

Bacteria associated with soft coral from Mot island - Nha Trang bay and their antimicrobial activities

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ABSTRACT

Microbial communities associated with invertebrates had been considered as a new source of bioactive compounds. The soft coral associated bacteria in Mot island, Nha Trang bay were isolated, extracted and assessed for antagonistic activity against human and coral pathogens, the strongly active strains were identified by 16S rRNA analysis. The soft coral associated bacterium SCN10 had abcd antibacterial pattern which was named for inhibition towards *Bacillus subtilis* (pattern a), *Escherichia coli* (pattern b), *Salmonella typhimurium* (pattern c) and *Serratia marcescens* (pattern d). It was the nearest strain to the well-known antibiotic producer *Bacillus amyloliquefaciens* with 99% sequence similarity. Whereas strain SCL19 had abde pattern which means inhibition of the growth of *B. subtilis*, *E. coli*, *S. marcescens* and *Vibrio parahaemolyticus* (pattern e). This strain SCL19 affiliated with *Bacillus* sp. strain A-3-23B with 99.8% identity. In addition to antimicrobial activity to the aforementioned tested bacteria, the isolate SCX15 also inhibited *Vibrio alginolyticus* (pattern f) and *Candida albicans* (pattern g), so this isolate possessed abcdefg antimicrobial pattern. The coral associated isolate SCX15 was identified as *Bacillus velezensis* with 99% sequence similarity. Among the 78 screened strains, 25 isolates possessed antibacterial activity against at least one of seven tested microorganisms and exhibited 12 different types of antimicrobial activities, suggesting that they can produce many different natural substances with antibacterial activity.

Keywords: *Bacillus* sp., antimicrobial activity, soft coral associated bacteria.

INTRODUCTION

Recent data showed that abundant and diverse microorganisms associated with healthy corals or diseased corals play an important role in the nutrient and diseases resistance in healthy coral [1]. Biological activity of coral-associated bacteria is not only restricted to activity against pathogenic bacteria, fungi, and viruses but has also been found against tumor cell lines, for example the cyclic thiodepsipeptide thiocoraline which showed an antitumor activity and was obtained from *Micromonospora* sp. strain derived from a soft coral [2]. Therefore, study of marine microorganisms associated with soft corals can lead to answering the question about the host and pathogen or can open new interesting research in terms of exploiting new drugs from nature. Due to human diseases such as cancer, microbial infections and inflammatory processes, as well as rapid increase in the number of antimicrobial resistant bacteria, it has become increasingly urgent to search for a new drug.

Analysis of the diversity of marine microorganisms via molecular techniques has made many advances for last decades: Many unique bacteria that were detected by DNA sequences could not be grown on agar plates and in culture media under standard conditions [3]. Although the diversity of marine microorganisms can be effectively analyzed using DNA fragments, traditional methods and cultivation of pure culture isolates are still needed in order to determine the ability to produce antimicrobial compounds and determine their biological activities. More than 30% of the twenty-two different isolates were novel species and new genera, the main bacteria groups were identified as *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* by using culture-based method, 16sRNA gene sequence, biochemical testing and antimicrobial susceptibilities to analyze the diversity of marine microorganisms in mucus of the hard coral *Fungia scutaria* in the Red Sea [4]. As the same method was applied for cold water coral *Lophelia pertusa* in the deep sea [5], the result showed that

Gammaproteobacteria was the most abundant associated bacteria group, *Betaproteobacteria* and the *Actinobacteria* were also found from cold water coral *L. pertusa*.

