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1 **Phylogenetic diversity and activity screening of cultivable actinobacteria isolated**
2 **from marine sponges and associated environments from the western coast of**
3 **India**
4

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24 inhibition; Molecular phylogeny.
25

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26 **Abstract**

27 Phylogenetic diversity of cultivable actinobacteria isolated from sponges (*Haliclona* spp.)
28 and associated environments of intertidal zones, along the northern parts of west coast of
29 India, were studied using 16S rRNA gene sequences. A subset of actinobacteria were
30 screened for three activities, namely predatory behavior, antibacterial activity and enzyme
31 inhibition. We recovered 237 isolates of actinobacteria belonging to 19 families and 28
32 genera, which could be attributed to 95 putative species using maximum likelihood partition
33 and 100 putative species using Bayesian partition in Poisson Tree Processes. Although the
34 trends in the discovery of actinobacterial genera isolated from sponges was consistent with
35 previous studies from different study areas, we provide first report of nine actinobacterial
36 species from sponges. We observed widespread non-obligate epibiotic predatory behavior in
37 eight actinobacterial genera and we provide first report of predatory activity in
38 *Brevibacterium*, *Glutamicibacter*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus* and *Rothia*.
39 Sponge associated actinobacteria showed significantly more predatory behavior than
40 environmental isolates. While antibacterial activity by actinobacterial isolates mainly affected
41 Gram-positive target bacteria with little to no effect on Gram-negative bacteria, predation
42 targeted both Gram-positive and Gram-negative prey with equal propensity. Actinobacterial
43 isolates from both sponge and associated environment produced inhibitors of serine proteases
44 and angiotensin converting enzyme. Predatory behavior was strongly associated with
45 inhibition of trypsin and chymotrypsin. Our study suggests that sponge and associated
46 environment of western coast of India are rich in actinobacterial diversity with widespread
47 predatory activity, antibacterial activity and production of enzyme inhibitors. Understanding
48 diversity and associations among various actinobacterial activities, with each other and the
49 source of isolation, can provide new insights in marine microbial ecology and provide
50 opportunities to isolate novel therapeutic agents.

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51 **INTRODUCTION**

52 The marine ecosystem is not only diverse with respect to microorganisms found in it but also
53 the natural products being synthesized by these microorganisms (Ward and Bora, 2006;
54 Taylor et al., 2007; Lam, 2006). Actinobacteria are among the taxa rich in secondary
55 metabolites (Barka et al., 2016) and are widely distributed in diverse habitats including soil,
56 marine and freshwaters and sediments (Ward and Bora, 2006; Taylor et al., 2007; Tan et al.,
57 2015; Brasel et al., 2019; Mincer et al., 2002; Kokare et al., 2004). They are also not
58 uncommon in extreme environments (Jose and Jebakumar, 2014; Pathom-Aree et al., 2006;
59 Mohammadipanah and Wink, 2016; Shivlata and Tulasi, 2015; Riquelme et al., 2015; Yang
60 et al., 2015) and are also found as endobiotic symbionts of higher organisms (Taylor et al.,
61 2007; Li et al., 2015; Mahmoud and Kalendar, 2016; Trujillo et al., 2015). They belong to the
62 phylum Actinobacteria and represent one of the major phyla within the bacterial domain
63 (Goodfellow, 2015). They are aerobic, spore forming, Gram-positive bacteria, which often
64 produce diffusible pigments, and occur as cocci or rods, branched filaments, aerial or
65 substrate mycelium (Goodfellow, 2015). The marine ecosystems are believed to have a wide
66 range of unexplored diversity of actinobacteria (Montalvo et al., 2005) and their metabolites
67 (Taylor et al., 2007; Lam, 2006; Manivasagan et al., 2005) with diverse biological activities
68 like anticancer (Olano et al., 2009), anti-inflammatory (Trischman et al., 1994), antibiotic
69 (Pimentel-Elardo et al., 2010; Cheng et al., 2015; Gandhimathi et al., 2008), cytotoxic
70 (Abdelfattah et al., 2016) and enzyme inhibitory (Manivasagan et al., 2015; Imada, 2005)
71 activity. Watve et al. (2001) estimated that the genus *Streptomyces* alone is capable of
72 producing up to 10^5 different metabolites, majority of which remain unexplored. Of 23,000
73 medicinally important metabolites produced by marine microorganisms 70% are contributed
74 by actinobacteria (Mahapatra et al., in press). Till date, eight genera of actinobacteria have

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75 been reported to produce secondary metabolites and 267 products have been reported from 96
76 marine actinobacteria (Subramani and Sipkema, 2019)[31].

77 Ecologically it is difficult to understand the production of extracellular metabolites or
78 enzymes by aquatic bacteria, since any molecule secreted outside the cell can be quickly
79 washed off. Extracellular products could be useful to the producer only in viscous or partially
80 enclosed environments. In the marine environment, sponges are likely to provide such closed
81 environment for bacteria. Sponges are filter feeders and collect small nutrient particles
82 including bacteria. This makes the environment locally nutrient rich in an otherwise
83 oligotrophic surroundings. Bacteria, especially actinobacteria, isolated from these sponges
84 may live in a symbiotic relationship that helps the host in defense against predation, sponge
85 skeleton stabilization, translocation of metabolites and help in nutritional process (Taylor et
86 al., 2007; Li et al., 2015; Montalvo et al., 2005; Pimentel-Elardo et al., 2010; Cheng et al.,
87 2015; Gandhimathi et al., 2008; Lee et al., 2009; Thomas et al., 2010). In addition, since
88 sponges are sessile and lack other anti-predator defenses, secondary metabolites of bacteria
89 can provide them with chemical defense (Lee et al., 2001). Therefore, we expect more
90 secondary metabolite related activities from sponge-associated actinobacteria.

91 Sponge-associated actinobacteria are likely to have another ecological role. Among
92 actinobacteria at least three genera, namely *Agromyces*, *Streptomyces* and *Streptoverticillium*,
93 are shown to be predators that kill and feed on other live bacterial cells (Casida, 1980; 1983;
94 Kumbhar et al., 2014). Kumbhar and Watve (2013) argued that antibiotic activity might have
95 evolved primarily as a weapon in predation. However, the expression of secondary
96 metabolites during predation may be independent of antibiotic expression in pure culture; the
97 latter is likely to have evolved for mutualism with higher animal or plant hosts (Harir et al.,
98 2018; Van der Meij et al., 2017). Further, for a niche of predation in association with sponge,
99 the predatory species needs to protect itself from the digestive enzymes of the sponge as well

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100 as its own enzymes used for predation. Therefore, predatory actinobacteria are also expected
101 to have efficient inhibitors of lytic enzymes.

