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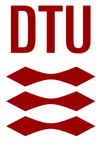

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1 **Influence of niche-specific nutrients on secondary metabolism in *Vibrionaceae*.**

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15
16 **Running title:** Bioprospecting in *Vibrionaceae*.
17 **Keywords:** bioprospecting, chitin, *Vibrionaceae*, antibacterial compounds
18
19 **List of abbreviations:** GlcNAc: N-acetylglucosamine; ChiS: Chitin catabolic cascade Sensor histidine
20 kinase; WGS: Whole Genome Sequence; SSBC Sea Salt Broth and Chitin medium WDA: Well
21 Diffusion Assay; CAS assay: Chrome Azurol S assay; AHL: Acyl-Homoserine Lactones.

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22 **ABSTRACT**

23 Many factors, such as substrate and growth phase, influence biosynthesis of secondary metabolites in
24 microorganisms. Therefore, it is crucial to consider these factors when establishing a bioprospecting
25 strategy. Mimicking the conditions of the natural environment has been suggested as a means of
26 inducing or influencing microbial secondary metabolite production. The purpose of the present study
27 was to determine how bioactivity of *Vibrionaceae* was influenced by carbon sources typical of their
28 natural environment. We determined how mannose and chitin as compared to glucose influenced the
29 antibacterial activity of a collection of *Vibrionaceae* strains isolated because of their ability to produce
30 antibacterial compounds, but that in subsequent screenings seemed to have lost this ability. The number
31 of bioactive isolates was two and 3.5 folds higher when strains were grown on mannose and chitin,
32 respectively, as compared to glucose. As secondary metabolites are typically produced during late
33 growth, potential producers were also allowed 1-2 days of growth before exposure to the pathogen.
34 This strategy led to three-fold increase in the number of bioactive strains on glucose and eight-fold
35 increase on both chitin and mannose. We selected two bioactive strains belonging to species where
36 antibacterial activity had not previously been identified. Using UHPLC-HRMS and bio-assay-guided
37 fractionation, we found that the siderophore fluvibactin was responsible for the antibacterial activity of
38 *Vibrio furnissii* and *Vibrio fluvialis*. These results suggest a role of chitin in the regulation of secondary
39 metabolism in vibrios and demonstrate that considering bacterial ecophysiology during development of
40 screening strategies will facilitate bioprospecting.

41

42 249 words

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43 **SIGNIFICANCE**

44 A challenge in microbial natural product discovery is the elicitation of the biosynthetic gene clusters
45 that are silent when microorganisms are grown under standard laboratory conditions. We hypothesized
46 that since the clusters are not lost during proliferation in the natural niche of the microorganisms, they
47 must, under such conditions, be functional. Here, we here demonstrate that an ecology-based approach
48 in which the producer organism is allowed a temporal advantage and where growth conditions are
49 mimicking the natural niche remarkably increases the number of *Vibrionaceae* strains producing
50 antibacterial compounds.

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51 **INTRODUCTION**

52 Following the first era of discover of bioactive compounds from natural sources, high throughput
53 screenings of compound libraries produced by combinatorial chemistry and rational drug design were
54 preferred over natural product discovery (1). Disappointingly, the discovery rate of this approach was
55 much lower than expected and the lack of new leads triggered a return to search for novel bioactive
56 molecules from microorganisms (1, 2).

57 Recent progress in genome sequencing and mining has demonstrated a significant number and degree
58 of diversity in microbial biosynthetic gene clusters. However, this potential can often not be unfolded
59 and detected under standard laboratory conditions (3, 4) and, today, one challenge in discovery of
60 natural products is to elicit these silent/cryptic biosynthetic gene clusters. The One Strain MAny
61 Compounds (OSMAC) method, where strains are cultivated in a range of growth conditions, has been
62 suggested as a solution (5).

63 Secondary metabolites are likely to play many different roles in natural bacterial behavior, including
64 antagonistic interactions and intercellular communication (6, 7). Hence, elicitation of the expression of
65 silent biosynthetic gene clusters could rely on re-creating the natural environmental conditions in the
66 research laboratory (8–10). With this in mind, Seyedsayamdost (11) demonstrated that two previously
67 silent biosynthetic gene clusters in *Burkholderia thailandensis* could be elicited by low concentrations
68 of molecules of microbial origin. Also, antibacterial compounds have been shown to be produced by
69 marine bacteria only when they were cultivated under conditions mimicking their natural intertidal
70 environment (12–14).

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71 Following the increasing interest in natural products from the marine environment during the last
72 decades of the 20th century, several groups are now pursuing methods for the identification and
73 production of natural product in marine microorganisms (15, 16). Our group took part in the global
74 marine research expedition Galathea 3 (http://www.galathea3.dk) with the aim of, on a global scale,
75 isolating marine bacteria with bioactivity potential. We cultured microorganisms on marine agar and
76 subsequently screened all colonies for antagonism against the fish pathogen *Vibrio anguillarum,* which
77 is very sensitive to antibacterial compounds produced by marine bacteria. We isolated approximately
78 three hundred bioactive *Vibrionaceae* strains (17). During re-screening, only 39 strains retained their
79 antagonistic activity (18). We isolated the potent antibiotics holomycin and andrimid from *V.*
80 *coralliilyticus* and *Photobacterium galatheae*, respectively (9, 18), as well as modulators of virulence
81 in *Staphylococcus aureus*, such as ngercheumicins F, G, H, I (19), nigribactin (20) and solonamide B
82 (21). However, we were challenged by the marked reduction in bioactivity during re-screening.

