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Communication within East Antarctic Soil Bacteria

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21 Antarctica | Bacterial communication | Quorum sensing | Homoserine lactone | Biosensor |

22 Survival mechanism | Soil bacteria |

23

24 **ABSTRACT**

25 Antarctica, being the coldest, driest and windiest continent on Earth, represents the most
26 extreme environment a living organism can survive in. Under constant exposure to harsh
27 environmental threats, terrestrial Antarctica remains home to a great diversity of
28 microorganisms, indicating that the soil bacteria must have adapted a range of survival
29 strategies that require cell-to-cell communication. Survival strategies include secondary
30 metabolite production, biofilm formation, bioluminescence, symbiosis, conjugation,
31 sporulation and motility, all of which are often regulated by quorum sensing (QS), a type of
32 bacterial communication. Up to now, such mechanisms have not been explored in terrestrial
33 Antarctica. In this study, LuxI/LuxR-based quorum sensing (QS) activity was delineated in
34 soil bacterial isolates recovered from Adams Flat, in the Vestfold Hills region of East
35 Antarctica. Interestingly, we identified the production of potential homoserine lactones
36 (HSLs) ranging from medium to long chain length in 19 bacterial species using three
37 biosensors, namely *Agrobacterium tumefaciens* NTL4, *Chromobacterium violaceum* CV026
38 and *Escherichia coli* MT102, in conjunction with thin layer chromatography (TLC). The
39 majority of detectable HSLs were from gram-positive species not previously known to
40 produce HSLs. This discovery further expands our understand of the microbial community
41 capable of this type of communication, as well as providing insights into physiological
42 adaptations of microorganisms that allow them to survive in the harsh Antarctic environment.

43

44 **IMPORTANCE**

45 Quorum sensing, a type of bacterial communication, is widely known to regulate many
46 processes including those that confer survival advantage. However, little is known about
47 communication by bacteria residing within Antarctic soils. Employing a combination of
48 bacteria biosensors, analytical techniques, and genome mining, we found a variety of

49 Antarctic soil bacteria speaking a common language, via the LuxI/LuxR-based quorum
50 sensing, thus potentially supporting survival in a mixed microbial community. This study
51 reports potential quorum sensing activity in Antarctic soils and has provided a platform for
52 studying physiological adaptations of microorganisms that allow them to survive in the harsh
53 Antarctic environment.

54

55 INTRODUCTION

56 Terrestrial Antarctica represents one of the most extreme environments a living organism can
57 survive in (1, 2). Precipitation is limited (3), and of this, the majority falls as snow and ice
58 crystals that do not melt but build up over time to form massive ice sheets. Cold, dense air
59 from these elevated ice-built surfaces continuously sinks downhill and is replaced by
60 subsiding air from above, resulting in strong winds that increases the aridity of Antarctica.
61 The high elevation together with reflection of sunlight by the snow and ice cover, not
62 allowing heat energy absorption, contribute to the low temperatures reaching -89 °C once in
63 1983 (4, 5). Antarctica experiences total darkness during winter, while during summer, it
64 receives 24-hour-sunlight, and with this, damaging UVB rays (1, 6). The harsh environmental
65 conditions of Antarctica have consequently shaped the simple trophic structures present in
66 Antarctic soil ecosystems (7). The lack of vascular plants (except for the Antarctic Peninsula),
67 the small proportion of macroinvertebrates and the low water availability, leads to extremely
68 low organic matter content (8, 9).

69

70 Despite the harsh conditions, terrestrial Antarctica remains home to a great diversity of
71 microorganisms, ranging from phyla frequently observed in soils from temperate regions,
72 such as Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (2, 8, 10) through to
73 Candidate phyla which have no cultured representatives (9, 11, 12). From an ecological

74 standpoint, these Antarctic microorganisms may have evolved under the environmental
75 stressors such as high UV exposure during summer, complete absence of light during winter,
76 frequent freeze-thaw cycles, osmotic limitation and nutrient starvation, to possess unique
77 biochemical adaptations that confer them a selective advantage in the seemingly inhospitable
78 terrain (9-11, 13, 14). These adaptations are likely to incorporate a variety of survival
79 strategies such as secondary metabolite production (15-18), biofilm formation (19-21),
80 bioluminescence, symbiosis, conjugation, sporulation and motility, all of which are often
81 regulated by quorum sensing (QS), a type of bacterial communication (22-24).

82

83 The most widely reported QS system is the LuxI/LuxR system (25, 26) that utilizes
84 homoserine lactones (HSLs) as signal molecules. HSLs are a diverse group of molecules
85 synthesized by the LuxI synthase enzyme. The typical structure of HSL consists of a
86 conserved homoserine lactone ring and a side chain of variable length that can undergo
87 various types of modification such as substitution, hydroxylation and carboxylation at the C-3
88 position, allowing the signalling molecules to be species-specific (27). The side chain of
89 HSLs is usually a fatty acyl group but new classes of HSLs containing p-coumaryl group (28),
90 cinnamoyl group (29) and aryl group (30, 31) have also been found to be produced and
91 detected by LuxI/LuxR homologs. In addition, LuxR were found to be capable of binding
92 non-HSL signalling molecules derived from other organisms, such as cyclic diguanylate (c-
93 di-GMP), furanones, and diketopiperazines (DKPs) (32), overcoming communication barriers
94 between species, or even kingdoms.

95

96 While the LuxI/LuxR system was previously thought to be exclusive to gram-negative
97 bacteria, knowledge on the distribution of this QS system among microbes is growing, with
98 HSL activity now reported in Archaea (33-35), in gram-positive marine bacteria (36, 37),

99 gram-positive rhizobacteria from a mangrove swamp (38) and gram-positive bacteria from
100 hypersaline microbial mats (39). In Antarctica, a limited number of HSL-based QS studies is
101 being restricted to marine environments (24, 40). The occurrence of HSL activity in
102 Antarctic soil bacteria is not known, thus major gaps remain in the understanding of the role
103 that quorum sensing may play in nutrient-poor, cold desert environments (27).

104

105 Here, we investigated the potential of HSL-based quorum sensing by Antarctic soil bacteria,
106 specifically from a hyper-arid site named Adams Flat, in the Vestfold Hills region. Adams
107 Flat soils are comprised of a microbial diversity with dominance by the phylum
108 Actinobacteria (41) ([Supplementary Figure 1](#)), combined with a high proportion of bacteria
109 with the genetic capacity for diverse secondary metabolite production (15). Microbial
110 communities in Adams Flat were also recently shown to be carrying out significant levels of
111 atmospheric chemosynthesis to support primary production (41), hence suggesting the need
112 to evolve with novel functionalities in order to survive in a barren desert environment.

113

114 **RESULTS & DISCUSSION**

115 **Culturable diversity of Adams Flat soils.** In total, 81 isolates were cultured that represented
116 21 different bacterial strains (Table 1). These 21 strains, belonging to 18 species, spanned
117 four soil phyla (Figure 1), with the majority being members of Actinobacteria (58%),
118 followed by Firmicutes (31%), Bacteroidetes (7%) and Proteobacteria (4%), which are the
119 four most well-characterised phyla as they are those most readily culturable (2, 42). These
120 phyla are ubiquitous and common in desert environments, including Antarctic soils (8, 20).
121 The bacterial 16S rDNA sequences have been submitted to GenBank Database and the
122 accession number is MN365277-MN365297.

123

124 Similar to the total 16S community data of three different Adams Flat soil samples
125 determined via 454 pyrosequencing in (41), Actinobacteria were dominant here (Figure 1),
126 which is in line with knowledge of their inherent flexibility by virtue of harbouring wide
127 metabolic capacities and survival mechanisms (12, 15, 20), thus giving them the selective
128 advantage to dominate over other phyla in harsh environments. This phenomenon is not
129 exclusive to just arid deserts but many other extreme environmental settings such as volcanic
130 areas, dark caves and marine environments (43-45). The Actinobacteria community in these
131 extreme habitats were found to produce various broad-spectrum antibiotics, enzymes, anti-
132 tumor agents and immunomodulators. In fact, since discovery of the first antibiotic till today,
133 80% of the important drugs are sourced from members of Actinobacteria, mainly from the
134 genus *Streptomyces* (46).