Bacteria associated with six stony corals (*Platygyra* sp., *Porites* sp., *Fungia granulosa*, *Favia* sp., *Stylophora* sp. and *Pocillopora* sp.) and two soft corals (*Rhytisma fulvum fulvum* and *Xenia* sp.) were studied for antimicrobial activity, the results showed that bacteria associated with stony corals have a higher percentage of activity as compared with bacteria associated with soft corals [6]. By cultivable method, a total of 36 bacteria were recovered and identified from the black coral, they consisted of three bacterial phyla - *Firmicutes*, *Actinobacteria* and *Alphaproteobacteria* and 24 fungal isolates affiliated to four fungal orders - *Eurotiales*, *Hypocreales*, *Pleosporales* and *Botryosphaeriales*. High proportion (51.6%) of microbial isolates displayed distinct antibacterial and antifungal activities [7]. The community and antimicrobial activity of culturable coral associated bacteria were examined in gorgonian, a total of 76 bacterial isolates from four species of East Vietnam Sea gorgonians were retrieved and identified as 21 species of 7 genera, and *Bacillus* was the most abundant and antimicrobial potential isolates [8]. *Pseudoalteromonas* spp., *Vibrio* spp. were the most dominant culturable bacteria and displayed good antimicrobial activity against a range of other cultured isolates from coral *Acropora millepora* in Great Barrier Reef [9]. Associated bacteria from hard coral *Acropora digitifera* were recovered, namely *Firmicutes*, *Gammaproteobacteria* and *Actinobacteria*, both coral mucus isolates and coral tissue isolates showed antimicrobial activity; this study revealed the presence of actinobacteria in both the coral mucus and the coral tissue, which had high activity against pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* [10]. Cultivable bacterium *Roseobacter* sp., isolated from mucus of the Mediterranean coral *Oculina patagonica* displayed a broad spectrum of antimicrobial activity against the coral

pathogens *Vibrio coralliilyticus*, *Thalassomonas loyana*, this paper suggested the concept of a probiotic effect on microbial communities to the coral holobiont [11]. Anthozoa orders Alcyonacea (soft corals) and Gorgonacea (sea fans) were being studied as promising sources of bioactive compounds with a number of species which produced antitumor, anti-HIV compounds as *Klyxum simplex*, *Lobophytum sp.*, *Sarcophyton crassocaule*, *Sinularia flexibilis*, *Clavularia sp.* [12].

In Vietnam, the soft coral *Lobophytum sp.* was shown as a host for isolation of a new squalene which showed inhibition of lung and colon cancer cell lines, also 3 cembrane diterpenes and 2 sterols were found [13]. Several new natural compounds and known compounds showed inhibition of many cancer cell lines from the soft coral *Lobophytum laevigatum* [14, 15]. A compound belonging to steroid group named polyoxygenated steroid was anti-inflammatory compound from the soft

coral *Sarcophyton pauciplicatum*, and other cembranoid diterpenoids which possessed anti-inflammatory capacity were from the soft coral *Lobophytum crassum* [16, 17]. Norditerpenoids were reported from the soft coral *Sinularia maxima* from Nha Trang, Khanh Hoa [18].

To the best of our knowledge, with limited reports mentioned above, in Vietnam study of coral associated bacteria has not been conducted for the purpose of investigation of new compounds or bioactive compounds. This present study was the first search for a new source for antimicrobial compound producers from Vietnamese seawater.

METHODS

The specimen sampling

The living corals (fig. 1) were collected through diver with SCUBA at ca. 6 meters in depth at 109°16'22.9" longitude, 12°10'54.8" latitude, at Mot island - Nha Trang bay in September 2018.



Figure. 1. Specimens from Mot island

The sea water temperature at the time of sampling was 27°C±1, salinity was 31‰. The living soft coral was collected in a sterile dark PE bag, then stored in an ice box and quickly transferred to the Marine Ecology Department - Institute of Oceanography for further experiments.

Isolation of coral associated bacteria - SCN, SCL, SCX and tested strain *Vibrio sp.*

Pre-sampling of specimens, culture and total number of heterotrophic bacteria counts were carried out according to Pham Thi Mien et al., [19]. In this study, sterile 0.45 µm filtered seawater collected at the sampling site was used to dilute the sample, and the R2A

medium (Merck), M2 medium [20] and MA (HiMedia) were used for cultivation of coral associated bacteria. The cultured isolates were named from 1 to n according to their name hosts SCN, SCL and SCX.

