



## Influence of Niche-Specific Nutrients on Secondary Metabolism in Vibrionaceae

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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.

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

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.

DRAFT

## 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).

71 Following the increasing interest in natural products from the marine environment during the last  
72 decades of the 20<sup>th</sup> 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 galathea*, 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

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

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<sub>2</sub>O 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<sub>2</sub>O 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)<sub>2</sub> operon.** The *chiS* (VC0622) gene and the  
133 (GlcNAc)<sub>2</sub> operon (VC0611-VC0620) of *Vibrio cholerae* were searched against a custom-built



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

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<sub>2</sub>O (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<sub>2</sub>O 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]<sup>+</sup>  
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

177 S0821 culture extracts were fractionated by Mixed-Mode Anion Exchange SPE on an Oasis MAX  
178 cartridge (Waters, Milford, MA, 30  $\mu$ m, 30 mg, 1 mL). The sample was dissolved in 400  $\mu$ L of 3:1  
179 H<sub>2</sub>O: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<sub>2</sub>O:MeOH (F1), 2 mL of 1:1 H<sub>2</sub>O:MeOH (F2),  
181 2 mL of MeOH (F3), 1 mL of H<sub>2</sub>O and 1 mL of 3:1 H<sub>2</sub>O:MeOH containing 1% formic acid (F4), 2 mL  
182 of 1:1 H<sub>2</sub>O: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  $\mu$ L  
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

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)<sub>2</sub> 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)<sub>2</sub> 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

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)<sub>2</sub> 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. furnissii* 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*.

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



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

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)<sub>2</sub> 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)<sub>2</sub> 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)<sub>2</sub> operon includes the gene encoding for the periplasmic (GlcNAc)<sub>2</sub>



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

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 catecholate 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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- 511

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)<sub>2</sub> 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)<sub>2</sub> 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.

531

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 <i>V. anguillarum</i>
S0821	<i>V. furnissii</i>	++
S1110	<i>V. fluvialis</i>	++
S1162	<i>V. fluvialis</i>	++
S1732	<i>Vibrio sp.</i>	+
S2043	<i>V. coralliilyticus</i>	+++
S2052	<i>V. coralliilyticus</i>	+++
S2054	<i>V. coralliilyticus</i>	+++
S2056	<i>V. coralliilyticus</i>	++
S2150	<i>Vibrio sp.</i>	+
S2600	<i>V. nigripulchritudo</i>	+
S2601	<i>V. nigripulchritudo</i>	+
S2604	<i>V. nigripulchritudo</i>	+
S4053	<i>V. coralliilyticus</i>	+++

535

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		Similarity
		<i>V. furnissii</i> S0821	<i>V. fluvialis</i> S1110	
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