135

136 The majority recovered isolates exhibited 99-100% sequence similarity to characterised
137 species (Table 1). Interestingly, potentially novel isolates were also recovered, including two
138 Bacteroidetes members, *Belliella* sp. (92% similarity), *Cesiribacter* sp. (96%) and a
139 Proteobacterium *Roseomonas* sp. (98%). The closest species match of isolates VN2, VN3 and
140 PR3 were *Belliella marina*, *Cesiribacter roseus* and *Roseomonas aquatica*, all of which are
141 gram-negative bacteria isolated from seawater, desert sand and drinking water, respectively
142 (47-49). The low sequence similarity (92%) of isolate *Belliella* sp. VN2 suggests that it may
143 represent a novel genus under the phylum Bacteroidetes. Further DNA-DNA whole genome
144 hybridisation, fatty acid analysis along with a range of biochemical tests are required to
145 confirm the novelty of these three new isolates.

146

147 Notable was the similarity of Soil 2 and Soil 3 in terms of the recovered taxa, whereas Soil 1
148 resulted in a distinct group of cultured members, primarily comprised of *Streptomyces* spp.

149 (Figure 1). Soil environmental factors are known to effect microbial community structure and
150 in fact, Soil 2 and Soil 3 had a similar alkaline pH of 8.7-8.8 (Supplementary Table 2)
151 whereas Soil 1 had a pH of 7.8.

152

153 The two soil samples (Soil 2 and 3) with higher soil fertility, defined as organic carbon,
154 nitrogen, and chloride content, had higher species richness over Soil 1. The diversity of
155 bacterial species recovered in Soil 2 and Soil 3, were substantially higher than Soil 1 cultures,
156 in which only two genera were successfully cultured. The limited microbial growth observed
157 for Soil 1 cultures, as shown by low colony counts, could be associated with the markedly
158 low phosphorus level ([Supplementary Table 1](#)), given that phosphorus is one of the vital
159 limiting nutrients in polar soils (9, 50). These results were consistent with previous work on
160 Antarctic soil biodiversity which used structural equation modelling to reveal that pH drives
161 microbial community structure in polar soil ecosystems, that is, which taxa would be present,
162 while soil fertility is positively correlated with bacterial species richness (9).

163

164 **HSL activity within Antarctic Soil Microbes.** Out of all 21 strain representatives recovered
165 from Adams Flat, only 17 isolates were tested for HSL activity due to lack of significant
166 growth in four isolates (*Hymenobacter* sp. S14, *Altererythrobacter* sp. VR2, *Cesiribacter* sp.
167 VN3 and *Roseomonas* sp. PR3) under the pre-set laboratory conditions for this study. Along
168 with the addition of eight Windmill Islands-recovered isolates, a total of 25 representative
169 species were subjected to bioassays to determine HSL activity (Table 2).

170

171 A diversity of presumptive HSL molecules was detected in 20/25 (80%) of grown to
172 stationary phase, a stage with high cell densities, that is associated with nutrient depletion and
173 accumulation of waste products that become toxic to the bacterial cells (51). Production of

174 these signalling molecules under unfavourable conditions suggest that they play a role in
175 regulating survival phenotypes. This explains why, production of secondary metabolites,
176 biofilm formation and virulence, usually occurs during stationary phase.

177

178 For Adams flat-recovered isolates, 13/17 (76%) of the tested bacterial isolates were positive
179 against at least one of the LuxI/LuxR-based QS biosensors (Table 2). Amongst the eight
180 Windmill Islands-recovered isolates, seven potential HSL producers were identified, three
181 belonging to Actinobacteria, with four others Proteobacteria. Biosensor CV026 only
182 produced violacein in response to *Pseudomonas aeruginosa* PAO1 culture (positive control),
183 but not with any other isolates that activated other biosensors used in this study. This could
184 be due to relatively low amounts of HSLs synthesised in the Antarctic cultures that has less
185 growth than cultures of temperate regions bacteria, especially CV026 was found to only
186 detect HSLs at higher concentrations (52).

187

188 Surprisingly, within all bacterial isolates displaying active compound production, 15 were
189 gram-positive bacteria spanning Actinobacteria and Firmicutes (Table 1). While HSL-based
190 QS is common in gram-negative Proteobacteria (53), to our best knowledge, there are only
191 four known reports of this QS system in gram-positive bacteria, two of which were of marine
192 origin (36, 37), one was from mangrove rhizospheres (38) and one isolated from microbial
193 mats (39). All the potential HSL producers isolated from these studies were also affiliated to
194 either the Actinobacteria or Firmicutes phylum. The widespread discovery of HSL activity
195 from bacteria spanning both gram-negative and gram-positive in Antarctic soils suggests
196 HSL-based QS systems may be used for communication by soil microbes.

197

198 It was previously thought that communication between gram-positive bacteria relies on short
199 peptides instead of HSLs as signalling molecules (54, 55) but, subsequently genes encoding
200 for peptide signals were found in gram-negative bacteria via *in silico* analyses (56) and HSL
201 activity has been reported in an expanding breadth of the gram-positive bacterial community
202 (36-39). With different species colonising the same habitat in proximity, chances of QS
203 regulatory cassette being transmitted between organisms via horizontal gene transfer (HGT)
204 are relatively high. This phenomenon suggested that bacteria may have co-evolved to be
205 multi-lingual with the presence of multiple QS systems, so that communication at multiple
206 distinct levels: intra-species, inter-species within the same genus, inter-species between
207 different genera and even inter-domain, is made easier (33-35).

208

209 **Profiling of presumptive HSL via TLC.** The extracted active compounds separated on thin
210 layer chromatogram were identified according to *R_f* values calculated based on distance
211 migrated by the molecules, along with tailing properties in comparison to a range of synthetic
212 HSL standards. Several active compounds appear on the TLC sheet after 8 h of incubation at
213 30 °C while some compounds were only visible after 18 h. These presumptive HSLs became
214 oversaturated at 18 hr, with several new compounds only becoming visible at this time point
215 (Figure 2). However, this does not reflect the concentration of a certain type of HSL being
216 produced as the biosensor *E. coli* MT102 has different sensitivities against different HSLs.
217 For instance, 0.009 ng of OHHL can induce green fluorescence by the biosensor while 113.2
218 ng of C12-HSL is required to induce the same amount of fluorescence (57). None of the 3-
219 oxo-derivatives, including those made synthetically, had tailing properties as proposed in
220 previous literature (58), except for N-(3-oxodecanoyl)-L-HSL (ODHL).

221

222 All detectable HSLs extracted in the present study ranged from C6- to C14-chain length. C7-
223 HSL appeared to be the potential signalling molecule of highest occurrence and was
224 produced by seven different isolates, affiliated to three phyla, namely, Actinobacteria,
225 Firmicutes and Proteobacteria (Table 1). A relatively long chain length may be required to
226 resist HSL hydrolysis that has been observed previously in alkaline conditions (59, 60),
227 especially given the alkaline pH of the soil in the present study (Supplementary Table 1). The
228 active compound produced by a Bacteroidetes isolate *Belliella* sp. VN2 was detected by
229 biosensor *A. tumefaciens* NTL4 but not by *E. coli* MT102, suggesting that the active
230 compounds may be 3-hydroxy-derivatives of HSL which cannot be detected by *E. coli*
231 MT102. However, it should be noted that confirmation of signal molecule identity would
232 entail employing mass spectrometry and nuclear magnetic resonance in follow-up studies.

233

234 Similar to HSLs detected in cultures of gram-negative bacteria, active compounds detected in
235 the cultures of gram-positive bacteria ranged from C6- to C11-HSLs. Gram-positive bacteria
236 that were identified as potential HSL-producers from previous studies (36-38) were found to
237 produce medium to long chain length active compounds ranging from C8 to C14-HSLs,
238 albeit the non-alkaline pH of their respective environmental setting, except for one
239 Actinobacterium isolated from a hypersaline microbial mat, which was found to produce C4-
240 HSL (39). Despite the relatively low detection of C4-HSL in various studies, it should be
241 noted that this does not necessarily suggest C4-HSLs are not in favour, in fact, these
242 compounds could simply be produced in concentrations ineffective for standard bioassays.