A branch of bleached coral *Acropora sp.* at the same sampling site was sampled for isolating *Vibrio sp.* with selected media TCBS (HiMedia) and CHROMagar™ (France). The strain *Vibrio parahaemolyticus* was confirmed following the manufacturer's conclusion with blue green color on TCBS and mauve color on CHROMagar™. The strain *Vibrio alginolyticus* was found in yellow color, large colony and changed medium's color in TCBS and colorless on CHROMagar™. These occasional

pathogenic bacteria *V. parahaemolyticus* and *V. alginolyticus* are used as tested strains in this present study and preserved in glycerol 20% at minus 80°C.

Antimicrobial tests

Pure strains were tested for antimicrobial activities by well diffusion method on Mueller-Hinton Agar (MHA-HiMedia, India) according to Bauer et al., [21] with five standardized indicators *Bacillus subtilis* ATCC6633, *Salmonella typhimurium* ATCC6994, *Escherichia coli* 0157, *Serratia marcescens* PDL100 ATCCBAA-632 and the yeast *Candida albicans* ATCC10231. The isolated strains were streaked in Marine Agar (HiMedia) plates for 3–5 days and then inoculated into 300 ml Erlenmeyer flasks containing 100 ml BM medium (yeast extract: 1 g/l, beef extract: 1 g/l, tryptone: 2 g/l, glucose: 10 g/l, up to 1,000 ml with filtered sea water). After 72 h of incubation at 30°C with shaking at 120 rpm, the bacterial cells and the supernatants were homogenized by using an ultrasonic processor for breaking the cells in 30 s. The homogenized broth was extracted by ethyl acetate 1/1 (v/v). Crude extracts were dried and re-suspended in 1 ml of methanol (Merck). The methanolic extracts (30 µl) were inoculated into four available wells on MHA containing indicator bacteria. The same amount of methanol without extract was used as negative control. All the plates tested with *B. subtilis*, *S. typhimurium*, and *E. coli* were incubated at 37°C for 24 h. The plates tested with *S. marcescens*, *V. parahaemolyticus* and *V. alginolyticus* were incubated at 25°C for 24 h. The yeast *C. albicans* was incubated at 30°C for 24 h. The zone of inhibition was measured and expressed in the mean of the four wells excluding the well diameter, and no inhibition zone was observed around negative control wells.

Identification of potential isolates

Antibacterial active strains were identified by traditional methods, Gram determination was based on the result of KOH reaction [22]. Some antibiotic-producing strains were identified by the 16S rRNA gene analysis according to Pham et al., [23]. Universal primer set of 27F, 1500R was used for amplification of 16S rRNA gene employing the PuReTaq™

Ready-To-Go™ PCR Beads (Heathcare) with a total volume of 25 µl including 5 µl DNA templates (50 ng), 10 ppmol 27F, 10 ppmol 1500R and DNA free H₂O (Sigma). The PCR reaction conditions include initial denaturation (2 minutes at 94°C), followed by 30 primer annealing cycles (40 seconds at 50°C), extended primer extension (90 seconds at 72°C), followed by denaturation (1 minute at 42°C), and final primer annealing (1 minute at 42°C), then extended final extension (5 minutes at 72°C). The PCR products were sequenced with 342f (5'-TACGGGAGGCAGCAG-3'), 790f (5'-GATACCCTGGTAGTCC-3'), and reverse 543r (5'-ATTACCGCGGCTGCTGG-3'). The sequences were aligned with SeqMan™ II (DNAStar) and compared with the highest 16S rRNA gene homologues on the gene bank (National Center for Biotechnology Information NCBI) using Nucleotide BLAST/NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [24].