83 We reasoned that one cause for this loss of activity could be that significant secondary metabolite
84 production mostly occurs during the late exponential and in the stationary phase of microbial growth,
85 and we hypothesized that the bio-discovery rate could be increased if the producing organisms were
86 allowed more time to grow before being exposed to the target organism. In the initial screening and
87 isolation, colonies were allowed to grow for 3-5 days before being tested (17), but this temporal
88 advantage was not given during the re-screening (18). We also questioned whether the use of naturally
89 co-occurring substrates such as mannose and chitin would restore bioactivity. Mannose is ubiquitous in
90 the marine environment where it is commonly used by algae for protein glycosylation and production
91 of extracellular polysaccharides (22, 23). Chitin is the most abundant organic molecule in the marine
92 environment, being a component of the exoskeleton of crustacean and zooplankton (24). It is a

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93 polysaccharide composed of *N*-acetylglucosamine (GlcNAc) units. *Vibrionaceae* are considered among
94 the major actors in marine chitin catabolism and the chitin utilization pathway is conserved within the
95 family (25, 26). In *V. cholerae* chitin and derivatives can regulate the expression of genes involved in
96 chitin metabolism (27) but also in biofilm formation and in virulence (28). In *V. coralliilyticus*, growth
97 on chitin doubles the yield of the antibiotic andrimid in comparison to glucose (9).

98 The aim of this study was to determine to which extent the use of substrates naturally present in the
99 niche of isolation and the growth phase of the producer could restore (or induce) the biosynthesis of
100 antibacterial compounds in a collection of 295 *Vibrionaceae* isolates. The number of antagonizing
101 strains was greatly increased when the assay was performed on chitin and up to eight folds higher when
102 the potential producers were given a temporal advantage over the target strain.

103

104 **MATERIAL AND METHODS**

105 **Bacterial strains.** Two hundred and ninety-five *Vibrionaceae* strains were isolated during the Danish
106 Galathea 3 global research expedition (17). Strains were selected based on their ability to inhibit the
107 growth of *Vibrio anguillarum* and identified as *Vibrionaceae* based on their 16S rRNA gene sequences
108 (17). Species affiliation of strains producing antibacterial extracts (see below), which had not been
109 previously assigned to a species by multilocus sequence analysis, was carried out by analysis of the *fur*
110 gene (29). The *fur* gene sequences were retrieved from whole genome sequences (WGSs) or
111 sequencing of PCR products obtained as described elsewhere (29).

112 **Preparation of colloidal chitin.** Colloidal chitin was prepared following a modified version of the
113 method published by Hsu and Lockwood (30). Ten grams of practical grade shrimp shell chitin (Sigma

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114 C9213) was added to 400 mL of 37% HCl at 4°C and stirred at this temperature for 6 hours. The
115 solution was poured into 4 L of cold H_2O and incubated overnight at 4°C, before it was neutralized
116 with solid NaOH. After centrifugation (6000 g for 10 minutes), supernatant was discarded and the
117 chitin pellet was suspended in 500 mL of H_2O and autoclaved. The concentration of colloidal chitin
118 was calculated from the dry weight (100°C) of a subsample.

119 **Screening of *Vibrionaceae* strains for antibacterial activity.** Square Petri dishes containing 20 g/L
120 Sea Salts (Sigma S9883), 3 g/L casamino acids (BD 223050), 15 g/L agar (AppliChem A0949) and
121 either 2 g/L of colloidal chitin or 2 g/L of mannose were prepared. As control, the same was done with
122 the same medium used in the original screening procedure (30 g/L Instant Ocean, 3 g/L casamino acids,
123 4 g/L glucose, 10 g/L agar) (17). Bacterial strains were grown overnight, aerated (200 rpm) at room
124 temperature in half strength YTSS (½ YTSS) (31). One microliter of each culture was spotted onto the
125 three media. On each plate 35 strains were spotted in rows, where the distance between two strains was
126 20 mm horizontally and 15 mm vertically. Each plate was produced three times. On one plate, 1 µL of
127 an overnight culture of the target strain *Vibrio anguillarum* 90-11-287 grown in ½ YTSS was spotted
128 simultaneously at a distance of 5 mm from the potential producers of antimicrobial compound. On the
129 second copy of each plate an identical process was performed after 24 hours and on a third plate after
130 48 hours. Plates were incubated at 25°C and examined 24/48 hours after the target strain had been
131 spotted. A biological replicate was performed for the isolates being bioactive in the first screening.

132 ***In silico* analysis of the distribution of *chiS* and $(GlcNAc)_2$ operon.** The *chiS* (VC0622) gene and the
133 $(GlcNAc)_2$ operon (VC0611-VC0620) of *Vibrio cholerae* were searched against a custom-built

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134 database using MultiGeneBlast (32). For the preparation of the database, genome sequences were
135 downloaded from the GenBank database (Figures 2 and 3).

136 **Extraction of bioactive compounds from liquid cultures.** All strains showing a consistent bioactivity
137 were grown aerated (200 rpm) in 10 ml of 2% Sigma Sea Salts solution with 0.3% casamino acids and
138 0.2% colloidal chitin (SSBC) for 48 hours at 25°C. Cultures were extracted with an equal volume of
139 HPLC-grade ethyl acetate (EtOAc) for 20 minutes. The organic phase was transferred to fresh vials and
140 evaporated until dryness under a stream of nitrogen. Extracts were dissolved in 250 µL methanol
141 (MeOH) and stored at -20°C until further analysis. The activity of the extracts against *Vibrio*
142 *anguillarum* 90-11-287 was tested in a well diffusion agar (WDA) assay (33).

143 **Genome sequencing and bioinformatics analysis.** High purity DNA was obtained for *V. furnissii*
144 S0821 and *V. fluvialis* S1110 by repeated phenol:chloroform:isoamyl alcohol purification followed by
145 RNase treatment and DNA precipitation, as described previously (34). Quantification was performed
146 on a NanoDrop Spectrometer (Saveen Werner, Sweden) and a Qubit 2.0 Analyzer (Invitrogen, United
147 Kingdom). Construction of 500 bp libraries and 100 bp paired-end sequencing of genomes were
148 performed by the Beijing Genome Institute (Hong Kong) on a HiSeq2000. Sequencing data were
149 assembled to contigs in CLC Genomic Workbench (CLC Bio, Aarhus, Denmark) using the *de novo*
150 assembly algorithm. The draft genomes of strains S0821 and S1110 were annotated with the NCBI
151 Prokaryotic Genome Annotation Pipeline (PGAP) (35) and submitted to antiSMASH 2.0 (36) and
152 BAGEL3 (37) for analysis of biosynthetic gene clusters.

