Marine Vibrionaceae as a reservoir for bioprospecting and ecology studies

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Marine *Vibrionaceae* as a reservoir for bioprospecting and ecology studies.

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Cover: the Mediterranean Sea at Hyères, France (Sonia Giubergia)

PREFACE

The present PhD study has been conducted at the Department of Systems Biology and at the Novo Nordisk Foundation Centre for Biosustainability at the Technical University of Denmark from May 2013 to April 2016 under the supervision of Professor Lone Gram.

The PhD was supported by an Early Stage Research Grant from the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program FP7 People-2012-ITN, under grant agreement No. 317058, "BacTory".

The work resulted in the preparation of one book chapter and three research articles, which are included in this thesis. Furthermore, collaborative research efforts resulted in two co-authorships (not included in this thesis).

Sonia Giubergia

April 2016

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I would also like to thank Colin, Odette and Zalan from Microdish for making my two weeks stay in Utrecht a lovely time. Thanks to all the Bactories: although my work was not based at CfB, I really enjoyed the time I spent with you all. In particular, thanks to Isotta for being a great confidant and flat mate ("caffettino?"), although I will never forgive her for drowning my beloved orchid.

Thanks to my parents, siblings and the rest of the "troop" for always making me feel close to them despite I was two thousands kilometers away (Grazie ai miei genitori, fratelli e al resto della truppa per avermi sempre fatta sentire vicina a loro nonostante io fossi a duemila chilometri di distanza). Finally, thanks to my beloved Tobi for supporting and encouraging me all the way, you were always by my side in spite of the distance between us. Danke!

ABSTRACT

The exploration of biodiversity ("bioprospecting") provides mankind with an immense pool of novel organisms, molecules and information, which can be exploited for the development of innovative biotechnological processes and new ways to treat diseases. In the past decades, the marine environment emerged as an untapped source of biodiversity, and this study investigated the marine bacterial family *Vibrionaceae* ("vibrios") for its potential as reservoir of novel biodiversity and of species relevant for the ecology of the marine environment.

The characterization of a novel species, *Vibrio galatheae*, contributed to the understanding of the phylogeny and diversity of *Vibrionaceae*, while the use of growth conditions mimicking the niche of isolation showed that substrates that are abundant in the marine environment significantly influence the metabolism of vibrios. Indeed, during a screening of approximately three hundred strains, the number of vibrios capable to inhibit the growth of a fish pathogen was nearly doubled when isolates were grown on chitin, the most abundant polymer in the marine environment, as compared to when they were grown on mannose or glucose. This observation led to investigate at the transcriptome level the effects of chitin on the two vibrios *Vibrio coralliilyticus* and *Photobacterium galatheae*. It was shown that the dynamics of chitin colonization and utilization in these two species are similar to those reported for the well-characterized chitin colonizer *Vibrio cholerae*. Bacteria reach chitinous surfaces by chemotaxis before adhering to it and completing their chitin degradation/utilization program. The complementation of this information with the metabolomic profiles of the strains suggested a possible role of secondary metabolites in chitin colonization, although further work is required to elucidate whether they are produced to antagonize competitors or to communicate with other colonizers and/or a potential host.

In conclusion, this PhD study adds to the knowledge of *Vibrionaceae* as an untapped reservoir of biodiversity and important players in the ecology of the marine environment. Studying microbial eco-physiology is important not only for the development of ecological models, but also as foundation for

bioprospecting studies, where this knowledge may be used, for example, to elicit silent biosynthetic gene clusters during natural product discovery.

RESUMÈ (DANISH)

Udforskningen af biodiversitet ("bioprospecting") forsyner menneskeheden med en umådelig stor pulje af nye organismer, molekyler og information der kan bruges med det formål at udvikle innovative, bioteknologiske processer samt nye måder at behandle sygdomme på. Over de seneste årtier har særligt det marine miljø fremstået som en uudnyttet kilde til biodiversitet. Dette studie har undersøgt den marine bakteriefamilie *Vibrionaceae* (vibrioer) for dens potentiale som et reservoir af ukendt biodiversitet og af arter med relevans for det marine miljøs økologi.

Karakteriseringen af en ny art, *Vibrio galatheae*, bidrog til forståelsen af *Vibrionaceae*-familiens fylogeny og diversitet, imens anvendelsen af vækstbetingelser der efterligner det miljø, hvori bakterierne blev isoleret, viste at substrater der er hyppigt forekommende i det marine miljø har en signifikant effekt på vibrioers metabolisme. Det viste sig, ved at screene tre hundrede stammer, at antallet af vibrioer der var i stand til at inhibere væksten af en fiskepatogen bakterie var næsten dobbelt så høj når isolaterne blev dyrket på kitin (den hyppigst forekommende biologiske polymer i det marine miljø) i forhold til når de blev dyrket på mannose eller glukose. Denne observation lå til grund for transkriptom-undersøgelser af kitins effekt på de to vibrioer *Vibrio coralliilyticus* og *Photobacterium galatheae*. Det blev påvist at mekanismer relateret til kolonisering og udnyttelse af kitin i disse to arter er tilsvarende dem der er kendt fra den velbeskrevne kitin-koloniserende bakterie *Vibrio cholerae*. Bakterier finder kitinholdige overflader ved kemotaksi før de klæber til overfladen og eksekverer deres kitinnedbrydnings- og udnyttelsesprogram. Komplementeringen af denne information med metabolismeprofiler af de undersøgte stammer antydede at sekundære metabolitter spiller en rolle i kitinkolonisering. Videre undersøgelser er dog nødvendige for at bestemme, hvorvidt de produceres for at antagonisere konkurrenter eller for at kommunikere med andre kolonisatorer og/eller en potentiel vært.

Dermed bidrager dette ph.d.-studium til vores viden om *Vibrionaceae* som et uudnyttet reservoir af biodiversitet og som vigtige aktører i det marine miljøs økologi. Studier i mikrobiel økofysiologi er ikke alene vigtige for at udvikle økologiske modeller, men også som et fundament for studier i bioprospecting,

hvor sådan viden f.eks. kan anvendes til at elicitere ellers ikke-udtrykte biosyntetiske gen-clustre i forbindelse med opdagelsen af nye naturprodukter.

PUBLICATIONS

Included in this thesis:

S. Giubergia, C. Schleissner, F. de la Calle, A. Pretsch, D. Pretsch, L. Gram and M. Schmidt Thøgersen. 2016. Screening microorganisms for bioactive compounds. Chapter to be included in the book "The marine microbiome – an untold resource of biodiversity and biotechnological potential". *Springer. In press*.

S. Giubergia, C. Phippen, C.H. Gotfredsen, K.F. Nielsen and L. Gram. 2016. Influence of niche-specific nutrients on secondary metabolism in *Vibrionaceae*. *Accepted for publication in "Applied and Environmental Microbiology"*.

S. Giubergia, C. Phippen, K.F. Nielsen and L. Gram. 2016. Growth on chitin impacts the transcriptome and metabolite profiles of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753. *Manuscript in preparation*.

S. Giubergia, H. Machado, R.V. Mateiu and L. Gram. 2016. *Vibrio galatheae* sp. nov., a member of the family *Vibrionaceae* isolated from a mussel. *International Journal of Systematic and Evolutionary Microbiology 66, 347-352*.

Collaborative articles not included in this thesis:

H. Machado, **S. Giubergia**, R.V. Mateiu and L. Gram. 2015. *Photobacterium galatheae* sp. nov., a bioactive bacterium isolated from a mussel in the Solomon Sea. *International Journal of Systematic and Evolutionary Microbiology 65, 4503-4507*.

H. Machado, J. Cardoso, **S. Giubergia**, K. Rapacki and L. Gram. 2016. *fur*IOS: a web-based tool for identification of *Vibrionaceae* species using the *fur* gene. *Submitted to BMC Microbiology*.

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1 INTRODUCTION AND OUTLINE

Mankind has for centuries made use of microorganisms and of molecules produced or derived from them. Microorganisms are used in biotechnological applications such as fermentations, bio-remediation and wastewater treatment. Microbial enzymes are used as biocatalysts in the food, detergent, textile and chemical industries (1), and small compounds produced by microorganisms represent a notable portion of all new molecular entities that are not of mammalian origin (2). The exploration of biodiversity ("bioprospecting") is therefore needed for the identification of new microorganisms, enzymes or other molecules, which can be used to improve the above-mentioned processes and applications and, possibly, to develop new ones.

Our group at the Technical University of Denmark studies marine bacteria as source of small molecules for therapeutic applications, as bio-control agents for the aquaculture industry and for their role in the ecology of the marine environment, e.g. their interaction with algae. We have a collection of marine bacteria that were isolated from locations all over the world during the global research expedition Galathea 3 (www.galathea3.dk) (Figure 1) based on their ability to inhibit the growth of the fish pathogen *Vibrio anguillarum* (3).

Figure 1 Route of the Galathea 3 global research expedition. From http://www.galathea3.dk.

All of the approximately 500 strains are Gram-negative, and three hundred of them belong to the family *Vibrionaceae*. This represents more than half of the whole collection. Whilst the *Vibrionaceae* family is well researched for

its pathogenic and symbiotic roles, its potential as source of bioactive compounds is emerging only now (4).

Before this PhD project was started, a number of antibacterial and anti-virulence compounds were isolated and identified from the Galathea 3 *Vibrionaceae* collection. However, most of the strains seemed to have lost their ability to produce antibacterial compounds since their antagonistic activity against *V. anguillarum* could not be detected in the screening assay used in the laboratory (5). However, we knew such strains had the potential for the biosynthesis of these compounds because this ability was the one and only criterion of selection during the isolation procedure. This potential was also confirmed by the analysis of the genomes of several strains from the collection. In several cases, this analysis demonstrated the presence of multiple loci dedicated to the biosynthesis of secondary metabolites in a single genome (6), indicating the potential of a single strain for the production of several potentially bioactive compounds.

In this PhD project I focused on "waking up" the biosynthetic potential of this family. I based my work on the assumption that secondary metabolites have ecological functions in Nature, e.g. defense and communication purposes. Therefore, as a number of research groups have been doing in recent years, I tried to elicit the expression of biosynthetic genes by growing the strains in conditions that simulate their natural environment, namely using substrates that are abundant in the sea. Also, in the initial screening and isolation procedure, bacterial colonies were tested for antibacterial activity after 3-7 days of incubation, hence allowing the producer to reach stationary phase where secondary metabolites are typically produced. Therefore, I also embarked on designing a screening strategy that would mimic the first procedure.

Small molecules of microbial origin are the subject of **Chapter 2** ("Research on microbial natural products – merging ecology and bioprospecting"), where I provide a general overview about microbial natural products, with a focus on their importance for therapeutic application, the basis of their biosynthesis and their ecological role. Examples of molecules of terrestrial and marine origin, with a particular focus on the latter one, are provided. This topic is treated more thoroughly in **Article 1**. In the second part

of Chapter 2, I describe strategies currently in use to access silent biosynthetic gene cluster, introducing the rationale behind the work presented in **Articles 2** and **3**.

The use of natural substrates to manipulate the biosynthesis of secondary metabolites enabled also the gain of insights about the role of *Vibrionaceae* in marine ecology, with a particular focus on their role as degraders of chitin, which is the most abundant polymer in the marine environment. This is introduced in **Chapter 3** ("The *Vibrionaceae*"), where the diversity and phylogeny of *Vibrionaceae* are discussed, too, as introduction to **Article 4**, in which a novel species is described.

In **Chapter 4** ("Conclusions and perspectives") conclusive remarks and future perspective are presented, followed by **Chapter 6**, which contains the full-length research articles that were prepared as part of this PhD project.

2 RESEARCH ON MICROBIAL NATURAL PRODUCTS – MERGING ECOLOGY AND BIOPROSPECTING

Starting from the first antibiotic ever discovered, penicillin, produced by the filamentous fungus *Penicillum rubens* (7, 8), microbial secondary metabolites have been key components with therapeutic application in human and animal health. These small molecules (<1500 Da) exhibit a range of bioactivities: they are employed because of their antibiotic, anti-inflammatory, anti-parasitic, immunosuppressant and anti-cancer properties, among others.

Towards the end of the twentieth century, however, screening of molecules synthesized by combinatorial chemistry was preferred over isolation and testing of compounds from natural sources. Despite huge economical investments from pharmaceutical companies, this strategy did not provide as many lead compounds as expected, and today there is a renewed interest in microbial natural products (9–11). A recent analysis by Patridge and colleagues (2) showed that approximately half of all new molecular entities that are not of mammalian origin are of microbial origin ("bacteria" and "fungi" in Figure 2). In parallel, strong experimental evidence emerged, that secondary metabolites have major roles in microbial intra- and interspecies interactions in Nature (12, 13). For the most part, however, the role of these molecules in Nature has not been experimentally proven and this represents one of the intriguing questions in biology.