Data analysis

All data were processed on excel software.

RESULTS AND DISCUSSION

Abundance of associated bacteria in culture-based methods

The number of isolates is shown in table 1. The total number of associated bacteria in three coral tissues was a bit higher than that in ambient seawater. A total of aerobic microorganisms in 1 g fresh coral were counted as at least 8.93×10^5 cfu/g from SCX coral in R2A medium, whereas the same amount was counted from SCN and SCL in R2A medium. The number of isolates from M2 medium from three corals is mostly similar and a bit higher than that from R2A medium (table 1). At a dilution of 10^{-4} , this study recorded less than 30 colonies and most of them had the same shape and size. The cultured isolates from SCN, SCL and SCX were 25, 23, and 30 isolates respectively.

M2 medium contained glycerol, arginine, K₂HPO₄, MgSO₄ and natural seawater which had been introduced to isolate actinomycetes from ocean sediment [20], this medium was reported as the most efficient medium among the 12 media used to isolate marine

microorganisms with 26 isolates [25]. Microorganisms consisted of 40 to 60% of the total biomass of their host and reached more than 10^9 cells/cm³, which was three to four times higher than that of marine bacteria with 10^6 ml/l [26]. Our other study of fresh hard coral associated bacteria reported 1.3×10^6 cfu/g wet samples with culture-based method, while the total number of heterotrophic bacteria counted directly from fluorescence dye method was 2.1×10^8 cells/g [19]. In addition, bacteria were also isolated from marine sediment, at the same sampling site for sponge in this study, we reported that heterotrophic bacteria in 1 g sediment from Mot island were 1.5×10^6 cfu/g [27]. Microorganisms that lived with the hosts,

such as the sponges, urchins, corals, mollusks, or other invertebrates had been reported to have a very high specificity and deeply depended on their hosts. Moreover, they also have inherent characteristics of microorganisms such as nutrient requirements, culture conditions and so on, of course, initial host states decided the effectiveness of the isolation of bacteria. Those important impact factors have a huge influence on the number of isolated microorganisms and type of isolates as well. It is the fact that our understanding of marine microorganisms can be as little as 0.01% of ecological, genetic and biological characteristics, while cultivable microbes can be estimated to be about 0.1% of species which were found so far [28].

Table 1. Number of isolates from different media

Host	R2A (cfu/g (ml))	M2 (cfu/g (ml))	Number of isolates on M2
SCN	1.21×10^6	4.82×10^6	25
SCL	1.37×10^6	4.77×10^6	23
SCX	8.93×10^5	4.45×10^6	30
Water habitat	7.91×10^5	8.36×10^5	-

Note: “-”: No identification.

Antagonistic potential of the isolates

In this study, the antibacterial patterns a, b, c, d, e, f, g were placed respectively for inhibition of *B. subtilis*, *E. coli*, *S. typhimurium*, *S. marcescens*, *V. parahaemolyticus*, *V. alginolyticus*, and *C. albicans* (fig. 2). The active strains for antimicrobial activities were 28.0, 34.7 and 33.4 % for isolates from SCN, SCL and SCX respectively (fig. 2A). Among 25 active strains, the antimicrobial activity is observed in 12 patterns, there were 7 strains inhibiting only Gram-positive indicator *B. subtilis* (pattern a) and three isolates (SCN3, SCL12, SCX20) possessed antimicrobial activity against only one Gram negative indicator *S. typhimurium* (pattern c). Three isolates showed the inhibition of Gram positive and Gram-negative indicators with patterns ab and ac, whereas two isolates SCL10 and SCX7 inhibited set of Gram-negative indicators *E. coli*, *S. typhimurium*, *S. marcescens* with antimicrobial pattern bcd. The last seven antimicrobial patterns were relative to one isolate.