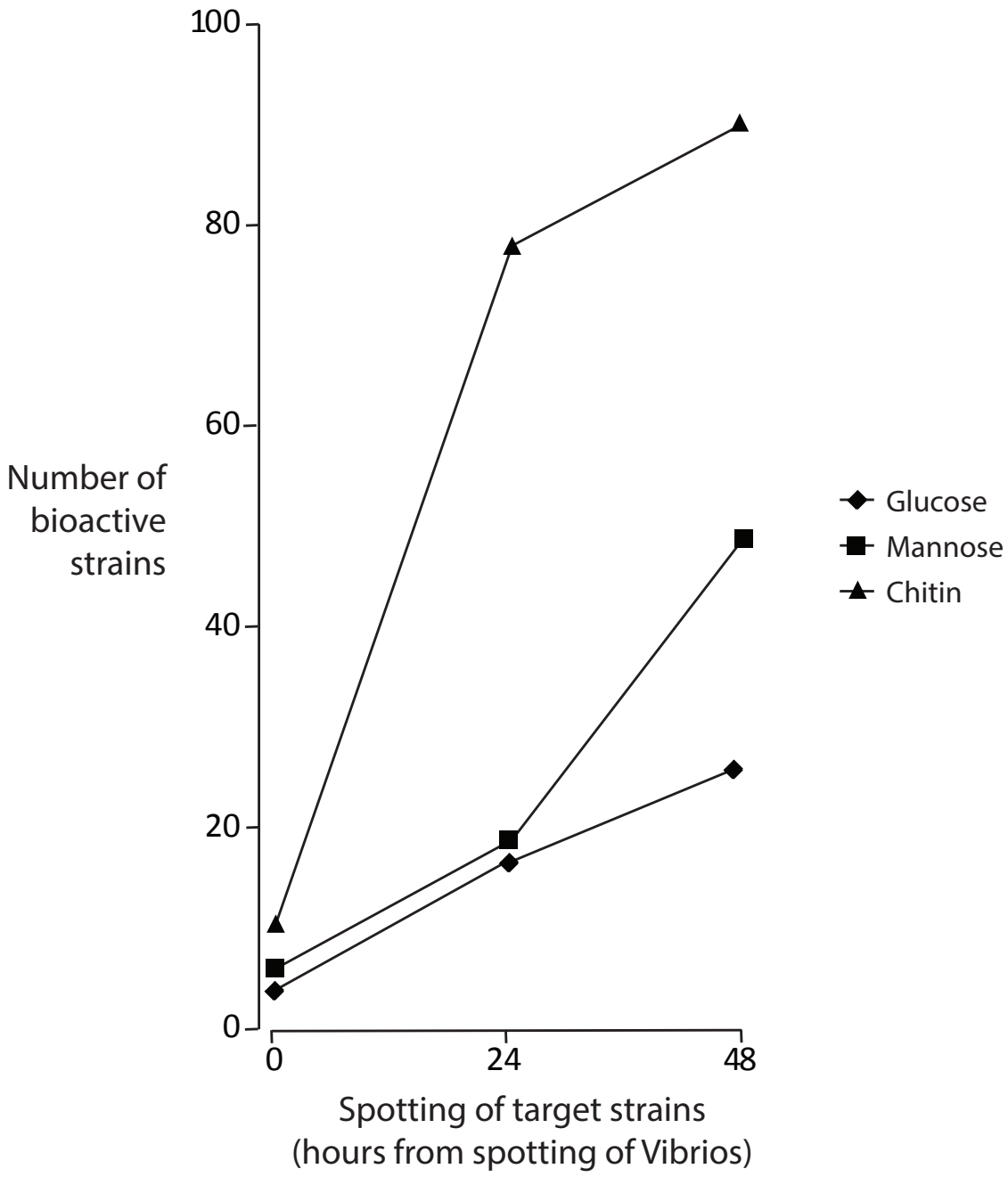
542 \* for *V. furnissii* S0821: two clusters with 29% gene similarity to the O&K antigen BGC; for *V. fluvialis* S1110: two clusters with 3 and  
 543 18% gene similarity to the O&K antigen BGC □ for *V. furnissii* S0821: one cluster with 4% gene similarity to the xantholipin BGC; for *V.*  
 544 *fluvialis* 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

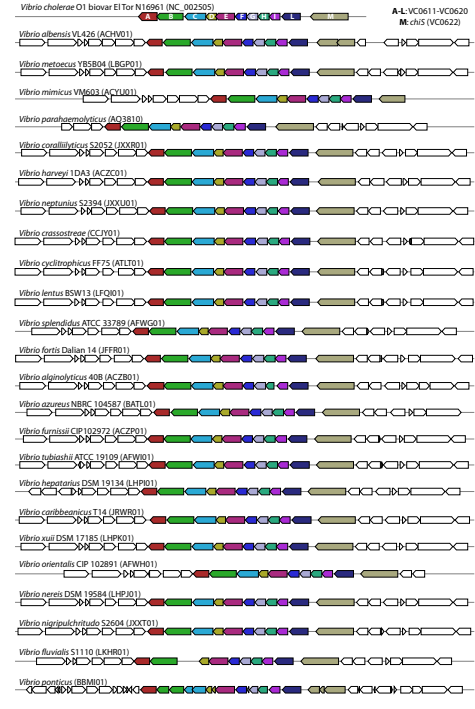
547 **Table 3.** Siderophore (column “CAS assay”) and antibacterial activity of the raw extract from a culture  
548 of *V. furnissii* S0821 and of the six derived fractions (F1-F6). The addition of a siderophore to the CAS  
549 solution causes a change in color from dark blue to orange-yellow. Activity against *V. anguillarum* is  
550 measured as the diameter of inhibition zones.