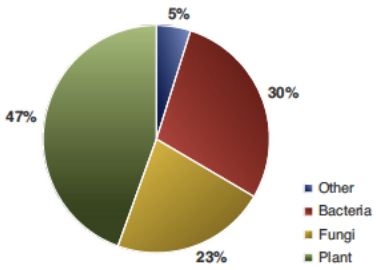


Figure 2 New molecular entities of non-mammalian origin, divided by environmental source. From (2)

The characterization of natural products at several levels (e.g. biosynthesis, structure, bioactivity, ecological role) is therefore important since it provides molecules that could be used to treat human and animal diseases as well as insights into microbial ecology.

2.1 Microbial bioactive secondary metabolites

2.1.1 Sources of bioactive compounds – the marine environment

Traditionally, soil filamentous fungi and Gram-positive Actinobacteria have provided the majority of bioactive compounds of microbial origin (2, 14, 15). However, in recent years, technological advances enabled the isolation and exploitation of microorganisms from other environments, including deserts, oceans and the cryosphere (16).

The marine environment has emerged as an untapped source of producers of bioactive natural products, which is not surprising when considering that 70% of the Earth's surface is covered by oceans and thirty-three of the thirty-five known animal phyla are present in this environment (17). In the micro-scale, it is estimated that there are up to 10^6 and 10^9 microbial cells per milliliter of seawater and ocean-bottom sediment, respectively (18). Hence, marine microorganisms represent an immense resource to be explored in the search for bioactive natural products. Indeed, hundreds of molecules of microbial origin have already been isolated from the marine environment. The total number of compounds isolated from microorganisms is still relatively small as compared to the number of those isolated from marine invertebrates, but the percentage of molecules showing some kind of bioactivity is higher (Figure 3) (19). Furthermore one must consider that marine microorganisms have started to draw the attention of natural product researchers only in the past two decades, whilst marine invertebrates have been investigated for much longer. Hence, the overall picture might change in the next years.

Several bioactive molecules that were initially thought to be produced by marine invertebrates are actually biosynthesized by their associated microbiota. The emblematic case is possibly the anticancer and anti-Alzheimer's compound bryostatin 1, which was originally isolated in extracts from the bryozoan *Bugula neritina* (20). Later, the genetic information required for its biosynthesis was

found in the DNA of the bacterial symbiont *Candidatus* Endobugula sertula (21, 22).

Most of the bioactive compounds produced by marine bacteria have been isolated from Actinobacteria and Cyanobacteria (23). Whilst in the past marine Gram-positive bacteria were emphasized as sources of natural products much more than Gram-negative bacteria (24), today the group (e.g. Proteobacteria and Bacteroidetes) is being reevaluated (6, 25, 26), and Gram-negative bacterial groups such as the *Roseobacter* clade, the genus *Pseudoalteromonas* and the *Vibrionaceae* family (the subject of this PhD project) have provided novel bioactive compounds.

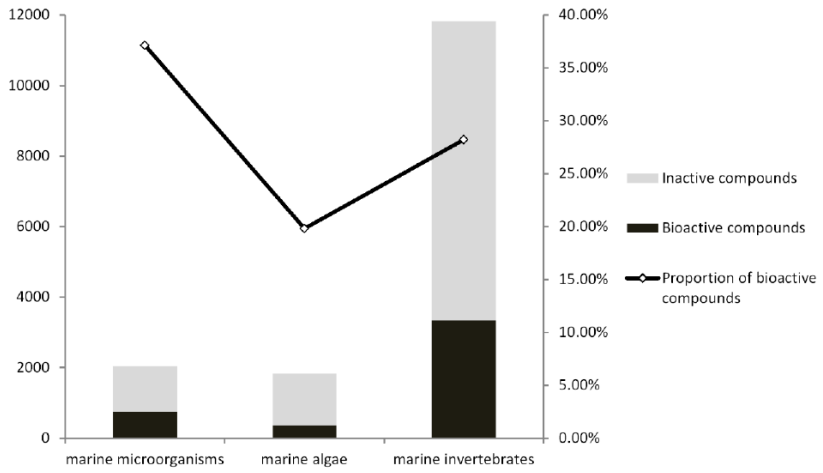


Figure 3 Number of compounds isolated from marine microorganisms, marine algae and marine invertebrates, respectively. The analysis was done based on a database compiled by the authors of the study. From (19).

Marine natural products have chemical features that distinguish them from molecules isolated from terrestrial environments, such as the incorporation of the halogen atoms bromide or chloride (Figure 4) (27, 28). This is the result of millions of years of evolution in an environment where factors like pressure, salinity and temperature led to the selection and maintenance of unique biosynthetic pathways (18, 29).

In some cases, molecules that are produced by terrestrial microorganisms have also been isolated from marine bacteria. This is the case of the potent antibiotic andrimid (Figure 4), which was isolated from at least four terrestrial

species (30–33) as well as from the marine strains *Vibrio coralliilyticus* S2052 (5) and *Vibrio splendidus* SWAT-3 (34). Another example is the antibiotic holomycin (Figure 4), which was isolated first from the terrestrial species *Yersinia ruckeri* (35) and *Streptomyces clavuligerus* (36) and later from the marine *Photobacterium galatheae* (5). The presence of the same genetic information in organisms that live in distinct environments and are distantly related from a phylogenetic point of view probably originates from an ancestral biosynthetic gene cluster that was transferred via horizontal gene transfer (37, 38). The gene cluster encoding for the biosynthetic machinery necessary for the production of andrimid in *Pantoea agglomerans* is flanked by a pseudogene with homology to transposases, which might explain its detection in the genomes of unrelated species (38).

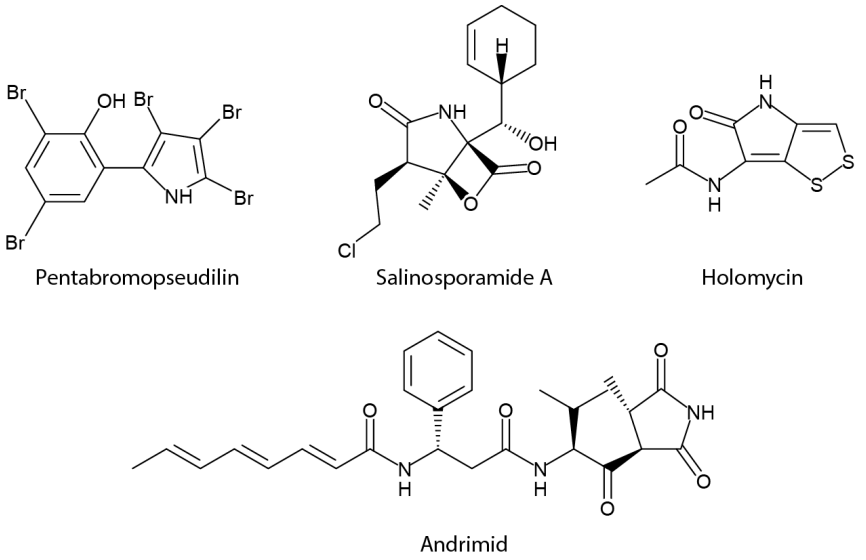


Figure 4 Chemical structures of the marine halogenated compounds pentabromopseudilin and salinosporamide A, and of the cosmopolitan antibiotics holomycin and andrimid.

Several compounds of marine origin have been approved by the Food and Drug Administration (FDA) for clinical use or are undergoing clinical trials. For several of them, the real producer (invertebrate or microorganism) has not been identified yet (Table 1).

Table 1 List of marine-derived compounds that are approved by FDA or that are in Phase I, II or II of drug development as of December 2015. ADC: Antibody Drug Conjugate. Modified from http://marinepharmacology.midwestern.edu/clinPipeline.htm.

Clinical status	Compound name	Trademark	Marine organism	Chemical class	Disease area
FDA-Approved	Brentuximab vedotin (SGN-35)	Adcetris®	Mollusk/cyanobacteri	ADC	Cancer
	Cytarabine (Ara-C)	Cytosar-U®	Sponge	Nucleoside	Cancer
	Eribulin Mesylate (E7389)	Halaven®	Sponge	Macrolide	Cancer
	Omega-3-acid ethyl esters	Lovaza®	Fish	Omega-3 fatty acids	Hypertriglyceridemia
	Ziconotide	Prialt®	Cone snail	Peptide	Severe Chronic Pain
	Vidarabine (Ara-A)	Vira-A®	Sponge	Nucleoside	Antiviral
	Trabectedin (ET-743)	Yondelis®	Tunicate	Alkaloid	Cancer
Phase III	Plitidepsin	Aplidin®	Tunicate	Depsipeptide	Cancer
	Tetrodotoxin	Tectin®	Pufferfish	Guanidinium alkaloid	Chronic Pain
Phase II	ABT-414 EGFRvIII - MMAF	NA	Mollusk/ cyanobacterium	ADC	Cancer
	DMXBA	NA	Worm	Alkaloid	Schizophrenia, Alzheimer Disease, Attention Deficit Hyperactivity Disorder
	Glembatumumab Vedotin (CDX-011)	NA	Mollusk/ cyanobacterium	ADC	Cancer
	Lurbinectedin (PM01183)	NA	Tunicate	Alkaloid	Cancer
	AGS-16C3F	NA	Mollusk/ cyanobacterium	ADC	Cancer
Phase I/II	Lifastuzumab vedotin DNIB0600A	NA	Mollusk/ cyanobacterium	ADC	Cancer
	Pinatuzumab vedotin (DCDT-2980S)	NA	Mollusk/ cyanobacterium	ADC	Cancer
	Polatuzumab vedotin (DCDS-4501A)	NA	Mollusk/ cyanobacterium	ADC	Cancer
Phase I	GSK2857916	NA	Mollusk/ cyanobacterium	ADC	Cancer
	ASG-67E	NA	Mollusk/ cyanobacterium	ADC	Cancer
	ASG-15ME	NA	Mollusk/ cyanobacterium	ADC	Cancer
	ASG-22ME	NA	Mollusk/ cyanobacterium	ADC	Cancer
	Bryostatin	NA	Bryozoan/ bacterium	Macrolide lactone	Cancer
	DSTP3086S	NA	Mollusk/ cyanobacterium	ADC	Cancer
	HuMax®-TF-ADC	NA	Mollusk/ cyanobacterium	ADC	Cancer
	Marizomib (Salinosporamide A; NPI-0052)	NA	Bacterium	Beta-lactone-gamma	Cancer
	MLN-0264	NA	Mollusk/ cyanobacterium	ADC	Cancer
	PM060184	NA	Sponge	Polyketide	Cancer
	SGN-CD19A	NA	Mollusk/ cyanobacterium	ADC	Cancer
	SGN-LIV1A	NA	Mollusk/	ADC	Cancer

2.1.2 Biosynthesis

Microbial secondary metabolites belong to a range of chemical classes, including non-ribosomal peptides, polyketides, alkaloids and terpenoids. Their biosynthesis is catalyzed by enzymes and proteins encoded by genes which are often organized in a single locus, the so-called "biosynthetic gene cluster" (BGC). Beside the information required for the biosynthesis itself, BGCs often contain also genes encoding for: i) regulatory elements (e.g. transcriptional regulators) that control the expression of the biosynthetic genes, ii) tailoring enzymes that modify the molecule produced by the biosynthetic enzymes (e.g. glycosylation, alkylation and oxidation) and/or iii) for cellular components involved in the resistance to the produced compounds, when these compounds are toxic for the producer – a sort of "self-protection" (see below).

Non-ribosomal peptides and polyketides are two classes of molecules which are encountered often when working with secondary metabolites from marine microorganisms, and which have been intensively studied. In both cases, the biosynthesis occurs through multifunctional protein complexes called non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), respectively. The organization and general mechanism of NRPS and PKS are comparable, with active domains that are organized in modular structures. The growing molecule is transferred from one module to the next one, where a new building block is added. The building blocks used by NRPSs are amino acids (usually < 20) that are condensed by transamination in an mRNA-independent process (39). For PKSs, building blocks are acetate and propionate, and the different units are bound together through condensation reactions (40).

Hybrid pathways that include both PKS and NRPS domains are widespread in Nature. One example is again andrimid, where a PKS module builds a polyunsaturated fatty acid that is then connected by NRPS modules to the amino acids phenylalanine, valine and glycine (Figure 5) (41).