Among the 78 isolates tested, seven, eight and ten strains from SCN, SCL and SCX

respectively exhibited antibacterial activities and 32% of the isolates were antibacterial active strains. Antimicrobial activity of the active strains is shown in figs. 3–5 with the mean and standard deviation. In Figure 3, there were six antibacterial patterns: Inhibition of Gram-positive *B. subtilis* (pattern a - SCN1, SCN25), inhibition of Gram-negative indicator *S. typhimurium* (pattern c - SCN3), inhibition of *B. subtilis* and *E. coli* (pattern ab - SCN6), inhibition of *E. coli* and *S. typhimurium* (pattern bc - SCN17), inhibition of *B. subtilis* and *S. typhimurium* (pattern ac - SCN18) and the pattern abcd - SCN10 with inhibition of *B. subtilis* (pattern a), *E. coli* (pattern b), *S. typhimurium* (pattern c) and *S. marcescens* (pattern d).

The antimicrobial activity of associated bacteria which were isolated from the coral SCL was divided into 7 patterns (three isolates SCL7, SCL22, SCL26 - a, two isolates SCL13, SCL25 - ac, one isolate SCL12 - c, one isolate SCL11 - ae, one isolate SCL4 - cd, one isolate SCL10 - bcd, and isolate SCL19 - abde).

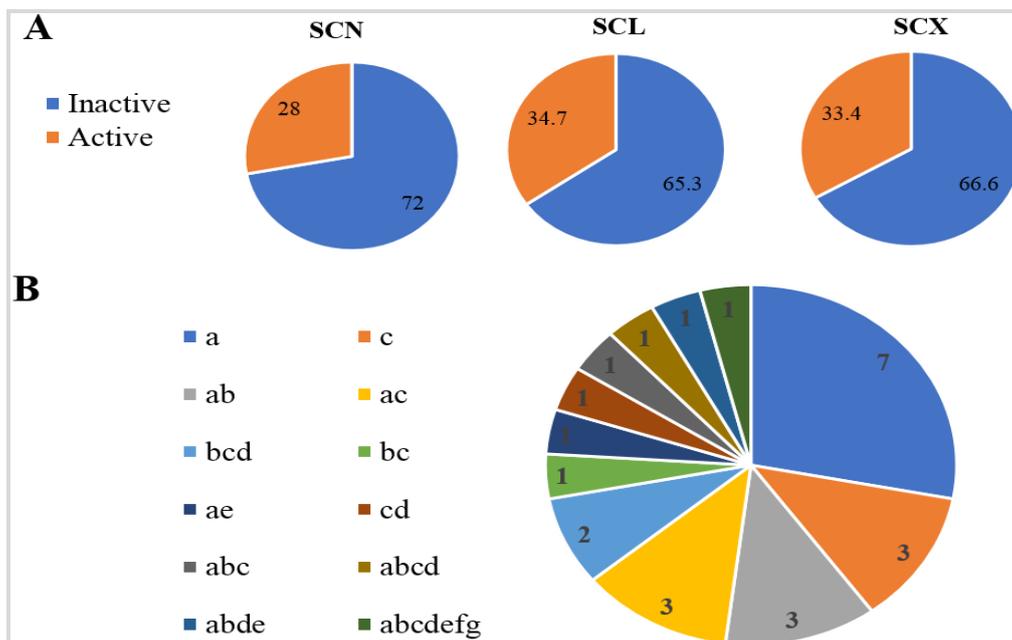


Figure 2. Percentage (%) of active antimicrobial producer strains from three corals (A) and antimicrobial patterns of all active isolates (B)

Notes: Patterns a, b, c, d, e, f, g represent the inhibition of *B. subtilis*, *E. coli*, *S. typhimurium*, *S. marcescens*, *V. parahaemolyticus*, *V. alginolyticus*, and *C. albicans*, respectively.