153 **UHPLC-HRMS.** Ultra-high Performance Liquid Chromatography-High Resolution Mass
154 Spectrometry (UHPLC-HRMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent

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155 Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Separation was obtained
156 on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 µm) with a linear gradient
157 consisting of H_2O (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B
158 and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and
159 remaining for 3 min (0.35 mL/min, 60 °C). An injection volume of 1 µL was used. MS detection was
160 performed on either an Agilent 6545 QTOF MS equipped with Agilent Dual Jet Stream electrospray
161 ion source with a drying gas temperature of 160 °C, gas flow of 13 L/min, sheath gas temperature of
162 300 °C and flow of 16 L/min, or an Agilent 6540 QTOF MS equipped with Agilent Dual Jet Stream
163 electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 L/min, sheath gas
164 temperature of 300 °C and flow of 12 L/min. Capillary voltage was set to 4000 V and nozzle voltage to
165 500 V. Mass spectra were recorded at 10, 20 and 40 eV as centroid data for *m*/*z* 85–1700 in MS mode
166 and *m*/*z* 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Lock mass solution in 70:30
167 MeOH:H_2O was infused in the second sprayer using an extra LC pump at a flow of 15 µL/min using a
168 1:100 splitter. The solution contained 1 µM tributylamine (Sigma-Aldrich) and 10 µM Hexakis(2,2,3,3-
169 tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The $[M + H]^+$
170 ions (*m*/*z* 186.2216 and 922.0098 respectively) of both compounds was used.

171 **Influence of culture conditions on bioactivity and characterization of the antibacterial**
172 **compound.** Extracts from the cultures *V. furnissii* S0821 and *V. fluvialis* S1110 were analyzed by
173 UHPLC-HRMS as described above. Extracts from the strains grown in SSBC supplemented with 0.1
174 g/L ferric citrate were also prepared and analyzed. For the bioassay-guided fractionation, fifty cultures
175 of strain S0821 grown in 10 mL SSBC for 48 hours were extracted with equal volume of EtOAc,
176 extracts were pooled together and evaporated until dryness under nitrogen. Portions of the pooled

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177 S0821 culture extracts were fractionated by Mixed-Mode Anion Exchange SPE on an Oasis MAX
178 cartridge (Waters, Milford, MA, 30 µm, 30 mg, 1 mL). The sample was dissolved in 400 µL of 3:1
179 H_2O:MeOH containing 2% ammonium hydroxide then directly loaded onto a conditioned SPE column.
180 The column was sequentially eluted with 2 mL of 3:1 H_2O:MeOH (F1), 2 mL of 1:1 H_2O:MeOH (F2),
181 2 mL of MeOH (F3), 1 mL of H_2O and 1 mL of 3:1 H_2O:MeOH containing 1% formic acid (F4), 2 mL
182 of 1:1 H_2O:MeOH containing 1% formic acid (F5) and finally 2 mL of MeOH containing 1% formic
183 acid (F6). The fractions were dried under a stream of nitrogen before being resuspended in 200 µL
184 MeOH. Fractions were tested for antibacterial activity in a WDA assay and for siderophore activity in a
185 Chrome Azurol S (CAS) assay (38). Extracts were mixed with CAS solution in 1:1 ratio and the color
186 change from blue to orange, indicating siderophore activity, was checked after 15 minutes and 24
187 hours.

188 **Nucleotide sequences accession numbers.** Sequence data generated in this study were deposited in
189 GenBank under accession numbers LKHS00000000 (WGS of strain S0821), LKHR00000000 (WGS of
190 strain S1110) and KT952522-26 (*fur* gene sequences of strains S1162, S1732, S2054, S2056 and
191 S2150, respectively).

192

193 **RESULTS**

194 **Screening of strains for antibacterial activity.** Of the 295 *Vibrionaceae* strains, four isolates
195 antagonized *V. anguillarum* when grown on the glucose medium, six when grown on the mannose
196 medium and eleven when grown on the chitin medium using a procedure where the potential producers
197 were not given any temporal advantage over the target strain. When the target strain was spotted

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198 twenty-four hours after the potential bioactive strains, six isolates were bioactive on glucose, nineteen
199 on mannose and seventy-eight on chitin. Twenty-six, forty nine and ninety-one strains were bioactive
200 on glucose, mannose and chitin, respectively, when the target strain was spotted with a forty-eight hour
201 delay (Figure 1). Examples of one plate and of the behavior of one strain (*V. furnissii* S0821) over time
202 on the mannose and the chitin-based media are shown in Figure S1.

203 Ethyl acetate extracts from the 91 antagonizing strains grown in chitin containing liquid medium for 48
204 hours were tested in a well-diffusion assay against *V. anguillarum*. Extracts from *V. coralliilyticus*
205 (strains S2043, S2052, S2054, S2056 and S4053), *V. nigripulchritudo* (S2601, S2600 and S2604), *V.*
206 *fluvialis* (S1110 and S1162), *V. furnissii* (S0821) and two *Vibrio* sp. (S1732 and S2150) inhibited the
207 growth of *V. anguillarum* (Table 1). The strongest inhibition (i.e. the largest inhibition zone) was
208 observed in extracts from the *V. coralliilyticus* strains. The extracts from the *V. furnissii* and *V. fluvialis*
209 strains were moderately growth inhibitory based on the size of the clearing zone. The remaining
210 extracts exhibited a weak antibacterial activity.