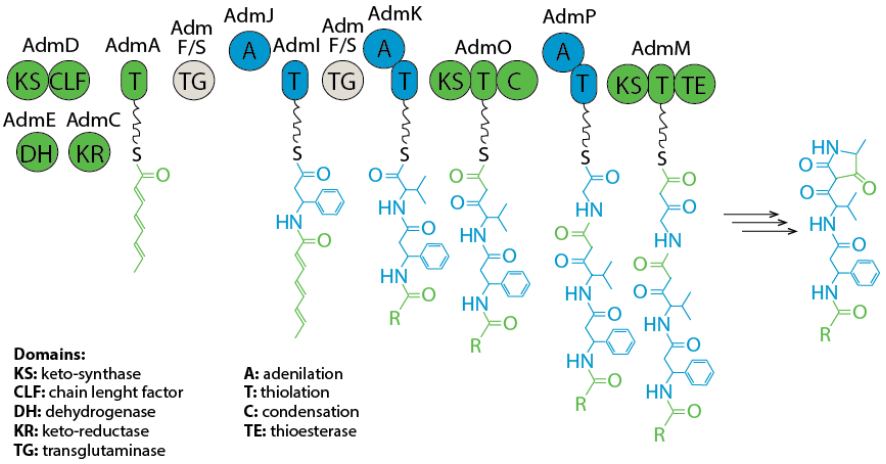


Figure 5 Biosynthesis of the antibiotic andrimid. Both PKS (green) and NRPS modules (blue) are required. Modified from (41).

Terpenoids are biosynthesized by terpene synthases using precursors built from dimethylallyl diphosphate and isopentenyl diphosphate (Figure 6), which are joined in a head-to-tail fashion before being further processed (e.g. in cyclization reactions). Terpenoids are produced by a variety of organisms, including plants, and more than 35.000 different structures have been reported (42). In microorganisms, terpenoids have been isolated mostly from fungi (43), but the biosynthetic potential is widespread in cyanobacteria (44) and other Gram-negative bacteria (45).

Alkaloids are nitrogen-containing compounds, in which the nitrogen atoms originate from amino acids either via transamination reactions or by direct incorporation of derivatives of amino acids such as ornithine, lysine, phenylalanine, tryptophan or histidine (42). The nitrogen-containing alkaloid core can be bound to other building blocks, such as amino acids, polyketides or terpenoids. As example, the iron-chelating molecule fluvibactin, isolated from *Vibrio* spp., contains the alkaloid norspermidine, three residues of 2,3-dihydrobenzoic acid and one residue of threonine (Figure 6) (46). Alkaloids have been isolated from several marine microorganisms, including filamentous fungi (15), cyanobacteria (47), *Pseudoalteromonas* spp. (48), *Vibrio* spp. (4) and recently also from previously uncultured bacteria (26).

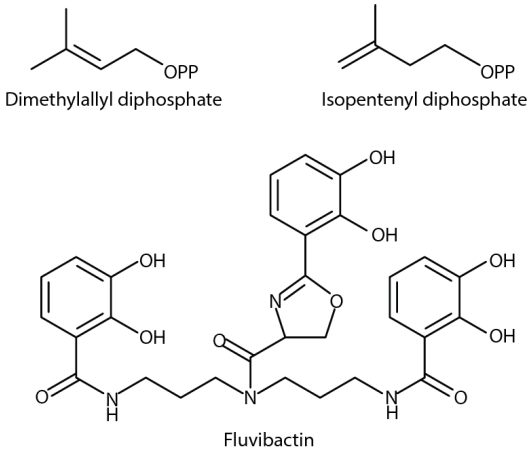


Figure 6 Chemical structure of the terpenoids building blocks dimethylallil diphosphate and isopentenyl diphosphate and of the iron-chelating compound fluvibactin, which has an alkaloid backbone.

2.1.3 Ecological role

In Nature, microorganisms live in complex communities that are highly dynamic with regard to their composition. Furthermore, they are constantly exposed to different and challenging environmental conditions: marine microorganisms are likely to encounter a great variety of habitats and niches because they are transported for considerable distances by oceanic currents (49). In order to increase their chances of survival, they produce a diverse pool of molecules that are involved in a range of phenomena, including defense against predators, antagonism, communication, nutrient uptake and protection against UV/oxidative stress.

Defense against predators. Microorganisms produce molecules that can: i) act as deterrent for bacterivorous/fungivore organisms, such as protozoa and nematodes, or ii) in case of symbiosis, protect their host from predators. As examples, it was shown that freshwater isolates of *Janthinobacterium lividum* and *Chromobacterium violaceum* kill bacterivorous nanoflagellates by producing the alkaloid violet compound violacein (50), while *Pseudoalteromonas tunicata* and a *Pheobacter* sp. can prevent fouling organisms from settling on their host, the alga *Ulva australis* (51).

Antagonism. The ability to produce molecules (antibiotics) capable to kill or slow the growth of competitors confers a strong advantage to micro-organisms

in low nutrients and/or colonization settings (13), such as during settlement on biotic surfaces (52). For example, an antibiotic producing marine strain (*Vibrio* sp. SWAT-3) completely inhibits the colonization of particles by *Vibrio cholerae* (34). Several examples of strains that produce antibacterial compounds only when challenged by other micro-organisms have been discovered (53, 54).

As mentioned earlier in the chapter, BGC often include genes encoding cellular components that are involved in the mechanism of resistance, and this is very common for BGC encoding for the biosynthesis of antibiotics. One example is the BGC for the above-mentioned antibiotic andrimid, which inhibits fatty acids biosynthesis by targeting acetyl-CoA carboxyltransferases. The BGC includes one gene encoding for an acetyl-CoA carboxyltransferase bearing a mutation that prevents the binding of andrimid (33, 41). Resistance genes are subject to horizontal gene transfer, which occurs also at inter-species level (55–57), and are widespread in both soil (58) and the marine environment (59).

Communication. In natural settings, antibiotics are rarely present in concentrations that are high enough to exert any growth inhibitory effect towards neighbor microorganisms. In the marine environment, water fluxes and currents quickly disperse any molecular gradient. Hence, it is thought that antibiotics might also have other roles, including cell-to-cell communication and enhancement of virulence (12, 13).

Bacterial communication relies also on a particular mechanism, the so-called quorum sensing (QS), in which bacteria produce small diffusible molecules called auto-inducers. These molecules are, for examples, *N*-acyl homoserine lactones (AHLs), AI-2 and diketopiperazines in Gram-negative bacteria (60) and small peptides in Gram-positive bacteria (61). When autoinducers reach a threshold concentration, population-wide behaviors (e.g. biofilm formation, antibiotic production, bioluminescence) are triggered (62).

The *luxI*/*luxR* QS system of the marine bacterium *Vibrio fischeri* (Figure 7) has been the first one to be thoroughly characterized. The *luxI* gene encodes for an *N*-3-oxohexanoyl-homoserine lactone (3OC6HSL) synthase, while LuxR is a 3OC6HSL-dependent transcriptional activator. When 3OC6HSL (the autoinducer) in the environment reaches a threshold concentration, LuxR is activated and the *lux* locus is transcribed. Such locus includes the *luxI* gene as well as genes encoding for the luciferase and the other proteins required for

bioluminescence, which is characteristic of *V. fischeri*, to occur (63). *luxI*/*luxR* type QS system are widespread among bacteria (62).

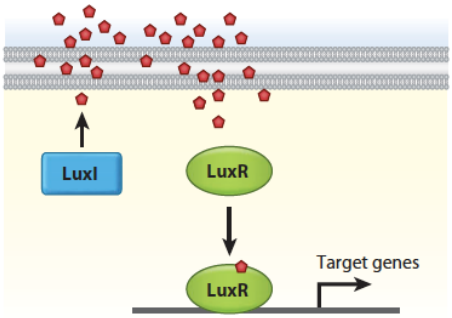
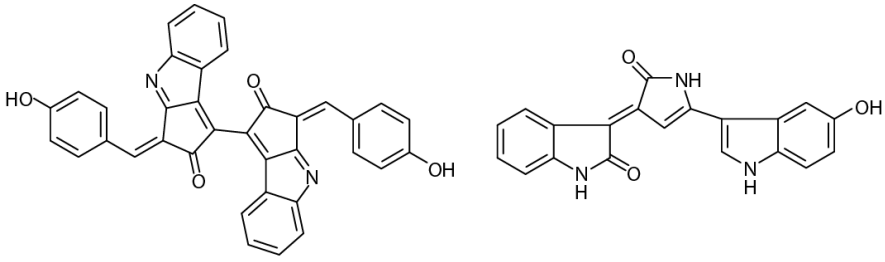


Figure 7 Scheme of the Gram-negative LuxI/LuxR type QS system. When the concentration of the autoinducer molecules (red pentagons) reaches a threshold concentration, the LuxR transcriptional regulator is activated and the expression of target genes is induced. From (62).

Nutrient uptake. Microorganisms have developed strategies to increase their chances to access nutrients whose concentration in Nature is often low. Among these strategies is the biosynthesis of siderophores, molecules that can solubilize, chelate and transport ferric iron into cells. Iron is required for several cellular processes, such as respiration and photosynthesis. When iron concentration in the surrounding environment is low, e.g. in the ocean where the average concentration of dissolved iron is below 0.2 nM (64), microorganisms secrete siderophores. The complex ferric iron-siderophore is recognized by membrane receptors and transported inside the cell. These transporters are present also in populations of "cheaters" that do not synthesize siderophores but benefit from their activity and presence in the environment (65), as exemplified by the fact that previously uncultured bacteria could be brought into culture by adding a siderophore-producing strain to the isolation plate (66). Some siderophores have been described also for other types of activity, such as protection from oxidative stress (67, 68), modulation of virulence (69) and antibacterial, as we saw during this PhD project for the above-mentioned compound fluvibactin (Article 2).

Stress protection. In Nature, microorganisms often experience oxidative stress, for example due to reactive oxygen species produced as immune response by colonized/infected hosts or to exposure to UV light. Indeed, because of its high

energy content, UV light causes oxidation of cellular components in living organisms. One way microorganisms tackle this is through the production of molecules (pigments) containing a high number of conjugated double bonds that absorb the energy from UV light. For example, the yellow to brown pigment scytonemin (Figure 8) is excreted and deposited in the extracellular sheath of a number of cyanobacteria (70). A similar role has been suggested for the above-mentioned compound violacein (71) (Figure 8), which is produced by a range of bacteria, including *Janthinobacterium lividum* (50) and *Pseudoalteromonas luteoviolacea* (72). Other bacterial pigments thought to have a similar role are carotenoids, melanins, xanthomonadines and prodiginines.



Scytonemin Violacein

Figure 8 Chemical structures of the pigments scytonemin and violacein.

2.2 How to access the biosynthetic potential

The genomic era triggered a new approach to natural products research and ecology studies. Genome sequences provide us with an unprecedented amount of information helpful during experiment design and to validate ecological model and networks. To date, nearly five thousand complete prokaryotic genomes are publicly available, and this number is more than ten times larger when considering also non-closed genomes (http://www.ncbi.nlm.nih.gov/genome/browse/). The analysis (mining) of few of these genomes revealed that the natural products isolated so far represent just a small fraction of the potential harbored by microorganisms (6, 49, 73), and that also groups that traditionally have not been exploited in natural product discovery, such as anaerobic bacteria, represent an immense reservoir of secondary metabolites (74). Cimermancic and colleagues (75) analyzed 1154 prokaryotic genomes and found that they contain a large number of families of BGC of unknown function. This indicates that much more effort must be done

in order to exploit this huge potential and, in view of the ecological role of microbial small molecules, to understand their function in Nature.

A challenge in natural product discovery is that several BGC are silent (or cryptic), meaning that they are not expressed under standard laboratory conditions. The reason for this could be that different compounds are produced under different environmental conditions, ensuring an advantage over competing organisms (76). Several approaches have been developed to overcome this problem. Some of them are based on knowledge about the physiology and ecology of a microorganism and rely on the elicitation of the expression of the BGC in the natural host, while others are based on molecular biology techniques, which enable to manipulate the genetic context/regulation of the BGCs.

2.2.1 Physiology and/or ecology-based strategies

In Nature, secondary metabolites have a number of ecological functions (see above) and are often produced during specific growth stages and/or when encountering external stimuli. Therefore, one approach to induce the expression of BGC in laboratory setups is to simulate the growth conditions that the potential producers might encounter in their natural environment.