102 In this study, we prepared an inventory of cultivable actinobacteria from sponges and
103 associated environments of intertidal zones along the northern parts of west coast of India and
104 studied their molecular diversity based on 16S rRNA gene sequences. We screened a subset
105 of randomly selected cultures for predatory activity, antibiotic production and enzyme
106 inhibition and tested their associations with each other and with the isolation source to test
107 the hypotheses mentioned earlier.

108

109 MATERIALS AND METHODS

110 Sample collection

111 Small tissue samples (less than one gram) of marine sponges (*Haliclona* spp.) were collected
112 at the time of low tide along Maharashtra and Goa coast (18–15ºN and 73–74ºE) of India
113 during April 2014 to October 2018 without damaging the sponge or its associated
114 environment. Specimens were rinsed and flushed with sterile media to remove debris and
115 loosely attached microbes. Each sponge sample was collected in labeled polystyrene tubes
116 with lids containing sterile Poor Ravan Saline (Watve et al., 2000) and ZoBell Marine broth
117 (ZoBell, 1941). Sediment, water and air samples were collected from the same environment
118 as that of the sponge and were collectively considered as environmental samples. The
119 samples were brought to laboratory maintaining cold chain and were immediately processed
120 for microbial culturing.

121

122 Isolation and maintenance of cultivable actinobacteria

123 Each sample was subjected to pre-heat treatment at 60°C for 15 minutes to eliminate non-
124 sporulating bacteria. Sponge tissue (0.1 cm^3) was homogenized in sterile medium and

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125 vortexed for 5 minutes. Tubes were left undisturbed for two minutes. From the resulting
126 supernatant serial 10 fold dilutions upto 10^{-5} were made and 0.1 ml sample was spread into
127 triplicates on petri plates containing sterile medium. We used two media the Zobell Marine
128 Agar (ZMA) and Poor Ravan Saline Agar (PRSA) with and without antibiotic
129 chloramphenicol (25 μg/ml). Plates were incubated at 30°C for 7 days in the case of ZMA
130 and 21 days for PRSA. Plates were observed regularly for the growth of actinobacteria.
131 Bacterial colonies that showed resemblance to actinobacteria under light microscope were
132 purified several times on the respective media. In all 237 actinobacterial isolates were
133 selected and were re-streaked for making pure cultures. Colonies were labeled as per
134 Maharashtra Gene Bank (MGB) project code and preserved on ZMA slants at 4°C for further
135 use. Similarly, glycerol (18%) stocks were prepared and maintained at -20°C for long term
136 storage. Actinobacterial cultures are deposited in the Microbial Culture Collection (MCC) of
137 National Centre for Microbial Resource, National Center for Cell Sciences, Pune, India
138 (accession numbers are provided in the Supplementary Table S1).
139
140 **Genetic identification, phylogeny and species delimitation**
141 Actinobacterial isolates were outsourced for near complete 16S rRNA gene sequencing. Gene
142 sequences used for the study are deposited in the GenBank database under the accession
143 numbers MN339687–MN339897 and MT598037–MT598065 (Supplementary Table S1)).
144 Sequences were checked in BLAST (Altschul et al., 1990) to find the closest sequences
145 available in the GenBank database (http://www.ncbi.nlm.nih.gov). Four species of
146 Firmicutes, namely *Bacillus paralicheniformis* (MCC 6306), *B. thuringiensis* (MCC 7835),
147 *B. subtilis* (MCC 6386) and *B. halotolerans* (MCC 8381), were used as outgroups (GenBank
148 accession numbers MN339894–MN339897 respectively).

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149 Gene sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA
150 7 (Kumar et al., 2016). Final aligned matrix had 1595 sites. Best nucleotide substitution
151 model was determined using ModelFinder (Kalyaanamoorthy et al., 2017) based on Bayesian
152 information criterion (Schwarz, 1978; Nei and Kumar, 2000). Maximum likelihood analysis
153 was performed in IQ-TREE (Nguyen et al., 2015) with ultrafast bootstrap support (Hoang et
154 al., 2018) for 1000 iterations. Phylogenetic tree was edited in FigTree v1.4.2 (Rambaut,
155 2009).

156 To understand putative number of actinobacterial species we performed species
157 delimitation based on Poisson Tree Processes (Zhang et al., 2013) with maximum likelihood
158 partitioning (mPTP) and Bayesian partitioning (bPTP). Maximum likelihood tree was used to
159 delimit species by setting the parameter values as follows: MCMC generations = 100,000,
160 Thinning = 100, Burn-in = 0.1 and seed = 123.

161 We have identified all isolates up to genus level, while operational taxonomic units, in
162 terms of putative species, are provided based on mPTP and bPTP methods (see
163 Supplementary Table S1). Only in the text, some isolates are assigned to known species
164 based on BLAST search and sequence identity more than 99%.

165

166 **Screening for activities**

167 Out of 237 actinobacterial isolates, 50 isolates were randomly selected for screening of three
168 activities, namely predation, antibiotic production and production of enzyme inhibition.

169

170 **Target bacteria used for predation and antibiotic screening**

171 Test bacteria, used for checking actinobacterial predation and antibiotic production, were
172 obtained from MCC and National Collection of Industrial Microorganisms (NCIM), National
173 Chemistry Laboratory, Pune, India. Fourteen bacteria, namely *Acetobacter pasterianus*

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174 (NCIM 2317), *Alcaligenes fecalis* (NCIM 2262), *Bacillus subtilis* (NCIM 2063),
175 *Enterobacter fecalis*, *Escherichia coli* (NCIM 2184), *Klebsiella pneumonae* (NCIM 2957),
176 *Micrococcus luteus* (NCIM 2673), *Mycobacterium smegmatis* (NCIM 5138), *Proteus*
177 *vulgaris* (NCIM 2172), *Pseudominas aeruginosa* (NCIM 5029), *Salinicoccus roseus* (MCC
178 7574), *Salmonella enterica* (NCIM 2501), *Serretia marcescens* (NCIM 2919) and
179 *Staphylococcus aureus* (NCIM 2121), were used as target species for screening.
180

181 **Screening for actinobacterial predatory growth**

182 Growth of predator with the zone of clearance on prey cells was considered as predation as
183 defined earlier (Kumbhar et al., 2014). The method for the preparation of prey cells was
184 modified from Kumbhar et al. (2014). Pure cultures of the prey species were inoculated on
185 nutrient agar plates to check the purity and were later re-inoculated in nutrient broth.
186 Inoculated flasks were incubated at 37°C for 24 h. Broth was centrifuged at 7000 rpm for 10
187 minutes to concentrate cells using Eppendorf centrifuge 5810R. Cells were washed thrice
188 with sterile distilled water to remove traces of nutrient broth. Pellet was suspended in saline
189 to obtain a thick suspension of optical density of 1.0 at 600 nm. Lawn of prey cells was
190 spread on water agarose plate and plates were incubated at 37°C for 40 minutes.
191 Actinobacterial culture was spot inoculated on pre incubated plates. These plates were
192 incubated at room temperature for 48–72 h at 30°C. Plates with plaque were examined
193 visually and by using 4x and 45x magnification under light microscope. Prey and predator
194 control plates were used for comparison. Each experiment consisted of triplicate sets of
195 plates, as well as one predator control for testing growth of actinobacterial predator without
196 prey. In addition, there was a prey control to demonstrate viable and independent growth of
197 prey without predator. In either controls there was no zone of clearance indicating there was
198 no predation in the presence of predator or prey alone.