243

244 Members of Proteobacteria yielded the highest number of detectable compounds, with the
245 production of a minimum of four potential HSLs by *Paracoccus* sp. NBH48 and five
246 potential HSLs by *Novosphingobium* sp. NBM11. This is not surprising given that

247 LuxI/LuxR-based QS is common within Proteobacteria, and hence is able to make full use of
248 this communication system. The highest diversity of presumptive HSLs in Proteobacterial
249 isolates was also reported in another study (38), compared to members of Actinobacteria and
250 Firmicutes, which are relatively unfamiliar with this language. Despite multiple active
251 compounds produced by *Novosphingobium* sp. NBM11, they were not detectable with the
252 broad range-detectivity biosensor *A. Tumefaciens* NTL4.

253

254 **Genome sequences of Windmill Islands isolates.** Of the eight genomes examined, six were
255 high-quality assemblies, displaying high contiguity (L50=1; N50>3 Mb), and completeness
256 (>97% complete) (Supplementary Table 1). They were the *Streptomyces* sp. NBSH44,
257 *Sphingomonas* sp. NBWT7, *Pseudoarthrobacter* sp. NBSH8, *Klenkia* sp.. NBWT11,
258 *Novosphingobium* sp. NBM11, and *Frigoribacterium* sp. NBH87. The genomes of
259 *Paracoccus* sp. NBH48 and *Azospirillum* sp. INR13 diverged from high-quality indicator
260 range for completeness (83% and 65% completion, respectively), and exhibited
261 fragmentation (12 and 50 contigs, respectively) and low coverage (22 and 18, respectively).

262

263 **Genetic basis for QS in Antarctic isolates.** Despite active detection of signalling molecules
264 in bioreporter assays in the present study, LuxI/LuxR homologs were not found in genomes
265 of all these isolates. By searching for HSL synthase COG category COG3916 and the IPR
266 codes of domains associated with autoinducer synthase ([Supplementary Table 3](#)), we
267 identified one LuxI homolog each in the gram-negative Proteobacteria genomes of
268 *Paracoccus* sp. NBH48 (A1PzeaV1_2917) and *Novosphingobium* sp. NBM11
269 (A2NstyV1_00719). No *luxI* genes were found in any of the available genomes of gram-
270 positive Actinobacteria, despite active HSL production detected in the *E.coli* MT102
271 bioassay. The same scenario has been reported by (39), where no HSL synthase genes were

272 identified within gram-positive genomes of Actinobacteria and Firmicutes. The remaining
273 two Proteobacterial isolates (INR13 and NBWT7) that showed HSL activity in the
274 bioreporter assays, had no signs of *luxI* genes in their genomes, suggesting that these
275 LuxI/LuxR sequences may not be very well conserved in Antarctic isolates due to adaptation
276 and evolution under constant environmental stressors. For instance, there has been multiple
277 autoinducer synthase homologs found since the first discovery of LuxI in *Vibrio fischeri*.
278 These include AinS, LuxM and VanM (61, 62). However, none of these homologs were
279 found in the eight available genomes. These Antarctic isolates could also employ other QS
280 systems such as peptide-based signalling and use of autoinducer-2 signals to communicate,
281 but this study only focuses on LuxI/LuxR-based QS, therefore did not went further into these
282 QS systems.

283

284 Based on PROKKA-annotation, the LuxI homolog found in isolate NBM11
285 (A2NstyV1_00719) is an isovaleryl-HSL synthase, which synthesises an unusual branched-
286 chain HSL signal (29). Phylogenetic analyses (Figure 3) further suggest that the
287 *Novosphingobium* sp. M11 LuxI homolog may represent a novel autoinducer
288 synthase that does not produce the common acyl-HSL, as shown by it clustering with the
289 CoA-utilizing autoinducer synthases that encode for atypical HSL signals. This may explain
290 the lack of detection of HSL production by the biosensors CV026 and NTL4, which only
291 detect normal acyl-HSLs (28, 29, 63). However, this does not exclude the possibility that
292 HSL signals were produced in levels beyond biosensor NTL4 detection limits and thus
293 resulted in false negative. On the other hand, LuxI sequence retrieved from isolate
294 *Paraococcus* sp. NBH48 (A1PzeaV1_2917) clustered with that of *Paracoccus zhejiangensis*,
295 suggesting conservation within the same genus.

296

297 Multiple sequence alignments of putative *luxI* in *Paraococcus* sp. NBH48 (A1PzeaV1_2917)
298 and *Novosphingobium* sp. NBM11 (A2NstyV1_00719) against *Vibrio fischeri* resulted in low
299 identity similarity, 24.53% and 38.27%, respectively ([Supplementary Figure 2](#)). However,
300 the eleven critical residues reported to be key sites in LuxI family proteins (64): R-25, E-44,
301 D-46, D-49, D-60, G-67, R-70, R-104, A-133, E-150 and G-164, were conserved in these two
302 LuxI sequences, except for D-46, D-60, A-133 and E-150 in isolate NBH48. LuxI sequence
303 found in isolate NBM11 showed conservation in all the eleven key residues except for E-150,
304 which was reported to be less well-conserved (64). Thus, these LuxI homologs are still able
305 to carry out critical enzymatic activity required to catalyse HSL production regardless the low
306 conservation consensus and using a different substrate, coenzyme-A (CoA)-activated acid,
307 instead of the usual acylated-acyl carrier protein-activated acid to synthesise HSLs.

308

309 A total of 135 potential LuxR sequences were extracted by searching for LuxR-associated
310 IPR codes ([Supplementary Table 3](#)). However, only five of these, derived from three
311 genomes, were predicted to have the ability to bind HSL ligands as determined by COACH.
312 As such, these five sequences were the only LuxR sequences subjected to further analysis, in
313 order to exclude other non-LuxR sequences that were picked up during IPR code search due
314 to the common HTH domain they possess. LuxR homologs are problematic to retrieve from
315 full genomes due to the generic domain it possesses, a DNA-binding, helix-turn-helix (HTH)
316 domain which is also present in numerous non-LuxR regulators (65). Horizontal gene transfer
317 between microorganisms could further complicate genome mining as this could result in
318 increased variabilities (and thus decreased homology) in the LuxI/LuxR homologs due to
319 permutation and combination.

320

321 Within the three genomes in which LuxR homologs were found, three LuxR homologs were
322 in the gram-negative Proteobacteria isolates, *Paracoccus* sp. NBH48 (A1PzeaV1_2919 and
323 A1PzeaV1_02748) and *Novosphingobium* sp. NBM11 (A2NstyV1_00759), while two LuxR
324 homologs were derived from the gram-positive Actinobacteria member, *Klenkia* sp.
325 NBWT11 (A2MmarV1_04386 and A2MmarV1_04387).

326

327 Multiple sequence alignments of these putative LuxR homologs against *V. fischeri* displayed
328 conservation in a number of amino acid residues, including those ones that were identified as
329 important residues in LuxR regulatory activity (66, 67), such as W-66, Y-70, D-79, P-80, V-
330 109, G-117, G-121, L-183, T-184, R-186, E-187, L-191, A-192, G-197, I-203, I-206, L-207,
331 T-213, V-214, H-217, L-218, K-224 and R-230 (Supplementary Figure 3). Conservation of
332 these critical residues in *luxR* sequences of the gram-positive isolate *Klenkia* sp. NBWT11
333 showed that LuxR homologs of gram-positive bacteria are similar to that of gram-negative
334 bacteria. The high bootstrap values from maximum likelihood trees between putative LuxR
335 homologs of NBWT11 (A2MmarV1_04387) with TraR homologs of *Agrobacterium* spp.
336 (Bootstrap value 78), and (A2MmarV1_04386) with putative LuxR homologs from
337 *Sphingobium* sp. (WP_119748565.1) and *Novosphingobium* sp. (WP_043976500.1 and
338 A2NstyV1_00759) (Bootstrap value 81), further emphasised that horizontal gene transfer is
339 the likely source of acquisition of this QS cassette in gram-positive community (Figure 3).

340

341 **Potential traits regulated by LuxI/LuxR.** Genes in the vicinity of putative LuxI/LuxR
342 homologs were found to encode for functions that provide survival advantage. The *luxI/luxR*
343 cassette in NBH48 (A1PzeaV1_2917/A1PzeaV1_2919) were found in separate strands, in a
344 convergent direction $\overleftrightarrow{IR}$ (Table 3), with virulence and heavy metal transporter genes
345 downstream of each *lux* genes in their respective contigs. The virulence factor virB4 that was

346 identified here, has been shown to increase cell survival by inhibiting apoptosis upon stimuli
347 (68) and mediates plasmid conjugation (69). Mercury and copper transporters are known to
348 confer resistance to heavy metals that can be toxic to the bacteria. There was no LuxI
349 homolog found near the other LuxR homolog in NBH48 (A1PzeaV1_02748) but a
350 cardiolipin synthetase was adjacent to it, on the opposite strand. Cardiolipin has been
351 reported to mediate various signalling pathways (70), and thus, may be working in tandem
352 with this LuxR homolog in $\tilde{I}\tilde{R}$ arrangement.