One strain many compounds (OSMaC). Schiewe and colleagues (77) proposed an approach, in which culture parameters (e.g. media composition, pH, aeration) are manipulated. They named the approach "One Strain, Many Compounds (OSMaC). Using the OSMaC strategy, Bode *et al* (78) isolated more than one hundred compounds from six microorganisms. Parameters can be varied based on previous knowledge about the strain or the environment of isolation, and in some cases the adjustment of several growth parameters at once can be required to achieve elicitation of BGCs (79). Variations must be established on a case-by-case basis, as it is not possible to generalize and assume *a priori* that growth conditions that worked with one strain will work with another one as well. Some marine bacterial strains produce antibacterial compounds only when grown in cultivation systems simulating the intertidal environment of isolation, such as agar-coated rolling bottles (80) or "air-membrane surface" and

"rotating disc" bioreactors (81, 82). The use of low phosphate concentrations, similar to those encountered in the marine environment, drastically changed the profile of secondary metabolites in a *Pseudovibrio* sp., including the induction of the biosynthesis of the antibiotic tropodithietic acid (83).

Our group demonstrated that shrimp chitin, which is the most abundant polymer in the marine environment, has a pronounced effect on the secondary metabolism of the marine bacterium *Vibrio coralliilyticus*. When grown on chitin, *V. coralliilyticus* doubles the amount of the antibiotic andrimid produced per cell as compared to when it is grown on glucose (84). As detailed in Chapter 3, genome analysis revealed that the ability to catabolize chitin is a common feature among members the *Vibrionaceae* family, of which *V. coralliilyticus* is a member. Hence, in this PhD project we investigated whether chitin affects the production of antibacterial compounds also in other members the *Vibrionaceae* family. We have included in the study other substrates (i.e. glucose and mannose) that are also abundant in the marine environment, since they are used by algae for protein glycosylation (85–87). We found more antibacterial strains on mannose than on glucose, but the highest effect was seen on chitin (Article 2) (Figure 9). In a follow-up study, we demonstrated using transcriptomics that indeed chitin affects the expression of a number of BGCs in *Vibrio coralliilyticus* and in *Photobacterium galatheae*.

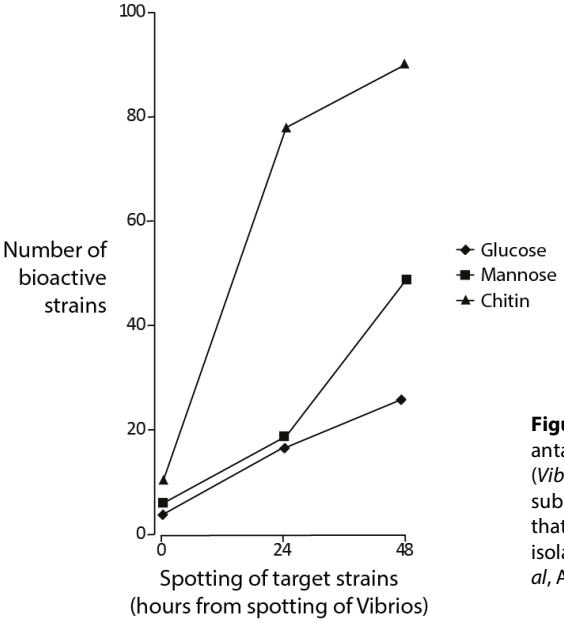


Figure 9 Number of *Vibrionaceae* strains antagonizing the growth of a target strain (*Vibrio anguillarum*) on three different substrates (glucose, mannose and chitin) that are abundant in the environment of isolation of the strains. From (Giubergia *et al*, Article 2).

Co-cultivation. The rationale that lies behind the use of co-cultivation to elicit the expression of BGCs is the ecological role that some secondary metabolites have in Nature, i.e. defense, competition and communication. When some microorganisms are exposed to others, they vary their secondary metabolite repertoire. Traxler and colleagues (88) reported this kind of behavior using MALDI-TOF imaging mass spectrometry; they showed that its exposure to other actinomycetes leads to a significant qualitative variation in the secondary metabolite profile of *Streptomyces coelicolor*. A variety of compounds with a range of bioactivities have been isolated using the co-cultivation approach, as reported in the recent reviews (53) and (89).

Also the study of Charusanti and colleagues (90) is based on co-cultivation. They adaptively evolved a *Streptomyces clavuligerus* strain in presence of the human and animal pathogen *Staphylococcus aureus*. After months of evolution, *S. clavuligerus* started to produce the antibiotic holomycin (which was not detected in culture extracts from the non-evolved control). Finally, although in his study he did not use direct co-cultivation, Seyedsayamdost (91) considered the dynamics of microbial inter-cellular communication in Nature and exposed the soil bacterium *Burkholderia thailandensis* to low concentrations of antibiotics and other molecules of microbial origin. With this strategy, he obtained the elicitation of previously cryptic BGCs.

2.2.2 Other strategies

Manipulation of regulatory elements. Requirements for this strategy are: i) culturability of the producer/host and ii) availability of a molecular toolbox for its genetic manipulation. One way to elicit the expression of BGCs is to modify or replace the natural promoters of the biosynthetic genes with inducible, strong or constitutive promoters. This has been done, for instance, by Olano and colleagues (92), who obtained the elicitation of a NRPS and a PKS-NRPS in *Streptomyces albus* J1074 by inserting a constitutive promoters upstream of the two BGCs.

Alternatively, transcriptional (e.g. repressors or activators) or translational (e.g. ribosome or polymerase) elements can be manipulated. For example, the induction of the expression of putative activator genes that were

predicted to be part of the BGCs led to the discovery of aspyridones A and B in *Aspergillus nidulans* (93) and of the antibiotic and anticancer compounds stambomycins in *Streptomyces ambofaciens* (94). Interestingly, the same approach, once more in *A. nidulans*, revealed a regulatory cross talk between two BGCs. In their study, Bergmann and colleagues (95) put the expression of the regulator ScpR, whose encoding gene was part of the silent asperfuranone BGC, under the control of a constitutive promoter. This resulted in the expression of the asperfuranone BGC, but also of a NRPS-containing BGC.

In 1996, Shima *et al* observed that a single mutation in the ribosomal protein S12 conferring streptomycin resistance to *Streptomyces coelicolor* induced the biosynthesis of the antibiotic actinorhodin (96). This was possibly the first example of the use of "ribosomal engineering" in natural product discovery. Since then, this approach has been exploited in several other studies using: i) the same type of single mutation; ii) inducing mutations in other proteins, such as in the β-subunit of the RNA polymerase (*rpoB* gene) conferring rifampicin resistance (97, 98) or iii) using cumulative drug resistance mutations (99). Ribosomal mutations have been obtained also through chemical mutagenesis using diethyl sulphate (100, 101).

Metagenomic libraries. It is estimated the with the laboratory conditions and techniques currently in use, only 1% of all bacteria have been brought into culture, and the portion cultured is even lower (0.01-0.1%) for marine bacteria (102, 103). For approximately 70% of the known bacterial phyla, there is not a single culturable member (104). Therefore, metagenomic libraries, where environmental DNA is cloned into a host, represent a way to circumvent the issue of low culturability of microorganisms. The use of metagenomic libraries for sequence-based and functional bioactivity-based screenings not only enabled the isolation of novel bioactive compounds both from soil and marine-derived libraries (105–108), but also contributed to reveal the widespread occurrence and variety of microbial BGC in the environment (109–112). Finally, it enabled the elucidation of the pathway for the biosynthesis of a number of known bioactive compounds, such as the antitumor molecules psymberin, ecteinascidin 743 (Yondelis®), and the already mentioned bryostatin 1. For all these molecules, the genetic information required for their biosynthesis was extracted from metagenomic libraries from sponges (21, 22, 113, 114).

Heterologous expression. The major limiting factors for this approach are the genetic toolboxes necessary to cope with the size of BGCs (in some cases up to 100 kb), and the influence of the new genetic context on the expression of the cloned BGC (e.g. different codon usage compared to the original host or necessity to replace the BGC native promoter with a strong one optimized for the expression host). However, several strategies have been developed to overcome these limitations, and BGCs have successfully been cloned into a number of heterologous hosts, including *Escherichia coli*, *Pseudomas putida* and several *Streptomyces* species. One remarkable example is the cloning of a silent 67-kb NRPS BGC from the marine actinomycete *Sacchamonospora* sp CNQ-490 in the expression host *Streptomyces coelicolor*, which led to the isolation of the antibiotic taromycin A (115), but many more are reported in literature, as recently reviewed in (116) and (76).

3 THE VIBRIONACEAE

The *Vibrionaceae* (commonly called "vibrios") are a family of Gram-negative γ-proteobacteria that is widespread in aquatic environments, where they contribute to the cycling of organic matter (117). Vibrios are usually rod-shaped and motile by means of one or more polar flagella (Figure 10), chemoorganotrophic and relatively easy to isolate and cultivate (118). They are considered among the most prevalent culturable bacteria in the ocean (117). The family includes species that are pathogenic to humans and/or animals, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, species that are symbiotic, such as *V. fisheri* and *V. harveyi*, but the majority of vibrios are commensal or mutualistic species (119).

Vibrios have two chromosomes, with Chromosome I (Chr I, average size 3.0 to 4.2 Mb) larger than Chromosome II (Chr II, average size 0.8 to 2.4 Mb) (120). Chr I harbors most of the essential genes, whereas Chr II carries species-specific genes and has been suggested to play an important role in environmental adaptation (121, 122). The presence of two chromosomes is thought to facilitate rapid cell replication, which can take as little as ten minutes (121, 123).

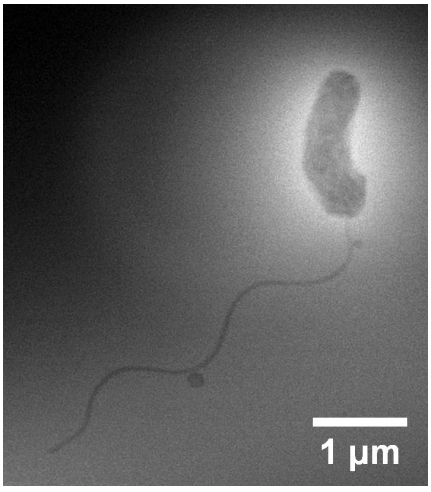


Figure 10 SEM micrograph of the type strain of *Vibrio galatheae*, the new species characterized in this thesis. The picture illustrates the curved rod-shaped morphology and a polar flagellum typical of members of the *Vibrionaceae* family. (Picture taken by Ramona Valentina Mateiu, Centre for Electron Nanoscopy - Technical University of Denmark).

3.1 Diversity and phylogeny

Based on 16S rRNA gene sequence analysis, the following genera are included in the *Vibrionaceae* family: *Aliivibrio*, *Enterovibrio*, *Grimontia*, *Photobacterium*, *Salinivibrio* and *Vibrio*. However, considering also phenotypic features, it has been suggested that the *Vibrionaceae* family should be split in four families: *Salinivibrionaceae* (genus *Salinivibrio*), *Enterovibrionaceae* (genera *Enterovibrio* and *Grimontia*), *Photobacteriaceae* (genus *Photobacterium*) and *Vibrionaceae* (genus *Vibrio*) (118, 124). The genus *Aliivibrio* is not included because it was proposed after the suggestion of the new families (125). However, practically no official agreement has been reached among taxonomist about which of the classification is the most accurate, and currently both of them can be found in literature. In this work, the definition based on the 16S rRNA gene sequence is considered.

As per February 2016, more than 120 *Vibrio* and 25 *Photobacterium* (the two most abundant genera in the family, see below) species are reported in the "Prokaryotic Nomenclature Up-to-date List" of the German Collection of Microorganisms and Cell Cultures (www.dsmz.de). Strain characterization is usually done using a classic polyphasic approach, in which the combination of different techniques provides genotypic, chemotaxonomic and phenotypic information. The gold standard for the measure of the genetic distance between different strains has been DNA-DNA hybridization (DDH), where two strains are considered as belonging to different species if their DNAs are less than 70% similar, although for vibrios the limit has been suggested to be increased to 80% (118). DDH was developed in the 1970s, but it is not easy to implement (126). Instead, the increasing availability of inexpensive sequencing technologies opened up the possibility to obtain gene or whole genome sequences. Therefore, the average nucleotide identity (ANI) has been proposed and has a resolution for species definition comparable to DDH. Genomes whose comparison leads an ANI value <95% are considered to be from different species (127).

An alternative way to infer genetic relatedness among strains is the comparison of their 16S rRNA gene sequences; however, in several cases, such as for vibrios, this approach does not provide the necessary resolution for

identification at the species level (126). Indeed, the threshold for 16S rRNA gene sequence based species differentiation is 97% similarity, but interspecies 16S rRNA-based relatedness among vibrios can be as high as 99%. Furthermore, in vibrios there are often several alleles of this gene (e.g. twelve in *V. fischeri*), which can lead to the assignment of a single strain to different species (128). For this reason, multilocus sequence analysis (MLSA) is preferred. In MLSA, hierarchical classification is based on a set of concatenated housekeeping genes (126). In vibrios, several genes have been proposed for this analysis, such as *rpoA*, *recA*, *pyrH* (124), *ftsZ*, *gapA*, *gyrB*, *mreB* and *topA* (129). Recently, the *fur* gene has been proposed as powerful unique phylogenetic marker for *Vibrionaceae* (130).