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199

200 **Screening for antibacterial activity using conventional cross streak method**

201 Selected actinobacterial cultures were screened for antibacterial activity by cross streak
202 method (Velho-Pereira and Kamat, 2011; Valli et al., 2012). Test organism was streaked as a
203 straight line along the diagonal of the petri dish with sterile ZMA medium. The isolated pure
204 colony of actinobacteria was inoculated as a single streak perpendicular to the central streak.
205 Streaking was done from the edge of the plate to the test organism growth line. Plates were
206 incubated at 37°C for 18 h. The microbial inhibition was observed by determining zone of
207 clearance around the sensitive organisms. Control plates of the same medium with the streak
208 of test bacteria and without the streak of actinobacteria growth was used to observe the
209 normal growth of the test bacteria.

210

211 **Screening for enzyme inhibitors**

212 Actinobacterial cultures were screened for their ability to inhibit the activity of serine
213 proteases and angiotensin converting enzyme (ACE). Three different serine proteases i.e.,
214 Subtilisin, Trypsin and α-Chymotrypsin were used for screening of inhibitory activity.
215 Protease inhibitor activity was studied using unprocessed X-ray films and spot-test method
216 (Cheung, 1991) with modifications. As described by Tripathi et al. (Tripathi et al., 2011),
217 dilutions of pure enzyme were first spotted on gelatine coated films. Lowest dilution showing
218 complete clearance (indicating complete digestion of gelatine) was chosen for further studies.
219 Pure enzyme (100 µg/ml) was incubated with equal quantity of cell free supernatant of
220 actinobacterial isolates for 10 minutes and transferred to untreated X-ray-Fuji Medical X-ray,
221 HRU grade-films. The mixtures were allowed to react for 15 minutes at room temperature
222 and results were recorded after washing the x-ray films under running water. Unprocessed X-
223 ray films contain a layer of gelatine on their surface, which acts as a substrate for various

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224 proteolytic enzymes. Degradation of gelatine gives a clear zone at the site of activity. Thus,
225 upon action of the proteases, clear zones were seen on unprocessed x-ray films, at the site of
226 inoculation, whereas, if the gelatine layer remains intact, no clearance is observed. No
227 clearance on the films indicated presence of protease inhibitors.

228 ACE acts on a specific substrate N-Hyppuryl-His-Leu (HHL) to liberate hippuric acid
229 and His-Leu. Liberated hippuric acid was detected spectrophotometrically. Upon reaction of
230 the enzyme with ACE inhibitors, the enzyme becomes inactive and this is measured in terms
231 of lower levels of hippuric acid released. Protocol suggested by Cushman and Cheung (1971)
232 was used with certain modifications and hippuric acid liberated was checked using method
233 suggested by Ng et al. (2008). Equal amount of ACE and cell free supernatants (10 µl each)
234 were allowed to react at 37°C. After 10 minutes 20 µl of HHL was added to the reaction
235 mixture and reaction was continued for 30 mins at 37°C. The reaction was stopped by
236 addition of 40 µl of 1 N HCl. Blank was prepared by addition of HCl before addition of the
237 substrate. Positive enzyme control was prepared by incubating enzyme with un-inoculated
238 broth. Liberated hippuric acid was extracted in 90 µl ethyl acetate by vigorous shaking. Ethyl
239 acetate layer was collected in a fresh vial and allowed to dry in water bath of 50°C. The
240 liberated hippuric acid was diluted in 150 µl distilled water and absorbance was checked at
241 228 nm. Zero was adjusted using distilled water. Test vials with more than 15% inhibition of
242 ACE were considered as positive for ACE inhibitor.

243

244 **RESULTS**

245 **Actinobacterial phylogenetic diversity in sponge and associated environment**

246 Actinobacteria from sponges and associated environments showed a rich phylogenetic
247 diversity (Figure 1). We obtained 237 actinobacterial isolates, from sponge and associated
248 environments, belonging to 19 families and 28 genera (Supplementary Table S1). Species

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249 delimitation based on mPTP suggested that these isolates belong to 95 putative species, while
250 bPTP suggested 100 putative species. The two species delimitation methods, mPTP and
251 bPTP, differed in the groups of species under genera *Micrococcus*, *Rhodococcus* and
252 *Streptomyces* (Supplementary Table S1). Air was generally devoid of actinobacteria and we
253 recovered only three isolates from air, belonging to genera *Brachybacterium*, *Brevibacterium*
254 and *Rhodococcus*, as compared to 39 isolates from water, 105 isolates from sediment and 90
255 isolates from sponge.

256 From sponges, 18 genera under 14 families belonging to 56 putative species (Table
257 1). From the sponge-associated environment, 22 genera under 15 families were recorded
258 belonging to 64 putative species as per mPTP and 65 putative species as per bPTP. A total of
259 12 genera under 9 families and 28 putative species based on mPTP and 25 putative species
260 based on bPTP were common to both sponge and associated environment.

261 Six genera, namely *Gordonia*, *Jonesia*, *Mycolicibacterium*, *Pseudonocardia*, *Rothia*
262 and *Serinicoccus* were isolated only from sponges (Table 1), which could be identified to
263 species *Gordonia terrae* (MCC 6452), *Jonesia denitrificans* (MCC 7852), *Mycolicibacterium*
264 *poriferae* (MCC 6242), *Pseudonocardia kongjuensis* (MCC 7930) and *Rothia terrae* (MCC
265 7823), *Serinicoccus marinus* (MCC 7935) respectively. Although 12 genera, namely
266 *Agrococcus*, *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Klenkia*, *Kocuria*,
267 *Microbacterium*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus* and
268 *Streptomyces*, were isolated from both sponges and associated habitats, most of these genera
269 had some putative species that were either exclusive to sponges or associated environments.
270 In particular, 7 species, *Brachybacterium muris* (MCC 7614), *Brevibacterium casei* (MCC
271 6140, MCC 6152, MCC 6176), *Kocuria rhizophila* (MCC 8384), *Nocardiopsis salina* (MCC
272 7931), *Rhodococcus zopfii* (MCC 7934), *Streptomyces smyrnaeus* (MCC 7924) and
273 *Streptomyces viridobrunneus* (MCC 7990), were recorded only from sponges.