353

354 The putative LuxI and LuxR homologs found in NBM11 were 42 000 nt apart, suggesting
355 they may not be working in tandem. The putative *luxI* in NBM11 (A2NstyV1_00719) were
356 flanked by several genes encoding for conjugal transfer proteins, which are essential for
357 passing on useful traits between microorganisms. No *luxR* gene was observed in vicinity to
358 the NBM11 *luxI*, but a HTH-type domain transcriptional regulator, MarR, was found
359 upstream. As for the NBM11 putative *luxR* (A2NstyV1_00759), a set of precursors of HSL
360 synthesis such as long chain fatty acid-CoA ligase and a few different acyl-CoA substrates,
361 were found upstream, in a $\tilde{R}\tilde{I}$ topology. Downstream were proteins involved in conjugation,
362 suggesting NBM11 *lux* genes were located on a conjugal plasmid, similar to *traI/traR* in *A.*
363 *tumefaciens* (71).

364 In NBWT11, two LuxR homologs (A2MmarV1_04386 and A2MmarV1_04387) were found
365 on the antisense strand in $\tilde{R}\tilde{R}$ topology. There was no LuxI homolog but an acetyl-CoA
366 synthetase and a spectinomycin and tetracycline transporter were located downstream of the
367 double LuxR homologs. This multiantibiotic efflux pump can confer antimicrobial resistance,
368 enhancing the bacteria competitive advantage (72). However, further experimental work is
369 required to determine if these genes were actively regulated by the adjacent *luxI/luxR* genes.
370 The lack of LuxI homolog but only precursors for HSL synthesis identified near the LuxR

371 homologs in NBWT11 suggests that this could potentially be a novel pathway of HSL-based
372 QS.

373

374 CONCLUSION

375 Bacteria surviving in East Antarctic soils are exposed to a number of environmental threats
376 that must drive a variety of survival responses that require cell-to-cell communication. Via
377 LuxR-based bioassays, this study reports profiles of the active signalling molecules produced
378 by both gram-negative and gram-positive bacteria within Antarctic soils, the latter which has
379 only been recently discovered to be capable of this communication system. As Antarctic
380 microbes represent a reservoir for novel functionalities and natural products, understanding
381 the basis of microbial communication, which is a pre-requisite to accomplish almost any
382 processes, is one of the first steps to manipulate microbes to our benefits. This study
383 has provided a platform for future studies further characterising the physiological adaptations
384 of microorganisms that allow them to survive under extreme Antarctic conditions.

385

386

387 MATERIAL & METHODS

388

389 **Sample Site and Description.** All soil samples were collected by the Australian Antarctic
390 Division (AAD), between 2005 and 2012, and were stored at -80 °C until further analysis (9,
391 73). This includes nine sites across East Antarctica spanning two different regions, the
392 Windmill Islands and Vestfold Hills ([Supplementary Figure 1](#)). Soils from Vestfold Hills are
393 hyper-arid as compared to arid soils in the Windmill Islands. This means Vestfold Hills have
394 lower levels of organic carbon, nitrogen and moisture (9, 41, 74). As no cultivation studies
395 have been performed for Adams Flat (68°33'S, 78°1'E), three soil samples, (designated as

396 Soil 1, 2 and 3 in this paper) that exhibited a diversity in measured soil factors, of organic
397 chemical contents and physical parameters including pH and moisture were selected for
398 cultivation ([Supplementary Table 1](#)).

399

400 **Cultivation onto Standard Media.** Soil inocula were prepared from the three Adams Flat
401 soil samples by adding 1 g soil to 5 ml 0.9% sterile saline. The soil suspension was serially
402 diluted (1:5) three times and 100 µl of each serial dilution was plated onto ¾ nutrient agar
403 (OxoidTM). Culture plates were left to incubate at RT (22 °C) for 30 days. Pure cultures were
404 established for each morphologically different isolate by successive sub-culturing onto ¾
405 nutrient agar (OxoidTM). All isolates were preserved in cryovials (MicrobankTM, Pro Lab
406 Diagnostics) at -80 °C for future use.

407

408 **DNA extraction and 16S rDNA Gene Sequencing of Isolates.** DNA extractions were
409 performed using an in-house modified bead beating protocol based on the
410 FastPrep[®] homogenisation system (MP Biomedicals, California). A single colony was
411 transferred into a 2 ml microcentrifuge tube containing 600 µl autoclaved Milli-Q[®] water and
412 0.5 g of both the 0.1 mm and 0.5 mm diameter glass beads (MoBio Inc, Carlsbad). The
413 mixture was homogenized in FastPrep[®] 120 cell disrupter, at speed setting 6.0 for 40 sec.
414 Following incubation at 95 °C for 5 min, the sample was centrifuged at 20800 x g for 3 min
415 and 400 µl of the supernatant was transferred to a new tube. This crude DNA extract was then
416 purified via an ethanol purification protocol as described in (75). The primer set 27F and
417 1492R (76) was used to amplify the bacterial conserved 16S rDNA genes in the extracted
418 DNA samples prior to sequencing via the Sanger method at the Ramaciotti Centre for Gene
419 Function Analysis (UNSW Sydney, Sydney). Obtained sequences were trimmed before

420 comparisons with reference sequences in the National Centre for Biotechnology Information
421 (NCBI) nucleotide database using BLASTn search tool.

422

423 **Data Availability.** The bacterial 16S rDNA sequences have been submitted to GenBank
424 Database and the accession number is MN365277-MN365297.

425

426 **Preliminary Screening for HSL-Producing Strains.** The bioreporter strains used
427 were *C. violaceum* CV026 (detects C4-, C6-, C8-, 3-oxo-C6- and 3-oxo-C8-HSLs) (52), *A.*
428 *tumefaciens* NTL4 (pZLR4) (detects C6 - C14-HSLs, all 3-oxo-HSLs, and 3-hydroxy-HSLs
429 of C6 – C10) (77) and *E. coli* MT102 (pjBA132) (detects C4 – C12-HSLs, 3-oxo-HSLs of
430 C6, C8 and C10) (57). The positive control used was *Pseudomonas Aeruginosa* PAO1. All
431 the above strains were kindly gifted by Dr. Onder Kimyon (School of Civil and
432 Environmental Engineering, UNSW, Sydney). Antibiotics (Sigma-Aldrich) were used at the
433 following concentrations: for *E. coli* MT102, Ampicillin (100 µg/ml); for *A.*
434 *tumefaciens* NTL4, Spectinomycin (50 µg/ml), and Tetracycline (4.5 µg/ml);
435 for *C. violaceum* CV026, Kanamycin (20 µg/ml).

436

437 HSL activity of bacterial isolates was tested using the biosensors *C. violaceum* CV026 and *A.*
438 *tumefaciens* NTL4 via the cross-feeding plate assay as described in (78), and by *E.*
439 *coli* MT102 green fluorescence protein (GFP)-based biosensor via the well-plate assay as in
440 (39). Briefly, each test isolate was streaked side to side with the biosensors CV026 and
441 NTL4 on ¾ nutrient agar supplemented with 40 µg /ml X-gal (Invitrogen). Self-
442 streaks of each biosensors served as negative controls and a streak of *P.*
443 *aeruginosa* PAO1 with the biosensors served as a positive control. These cross-feeding plates

444 were incubated at RT for a week and were monitored for positive HSL activity as indicated
445 by visible purple or blue pigmentation, respectively.

446

447 For the bioassay against *E. coli* MT102, test isolates were grown in 25 ml of ¼ nutrient
448 broth (OxoidTM) cultures at RT while shaking at 100 rpm until stationary phase,
449 at an approximate OD₆₀₀ of 0.5 – 1.0. Absorbance at 600 nm was measured right after
450 inoculation (T= 0) and subsequently after every 24 h for 7 d. Cell cultures were subjected to
451 ethyl acetate extraction, followed by incubation with *E. coli* MT102, and analysis as per (39).