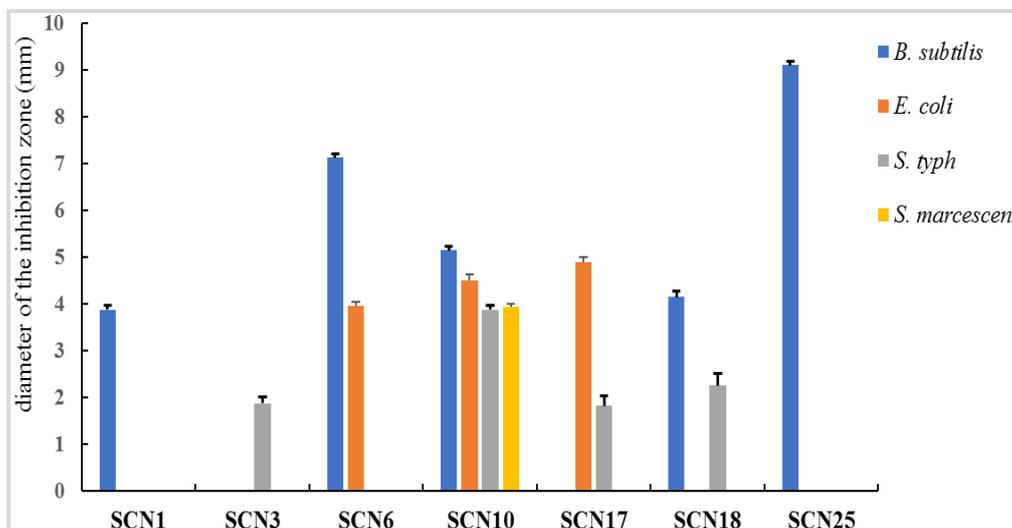


Figure 3. Antimicrobial activities of active strains from SCN coral

It was noteworthy that this study used the indicator strain *S. marcescens* PDL100 causing severe disease named white spot on reef building coral *Acropora palmate* at the Florida Keys, the United States [29]. In our other study [30] on bacteria from hard corals, 7/11 active

strains were reported in which strains B17, D1 inhibited growth of *B. subtilis*, *S. typhimurium*, and *E. coli*. In addition, four strains (B16, B17, B18 and D1) showed antibacterial activity against *S. typhimurium*. The inhibition level of soft coral associated bacteria in this study was

similar to that of isolates from mangrove sediment [31], when the clear zones received in both studies were quite weak. However, the results of other study showed that crude

extract from an octocoral-derived *Pseudoalteromonas* sp. strongly inhibited the growth of *B. subtilis* with average clear zone of 23 mm in diameter [32].