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274 With respect to both, the number of isolates and number of putative species,
275 *Streptomyces* was the most dominant genus, which was found in both sponges and associated
276 environments. *Nocardiopsis* was the second most common genus with two dominant species
277 *Nocardiopsis alba* (MCC 8385) followed by *N. dassonvillei* (MCC 7845). Among the genera
278 and species that were recorded only from the environment, we provide first record of species
279 such as *Aeromicrobium massiliense* (MCC 6739) and *Glutamicibacter mysorens* (MCC 7825)
280 from marine waters.
281

282 **Non-obligate epibiotic predatory activity**

283 Out of the total 50 actinobacterial isolates screened for non-obligate epibiotic predatory
284 activity, 26 isolates showed predation on at least one of the 14 target organisms
285 (Supplementary Table S2). Of the 26 isolates with predatory behavior, 17 preyed on Gram-
286 negative prey, 21 preyed on Gram-positive prey, while 12 preyed on both Gram- negative and
287 Gram-positive prey. There was no significant difference (Mann-Whitney U = 15, P = 0.2601)
288 in the frequency of actinobacterial predators on Gram-negative and Gram-positive prey
289 (Table 2). Most actinobacterial predators (n = 14) preyed on a single prey species while only
290 a few predators preyed on multiple prey species. A single predator of the genus *Streptomyces*
291 tpreyed on 8 prey species. There was a significant association between the source of isolation
292 (sponge or associated environment) and predatory behavior (χ^2 = 5265, P = 0.0218), where
293 the isolates from sponge showed proportionately more predatory behavior (Figure 2).

294 All eight isolates of *Streptomyces* used for screening showed predatory behavior and
295 preyed on both Gram-negative and Gram-positive prey (Supplementary Table S2). Out of 25
296 isolates of *Nocardiopsis*, 12 showed predatory behavior, out of which 5 preyed on Gram-
297 negative bacteria while 11 preyed on Gram-positive bacteria. Both the isolates of
298 *Micromonospora* preyed on Gram-positive prey while only one preyed on Gram-negative

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299 prey. Isolates belonging to genera *Brevibacterium*, *Glutamicibacter* and *Rhodococcus* preyed
300 only on Gram-negative prey while *Rothia* preyed only on Gram-positive prey.
301

302 **Antibiosis, antibacterial activity and growth inhibition**

303 Of the 50 actinobacterial isolates screened for antibacterial activity, 25 showed antibiosis
304 against at least one target organism (Supplementary Table S2). Of these 25 isolates, all
305 showed antibiosis against at least one of the Gram-positive target species, while only five
306 showed antibiosis against at least one of the Gram-negative organisms. The frequency of
307 antibacterial activity against Gram-positive organisms was significantly higher (Mann-
308 Whitney U = 1.5, P = 0.003) than those against Gram-negative organisms (Table 2). Most
309 antibacterial activities were broad spectrum with respect to the target organisms that they
310 affected. There were 10 actinobacterial isolates that showed antibiosis against two target
311 organisms, 6 isolates that affected 4 target species and 2 isolates that affected 6 target species.
312 There was no association between antibacterial activity and the source (sponge or associated
313 environment) of the isolation ($\chi^2 = 2.0129$, P = 0.1560).

314 Out of eight isolates of *Streptomyces* that were screened for antibacterial activity, five
315 showed antibiosis, of which two showed antibiosis against Gram-negative target species,
316 while all showed antibiosis against Gram-positive organisms. In the case of *Nocardiopsis*, of
317 the 25 isolates used for screening 17 showed antibiosis, of which all affected growth of
318 Gram-positive organisms, while only two affected growth of Gram-negative organisms.
319 Genus *Kytococcus* showed antibiosis that affected both Gram-positive as well as Gram-
320 negative organisms, while *Glutamicibacter* and *Rothia* showed antibiosis against Gram-
321 positive organisms only.
322

323 **Enzyme inhibition**

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324 Out of 50 actinobacterial isolates screened for inhibition of four enzymes, 30 isolates
325 inhibited at least one of the enzyme (Supplementary Table S2). Of these 30 isolates, 28
326 inhibited trypsin, 24 inhibited chymotrypsin, three inhibited angiotensin converting enzyme
327 (ACE) and only two inhibited subtilisin. Venn diagram of frequency of isolates inhibiting
328 different enzymes (Figure 3) suggested that five isolates inhibited only trypsin and one isolate
329 each inhibited chymotrypsin and ACE, while subtilisin inhibition was accompanied by
330 inhibition of other enzymes. No isolate inhibited all four enzymes. Out of 30 actinobacteria
331 that produced enzyme inhibitors, 19 produced two inhibitors, four produced three inhibitors
332 while seven produced only one of the four inhibitors. There was no association between the
333 enzyme inhibition and source of the actinobacterial isolate (χ^2 = 2.3386, P = 0.1262).

334 Out of eight isolates of *Streptomyces* seven produced enzyme inhibitors against
335 proteases, while 12 out of 25 isolates of *Nocardiopsis* produced enzyme inhibitors of which
336 11 produced against proteases and two produced against ACE (Table 3). One isolate of
337 *Actinomycetospora* inhibited activity of ACE.

338

339 **Associations between different activities**

340 Out of 50 actinobacterial isolates that were screened for activities, 39 showed at least one of
341 the three activities. Of these 39 isolates, 15 showed all three activities, while nine showed
342 predation as well as enzyme inhibition (Figure 4). There were only seven isolates that showed
343 predation and antibiotic production against the same target organism (Table 2) and all these
344 isolates belonged to genera *Streptomyces* and *Nocardiopsis*.

345 Antibiotic production showed no significant association with predation (χ^2 = 2.8846,
346 P = 0.0894) or any of the four enzyme inhibition (χ^2 = 2.0525, P = 0.1520). However, there
347 were significant associations between predation and protease inhibitors (Figure 5). There
348 were 24 isolates that showed both predation as well as inhibition of at least one enzyme and

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349 there was a significant association between the two activities (χ^2 = 26.172, P < 0.0001),
350 where predators proportionately produced more enzyme inhibitors than non-predators (Figure
351 5a). There were 23 actinobacterial isolates that showed predation as well as trypsin inhibition
352 and there was a significant association between the two (χ^2 = 23.165, P < 0.0001) with
353 predators more likely to produce trypsin inhibitors than non-predators (Figure 5b). Similarly,
354 24 actinobacteria were predators as well as inhibited chymotrypsin activity and there was a
355 significant association between the two (χ^2 = 42.604, P < 0.0001) with predators more likely
356 to produce chymotrypsin inhibitors than non-predators (Figure 5c).
357