452

453 **Thin layer chromatography profiling of presumptive HSLs.** Isolates that returned positive
454 in the preliminary screening were grown in larger batches of culture (450 ml) to
455 extract higher concentrations of HSLs during stationary
456 phase, using the aforementioned ethyl acetate extraction method. Growth curves were
457 determined for all non-sporulating isolates by measuring absorbance at 600 nm starting from
458 inoculation point and subsequently every 24 h for 3 weeks.

459

460 Culture extracts of 4- 40 µl, depending of the concentration of the extracts, were spotted
461 onto non-fluorescent reverse-phase C18 TLC glass plates coated with a silica gel matrix
462 (Analtech TLC uniplates, Sigma-Aldrich). The chromatogram was developed with a HPLC-
463 grade methanol and Milli-Q[®] water mixture (60:40 v/v) as described by (58). After
464 development, the solvent was evaporated before being overlaid with the GFP-based
465 biosensor *E. coli* MT102. A 5 ml overnight culture of *E.coli* MT102 was inoculated in 75 ml
466 of LB broth and grown to late exponential phase by incubation at 30 °C with shaking at 150
467 rpm for 4 h. The entire 80 ml of culture was added to 120 ml of agar-enriched LB medium to
468 give a final agar concentration of 0.8% (w/v) before pouring over the dried

469 chromatogram. The TLC plates were incubated O/N at 30 °C before visualization of
470 the active compounds using a GE phosphorimager (Fujifilm LPB filter set, 473 nm
471 laser) at an 8-h-and 18-h time point.

472 Chain lengths of the presumptive HSLs were roughly estimated by comparing
473 the *R_f* values and the shape of spots with a range of N-acyl-L-homoserine lactones, including
474 N-butyryl (C4)-, N-hexanoyl (C6)-, N-heptanoyl (C7)-, N-octanoyl (C8)-, N-decanoyl (C10)-,
475 N-dodecanoyl (C12)-HSLs, N-(3-oxo-hexanoyl)-L-HSL (OHHL), N-(3-oxo-octanoyl)-L-HSL
476 (OOHL), N-(3-oxo-decanoyl)-L-HSL (ODHL) and N-(3-oxo-dodecanoyl)-L-HSL (OdDHL)
477 (Sigma-Aldrich).

478 **Bacterial genome sequencing and assembly for eight Antarctic isolates.** In addition to
479 Adams Flat bacterial isolates recovered in this study, eight isolates from the collection
480 of bacteria recovered from East Antarctic Windmills Islands region (Supplementary Table
481 1) were included in the HSL screening assays due to availability of full genome sequences (N.
482 Benaud, Unpublished data). Genomic DNA was sequenced using the PacBio RS II
483 instrument with P6/C4 chemistry, and *de novo* assembly performed using FALCON v 1.8.6
484 (79). Assemblies were joined and circularized using Circlator v1.4.0 (80), then subjected to
485 two rounds of consensus polishing via the Arrow algorithm of the GenomicConsensus
486 package tool variantCaller v.2.2.1 (79). Annotations were performed on contigs using Prokka
487 v1.13 (81). Protein sequences were phylogenetically classified into clusters of orthologous
488 groups (COGs), assigned via the COG functional annotator module of WebMGA
489 (<http://weizhong-lab.ucsd.edu/webMGA/>) (82, 83). Genome assemblies were quality assessed
490 for completeness and contamination using CheckM v1.0.7 (84).

491

492 **Genome mining for LuxI and LuxR homologs.** Potential LuxI- and LuxR-containing
493 sequences within the eight genome sequences of Windmill Isolates were extracted
494 from InterproScan v5.25-64.0 (85). InterProScan integrates data from various major protein
495 signature databases: CATH-Gene3D, CDD, HAMAP, PANTHER, Pfam, PIRSF, PRINTS,
496 ProDomBlast, PROSITE (Patterns and profiles), SFLD, SMART, SUPERFAMILY and
497 TIGRFAMs. These genomes were searched for conserved domains within autoinducer
498 synthases and LuxR-type transcriptional regulators ([Supplementary Table 3](#)). The genomes
499 were also searched for acyl-homoserine lactone (AHL) synthase COG category COG3916 .

500 Ligand-binding site of the potential LuxR homologs extracted from genomes were performed
501 with COACH software (86, 87). Following structural prediction of the receptor via the I-
502 TASSER suite, potential ligands were determined based on structure of the binding sites via
503 five methods (TM-SITE, S-SITE, COFACTOR, FINDSITE and ConCavity). These potential
504 LuxI and LuxR were aligned against *V. fishcherii* LuxI (P12747) and LuxR (P12746),
505 respectively using Clustal Omega (88). It employs an mBED algorithm to calculate seeded
506 guide trees and HMM profile-profile techniques to generate alignments between sequences.

507

508 Phylogenetic trees were built via maximum-likelihood method based on 1000 bootstrap
509 replications, via PhyML (89). Reference amino acid sequences were retrieved from
510 UniProtKB database (90). Sequences to be passed through PhyML were first aligned using
511 MUSCLE (91) in full processing mode.

512

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522

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524 No competing financial interest exist. The authors declare that there is no conflict of interest
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526

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791 **Table 1.** Identification of isolates based on 16S rDNA sequence analysis.

Phyla	Isolate	Closest Species Match	Identity (%)	Accession no.
Adams Flat Isolate				
Actinobacteria	VN1	<i>Arthrobacter flavus</i>	100	MF541537.1
	PN6	<i>Arthrobacter tumbae</i>	99.8	MK696231.1
	PN2	<i>Arthrobacter tumbae</i>	99.9	KY753209.1
	N2	<i>Streptomyces anulatus</i>	100	MK530154.1
	R8	<i>Streptomyces badius</i>	99.3	MH482909.1
	S10	<i>Streptomyces flavogriseus</i>	99.8	KX358684.1
	N3	<i>Streptomyces pratensis</i>	100	MK484235.1
	N1	<i>Streptomyces globisporus</i>	100	MF980904.1
	VN7	<i>Streptomyces xinghaiensis</i>	98.8	KC493988.1
Bacteroidetes	VN2	<i>Belliella marina</i>	92.1	NR145847.1
	VN3	<i>Cesiribacter roseus</i>	96.1	NR109022.1
	S14	<i>Hymenobacter roseosalivarius</i>	98.8	NR029359.1
Firmicutes	PN5	<i>Bacillus luteolus</i>	98.7	KY849443.1
	PS1	<i>Jeotgalibacillus marinus</i>	99.6	LR218079.1
	PN4	<i>Jeotgalibacillus marinus</i>	99.6	LR218079.1
	VN4	<i>Paenisporosarcina antarctica</i>	99.7	CP038015.1
	PN9	<i>Paenisporosarcina quisquiliarum</i>	98.7	MH392666.1
	PN1	<i>Paenisporosarcina quisquiliarum</i>	99.5	NR_043720.1
Proteobacteria	VN5	<i>Solicibacillus silvestris</i>	100	MH973485.1
	VR2	<i>Altererythrobacter confluentis</i>	99.3	NR152077.1
	PR3	<i>Roseomonas aquatica</i>	97.8	NR042501.1
Total	21			
Windmill Islands Isolate^a				
Actinobacteria	NBH87	<i>Frigoribacterium endophyticum</i>	98.9	NR_134732.1
	NBWT11	<i>Klenkia marina</i>	97.2	NR_156810.1
	NBSH8	<i>Pseudarthrobacter phenanthrenivorans</i>	98.6	NR_074770.2
	NBSH44	<i>Streptomyces finlayi</i>	99.9	NR_043354.1
Proteobacteria	INR13	<i>Azosprillum zeae</i>	98	NR_043934.1
	NBM11	<i>Novosphingobium stygium</i>	98.1	NR_040826.1
	NBH48	<i>Paracoccus carotinifaciens</i>	99.7	NR_024658.1
	NBWT7	<i>Sphingomonas jeddahensis</i>	98.9	NR_158139.1
Total	8			

792 ^aWindmill Islands isolates were previously recovered and had their full genome 16S rDNA sequenced (N.

793 Benaud, Unpublished data).

794 ^bPotential novel bacterial species recovered are in bold.

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Table 2. Response of isolates to biosensors and HSL profiling of culture extracts.