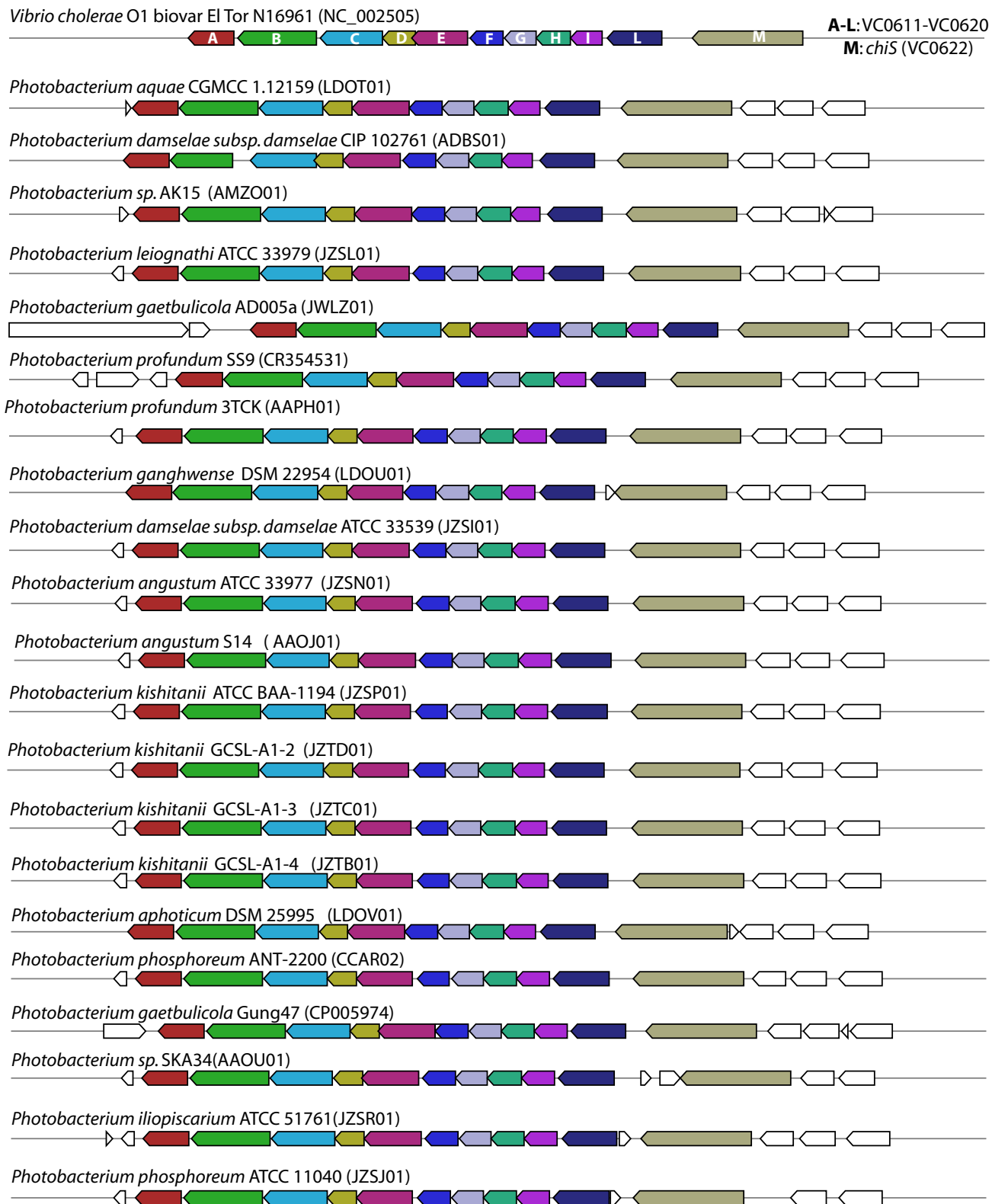
Sample	CAS assay	Inhibition zone (mm)
Raw extract	Yellow	20
F1	Blue	-
F2	Blue	-
F3	Blue	-
F4	Blue	-
F5	Dark orange	9
F6	Yellow	23
Blank	Blue	-

551

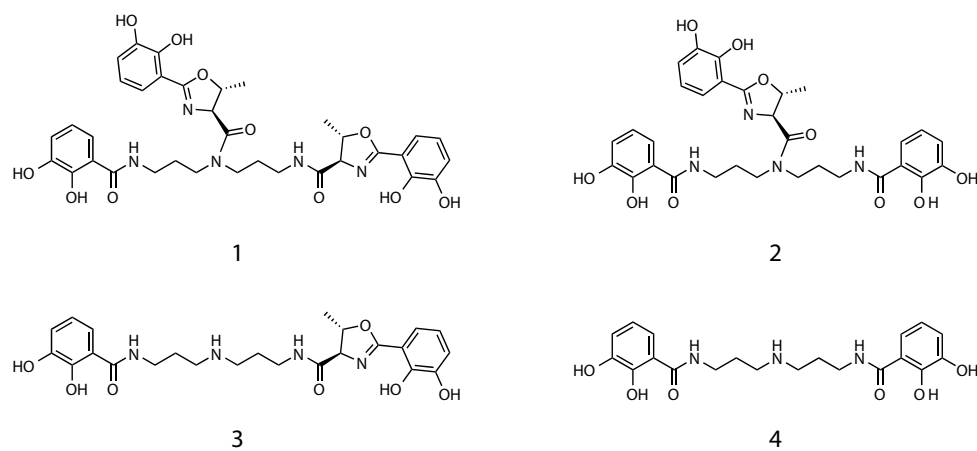








A



B