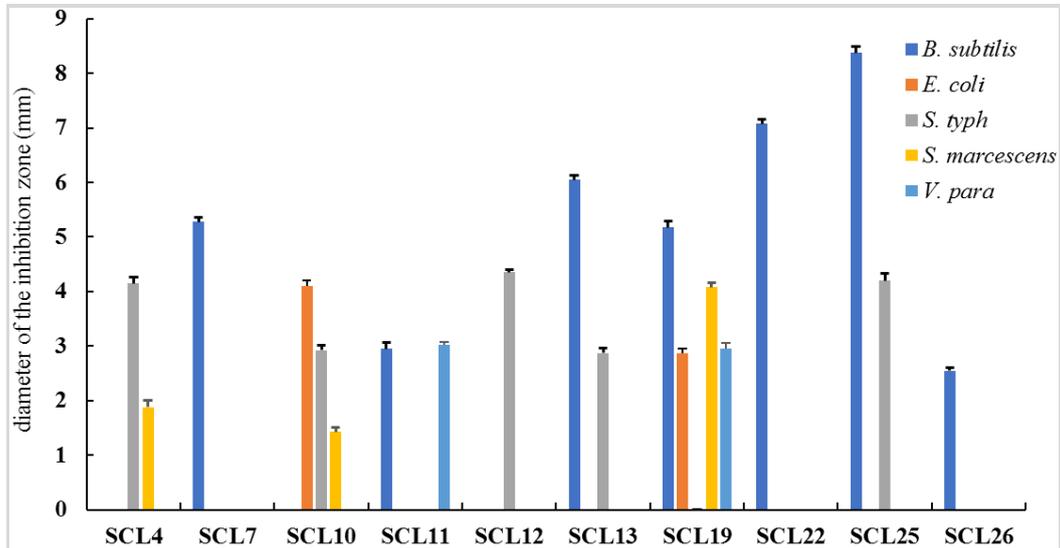


Figure 4. Antimicrobial activities of active strains from SCL coral

The antimicrobial active isolates from coral SCL are shown in fig. 4. Among seven isolates which inhibited *B. subtilis*, the isolate SCL25 had the strongest activity with the highest diameter of inhibition zone. Three isolates inhibited the coral pathogenic bacteria *S. marcescens* (pattern d), and showed

antimicrobial activity against *S. typhimurium* (SCL4), *E. coli*, *S. typhimurium* (SCL10), and *B. subtilis*, *E. coli*, *V. parahaemolyticus* (SCL19) respectively. The SCL11 inhibited *B. subtilis* and *V. parahaemolyticus* isolated from a bleached reef building coral which was set as an indicator strain in the present study.

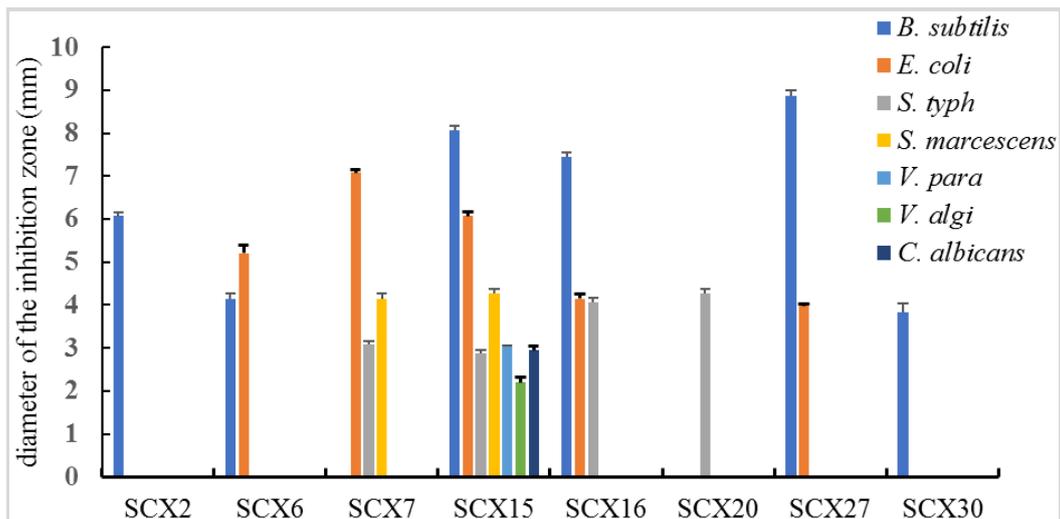


Figure 5. Antimicrobial activities of active strains from SCX coral

Among all active isolates, only strain SCX15 showed antifungal activity. This potent isolate illustrated an excellent source for antibiotic exploitation when it inhibited all tested strains, whereas the isolate SCX20 inhibited only *S. typhimurium* and SCX2, SCX30 inhibited only *B. subtilis*. The isolates SCX7 possessed antimicrobial activity against three Gram negative indicator tests (pattern bcd) consisting of human pathogen *E. coli* and reef building coral pathogen *S. marcescens*. Interestingly, seventy five percent of active strains isolated from the soft coral SCX showed inhibition of human pathogenic bacteria *E. coli* strain 0157.