Bacterial strain		NTL4 ^a	CV026	MT102	Potential HSLs produced ^b
<u>Adams Flat Isolate</u>					
Actinobacteria	<i>Arthrobacter</i> sp. VN1	+	-	+	C6-, C9-, C11-
	<i>Arthrobacter</i> sp. PN6	-	-	+	C9-
	<i>Arthrobacter</i> sp. PN2	+	-	+	C8-
	<i>Streptomyces</i> sp. N4	-	-	-	-
	<i>Streptomyces</i> sp. R8	-	-	+	3-oxo-C6-, C7-
	<i>Streptomyces</i> sp. S10	-	-	+	3-oxo-C8-
	<i>Streptomyces</i> sp. N3	-	-	-	-
	<i>Streptomyces</i> sp. N1	+	-	+	3-oxo-C6-, C7-
	<i>Streptomyces</i> sp. VN7	+	-	+	C7-, C8-, C10-
Bacteroidetes	<i>Belliella</i> sp. VN2	+	-	-	N/A
	<i>Cesiribacter</i> sp. VN3	N/A	N/A	N/A	N/A
	<i>Hymenobacter</i> sp. S14	N/A	N/A	N/A	N/A
Firmicutes	<i>Bacillus</i> sp. PN5	+	-	+	C7-, C8-, C10-
	<i>Jeotgalibacillus</i> sp. PS1	-	-	+	C8-
	<i>Jeotgalibacillus</i> sp. PN4	+	-	+	3-oxo-C8-
	<i>Paenisporosarcina</i> sp. VN4	+	-	+	C7-
	<i>Paenisporosarcina</i> sp. PN9	-	-	-	-
	<i>Paenisporosarcina</i> sp. PN1	-	-	-	-
	<i>Solicibacillus</i> sp. VN5	+	-	+	C7-, C8-
	<i>Altererythrobacter</i> sp. VR2	N/A	N/A	N/A	N/A
Proteobacteria	<i>Roseomonas</i> sp. PR3	N/A	N/A	N/A	N/A
	PAO1 (+ve control)	+	+	+	3-oxo-C8, 3-oxo-C10, 3-oxo-C12
Total		9	0	12	
<u>Windmill Islands Isolate</u>					
Actinobacteria	<i>Frigoribacterium</i> sp. NBH87	-	-	+	C6-, C9-, C10-
	<i>Klenkia</i> sp. NBWT11	-	-	+	C6-
	<i>Pseudarthrobacter</i> sp. NBSH8	-	-	-	-
	<i>Streptomyces</i> sp. NBSH44	+	-	+	3-oxo-C8-
Proteobacteria	<i>Azospirillum</i> sp. INR13	+	-	+	C6-
	<i>Novosphingobium</i> sp. NBM11	-	-	+	C6-, C8-, 3-oxo-C10-, 3-oxo-C12-
	<i>Paracoccus</i> sp. NBH48	+	-	+	3-oxo-C6-, C9, C10, C11, 3-oxo-C14-
	<i>Sphingomonas</i> sp. NBWT7	-	-	+	3-oxo-C10-
Total		3	0	7	

798 N/A Isolates did not have significant growth to be included in this bioreporter assay

799 ^aPreliminary screening via plate bioassays using strains NTL4 & CV026; Small-batch ethyl

800 acetate extraction using strain MT102

801 ^bProfiling of potential HSLs derived from large-batch ethyl acetate extraction, using strain

802 MT102 as an agar overlay on TLC plates

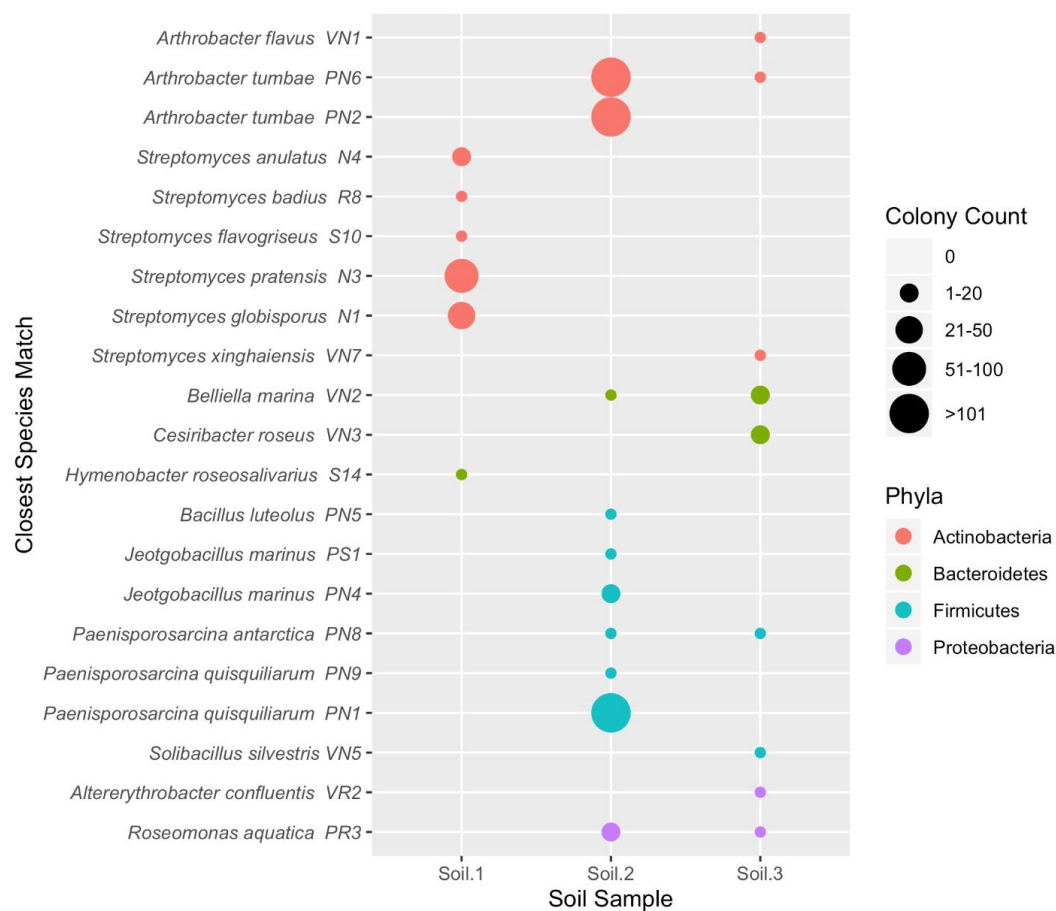
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Isolate	<i>luxI</i> locus tag	<i>luxI</i> position	<i>luxR</i> locus tag	<i>luxR</i> position	Gene topology	Genes downstream <i>luxI/luxR</i>
<i>Paracoccus</i> sp. NBH48	A1PzeaV1 _02917	10585.. 11214	A1PzeaV1 _02919	11565.. 12191	$\tilde{I}\tilde{R}$	Type IV secretion system protein virB4; Mercury & copper transporters
<i>Paracoccus</i> sp. NBH48	-	-	A1PzeaV1 _02748	27662.. 28333	$*\tilde{I}\tilde{R}$	Enzymes required in various sugar metabolism
<i>Novosphingobium</i> sp. NBM11	A2NstyV1 _00719	718438.. 719064	-	-	-	Conjugal transfer proteins
<i>Novosphingobium</i> sp. NBM11	-	-	A2NstyV1 _00759	760048.. 760800	$\tilde{R}\tilde{I}^*$	Conjugal transfer proteins
<i>Klenkia</i> sp. NBWT11	-	-	A2Mmar V1_04386	4465654. .4466271	$*\tilde{I}\tilde{R}\tilde{R}$	Spectinomycin and tetracycline efflux pump
<i>Klenkia</i> sp. NBWT11	-	-	A2Mmar V1_04387	4466278. .4466655		

804 **Table 3.** Topology of *luxI/luxR* genes in Windmill Island isolates.