358 **DISCUSSION**

359 Sponges and associated environment in northern parts of western coast of India are rich in
360 actinobacterial diversity with about 95 putative species under 19 families and 28 genera. We
361 recorded 13 species of actinobacteria only from sponges. Out of these, *Mycobacterium*
362 *poriferae* was originally described from marine sponge (Padgitt and Moshier, 1987), while
363 three species, *Gordonia terrae* (Elfalah et al., 2013; Santos et al., 2019; Montalvo et al.,
364 2005), *Brevibacterium casei* (Kiran et al., 2010) and *Koc uria rhizophila* (Palomo et al.,
365 2013), have been previously reported from sponges. To our knowledge, we provide first
366 report of nine species, namely *Brachybacterium murisi, Jonesia denitrificans*, *Nocardiopsis*
367 *salina*, *Pseudonocardia kongjuensis*, *Rhodococcus zopfii*, *Rothia terrae*, *Serinicoccus*
368 *marinus*, *Streptomyces smyrnaeus* and *Streptomyces viridobrunneus*, from marine sponges,
369 although some of them are known from marine habitats (Stach et al., 2003; Satheeja and
370 Jebakumar, 2011; Yi et al., 2004; Shinde et al., 2018).

371 *Streptomyces* was the most dominant genus among the isolates, which agrees with the
372 findings of Zhang et al. (2008). Genus *Nocardiposis*, with its two species *N. alba* and *N.*
373 *dassonvillei*, has been suggested (Bennur et al., 2015) as the second common genus after

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374 *Streptomyces* and that too agrees with our findings. Further, report of most genera, including
375 *Agrococcus*, *Arthrobacter*, *Brevibacterium*, *Kocuria*, *Microbacterium* and *Micrococcus*, from
376 sponges in our study are consistent with previous reports from other study areas including
377 South China Sea (Li et al., 2015), Yellow Sea (Zhang et al., 2008), Mediterranean Sea
378 (Cheng et al., 2015), coast of Florida in USA (Montalvo, 2005) and northern coast of Brazil
379 (Menezes et al., 2010) indicating that there are common trends in the discovery of
380 actinobacteria from sponges.

381 Among the first reports from marine environment from our study, *Aeromicrobium*
382 *massiliense* and *Glutamicibacter mysorens* are known from human fecal microbiota
383 (Ramasamy et al., 2012) and sewage (Nand and Rao, 1972) respectively. Presence of these
384 two species in the sediments along the collection site Harne (17.81ºN, 73.09ºE) likely
385 suggests fecal pollution in this area.

386 Although predation is a widespread behavior in bacterial kingdom, δ-proteobacteria
387 of the orders *Myxococcales* and *Bdellovibrionales* have received more attention (Jurkevitch,
388 2007) as compared to other taxa, especially the Gram-positive bacteria such as actinobacteria.
389 Among actinobacteria only three genera, namely *Agromyces*, *Streptomyces* and
390 *Streptoverticillium*, are known to have predatory behavior against other bacterial species
391 (Casida, 1980; 1983; 1988; Kumbhar et al., 2014; Zeph and Casida, 1986; Ibrahimi et al.,
392 2020). In the current study, for the first time, we show predation in six other genera of
393 actinobacteria, namely *Brevibacterium*, *Glutamicibacter*, *Micromonospora*, *Nocardiopsis*,
394 *Rhodococcus* and *Rothia*. Kumbhar et al. (2014) argued that predatory behavior is widespread
395 in genus *Streptomyces* and even in the current study we observed that all the isolates of
396 *Streptomyces* used for screening showed predation on Gram-positive as well as Gram-
397 negative prey.

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398 Since sponges are sessile and lack other anti-predator defenses, it has been suggested
399 that secondary metabolites of bacteria can provide sponges with chemical defense (Lee et al.,
400 2001; Kumbhar and Watve, 2013). However, we did not observe any significant association
401 between the source of actinobacterial isolation and antibiotic production, suggesting that
402 isolates even from environment were equally likely to produce antimicrobials as that of the
403 isolates recovered from sponges. However, there was a significant association between the
404 source of isolation and predatory activity, with proportionately more predators among the
405 isolates recovered from sponge. Ecologically this makes sense. As the sponges are filter
406 feeders and have regular intake of environmental bacteria, sponge associated actinobacteria
407 will have better predation opportunities. It is also possible that the predatory activity of
408 sponge associated actinobacteria, could have evolved as a mutualistic activity as it can defend
409 sponges from pathogenic bacterial invasions.

410 Actinobacteria are known to produce several enzyme inhibitors (Manivasagan et al.,
411 2015; Imada, 2005). However, for the first time we show a strong association between
412 predation and enzyme inhibition, specifically inhibition of trypsin and chymotrypsin, where
413 predators produced proportionality more enzyme inhibitors as compared to non predators.
414 Predators themselves are known to produce a variety of hydrolytic enzymes for degrading the
415 prey (Pérez et al., 2016). Therefore, it is possible that the production of enzyme inhibitors
416 safeguards their own cells from being target of the enzyme. It is also possible that enzyme
417 inhibitors also protect the actinobacteria from hydrolytic enzymes produced from the sponge
418 host and other microbiota.

419 An interesting observation that we made, when comparing the predation and antibiotic
420 production by actinobacteria, was that, while predation was equally effective against Gram-
421 positive as well as Gram-negative target species, antibiotic production was mainly effective
422 against Gram-positive bacteria. Recently, Ibrahimi et al. (2020) suggested that there are some

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423 bio-active secondary metabolites that co-cultured actinobacteria produce in the presence of
424 prey cells. It is therefore possible that studying the predatory behavior of actinobacteria and
425 predation specific metabolites could lead to discovery of novel therapeutic agents that are
426 more broad-spectrum.

427 Although actinobacteria are known to be rich in secondary metabolites, extracellular
428 enzymes and enzyme inhibitors, the ecological role of these extracellular bioactive molecules
429 is little known. We suggest that studying the ecological correlates of bioactivity and the inter-
430 correlation patterns of different types of bioactivity can be a useful tool in understanding the
431 ecological origins of bioactivity and testing alternative ecological hypotheses.
432

433 **CONCLUSION**

434 Sponges and associated environments of intertidal zones, along the northern parts of west
435 coast of India, are rich in actinobacterial diversity with 19 families and 28 genera, which
436 could be attributed to 95 putative species using mPTP and 100 putative species based on
437 bPTP methods. Although, at the genus level, the trends in the discovery of actinobacteria
438 isolated from sponges was consistent with previous studies from different study areas, we
439 provide first report of nine species, namely *Brachybacterium murisi, Jonesia denitrificans*,
440 *Nocardiopsis salina*, *Pseudonocardia kongjuensis*, *Rhodococcus zopfii*, *Rothia terrae*,
441 *Serinicoccus marinus*, *Streptomyces smyrnaeus* and *Streptomyces viridobrunneus*. Non-
442 obligate epibiotic predatory behavior was widespread among actinobacterial genera and we
443 provide first report of predatory activity in *Brevibacterium*, *Glutamicibacter*,
444 *Micromonospora*, *Nocardiopsis*, *Rhodococcus* and *Rothia*. Sponges associated actinobacteria
445 showed significantly more predatory behavior than environmental isolates, and we
446 hypothesize that predatory actinobacteria might provide sponges with defense against
447 pathogenic bacteria. While antibiotic produced from actinobacterial isolates affected Gram-