In the course of this PhD, strain S2757 was identified as potential novel *Vibrio* species. The strain was originally isolated from a mussel in the Solomon Sea (Solomon Island) (3). Analysis of the 16S rRNA gene placed the strain into the *Vibrionaceae* family and showed 98.5, 98.3, 98.2 and 97.8 % similarity with the gene sequences from *V. hepatarius*, *V. brasiliensis*, *V maritimus* and *V. tubiashii*, respectively. However, according to MLSA analysis (genes 16S rRNA, *gyrB*, *pyrH*, *recA*, *topA*), *V. tubiashii*, *V. sinaloensis*, *V. orientalis* and *V. xuii* were the most closely related species. Similar results were obtained also with the *fur* gene-based analysis (Figure 11). These discrepancies between the different analyses made challenging the choice of the related strains to be used for the phenotypic and chemotaxonomic characterization. However, combination of the MLSA and *fur* gene based phylogenetic trees with DDH and ANI analyses helped in this choice. Ultimately, phenotypic and chemotaxonomic analyses confirmed that strain S2757 is the type strain of a new species, which has been described as *Vibrio galatheae* (Article 4).

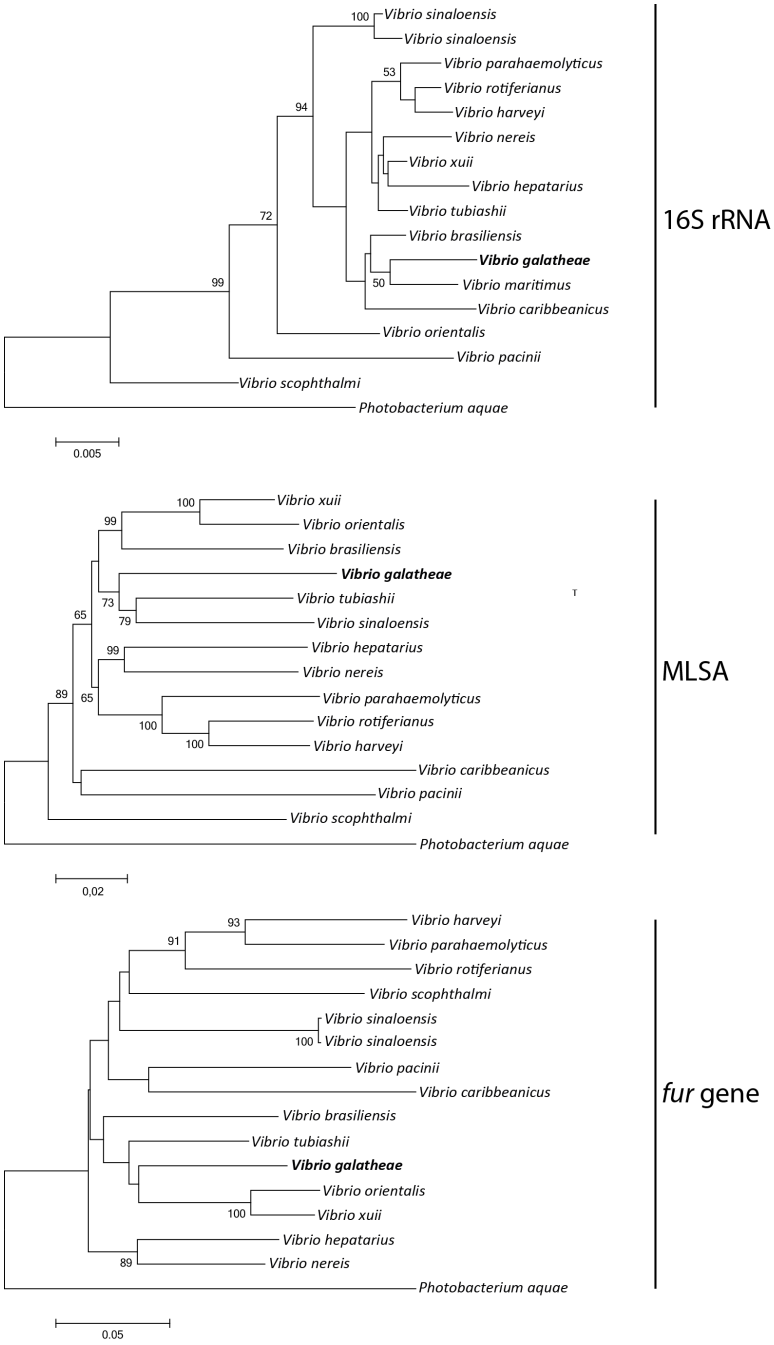


Figure 11 Phylogenetic analyses of *Vibrio galatheae*^{T} and related type strains based on partial 16S rRNA gene sequences (top), concatenated genes (16S rRNA gyrB, pyrH, recA, topA) (middle) and complete fur gene sequence (bottom). *Photobacterium aquae*^{T} was used as outgroup. Bars, from top to bottom, indicate 0.5, 2 and 5 % estimated sequence divergence, respectively.

3.2 Occurrence and ecological importance

Vibrionaceae 16S rRNA gene sequences constitute 0.8% of all bacterial sequences (6% of all γ-proteobacteria sequences) in the dataset of the Tara research expedition (http://oceans.taraexpeditions.org/en/), which includes metagenomics data from different locations all over the world. In this dataset, among *Vibrionaceae*, *Vibrio* and *Photobacterium* are the most abundant genera (73% and the 16% of all the vibrios sequences, respectively) (Figure 12). These values are in agreement with previous metagenomics studies (131), and slightly lower (about 1%) than values obtained in studies where a hybridization approach was used (132).

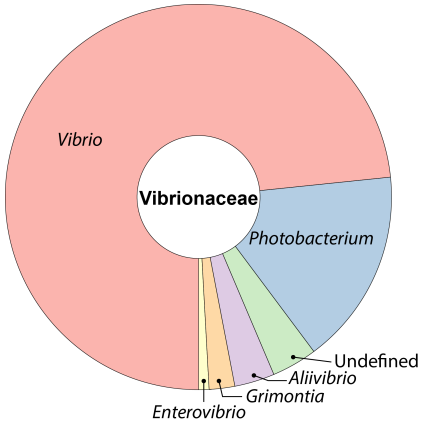


Figure 12 Relative abundance of the different genera of the *Vibrionaceae* family in the dataset of the Tara expedition based on 16S rRNA gene sequences. The genera *Vibrio* and *Photobacterium* are the most abundant. Modified from http://ocean-microbiome.embl.de/

Vibrios occur both as free-living cells and associated with abiotic and biotic surfaces. This is possible because of their ability to: i) utilize a range of nutrients (119) including aromatic hydrocarbons (133) and plastic films (134), ii) form biofilms even on non-nutritive surfaces (117, 118) and iii) switch to a dormant state (called "viable but not culturable", VBNC) when environmental conditions are not optimal for growth (117). Vibrios have been isolated from plastic debris in the ocean (135), from areas contaminated by oil spills (136), from sediments as well as from the exterior surface of marine organisms such as fish, sponge, corals and zooplankton (118). Vibrios are also part of the resident gut microbiome of some marine animals (137, 138), although in general they do not appear to have host preference (139).

The relative abundance of vibrios is strongly related to water temperature and salinity (140). They are more abundant in subtropical and tropical regions, but can be found at all latitudes (132). This could be a consequence of the increase of the sea surface temperature (SST) caused by global warming. Indeed, SST has been related to an increase in the number of vibrio-associated diseases outbreaks also at latitudes where such infections have not been observed earlier (141, 142). Other parameters, such as nutrient availability, affect vibrios abundance. For example, a single *Vibrio* sp. constituted half of the population of a bacterial bloom in the English Channel, which was correlated with an increase in the abundance of the diatom *Chaetoceros compressus* (143), whereas the detection frequency of species such as *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* positively correlates to the abundance of chitinous zooplankton (144).

Some species from the *Vibrionaceae* family have been intensively studied because of their role as symbionts or pathogens. *V. fischeri* and its host, the Hawaiian bobtail squid *Eupryma scolopes*, are one of the most studied host-bacteria symbiosis models (145). In this relationship, the light-emitting *V. fischeri* (see previous chapter) colonizes the light-organ of the squid, and the light it produces is used by the host in a camouflaging behavior ("counterillumination") to escape predators (145, 146). Pathogenicity is linked to the production of virulence factors, such as hemolysins, toxins, siderophores and proteases. The ability to produce these virulence factors is often maintained by cells in the VBCN state (117). Among human pathogens, *V. cholerae* is responsible for epidemics of the acute diarrheal disease cholera, which can be lethal if not treated properly: in 2014, more than two thousands deaths have been reported worldwide to the World Health Organization due to *V. cholerae* infections (www.who.int). The reoccurrence of epidemics has been linked to the ability of the bacterium to survive in a variety of environmental reservoirs and vectors, including sediments, birds, crustaceans, zooplankton and bivalves (147). *V. fluvialis* and *V. furnissii* are two species closely related to *V. cholerae*, and in recent years interest towards them has been growing because of their established causative role in gastroenteritis outbreaks (148, 149). The food-borne pathogens *V. parahaemolyticus* and *V. vulnificus* are often acquired through the ingestion of raw seafood, such as oysters. *V. vulnificus* can cause primary septicemia and is responsible for the majority of sea-food related deaths in the

United States (150). *Vibrio* spp. such as *V. anguillarum*, *V. harveyi*, *V. nigripulchritudo* and *V. vulnificus* are among the major causative agents of fish and shellfish diseases (e.g. "early mortality syndrome", "bright-red syndrome" or "luminous vibriosis" in lobsters and shrimps) and have caused enormous losses to the aquaculture industry over the years (151–153), while *V. coralliilyticus* and *V. shilonii* cause coral bleaching (154, 155).

3.3 *Vibrionaceae* and chitin

Chitin is a polysaccharide composed of (1→4)-β-linked units of the aminosugar *N*-acetylglucosamine (GlcNAc) (Figure 13). GlcNAc chains can be arranged in antiparallel, parallel or mixed configurations, originating α, β, and γ -chitin, respectively. After cellulose, chitin is the most abundant polymer on Earth, where it occurs as cellular component in several organisms such as fungi, molds, insects, diatoms and zooplankton. Despite estimations indicating that billions of tons of chitin are produced in the environment every year, with 10^{11} metric tons produced just in the aquatic biosphere (156), there are no reports of chitin accumulation in Nature (as reviewed in (157) and (158)). This is due to a rapid turnover of the polymer, with fungi and bacteria being the major players in the process.

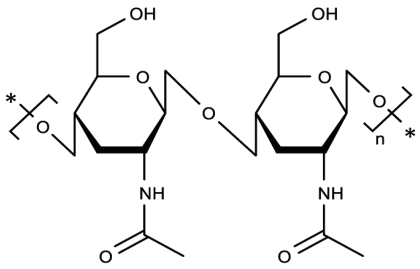


Figure 13 Structure of chitin.

Bacterial chitin degradation relies on the synthesis and secretion of chitin-hydrolyzing enzymes, and results in the production of molecules that serve as carbon and nitrogen sources for the entire food web (156, 159, 160). In the marine environment, unculturable bacteria harbor a great potential for chitin degradation (161) but, among culturable bacteria, vibrios are considered to be among the most chitinolytic, although the major studies to elucidate the phenomenon focused on a limited number of species, mostly *V. cholerae* and *V. furnissii*.

3.3.1 Association of *Vibrionaceae* with chitin surfaces

The association of *Vibrionaceae* with chitinous surfaces in the marine environment (e.g. copepods, shrimps, crabs) is well known (118, 159, 162) and attracted the attention of several researchers. One reason for this is the important role of this bacterial family in the ecology and turnover of marine chitin (see above). Another reason is the concern due to the direct link between this association and their role as pathogens. As mentioned earlier, the abundance of chitinous plankton directly influences the abundance of the pathogenic species *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (144). Cholera outbreaks correlate with copepod blooms, which in turn correspond to *V. cholerae* blooms (163, 164). Removal of plankton from water via filtration using a sari cloth led to a 48% reduction in cholera incidence (165).

It has been suggested that chitin metabolism is an ancestral feature in *Vibrionaceae* (166), and that their spread presence in the ocean is a consequence of their ability to degrade chitin (167). Preheim and colleagues (139) observed that vibrios associate with both live and dead chitin-containing specimens. However, the frequency of this association is higher on dead specimens and discarded carapaces, and the authors suggested a saprophytic lifestyle. Keyhani and Roseman (156) proposed a model for the establishment of the association between vibrios and chitin (Figure 14).