805 *Only precursors for HSL synthesis were found, rather than actual LuxI homolog

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807

808 **Figure 1. Presumptive bacterial species cultured from all three Adams Flat soil**809 **samples.** Soil 2 and Soil 3, with higher total organic content compared to Soil 1, recovered

810 the highest diversity of taxa. As expected, Actinobacteria dominated the culturable fraction,

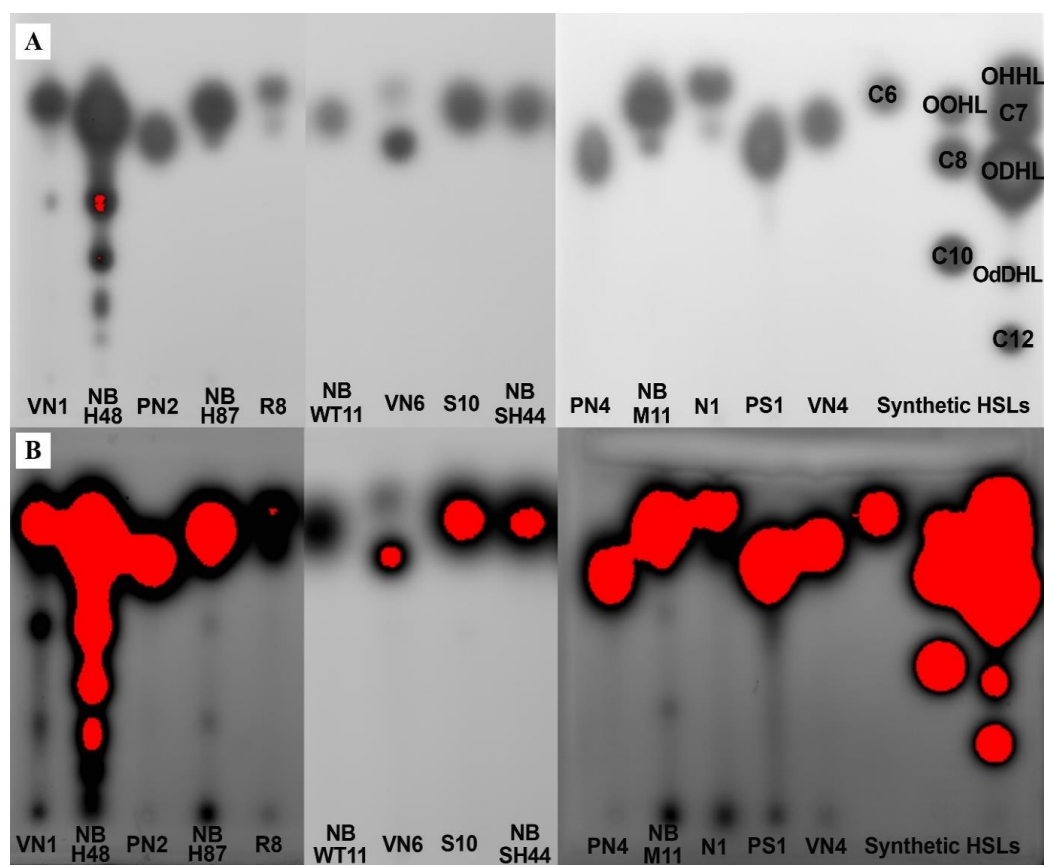
811 especially in Soil 1 and Soil 2.

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817 **Figure 2. Visualisation of active compounds with agar overlay of MT102 bioreporter**818 **incubated at 30 °C for (A) 8 h (B) 18 h.** Presumptive HSLs extracted from cell-free culture

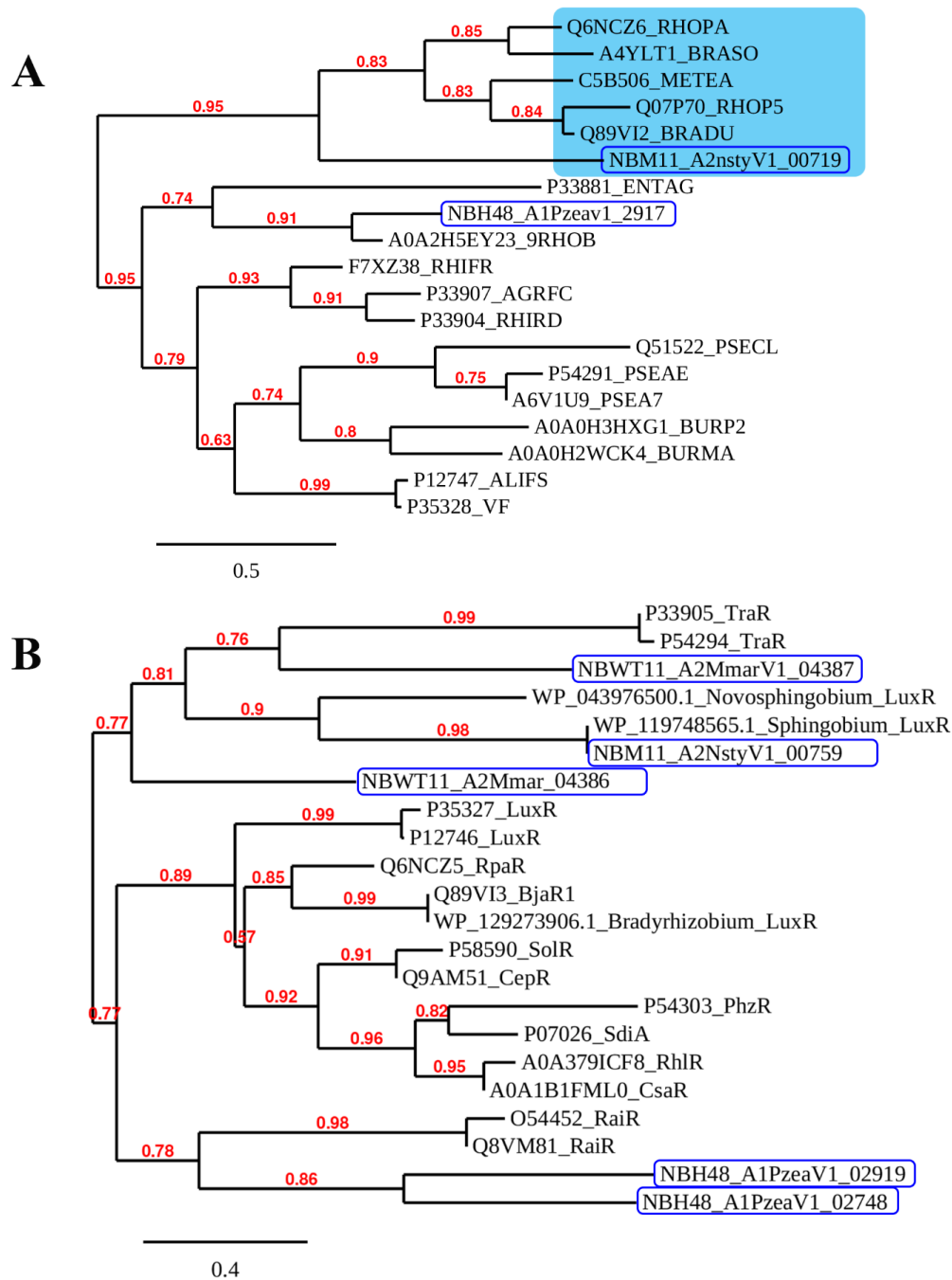
819 supernatants were separated by TLC developed with methanol/water (60:40 v/v). Culture

820 extracts for isolates and positive controls are presented (left to right).