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448 positive target bacteria with little to no effect on Gram-negative bacteria, predation targeted
449 both Gram-positive and Gram-negative prey with equal propensity, suggesting that study of
450 predation specific metabolites might provide novel therapeutic agents with broad-spectrum.
451 Actinobacterial isolates from both sponge and associated environment produced inhibitors of
452 serine proteases and angiotensin converting enzyme. Predatory behavior was strongly
453 associated with inhibition of trypsin and chymotrypsin, which might be helpful for the
454 actinobacteria for overcoming effects of proteolytic enzymes produced by sponge host and
455 other microbiota. Understanding diversity and associations among various actinobacterial
456 activities, with each other and the source of isolation, can provide new insights in marine
457 microbial ecology and provide opportunities to isolate novel therapeutic agents.
458

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459 **DATA AVAILABILITY**

460 Sequences of 16S rRNA gene of studied isolates are submitted to GenBank NCBI under the
461 accession numbers MN339687–MN339897 and MT598037–MT598065. Actinobacterial
462 cultures are deposited in the Microbial Culture Collection (MCC) of National Centre for
463 Microbial Resource, National Center for Cell Sciences, Pune, India (accession numbers are
464 provided in the Supplementary Table S1). All the data used for analysis is provided in
465 supplementary information (Supplementary Table S1 and Table S2).

466

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474

475 **AUTHOR CONTRIBUTIONS**

476 M.W., U.B. and N. Deshpande conceived and designed the study. U.B., N.S., K.H., A.P.,
477 U.L., T.G., K.P., A.J., R.S., H.V and V.T. performed the study. N. Dahanukar and M.W.
478 analyzed the data. N. Dahanukar, U.B. and M.W. wrote the manuscript with inputs from
479 other authors. All authors contributed to the proofreading of the manuscript.

480

481 **Supplementary information:** Supplementary Table S1 and Table S2 accompanies the online
482 version of the paper.

483 **Competing Interests:** The authors declare no competing interests.

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688 **Table 1.** Putative number of species of actinobacterial genera based on PTP and bPTP
689 methods isolated from sponge, associate environment and both sources.
690

Family	Genus	Sponge		Environment		Both	
		mPTP	bPTP	mPTP	bPTP	mPTP	bPTP
Actinomycetaceae	*Streptomyces*	23	23	24	25	12	11
Brevibacteriaceae	*Brevibacterium*	5	5	2	2	1	1
Cellulomonadaceae	*Cellulomonas*	0	0	1	1	0	0
Dermabacteraceae	*Brachybacterium*	1	1	2	2	0	0
Dietziaceae	*Dietzia*	0	0	1	1	0	0
Geodermatophilaceae	*Klenkia*	1	1	1	1	1	1
Gordoniaceae	*Gordonia*	1	1	0	0	0	0
Intrasporangiaceae	*Janibacter*	0	0	2	2	0	0
	Knoellia	0	0	1	1	0	0
	Terrabacter	0	0	1	1	0	0
Jonesiaceae	*Jonesia*	1	1	0	0	0	0
Kytococcaceae	*Kytococcus*	0	0	1	1	0	0
Microbacteriaceae	*Agrococcus*	1	1	1	1	1	0
	Curtobacterium	0	0	1	1	0	0
	Microbacterium	1	1	1	1	1	1
Micrococcaceae	*Arthrobacter*	1	1	1	1	1	1
	Glutamicibacter	0	0	2	2	0	0
	Kocuria	4	4	6	6	2	2
	Micrococcus	6	6	8	8	4	4
	Rothia	1	1	0	0	0	0
Micromonosporaceae	*Micromonospora*	1	1	1	1	1	1
Mycobacteriaceae	*Mycolicibacterium*	1	1	0	0	0	0
Nocardiaceae	*Rhodococcus*	2	2	2	2	2	1
Nocardioidaceae	*Aeromicrobium*	0	0	1	1	0	0
Nocardiopsaceae	*Nocardiopsis*	4	4	3	3	2	2
Ornithinimicrobiaceae	*Serinicoccus*	1	1	0	0	0	0
Pseudonocardiaceae	*Actinomycetospora*	0	0	1	1	0	0
	Pseudonocardia	1	1	0	0	0	0
	Total	56	56	64	65	28	25

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694 **Table 2.** Predation and antibiotic production by actinobacteria against the Gram positive and

695 Gram negative target species.

696

Target species	Predation	Antibiotic	Predation and Antibiotic by same actinobacterial isolate
Gram positive			
Mycobacterium smegmatis	3	12	0
Micrococcus luteus	8	5	0
Bacillus subtilis	1	24	1
Staphylococcus aureus	17	9	4
Salinicoccus roseus	9	3	0
Enterococcus faecalis	3	20	1
Gram negative			
Acetobacter pasterianus	7	0	0
Alcaligenes faecalis	3	1	1
Escherichia coli	2	5	0
Klebsiella pneumoniae	3	0	0
Proteus vulgaris	8	0	0
Salmonella enterica	2	0	0
Serratia marcescens	3	0	0
Pseudomonas aeruginosa	1	0	0

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699 **Table 3.** Frequency of actinobacterial isolates producing four different enzyme inhibitors.

700

Genus	Number of isolates	Frequency of isolates inhibiting				Isolates with at least one inhibition activity
		Subtilisin	Trypsin	Chymotrypsin	ACE	
Actinomycetospora	2	0	1	0	1	2
Agrococcus	1	0	0	0	0	0
Brevibacterium	1	0	1	1	0	1
Glutamicibacter	1	0	1	1	0	1
Jonesia	1	0	0	0	0	0
Kocuria	1	0	0	0	0	0
Kytococcus	1	0	1	0	0	1
Micrococcus	1	0	1	0	0	1
Micromonospora	2	0	2	2	0	2
Nocardiopsis	25	0	11	11	2	12
Pseudonocardia	1	0	0	0	0	0
Rhodococcus	4	0	2	1	0	2
Rothia	1	0	1	1	0	1
Streptomyces	8	2	7	7	0	7

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703 **FIGURE CAPTIONS**

704

705 **Figure 1.** Maximum likelihood phylogenetic tree of actinobacterial isolates based on
706 TIM3+F+I+G4 nucleotide substitution model (lnL of consensus tree: -18684.58). Firmicutes
707 belonging to genus *Bacillus* were used as outgroups.