211 **Distribution of *chiS* and (GlcNAc)$_2$ operon.** Given the pronounced increase in bioactivity when chitin
212 was used as growth substrate, we speculated that this could be due to simple substrate change (e.g.
213 catabolite repression) or to a direct involvement of chitin in the regulation. Since chitin is indeed
214 involved in regulation of phenotypes in *Vibrio* species (27, 39–41), we addressed the possible chitin-
215 dependent regulation of secondary metabolism in *Vibrionaceae*, possibly through the ChiS regulatory
216 system (see discussion). Therefore, we investigated the distribution of the *chiS* gene and of the
217 (GlcNAc)$_2$ operon in thirty-three genomes of vibrio species belonging to eight of the seventeen
218 proposed *Vibrio* clades (42) and to three of the four proposed *Photobacterium* clades (42). In total,
219 twenty-two *Vibrio* and eleven *Photobacterium* genomes were included in the analysis. This choice was

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220 driven by the quantity and the quality of the publicly available genome sequences. MultiGeneBlast-
221 based analysis showed that the *chiS* gene and the complete $(GlcNAc)_2$ operon are widely distributed in
222 both *Vibrio* and *Photobacterium* species, both being present in all analyzed species (Figure 2 and 3).

223 **Genome mining of *Vibrio furnissii* and *Vibrio fluvialis*.** Contig-based draft genomes of *V. furnissii*
224 S0821 and *V. fluvialis* S1110 were obtained by assembling the sequencing data in CLC Genomics
225 Workbench. The genome size was 5.0 Mb for *V. furnissii* S0821 and 4.5 Mb for *V. fluvialis* S1110.
226 antiSMASH analysis of the genomes found six putative biosynthetic gene clusters in *V. furnissii* S0821
227 and five in *V. fluvialis* S1110 (Table 2). Due to the phylogenetic relatedness of *V. furnisii* and *V.*
228 *fluvialis* (42) and the similarity of the antiSMASH results for the two strains, we thought it likely that
229 the antibacterial activity of the two extracts could be due to the same compound(s).

230 Both genomes harbored a biosynthetic gene cluster for the production of the quorum sensing auto-
231 inducer molecules acyl-homoserine lactones (AHLs) and biosynthetic gene clusters with a relatively
232 high gene similarity to those for the biosynthesis of ectoine, vibriobactin and aryl polyenes. A cluster
233 for bacteriocin production was identified in both strains, but the bacteriocin prediction tool BAGEL3
234 was not consistent with the antiSMASH results. Although BAGEL3 did predict the presence of one
235 bacteriocin gene cluster, it differed from the one predicted by antiSMASH. A BLAST-based homology
236 search using the bacteriocin amino acid sequences predicted in the two genomes (Table S1) as queries
237 revealed a high similarity (E value=0, homology>98%) with endopeptidases from the M23 superfamily
238 involved in cell wall biogenesis.

239 **Investigation on the antibacterial compound produced by *Vibrio furnissii* and *Vibrio fluvialis*.**
240 There are no reports in the literature describing antibacterial compounds in *V. furnissii* and *V. fluvialis*.

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241 Given the importance of these two species as human pathogens (43, 44), we focused on these strains to
242 determine the nature of the compound(s) responsible for the activity. Working under the hypothesis
243 that these closely related species likely produced similar antimicrobial compounds, the bioactive
244 extracts were dereplicated through a two phase approach: first, by comparison with extracts from
245 cultures of related strains, which did not display bioactivity in the well diffusion assay. Compounds
246 that were found in both the active and inactive strains were assumed to not be responsible for the
247 observed antibacterial activity. The remaining unassigned compounds were further dereplicated by
248 searching for all known compounds produced by *Vibrio* species found in AntiBase 2012, MarinLit
249 2012 and The Dictionary of Natural Products. Analysis of the dereplicated UHPLC-HRMS data
250 revealed the presence of an abundant compound with ions at *m/z* 623.2342 $[M+H]^+$ and 645.2158
251 $[M+Na]^+$ in extracts from cultures of *V. furnissii* S0821 and *V. fluvialis* S1110 which was tentatively
252 identified as the siderophore fluvibactin based on the accurate mass (mass deviation 0.96 ppm).
253 Subsequent MS/MS analysis, comparison with the literature UV spectrum as well as isolation and
254 NMR analysis confirmed this assignment (Figures S2-S5 and Tables S2-S3). The UHPLC-HRMS
255 analysis also found another abundant ion with *m/z* 404.1818, which was assigned to the known
256 compound 4 (*N*,*N*-bis-(2,3-dihydroxybenzoyl)-norspermidine (mass deviation 0.49 ppm) (Figure 4A
257 and 4B).
258
259 These compounds (fluvibactin and compound 4) were not detected when extracts were prepared from
260 *V. furnissii* S0821 and *V. fluvialis* S1110 grown in chitin medium supplemented with 0.1 g/L of ferric
261 citrate (Figure S6). These extracts were not inhibitory against *V. anguillarum* 90-11-287 (Figure S7).
262 The bioactive extract was then divided into fractions by Mixed-Mode Anion Exchange SPE. Only the
263 extract fraction containing the putative fluvibactin was inhibitory to *V. anguillarum* (Table 3 and

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264 Figure S8). A Chrome Azurol S assay performed on the same fraction confirmed the siderophoric
265 nature of the compound (Table 3). The use of anion exchange chromatography allowed for the
266 separation of fluvibactin from *N*-(3-oxo-decanoyl-*L*)-homoserine lactone (O-C10-HSL), which co-
267 eluted under the reverse phase conditions used for UHPLC-HRMS analysis. Fractions containing the
268 AHL (F3) (Figure S6) did not show bioactivity and the AHL was also found to be present in non-
269 bioactive iron supplemented cultures (Figure S7). O-C10-HSL was identified based on accurate mass,
270 retention time and the characteristic homoserine fragment ion at m/z 102.0549 ion (45).