First, free-living bacteria randomly collide with chitin particles or are chemo-attracted by chitin-derived oligosaccharides released in the marine environment by chitin containing organisms. For example, this is at the basis of the above-mentioned symbiosis between *V. fischeri* and the squid *E. scolopes*.

Indeed, *V. fischeri* reaches the light organ of the latter by following a gradient of chitin oligosaccharides through the entrance pore (168).

When bacteria reach the chitin surface, they adhere to it through specific membrane proteins (156), whose synthesis is common in vibrios (169, 170). The 53 kDa GbpA protein mediates adhesion of *V. cholerae* both to zooplanklton and to human epithelial cells by binding GlcNAc (171). A similar role has been demonstrated for a specific calcium-dependent lectin in *V. furnissii* (172). This lectin is part of a complex apparatus that monitors continuously the level of nutrients in the environment, and starts a de-adhesion process when it is low. Detachment is followed by chemotaxis to a nutrient-richer environment.

After adhesion to the chitin surface, bacteria start to produce several enzymes (e.g. chitinases, hexosaminidases) and proteins (e.g. porins and PTS systems) required to hydrolyze chitin, transport the hydrolysis products inside the cell and metabolize them. This is described in detail in the next section.

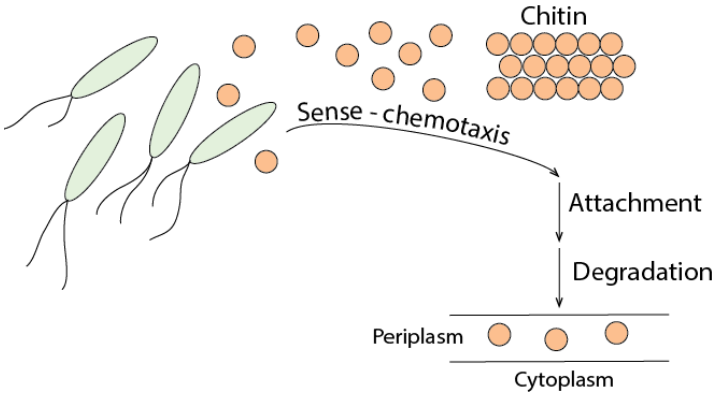


Figure 14 Model proposed by Keyhani and Roseman for the establishment of the association between vibrios and chitin. Bacterial cells either randomly collide with chitin particles or move towards them through chitin oligosaccharides-driven chemotaxis. This is followed by attachment to chitin particles and secretion of extracellular chitinases, which degrade chitin. The generated oligosaccharides are then transported into the cells and are ultimately converted to fructose-6-phosphate. Modified from (156).

3.3.2 Chitin catabolism in *Vibrionaceae*

Based on literature data, experimental data and *in silico* predictions Hunt and colleagues (166) developed a model for chitin metabolism that is valid for *V. cholerae* (Figure 15). In the same study, the authors showed that the required core genetic potential is spread among *Vibrionaceae*. Their genomic analysis was

done on a limited number of species, possibly due to a small number of whole genome sequences available at the time (year 2008). During this PhD work, we performed a similar analysis on a larger dataset and confirmed their findings (Articles 2 and 3). With respect to the *V. cholerae* model, chitin is initially cleaved by extracellular chitinases and other hydrolases into $(GlcNAc)_{2\text{-}6}$, but also into GlcNAc-GlcN, because actually not all chitin residues in Nature are acetylated. Distinct chitinases, with diverse substrate preference and specificity, are synthesized in the presence of different types of chitin (i.e. α/β-chitin) (173).

All hydrolysis products are transported by specific and non-specific porins into the periplasm, where $(GlcNAc)_{2\text{-}6}$ are degraded to $(GlcNAc)_{1,2}$ by chitodextrinases and acetylglucosaminidases, whilst GlcNAc-Glcn is deacetylated to $(GlcN)_2$. These disaccharides are then transported into the cytosol by ABC-type transporters and/or PTS systems. $(GlcNAc)_2$ and $(GlcN)_2$ are converted to 2(GlcNAc-6-phosphate), and then, together with the GlcNAc-6-phosphate generated during the uptake through the PTS, to acetate, ammonia and fructose-6-phosphate, which enters central metabolism.

The utilization of GlcNAc has been well characterized in *Escherichia coli*, where it depends on the *nagE-nagBACD* divergent operon. NagE is a GlcNAc transporter (PTS), NagB a GlcN-6-phosphate deaminase, NagA a GlcNAc-6-phosphate deacetylase and NagC a repressor that represses the operon when no GlcNAc is present in the environment (174, 175). NagD is a monophosphatase but its exact role in GlcNAc utilization has not been defined. In *V. cholerae* and *V. fischeri*, *nagE-nagAC* exists as a divergent operon on Chromosome I, *nagB* is on Chromosome II and *nagD* is not present (176–178). The GlcNAc PTS (NagE) is widespread not only among *Vibrionaceae* (166), but also among marine Firmicutes, α-Proteobacteria and other γ-Proteobacteria (167), indicating that a vast number of bacteria can uptake this molecule. In *V. fischeri*, NagC controls also a number of other genes required for chitin utilization and efficient colonization of *E. scolopes* (178).

All in all, it is estimated that chitin degradation requires between 50 and 100 genes (179, 180). Multiple copies of some of such genes have been observed within a genome, and this could be the result of gene duplication and horizontal gene transfer (166), which has been described also between distantly

related organisms (161). For several genes, transcription is induced by the presence of chitin and/or its derived oligosaccharides (167, 173, 177, 180–183). This phenomenon is in some cases under the control of the above-mentioned NagC repressor (178) and in other cases of the regulatory system ChiS, which is described in the following section.

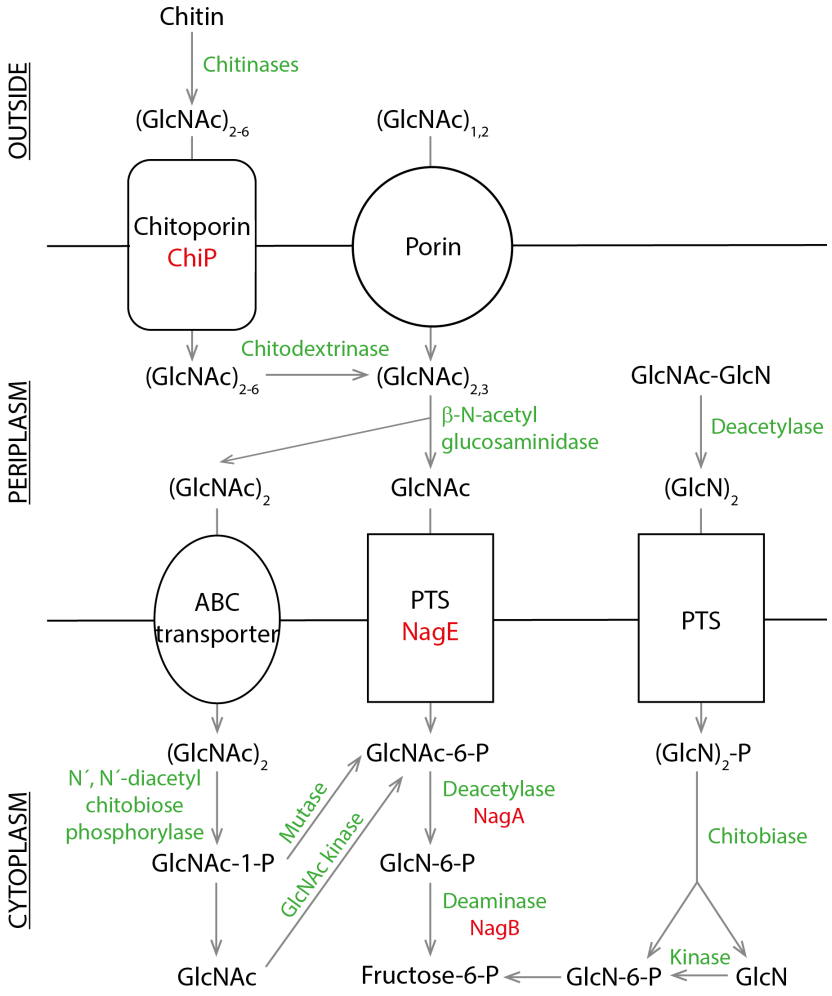


Figure 15 Chitin utilization pathway proposed for vibrios. Extracellular chitinases are responsible for the breakdown of chitin into GlcNAc oligosaccharides of different sizes, which are transported into the perisplasmic space by porins and/or specific chitoporins. In the perisplams, these chitin-derived oligosaccharides are further hydrolyzed to $(GlcNAc)_2$, GlcNAc and $(GlcN)_2$, which enter the cytoplasm through ABC transporters and PTS systems. In the cytoplasm, a number of enzymes participate to the conversion of these molecules to fructose-6-phosphate, which enters central metabolism. Green: enzyme catalyzing a reaction, red: protein name, when known. Adapted from (166).

3.3.3 ChiS: a chitin-dependent two-component signaling system

Li and Roseman (180) identified a two-component signaling system that controls the expression of genes required for chitin catabolism in *V. cholerae* and *V. furnissii* (Figure 16). In this system, the ChiS hybrid sensor kinase of the Arc B type is anchored to the inner membrane. In this type of sensors, the cytoplasm domain includes three domains: i) histidine kinase (HK); ii) receiver aspartate (RR) and iii) histidine-containing phosphotransferase (HPt). When the sensor is activated, a phosphoryl group from ATP is sequentially transferred to HK, to RR, to a His residue in HPt and, finally, to an Asp residue in the cognate response regulator that controls the transcription of target genes (184).

In the case of the ChiS system, when there is no chitin in the environment and, consequently, no $(GlcNAc)_{2\text{-}6}$ in the periplasmic space, a chitin oligosaccharide binding protein (CBP) acts as inactivator of ChiS by binding to its periplasmic domain. When, on the contrary, chitin is available, CBP complexes with $(GlcNAc)_2$ in the periplasmic space. This prevents its binding to ChiS and triggers the phosphorylation cascade that activates the cytoplasmic cognate response regulator (180). The response regulator associated to ChiS has not been identified so far.

In *V. cholerae*, the *chiS* gene (VC0622) is located downstream of a $(GlcNAc)_2$ catabolic operon (VC0620-VC0611) (180, 182). The operon encodes for the periplasmic CBP, for the ABC-type transporter necessary for the uptake of $(GlcNAc)_2$, for the periplasmic β–***N***–acetylglucosaminidase and for the enzymes required for the cytoplasmic part of chitin degradation (see Figure 13), excluding those encoded in the *nag* operon. The expression of the $(GlcNAc)_2$ operon, together with the expression of several other genes involved in chitin catabolism and in the synthesis of a pilus that facilitates adherence to chitin surfaces, is significantly reduced in *chiS* mutants, suggesting a ChiS dependent regulation (182). Beside regulation of chitin catabolism, ChiS has been directly linked to the regulation of natural competence in *V. cholerae* on chitin (185, 186). In this PhD project, we have shown that the *chiS* gene and the $(GlcNAc)_2$ operon are conserved in *Vibrio* and *Photobacterium* species and maintain their topological organization (Article 2). Hence, it is possible that the ChiS system might have a regulatory role in both genera.

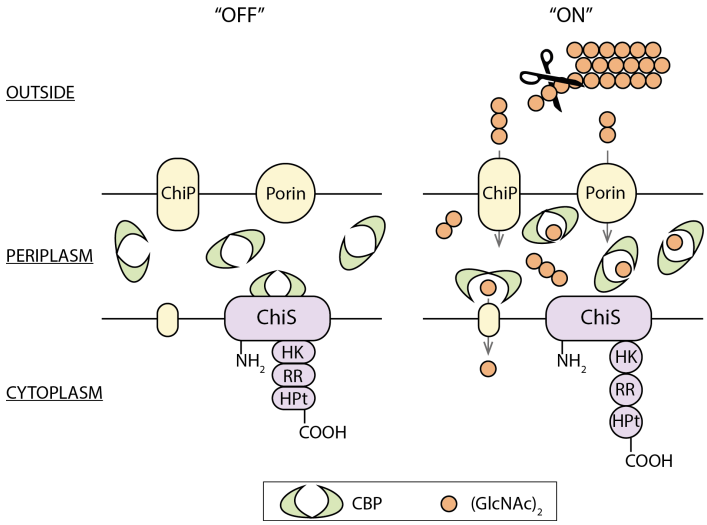


Figure 16 Model for the hybrid sensor ChiS. When no chitin is present in the environment (left side of the figure), a chitin oligosaccharides binding protein (CBP) is bound to the ChiS sensor, which is inactive. When chitin is present in the environment (right side of the figure), the CBP binds chitin oligosaccharides instead and ChiS is active. Adapted from (180) and (183).