Identification of potential strains

The isolate SCN10 was the closest to *B. amyloliquefaciens*, accession number MK086133.1, with 99% identity. Whereas isolate SCL19 affiliated with *Bacillus* sp. A-3-23B, accession number KT583498.1, with

99.8% identity, isolated from a soft coral *Alcyonium digitatum* from the Baltic Sea. The isolate SCX15 was identified as *Bacillus velezensis*, accession number CP042271.1, with 99% sequence similarity (table 2). A gorgonian associated *B. amyloliquefaciens* strain SCSIO 00856 had strong antibacterial and antilarval activities towards bryozoan *Bugula neritina* [33]. Also, the *Bacillus* sp. A-3-23B was reviewed as a potent antibiotic producer due to its antimicrobial activities [23]. As a member of genus *Bacillus* - sources of giant bioactive compounds, *B. velezensis* was used as plant growth promotion and biocontrol [34]. Seaweed associated *B. amyloliquefaciens* and *B. subtilis* had broad spectra of antibacterial activities against food pathogen *Aeromonas hydrophila*, *Vibrio harveyi*, *Vibrio vulnificus*, and *V. parahaemolyticus*. In addition, both bacilli also held the functional type I polyketide synthase gene [35, 36].

Table 2. Classification of antibiotic producing strains

Isolates	Characteristics	Antimicrobial patterns	Next relative strains from GenBank
SCN10	Gram positive, white, irregularly round, flat colony, long chains with rod shape.	abcd	<i>Bacillus amyloliquefaciens</i> Accession number: MK086133.1 Identity: 99%
SCL19	Gram positive, white, round, flat colony, long chains with rod shape.	abde	<i>Bacillus</i> sp. A-3-23B Accession number: KT583498.1 Identity: 99.8%
SCX15	Gram positive, off-white, irregularly round, flat colony, chain with rod shape.	abcdefg	<i>Bacillus velezensis</i> Accession number: CP042271.1 Identity: 99%

As far as we know, members of *Bacillus* species had shown as excellent sources of natural bioactive compounds. They were reported as a source of huge antimicrobial compounds so far [37]. For instance *Bacillus* species showed effective inhibition of all four tested microorganisms and they were reported as potential strains for biological active substance producers [23]. In addition, pure substances obtained from *Bacillus* sp. were capable of inhibiting some cancer cells and some important enzymes [38]. Most recently, *Bacillus* sp. strain BC028 isolated from the blue mussel *Mytilus edulis* was also reported as a new hexapeptide producer, this strain produced a natural biological active compound

considered as a new antibiotic, when it showed inhibition of the growth of opportunistic pathogen *Enterococcus faecalis* and pathogen *Staphylococcus aureus* with rather low inhibition concentration values of 8 μ M and 12 μ M, respectively [39]. In a study of the diversity of bacteria associated with the fungal coral *Fungia scutaria* in the Red Sea by combining 16S rRNA gene analysis and traditional culture methods, it was reported that more than 30% of isolates were new species, although molecular method can only determine the level of genetic similarity for those species \leq 98%. However, the authors have confirmed that they were indeed new species with a range of biochemical experiments, in which the most

notable new and highly reliable method was carried out by the comparison of susceptibility of antibiotics [4]. From the above results, some strains which were isolated from three corals showed the same antimicrobial patterns. Therefore, other biochemical characteristics need to be investigated to confirm whether microbial strains were actually different species. Significantly, all active species in this study could be considered as potential antibiotic producing strains and required further research.

CONCLUSION

This study reported the antimicrobial activity of 32% active bacteria associated with the three soft coral species from Mot island in Nha Trang bay. This was the first report on findings of soft coral associated bacteria from Vietnamese seawater, particularly all three potent strains showed inhibition of growth of human and coral pathogenic indicator microorganisms. It was suggested that the associated bacteria from this study were diverse in metabolites, they were more likely to produce antibiotics especially broad-spectrum antibiotics. They were considered as a good source for bioactive compounds.

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