821

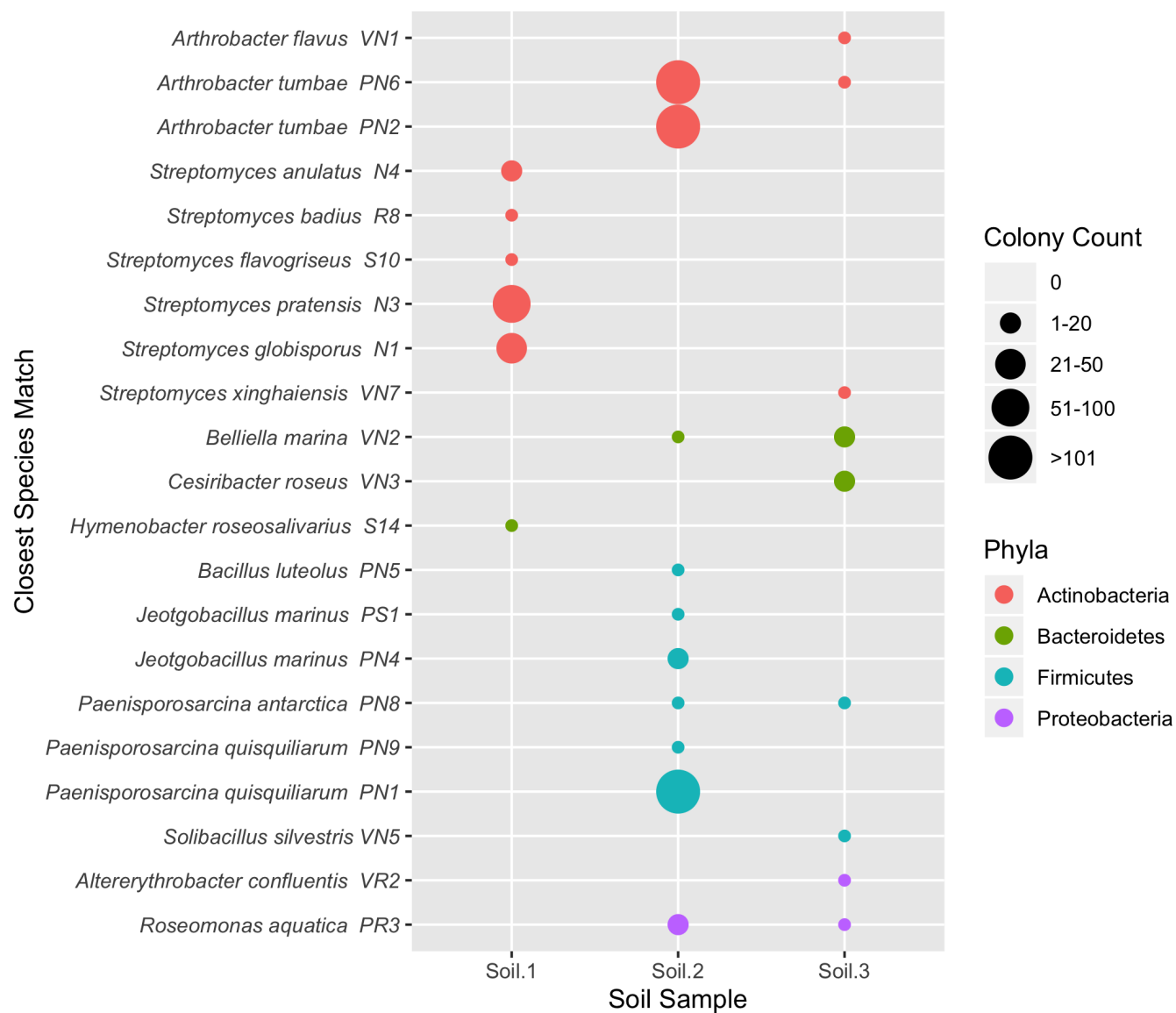
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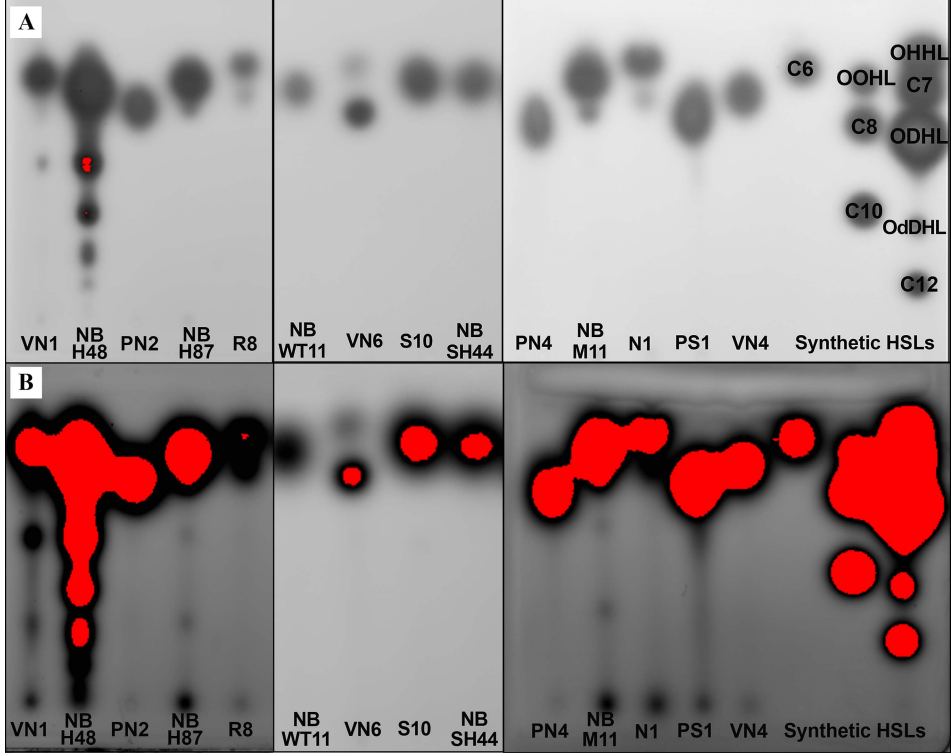
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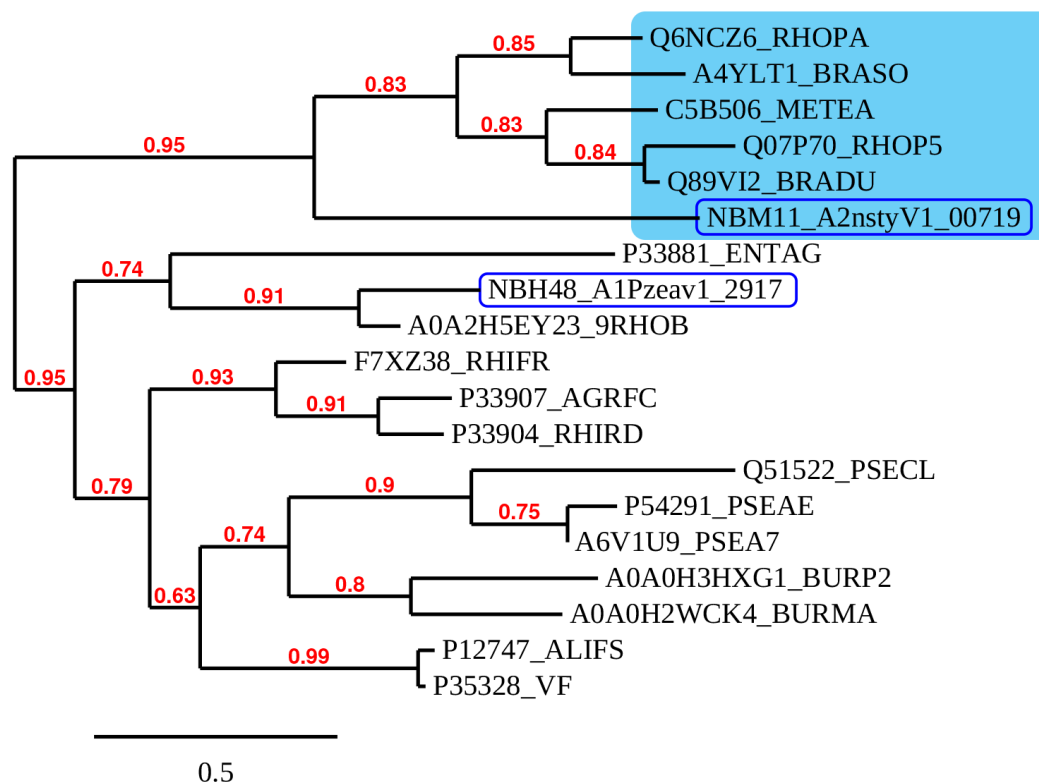


824 **Figure 3. Protein phylogeny of (A) HSL synthases (B) LuxR regulators.** The clade
825 shaded in blue are HSL synthases that produce atypical HSL signals from coenzyme-A
826 (CoA)-activated acids instead of the usual acylated-acyl carrier protein-activated acid. LuxI

827 and LuxR homologs retrieved from Windmill Islands isolate genomes are within blue boxes.
828 These maximum-likelihood trees were constructed with reference amino acid sequences of
829 HSL synthases and LuxR homologs retrieved from UniProtKB and NCBI database. Numbers
830 at nodes indicate bootstrap probabilities of 1000 bootstrap re-samplings. Bar, 0.5 and 0.4
831 substitutions per amino acid position for Phylogenetic tree (A) and (B), respectively.
832





A**B**