3.4 Bioactive compounds from *Vibrionaceae*

Vibrionaceae, like many other Gram-negative bacteria, have emerged as a reservoir of bioactive compounds with therapeutic applicability just in recent years (25). Analysis of their genomes with different prediction tools revealed great potential for secondary metabolism, but there is no correlation between genome size and biosynthetic capacity (6), as exemplified in Table 2, which includes the strains on which article 3 and 4 focus.

Table 2 Number of BGCs identified by the prediction tool antiSMASH (antibiotics and secondary metabolites analysis shell) in the genomes of the four strains that were the subject of Articles 3 and 4.

Strain	Genome size (Mb)	# BGCs (antiSMASH)
V. furnissii S0821	4.98	6
V. fluvialis S1110	4.52	5
V. coralliilyticus S2052	5.43	7
P. galatheae S2753	4.53	11

Vibrios are producers of the potent neurotoxic molecule tetrodotoxin (TTX), which has been isolated from several marine macroorganisms (e.g. pufferfish) hosting vibrios as part of their microbiota (187, 188). Consumption

of food containing TTX producers can lead to poisoning and, possibly, to death by paralysis due to inhibition of sodium channels. TTX is currently undergoing Phase III clinical trials for the treatment of cancer-related pain in substitution of morphine, avoiding opioid-like side effects (www.wexpharma.com). A number of other compounds, mostly non-ribosomal peptides, have been isolated from vibrios. These molecules exert a range of bioactivities, including anticancer, antifungal and anti-algal (4). Some examples are the antibacterial and cytotoxic compounds aqabamycins A-G (189) and the anticancer molecule kahalalide F (190).

Besides compounds with pharmaceutical potential, *Vibrionaceae* are also good producers of a range of ecologically important molecules such as acyl-homoserine lactones (191) and siderophores. Furthermore, many genomes from vibrios contain aryl polyene BGCs, whose exact role is not known but, given their structural similarity to protectants against oxidative stress, it has been speculated that this may be their function, too (75).

The work included in Article 3 focused on two strains, namely *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753. The former is a coral pathogen whose virulence is temperature-dependent (154). The antibiotic andrimid (see Chapter 2) has been isolated from strain S2052 (5, 84). Andrimid was not detected in extracts from cultures of closely related strains and its production in strain S2052 was doubled when chitin was used as substrate, indicating that different strains have developed distinct eco-physiological characteristics (84). *P. galatheae* S2753 is the type strain of a new species that has been described as the result a collaborative project during this PhD (192). It was isolated from a mussel, but no further work concerning its role in this association or its relationship with other marine organisms has been done, even on the closely related species *P. halotolerans*. *P. galatheae* S2753 produces the antibiotic holomycin (5) (see Chapter 2) and a number of compounds shown to reduce virulence in the pathogen *Staphylococcus aureus* by inhibiting its QS machinery. These compounds are the depsipeptides ngercheumicins F, G, H and I (193) and solonamides A and B (194).

4 CONCLUSION AND PERSPECTIVES

Bioprospecting provides an extraordinary amount of material and information useful for the development of biotechnological processes and applications. The exploration of new environments is a condition to access novel biodiversity. In this work, the marine *Vibrionaceae* family was studied for its potential as a source of novel biodiversity and for its ecological role in the marine environment. The ecology-driven investigation was based on the assumption that the understanding of the eco-physiology of an organism provides valuable information to be used in the full exploitation of its bioprospecting potential.

The characterization of a novel species, *Vibrio galatheae*, demonstrated that, although approximately one hundred and fifty *Vibrionaceae* species are already known, further work is needed to explore the biodiversity of the family, which would also add to the knowledge about its phylogeny, distribution and evolution. This and, in general, the exploration of microorganisms, will be facilitated by the continuous evolution of technologies that enable the access to hitherto unexplored locations and niches, and by the increasing knowledge in the field of microbial eco-physiology. This knowledge allows, for example, the development of culture media that enable the cultivation of previously unculturable microorganisms.

The use of niche-specific nutrients for the growth of cultures of vibrios in the laboratory proved to be an efficient strategy to manipulate the expression of gene clusters for the biosynthesis of small molecules. The regulatory mechanisms that are responsible for this phenotype remain to be uncovered; however, the results of this thesis support the hypothesis that environmental clues can influence the biosynthesis of small molecules, suggesting an ecological function for these molecules in Nature.

Knowledge about the ecology of an organism should be used in the rational choice of substrates and culture conditions that may elicit the production of microbial small molecules. For species that are often found associated with algae, fish or mussels possible growth substrates could be, for example, extracts from these marine macro-organisms. Genome analysis and

identification of specific metabolic pathways may also provide hints about substrate preference of microorganisms, as in the case of chitin for *Vibrionaceae*. Also recording the physico-chemical parameters of the place of isolation of microorganisms (e.g. temperature, salinity, pH, concentration of heavy metals or presence of oil spills) can provide useful information in the choice of growth parameters that may affect the biosynthesis of small molecules.

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6 RESEARCH ARTICLES

This section consists of the full-length research articles that were prepared as part of this PhD project. The articles are presented in the following order:

ARTICLE 1:

S. Giubergia, C. Schleissner, F. de la Calle, A. Pretsch, D. Pretsch, L. Gram and M. Schmidt Thøgersen. 2016. Screening microorganisms for bioactive compounds. Chapter to be included in the book "The marine microbiome – an untold resource of biodiversity and biotechnological potential". *Springer. In press*.

ARTICLE 2:

S. Giubergia, C. Phippen, C.H. Gotfredsen, K.F. Nielsen and L. Gram. 2016. Influence of niche-specific nutrients on secondary metabolism in *Vibrionaceae*. *Accepted for publication in "Applied and Environmental Microbiology"*.

ARTICLE 3:

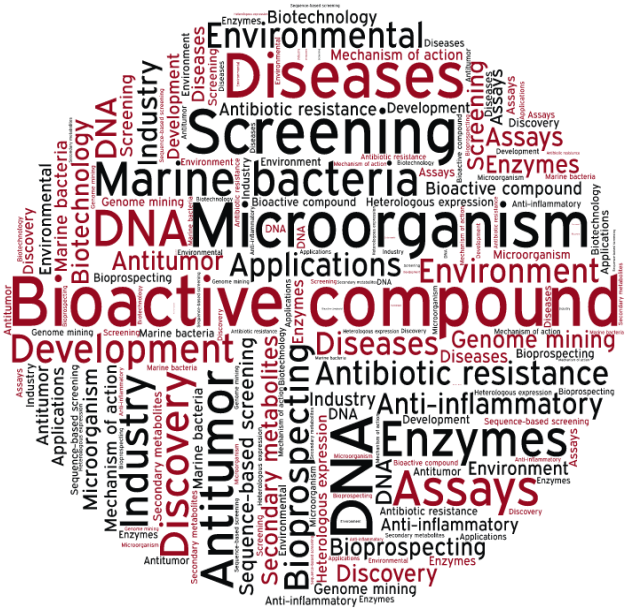
S. Giubergia, C. Phippen, K.F. Nielsen and L. Gram. 2016. Growth on chitin impacts the transcriptome and metabolite profiles of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753. *Manuscript in preparation*.

ARTICLE 4:

S. Giubergia, H. Machado, R.V. Mateiu and L. Gram. 2016. *Vibrio galatheae* sp. nov., a member of the family *Vibrionaceae* isolated from a mussel. *International Journal of Systematic and Evolutionary Microbiology 66, 347-352*.

ARTICLE 1

Screening microorganisms for bioactive compounds.



Screening microorganisms for bioactive compounds

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Abstract

Novel bioactive compounds are in high demand due to the development of microbial antibiotic resistance, increase in age-related diseases and requirements for optimized manufacturing processes. The bioactive compounds can act as specific antitumor, anti-inflammatory, and anti-fungal compounds as well as pharmaceuticals against metabolic diseases. Bioprospecting from marine microorganisms has a tremendous potential for the discovery of novel bioactive compounds as enzymes and complex secondary metabolites for industrial as well as for biotechnological and therapeutic applications. A bioprospecting process usually begins with the isolation of a native microorganism, extraction of compounds from a culture sample, or with the isolation of environmental DNA for either heterologous expression or sequencing. Extracts from live microorganisms can be screened for bioactivity in function-based screening assays, while DNA from either the isolated microorganisms or from the environment can be screened with sequence-based screening methods or genome mining. Once a desired activity has been detected, the bioactive compound should be purified, the structure should be elucidated, and preferably its mechanism of action should be described. In this chapter we give an overview of the bioprospecting process with special focus on compounds with therapeutic properties from marine microorganisms, and we evaluate some of the most commonly used strategies that have been used at different steps in the bioprospecting processes when searching for novel bioactive compounds.

1. THE NEED FOR NOVEL BIOACTIVE COMPOUNDS

During the last century, microorganisms have emerged as a valuable source of bioactive compounds with a wide range of biotechnological applications. Two major groups of such compounds are enzymes and compounds with bioactivity on bacterial, archaeal, or eukaryotic cells. Enzymes of microbial origin are used as bio-catalyzers in many different industries such as feed, detergent, textile, chemical and biofuel industries. Polymer-degrading enzymes such as cellulases and xylanases can be used for bioremediation and food processing purposes (Adrio & Demain 2014; Schmid et al. 2001). Compounds exhibiting bioactivity on bacterial, archaeal, and eukaryotic cells (from now on referred to as bioactive compounds) include microbial secondary metabolites with therapeutic properties such as antibiotic, antitumor, or anti-inflammation activity. Secondary metabolites belong to several different chemical classes of compounds, which among others include polyketides, non-ribosomal peptides, terpenes, and terpenoids. Bioactive compounds of microbial origin also include bacteriocins and bacterial polysaccharides. Bacteriocins are ribosomal peptides produced by bacteria to kill or inhibit the growth of closely related bacteria and are used in e.g. dairy and food industries to prevent food spoilage (Cleveland et al. 2001), however bacteriocins are also increasingly being recognized as potential alternatives to classical antibiotics (reviewed in Reen et al. 2015). Bacterial polysaccharides are used in food, cosmetic and pharmaceutical industries as well as in bioremediation and for the development of biomaterials and bioplastics (Nicolaus et al. 2010; Sutherland 2001).

The United Nations' Convention on Biological Diversity (CBD) defines biological prospecting, or just bioprospecting, as "the exploration of biodiversity for commercially valuable genetic and biochemical resources" (https://www.cbd.int). In other words, bioprospecting is the search for any product from Nature, such as genes, enzymes, chemical compounds or organisms for commercial purposes. In this chapter, we will focus on bioprospecting for small molecules of microbial origin with therapeutic properties.

The need for novel bioactive compounds is best exemplified by the rapid development and spread of antibiotic resistance in bacteria caused by the widespread use of antibiotics in both the clinic and the food production sector. Infections caused by antibiotic resistant bacteria are increasing (World Health Organization 2014) and this is of great concern and a threat to human and animal health, since known antibiotics are losing their effectiveness. Novel antibiotics, preferably with new mechanisms of action, are therefore urgently needed. Many compounds with antibacterial activity are of microbial origin, and some classes of such compounds may have the desired effect without being hampered by already known resistance mechanisms (reviewed in Clardy et al. 2006). As an initiative to discover and develop novel therapeutics, the Infectious Disease Society of America (IDSA) has launched the "10 x '20 Initiative" with the aim of bringing at least 10 new antibiotics to the marked before 2020 (http://www.idsociety.org/10x20/).

With the increase in average life expectancy of the human population, age-related diseases such as Alzheimer's disease, Parkinson's disease, osteoporosis, and several types of cancer are also requiring new and improved drugs to optimize treatment. Especially for Alzheimer's and Parkinson's diseases, the drugs used today only provide symptomatic relief but no cure.

1.1 Bioactive compounds from the marine environment

Groups of soil microorganisms such as *Streptomyces* and *Micromonospora* as well as several species of filamentous fungi have for years been the most commonly known microbial reservoirs of bioactive compounds (Bérdy 2005; Hoffmeister & Keller 2007; Keller et al. 2005; Watve et al. 2001). However, recent advances in novel bioprospecting technology are providing the opportunity to explore new habitats. These include the ocean, the deep sea, the cryosphere, and the deserts (Gerwick & Moore 2012; Molinski et al. 2009).