271

272 **DISCUSSION**

273 We investigated to what extent culture parameters could affect (restore or induce) the production of
274 antibacterial compounds in a collection of marine *Vibrionaceae* whose members were initially isolated
275 based on their ability to antagonize the fish pathogen *V. anguillarum*. However, in later re-screenings,
276 only approximately 10% of them retained the activity. With the use of substrates typical to the natural
277 niche of isolation and allowing potential producer strains to reach a late growth phase, we could restore
278 the bioactivity in one third of the strains. Allowing *V. fluvialis* and *V. furnissii* to reach a late growth
279 phase before exposure to the target strain led to the identification of the siderophore fluvibactin as
280 responsible for their antibacterial activity.

281 Different carbon sources can lead to significantly different profiles in microbial secondary metabolism
282 (5, 9, 46). In our investigation, we used three molecules (glucose, mannose and chitin) that are
283 abundant in the marine environment (22, 24) as substrate for marine *Vibrionaceae*. The number of

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284 bioactive (antibacterial) strains was nearly two and 3.5-fold higher when mannose and chitin were used
285 as carbon-source, respectively, as compared to glucose.

286 The high efficacy of chitin in restoring (or inducing) the production of antibacterial compounds in the
287 tested strains is in agreement with the ecology and lifestyle of *Vibrionaceae* that are adapted to live in
288 marine niches richer in this polysaccharide than in other carbohydrates (25, 26). Indeed, vibrios are
289 well known for their association with chitin-rich biotic surfaces, such as zooplankton (24, 47).
290 Chitinase genes and the chitin utilization pathway are conserved in *Vibrionaceae* (25, 26), and natural
291 competence is induced by chitin in *V. vulnificus* (48) and *V. cholerae* (49). In the latter, chitin affects
292 also chitin catabolism (27), biofilm formation and virulence (28, 50).

293 Chitin-dependent regulation of secondary metabolism mediated by the transcriptional regulator DasR
294 occurs in the soil bacterium *Streptomyces coelicolor* A3(2) (51). In vibrios, one possible mechanism for
295 a similar regulation could be through the two-component histidine kinase sensor ChiS, which has been
296 characterized in *Vibrio cholerae* and is activated by chitin derived oligosaccharides (27). In the
297 proposed model, a putative cognate receptor regulates the expression of target genes involved in the
298 above-mentioned phenomena (27). Hunt and colleagues (25) suggested that genes with high homology
299 to *chiS* (VC0622) and to some of the genes from the downstream $(GlcNAc)_2$ operon (VC0611-VC0613
300 and VC0616-VC0619) are widespread among *Vibrionaceae*. However, their genome analysis included
301 a limited number (ten) of species, possibly due to low availability of genome sequences at the time the
302 study was conducted. We performed a broader analysis and showed that, indeed, both *chiS* and the
303 complete $(GlcNAc)_2$ operon (VC0611-VC0620), which were detected in all analyzed genomes, are
304 very conserved and maintain their topological organization in *Vibrio* and *Photobacterium* species
305 (Figures 2 and 3). The $(GlcNAc)_2$ operon includes the gene encoding for the periplasmic $(GlcNAc)_2$

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306 binding protein which inactivates ChiS when chitin is not present in the environment (27). Given its
307 importance in *V. cholerae*, such a degree of conservation of genes hypothesized to be involved in the
308 ChiS regulatory system in *Vibrionaceae* indicates that chitin could serve a regulatory role in the whole
309 family. Certainly, chitin-dependent regulation of phenomena such as biofilm formation and
310 biosynthesis of antibacterial compounds would be advantageous during competition for nutrients with
311 other microorganisms in the marine environment.

312 Although the use of chitin restored or induced the production of antibacterial compounds in
313 approximately one third of the isolates, this approach was not effective with the majority of the strains,
314 even when they were allowed longer time before exposure to the target strain. Induction of
315 silent/cryptic biosynthetic gene clusters has been achieved by exposing bacteria or fungi to small
316 molecules produced by naturally co-occurring microorganisms (11, 52, 53). Similarly, it is likely that
317 molecules that were present in the local seawater used to prepare the medium for the original
318 screening/isolation procedure or that were produced by strains that were tested on the same plate ("co-
319 cultivated") might have elicited the biosynthesis of antibacterial compounds. Co-cultivation could
320 therefore also be a strategy to be used to induce the production of antibacterial compounds in our strain
321 collection.

322

323 Extracts of cultures from *V. furnissii* S0821 and *V. fluvialis* S1110 had antibacterial activity against *V.*
324 *anguillarum*. The bioactivity was present in all tested media, however on chitin the antagonistic activity
325 could be observed earlier than on the other media (data not shown). Genome analysis of the strains
326 provided a list of four biosynthetic gene clusters potentially responsible for the biosynthesis of the

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327 antibacterial compound. Three of them (AHL, ectoine and arylpolyenes) could be excluded as the cause
328 of bioactivity through testing of pure standards in WDA and analysis of UV/Vis spectra of the extracts
329 (Figures SI8- SI9).

330 The remaining predicted biosynthetic gene cluster had 72% gene similarity to the biosynthetic gene
331 cluster for the catechole siderophore produced by *V. cholerae* vibriobactin(54). Due to the phylogenetic
332 relatedness of *V. furnissii* and *V. fluvialis* with *V. cholerae* (42), we hypothesized that the identified
333 biosynthetic gene cluster encodes for the fluvibactin non-ribosomal peptide synthetase. Fluvibactin is a
334 siderophore produced by *V. fluvialis* (55), which differs from vibriobactin only in that it contains a
335 single L-threonine residue rather than two (Figure 4). Siderophores similar to fluvibactin can inhibit
336 bacterial and fungal growth (20, 56), and catechol iron chelators have also been suggested to protect
337 bacteria from oxidative stress (57, 58). Hence, beside the competitive advantage during surface
338 colonization due to the antibacterial activity of fluvibactin, producers of this compound might as well
339 be protected from oxidative stress, which is a prevalent phenomenon in the marine environment (59).

