table 4: Molecular characteristics of the marine fish intestinal isolates capable of siderophore production

Isolate code	Sources	Tentative identity	Closest gene bank relative	Max identity (%)	Genes bank accession number
AR1	Angelfish	*Pseudomonas* sp.	*Pseudomonas aeruginosa* strain ET05	99	MF187526
AR2	Angelfish	*Pseudomonas* sp.	*Aeromonas* sp. strain JDMASP8	99	MF187517
AR3	Smooth grouper	*Enterobacter* sp.	*Enterobacter aerogenes* A244438.1	99	MF187518
AR4	Angelfish	Proteus sp.	*Morganella morganii* strain HX08027	99	MF187519
AR5	Silverfish	*Proteus* sp.	*Proteus* sp. SBP10	99	MF187520
AR6	Angelfish	*Vibrio* sp.	*Vibrio fluvialis* strain FSP561/08	99	MF187521
AR7	Snapper	*Providencia* sp.	*Providencia stuartii* strain S2SA-	99	MF187522
AR8	Sleeper	*Vibrio* sp.	*Vibrio fluvialis* strain ATCC 33809	99	MF187523
AR10	Grunt	*Aeromonas* sp.	*Aeromonas caviae* strain AH07	99	MF187524
AR11	Sleeper	*Morganella* sp.	*Morganella morganii* strain FDAARGOS_172	99	MF187525

The siderophore producers were found to be antagonistic to at least one marine pathogen tested (Table 3). These novel marine fish intestinal bacterial strains listed in Table 4 will boost the existing literature on ecology of coastal marine fish intestinal microbiota in the Niger Delta. These organisms are different from the ones isolated in Japan by Sugita *et al.*[33] from intestinal tracts of different fish species. Sugita *et al.*[33] identified six bacterial species, *Enterovibri norvegicus*, *Photobacterium leiognathi*, *Photobacterium phosphoreum*, *Photobacterium rosenbergii*, *V. crassostrea* and *Vibrio scophthalmi* as possible candidates for use as probionts in fish aquaculture. The 10 siderophore producing strains with antibacterial ability isolated in this study are novel and have been deposited in gene bank with accession numbers MF187517-MF187526. The indigenous strains will be better adapted to local environment when used for aquaculture than imported strains. These isolates can be antagonistic to the pathogens in the intestine and in marine waters by making iron unavailable for their survival and produce some other antagonistic metabolites consequently leading to the pathogen's death. Marine micro-organisms are well-known for the production of novel bioactive metabolites (or natural products) which are believed to be a requirement for their survival in the sea to counter acute competition from other species. These metabolites are found to have wide pharmaceutical and biotechnological applications[34,35].

CONCLUSION

The study has revealed the existence of siderophore producing bacteria with strong antibacterial potential in the intestine of marine fish species. These organisms and their siderophores can further be characterized for use as siderophoregenic probiotics for iron nutrition and biocontrol in marine aquaculture.

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