708

709 **Figure 2.** Association between source of actinobacterial isolation on their predatory behavior.
710 There was a significant association between the source (sponge or associated environment) of
711 actinobacterial isolation and predation ($\chi^2 = 5.265$, $P = 0.0218$).

712

713 **Figure 3.** Venn diagrams combination of enzyme inhibitors produced by actinobacterial
714 isolates. Venn diagrams is not to scale.

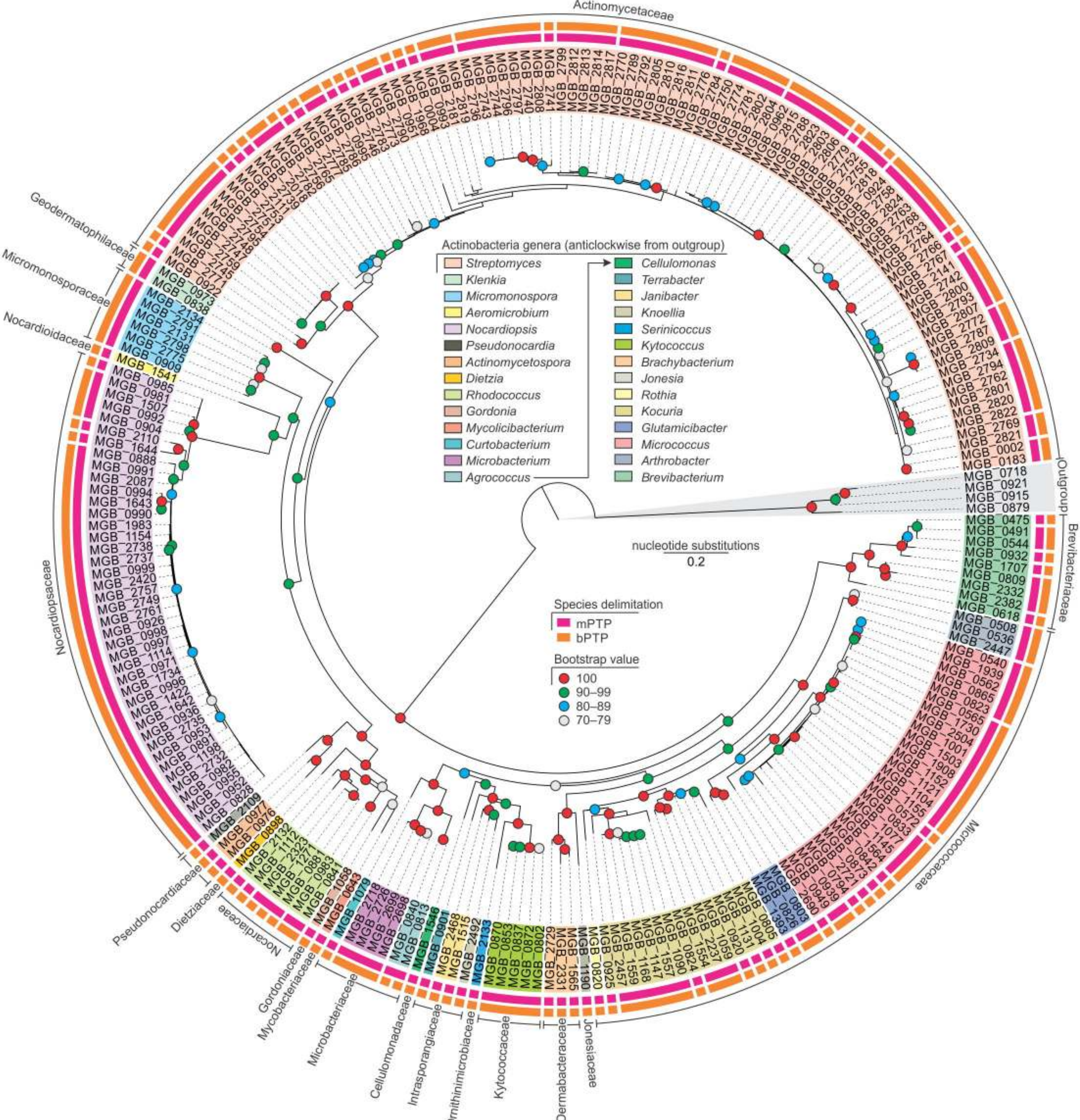
715

716 **Figure 4.** Venn diagrams of predation, antibiotic production and enzyme inhibition by
717 actinobacterial isolates. Venn diagrams is not to scale.

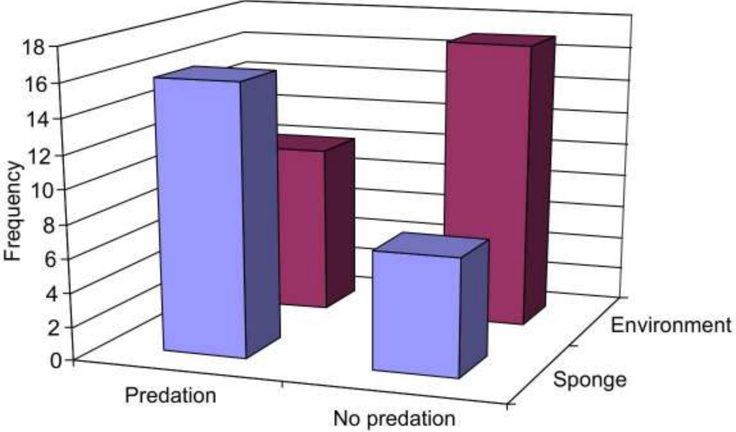
718

719 **Figure 5.** Association between enzyme inhibition and predation in actinobacterial isolates.
720 Predation was significantly associated with (a) inhibition of any one of the four enzymes
721 tested ($\chi^2 = 26.172$, $P < 0.0001$), (b) inhibition of trypsin ($\chi^2 = 23.165$, $P < 0.0001$) and (c)
722 inhibition of chymotrypsin ($\chi^2 = 42.604$, $P < 0.0001$).