340 **Conclusion**. We have shown that a rational choice of substrates typical of the niche of isolation of
341 microorganisms is a valid cultivation strategy to enhance the numbers of bioactive strains in a
342 screening step. Our results suggest a role of chitin in the production of secondary metabolism in
343 *Vibrionaceae*. The genomes of members of this family of bacteria harbor great potential for chitin
344 catabolism. Hence, genomic studies could predict which substrates other families of microorganisms
345 might prefer and, subsequently, lead to the elicitation of biosynthetic gene clusters. Also, allowing the
346 potential producing strain a temporal advantage (reaching stationary phase) is an important aspect to
347 consider when designing a screening strategy.

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356

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512 **LIST OF TABLES, FIGURES AND SUPPLEMENTARY INFORMATION**

513 **Table 1.** Antibacterial activity of 13 ethyl acetate extracts against *V. anguillarum* shown as the
514 diameter of clearing zones (+: between 1 and 15 mm; ++: between 16 and 25 mm; +++: over 25 mm).

515 **Table 2** Potential for the production of secondary metabolites from *V. furnissii* S0821 and *V. fluvialis*
516 S1110 based on AntiSMASH (upper part of the table) and Cluster Finder algorithms (lower part of the
517 table). In the "Similarity" column, the percentages on the left and on the right sides of each slash refer
518 to *V. furnissii* S0821 and to *V. fluvialis* S1110, respectively. BGC: biosynthetic gene cluster; NRPS:
519 non-ribosomal peptide synthetase

520 **Figure 1.** Number of bioactive *Vibrionaceae* strains (of 295 in total) on glucose (rhombus), mannose
521 (square) and chitin (triangle) allowing 0, 24 and 48 hours pre-growth of the potential producer before
522 exposing the target strain, *Vibrio anguillarum*.

523 **Figure 2.**Distribution of the *chiS* gene and of the $(GlcNAc)_2$ operon among *Vibrio* spp..
524 GenBank/EMBL/DDBJ accession numbers of the used genomes are indicated in brackets.

525 **Figure 3.**Distribution of the *chiS* gene and of the $(GlcNAc)_2$ operon (VC0611-VC0620) among
526 *Photobacterium* spp.. GenBank/EMBL/DDBJ accession numbers of the used genomes are indicated in
527 brackets.

528 **Figure 4 (A)** Structures of vibriobactin (**1**) (54), fluvibactin (**2**) (55), **3** and **4**. **(B)** UHPLC-HRMS Total
529 Ion Chromatogram (TIC) of the culture extract from *V. furnissii*. The peaks assigned to fluvibactin (**2**)
530 and compound **4** are highlighted.

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532 **Table 1.** Antibacterial activity of 13 ethyl acetate extracts against *V. anguillarum* shown as the

533 diameter of clearing zones (+: between 1 and 15 mm; ++: between 16 and 25 mm; +++: over 25 mm).

534

Strain	Species	Inhibition of *V. anguillarum*
S0821	*V. furnissii*	++
S1110	*V. fluvialis*	++
S1162	*V. fluvialis*	++
S1732	*Vibrio sp.*	+
S2043	*V. coralliilyticus*	+++
S2052	*V. coralliilyticus*	+++
S2054	*V. coralliilyticus*	+++
S2056	*V. coralliilyticus*	++
S2150	*Vibrio sp.*	+
S2600	*V. nigripulchritudo*	+
S2601	*V. nigripulchritudo*	+
S2604	*V. nigripulchritudo*	+
S4053	*V. coralliilyticus*	+++

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536 **Table 2** Potential for the production of secondary metabolites from *V. furnissii* S0821 and *V. fluvialis*
537 S1110 based on AntiSMASH (upper part of the table) and Cluster Finder algorithms (lower part of the
538 table). In the "Similarity" column, the percentages on the left and on the right sides of each slash refer
539 to *V. furnissii* S0821 and to *V. fluvialis* S1110, respectively. BGC= biosynthetic gene cluster; NRPS:
540 non-ribosomal peptide synthetase

Algorithm	Type of cluster	# clusters *V. furnissii* S0821	# clusters *V. fluvialis* S1110	Similarity
AntiSMASH	Hserlactone	1	1	
	Ectoine	1	1	66/66% ectoine BGC
	NRPS	1	1	72/72% vibriobactin BGC
	Arylpolyene	1	1	90/75% APE BGC
	Bacteriocin	1	1	
	Other	1	0	5% lipopolysaccharide BGC
Cluster Finder	Saccharide	2	3	*
	Putative	8	9	¤
	Fatty acid	2	2	
	Saccharide-Fatty acid	1	1	

541
542 * for *V. furnissii* S0821: two clusters with 29% gene similarity to the O&K antigen BGC; for *V. fluvialis* S1110: two clusters with 3 and
543 18% gene similarity to the O&K antigen BGC ¤ for *V. furnissii* S0821: one cluster with 4% gene similarity to the xantholipin BGC; for *V.*
544 *fluvialis* S1110: one cluster with 14% gene similarity to the O-antigen BGC and one cluster with 36% gene similarity to the vibrioferrin
545 BGC
546

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547 **Table 3.** Siderophore (column "CAS assay") and antibacterial activity of the raw extract from a culture

548 of *V. furnissii* S0821 and of the six derived fractions (F1-F6). The addition of a siderophore to the CAS

549 solution causes a change in color from dark blue to orange-yellow. Activity against *V. anguillarum* is

550 measured as the diameter of inhibition zones.

Sample	CAS assay	Inhibition zone (mm)
Raw extract	Yellow	20
F1	Blue	-
F2	Blue	-
F3	Blue	-
F4	Blue	-
F5	Dark orange	9
F6	Yellow	23
Blank	Blue	-

551

DRAFT

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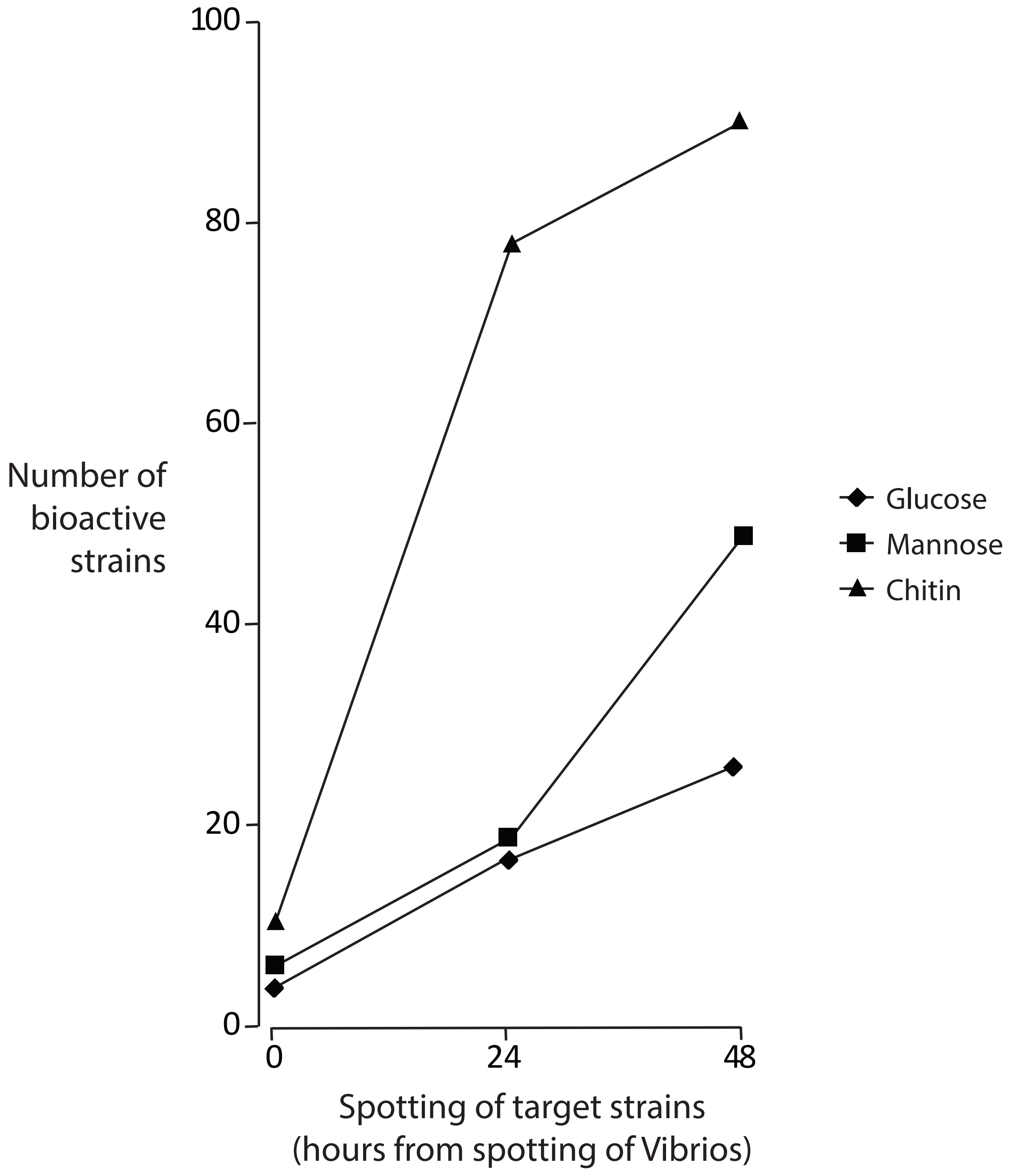

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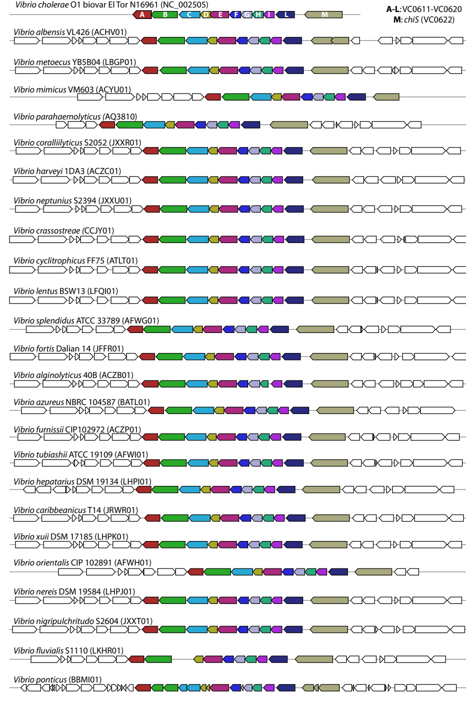