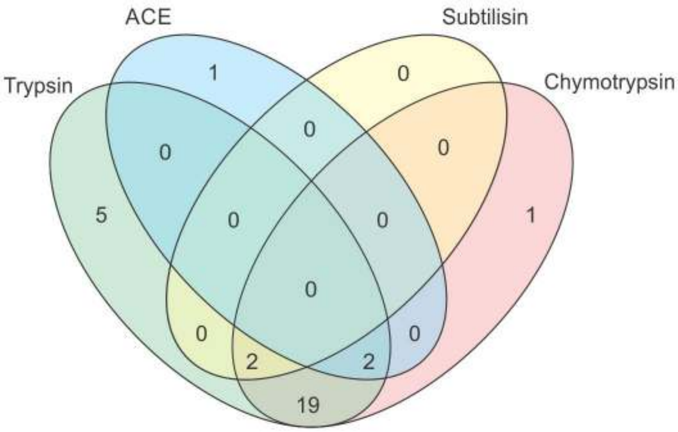
723

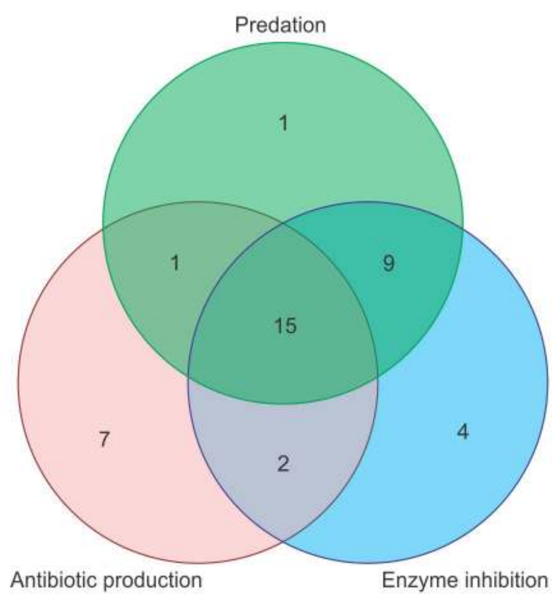


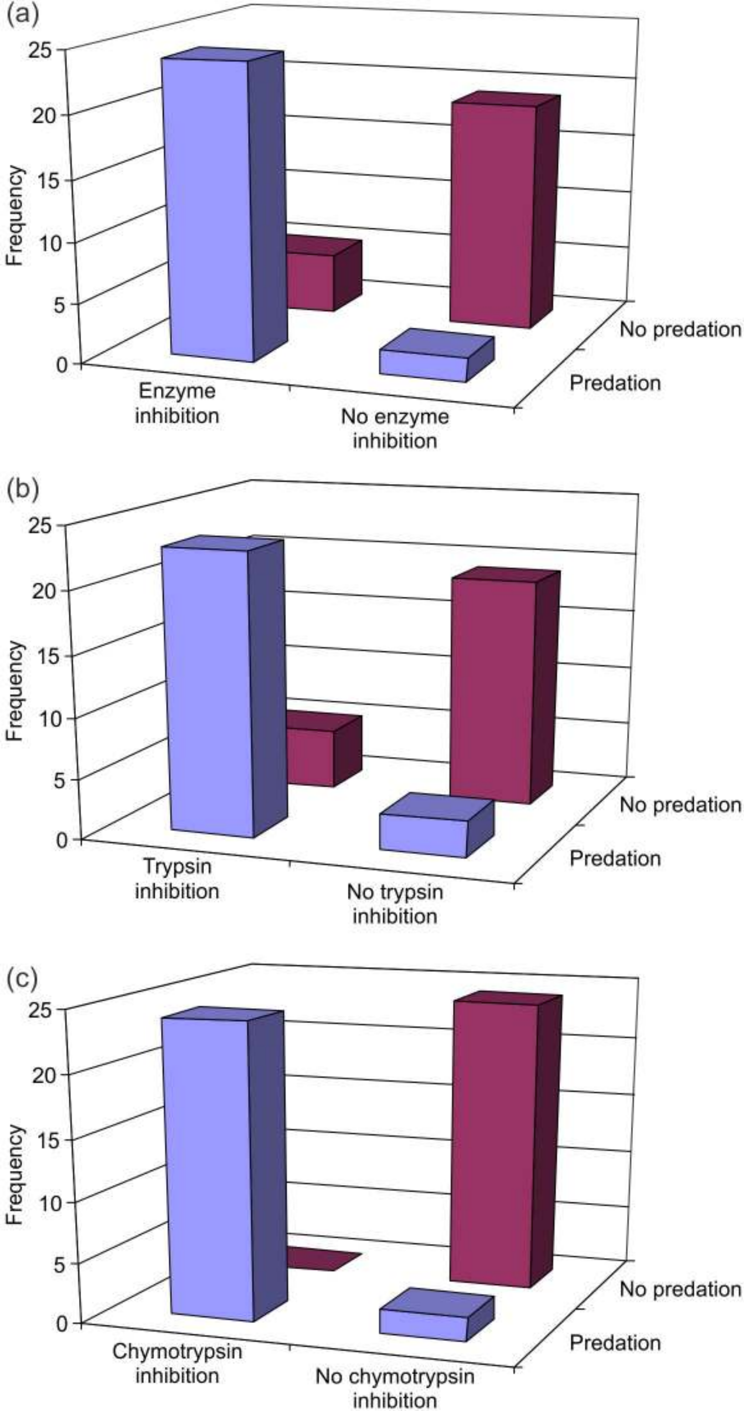
Actinomycetaceae
Geodermatophilaceae
Micromonosporaceae
Nocardioidaceae
Nocardiopsaceae
Pseudonocardiaceae
Dietziaceae
Nocardiaceae
Gordoniaceae
Mycobacteriaceae
Microbacteriaceae
Cellulomonadaceae
Intrasporangiaceae
Ornithinimicrobiaceae
Kytococcaceae
Dermabacteraceae
Jonesiaceae
Micrococcaceae
Brevibacteriaceae
Outgroup
Actinobacteria genera (anticlockwise from outgroup)
Streptomyces
Klenkia
Micromonospora
Aeromicrobium
Nocardiopsis
Pseudonocardia
Actinomycetospora
Dietzia
Rhodococcus
Gordonia
Mycolicibacterium
Curtobacterium
Microbacterium
Agrococcus
Cellulomonas
Terrabacter
Janibacter
Knoellia
Serinicoccus
Kytococcus
Brachybacterium
Jonesia
Rothia
Kocuria
Glutamicibacter
Micrococcus
Arthrobacter
Brevibacterium
nucleotide substitutions
0.2
Species delimitation
mPTP
bPTP
Bootstrap value
100
90–99
80–89
70–79



Frequency
18
16
14
12
10
8
6
4
2
0
Predation
No predation
Sponge
Environment


ACE
Subtilisin
Trypsin
Chymotrypsin
1
0
0
0
0
5
0
0
1
0
0
0
0
2
2
19


Predation
1
1
9
15
7
4
2
Antibiotic production
Enzyme inhibition


(a)
25
20
15
10
5
0
Frequency
Enzyme inhibition
No enzyme inhibition
No predation
Predation
(b)
25
20
15
10
5
0
Frequency
Trypsin inhibition
No trypsin inhibition
No predation
Predation
(c)
25
20
15
10
5
0
Frequency
Chymotrypsin inhibition
No chymotrypsin inhibition
No predation
Predation