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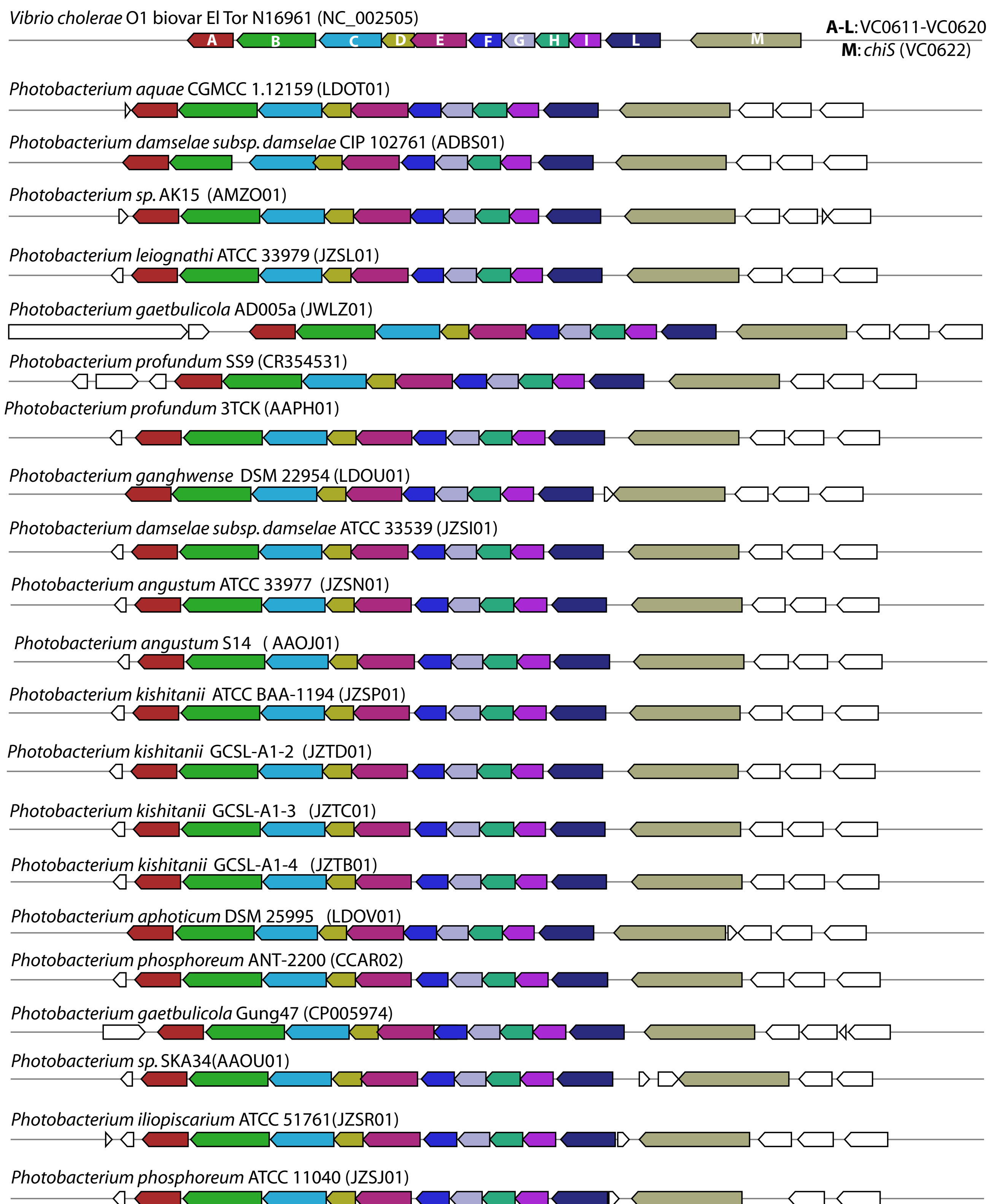

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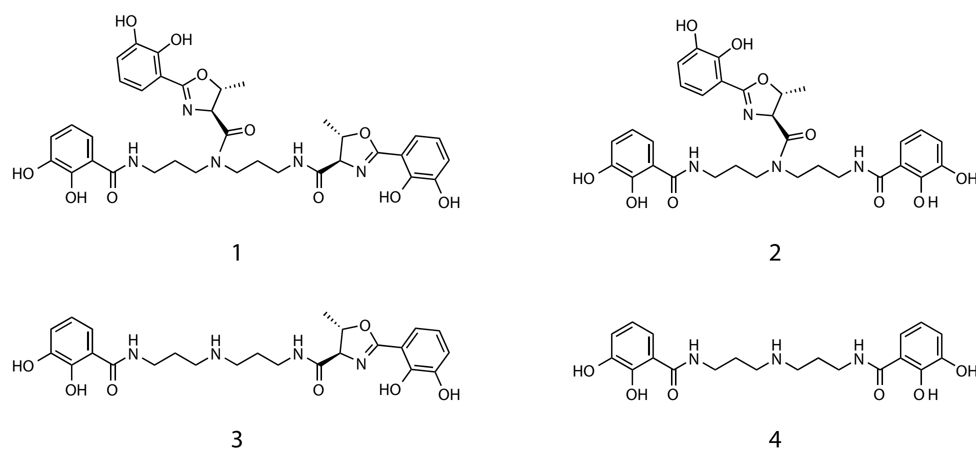

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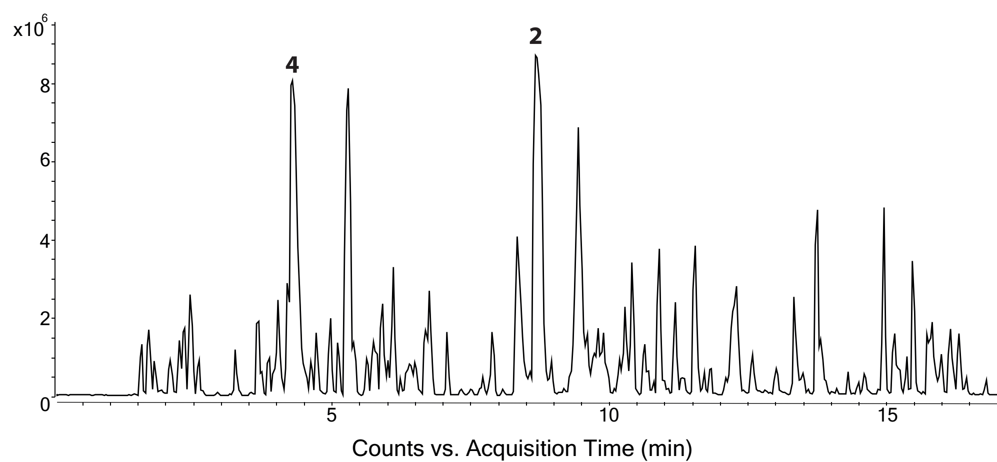

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