The ocean covers more than 70% of the Earth's surface and represents more than 95% of the total biosphere. Thirty-three of the thirty-five known animal phyla of life are present in the oceans and thirteen are exclusively marine phyla (Heip & McDonough 2012), making it an invaluable source of biodiversity potentially useful for bioprospecting purposes. The environmental

conditions of the oceans require selection and maintenance of unique biosynthetic pathways as a result of adaptation to pressure, salinity, temperature, oligotrophic conditions and unique chemical compounds (Fenical & Jensen 2006; Lozupone & Knight 2007). Several marine-derived natural products therefore have new chemical features as compared to molecules isolated from terrestrial environments, exemplified by the incorporation of the halogen atoms bromide or chloride (Gerwick & Moore 2012; van Pée 1996).

Early studies of the marine environment focused on natural products from invertebrates such as sponges, bryozoans, algae and corals, and led to the isolation of several classes of bioactive natural products including polyketides, non-ribosomal peptides, terpenes, and indoles (DeGroot et al. 2015; Proksch et al. 2002; Woodhouse et al. 2013). However, evidence is currently emerging, based on metagenomics of marine sponges and tunicates and their associate microbiota, that many of the compounds originally isolated from eukaryotic organisms are in fact of bacterial origin, as will be outlined below.

Many marine organisms have not developed an adaptive immune response and instead rely mostly on an immediate, innate immune system as the defense against pathogenic microorganisms. Also, many marine microorganisms have developed specialized strategies against predators. It is therefore expected that marine bacteria are capable of producing an array of anti-inflammatory compounds to better be able to evade the innate immune response of the host organism. For instance, several cyanobacteria produce such anti-inflammatory compounds, potentially targeting a broad range of molecular targets (Stevenson et al. 2002; Villa et al. 2010).

There are several cases of antitumor compounds originally isolated from marine invertebrates and tunicates and later identified in the genomes of bacteria (Lane & Moore 2012; Wilson & Piel 2013; Wilson et al. 2014). The similar chemical structure between safracin B and ecteinascidins (trabectedin, Yondelis®, the first European marine-derived compound approved for cancer treatments and developed by PharmaMar) has been corroborated to a genetic level by the identification of a NRPS gene cluster for the putative biosynthesis of ecteinascidins into a bacterial symbiont living into the ascidian cells (Rath et al., 2011). Another example is the anticancer and anti-Alzheimer's compound

bryostatin, which is currently undergoing clinical trials. Bryostatin was first identified in extracts from the bryozoan *Bugula neritina*, but the polyketide synthase (PKS) cluster putatively responsible for its biosynthesis has been identified in the bacterial symbiont *Candidatus* Endobugula sertula (Davidson et al. 2001). Similarly, the anti-tumor depsipeptide dolastatin 10 was first isolated from the sea hare *Dollabella auricularia* (Pettit et al. 1993) and later from the marine cyanobacterium *Symploca* sp. VP642 (Luesch et al. 2001). Also, the Gram-positive *Bacillus pumilus*, isolated from the marine sponge *Acanthella acuta*, produces diglucosyl-glycerolipids with anti-tumor properties (Ramm et al. 2004)

The Gram-positive actinobacteria, which are already known as an excellent reservoir of natural products in the terrestrial environment (Bérdy 2005), have also emerged as producers of novel bioactive compounds in the marine environment. Although several compounds have been isolated from marine species of *Streptomyces* (Khan et al. 2011; Manivasagan et al. 2014; Schleissner et al. 2011), probably the best-known example is the novel actinomycete genus *Salinispora*. This genus includes only three species, but has already provided a number of bioactive compounds, including the anticancer compound salinosporamide A and derivatives of the antibiotic rifamycin (Fenical & Jensen 2006). Other interesting actinomycete genera for drug discovery are *Saccharopolyspora* (Pérez et al. 2009), *Micromonospora* (Romero et al. 1997) or *Nocardiopsis* (PharmaMar, personal communication), which contain several putative biosynthetic gene clusters.

Marine Gram-negative bacteria are currently emerging as potential producers of such compounds and novel natural products have been discovered in marine cyanobacteria (Burja et al. 2001; Calteau et al. 2014; Tan 2007), and Proteobacteria (Proksch et al. 2002; Schäberle et al. 2010). For instance, the gammaproteobacterial genera *Pseudomonas*, *Pseudoalteromonas* and *Vibrio* have provided several compounds with bioactivities including antibacterial and anti-virulence activity (Bowman 2007; Chellaram et al. 2012; Gram et al. 2010; Mansson et al. 2011; Nielsen et al. 2012; Nielsen et al. 2014; Vynne et al. 2011; Whalen et al. 2015; Wietz et al. 2010). Furthermore, members of the alphaproteobacterial *Roseobacter* clade produce bioactive compounds,

including the antimicrobial compounds indigoidine (Cude et al. 2012) and tropodithietic acid (TDA) (Brinkhoff et al. 2004; Bruhn et al. 2007) (Figure 1).

2. BIOPROSPECTING FOR BIOACTIVE COMPOUNDS

Bioprospecting of microbial natural products can include the following steps:

1. Isolation and molecular characterization of microorganisms
2. Culture and extract preparation
3. Construction of metagenomic libraries
4. Screening for bioactivities
5. Chemical dereplication, compound purification and structure elucidation

A bioprospecting process is not linear, and many different approaches can be used. Sometimes the starting material is a live microorganism, wild type or recombinant, in other situations it is a crude extract from cultures of microorganisms, and in some cases it is a library of pure compounds. Here we will describe a classical methodology following the steps described above, well knowing that many researchers choose alternative strategies, and that one often has to re-visit a screen or an assay several times before a novel, pure bioactive compound can be presented.

It is important to note that the strategy chosen for bioprospecting is independent of the origin of the samples; hence, here we will provide examples that have been used in the discovery of natural products from microorganisms of both terrestrial and marine origin with a focus on the latter.

3. ISOLATION OF MICROORGANISMS

The first step in bioprospecting for microbial natural products is to isolate microorganisms or to purify microbial DNA from environmental samples. Any biological sample collection should be carried out in accordance with the Convention on Biological Diversity, the Cartagena Protocol and the Nagoya Protocol (https://www.cbd.int). These documents have been created to ensure the conservation of biological resources, their sustainable use and the equitable

and fair sharing of benefits deriving from the use of biological and genetic resources. Despite the fact that several countries in the world have signed the convention and the protocols, they have not been ratified by all of them. There should be an agreement between those collecting the biological material and the country where the sampling takes place.

The culturability of microorganisms is an important consideration in bioprospecting. With the current standard laboratory culture conditions, only approximately 1% of all bacteria have been brought into culture. For marine bacteria this number is thought to be even lower; between 0.01 and 0.1% (Gram et al. 2010; Kogure et al. 1979). The bioprospecting potential of microorganisms is therefore potentially overlooked since they simply have never been cultured in the laboratory. However, efforts are now being made to develop devices and techniques in order to increase microbial culturability or to use microbial DNA as the starting material.

In traditional isolation techniques, microorganisms from e.g. soil, water, or invertebrate samples are cultured on artificial substrates and strains able to grow into colonies are selected for further analyses. Selective media are often used to enrich for a desired group of bacteria. Thaker et al. (2013) succeeded in the isolation of scaffold-specific natural product producers; the assumption was that producers of antibiotic compounds must have a mechanism of self-resistance to the produced compounds to avoid suicide. Therefore, the addition of a specific antibiotic to the medium will select for resistant microorganisms, which would also be likely to produce other compounds with the same scaffold. Indeed, the addition of the glycopeptide antibiotic (GPA) vancomycin to the isolation medium led to the isolation of the novel GPA pekiskomycin.

Several strategies are currently being exploited to increase microbial culturability. When in their natural habitat, microorganisms are exposed to a multitude of factors that most often are not present in traditional laboratory culturing, including the presence of other microorganisms, specific substrates and growth factors (Marmann et al. 2014; Stewart 2012). Connon and Giovannoni (2002) succeeded in the high-throughput isolation of previously uncultured marine bacteria using the “dilution-to-extinction” approach based on the work of Button et al. (1993). The dilution of seawater samples to a

concentration of one to five bacterial cells per ml led to an increase in the number of isolated strains of up to three orders of magnitude compared to direct inoculum of water samples onto solid medium. In contrast, after plating serial dilutions of a microbial suspension obtained from marine environmental samples, D'Onofrio et al. (2010) observed that there were significantly more colonies on densely inoculated plates as compared to diluted ones. They hypothesized and proved that the growth of some of the isolates from the densely inoculated plates depended on neighboring colonies providing growth factors, in this case siderophores.

An alternative strategy to increase bacterial culturability is to introduce an inoculum of environmental cells into culture chambers spatially delimited by semi-permeable membranes, whose pore size prevents cells to pass through but enables free exchange of molecules with the external environment. The chamber is then incubated either at the original sampling location or in an artificial environment simulating it. Several variants of such chambers are available, such as the isolation Chip (ichip) (Nichols et al. 2010) which is miniaturized and optimized for high-throughput isolation of hundreds of strains in parallel, and the MicroDish Culture Chip, which consists of up to 180,000 culture areas 20 μm across on top of a porous base that allows for passage of nutrients from below when the chip is placed on e.g. an agar substrate (www.microdish.nl). The use of culture chambers has recently resulted in the isolation of the novel, previously uncultured species *Eleftheria terrae* (provisional name), which produces teixobactin, an antibiotic with a novel mode of action active against both *Staphylococcus aureus* and *Mycobacterium tuberculosis* (Ling et al. 2015).

In order to construct a collection of unique culturable strains, biotechnological methods for molecular de-replication, such as DNA fingerprinting, and identification based on sequencing of the 16S rRNA gene must be used. In the future, genome sequence based identification and de-replication is likely to be the method of choice.

4. CULTURE AND EXTRACT PREPARATION

When native organisms are successfully brought into culture, the next challenge is to unlock their bioactive potential. The artificial *in vitro* culture conditions are most often not identical to the original environment of the microorganisms, and chemical substances, signaling molecules and other compounds important for induction of gene expression might be missing.

In bioprospecting, screening for bioactivity is carried out using either live organisms or extracts from whole cultures or sub-fractions hereof (*i.e.* separation of biomass from the supernatant). In preparation of an extract, the choice of the solvent depends on the availability of information about the nature of the compound to be extracted. For non-polar compounds, organic solvents such as hexane and chloroform can be used, while for polar ones water, ethanol, or methanol are suitable. When there is no information about the nature of the compounds, extraction will have to be carried out by a trial and error approach: often medium polarity extractions with ethyl acetate or dichloromethane can be applied, or alternatively a mixture of solvents. In the case of large liquid cultures where liquid-liquid extraction would be challenging due to the big volumes to be handled, it is possible to add an adsorptive resin (e.g. DIAION HP 20®) to the culture: after 24-48 hours of incubation, the resin is separated from the cultures and the secreted metabolites can be extracted from its surface.

Microbial crude extracts are usually complex mixtures of compounds including cellular and media components. The most commonly used strategy to narrow down the complexity of the crude extract is bioassay-guided isolation, where the complexity is reduced by fractionation, and bioactive fractions are identified by means of a bioassay (Gerwick et al. 1994; Wietz et al. 2010). An alternative to working with crude extracts is to build libraries containing pure compounds, which are then screened for bioactivity. This approach, however, requires more resources as compared to the bioassay-guided isolation (Wagenaar 2008).

When it is not possible to bring the native microorganisms into culture, an alternative strategy is to extract DNA directly from the environment for metagenomic analyses. The isolated DNA can either be used to construct clone

libraries to be tested in bioactivity screening assays, or alternatively, the DNA sequences can be mined using software developed to search for biosynthetic gene clusters or other structures indicating potential production of bioactive compounds.

5. CONSTRUCTION OF METAGENOMIC LIBRARIES

The metagenomic approaches bypasses the microbial isolation and culturing steps, however, isolation of DNA directly from an environmental sample is a major challenge, especially when dealing with samples from extreme environments. Extremophilic microorganisms are often reluctant to standard lysis protocols developed for mesophilic microbes and, when lysed, they often release stable nucleases that will degrade the purified DNA (Simon & Daniel 2011).

When constructing a metagenomic library, DNA is purified directly from an environmental sample, fragmented, and cloned into a host organism using a selected vector. This vector can be either an expression plasmid for direct gene expression from small DNA fragments (Lynch & Gill 2006; Schmitz et al. 2008) or a larger plasmid such as BAC (bacterial artificial chromosome) plasmids. Larger plasmids are commonly used to insert and sequence large fragments of environmental DNA (eDNA) to search *in silico* for known or hypothetical genes or gene clusters (O'Connor et al. 1989; Shizuya & Kouros-Mehr 2001). However, it is important to keep in mind that many gene clusters encoding e.g. polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) hybrids are too large for classical heterologous expression and that novel expression systems should be developed for expression of large gene clusters (>50 kb).

In the preparation of expression libraries for smaller genes or gene clusters, both the vector and the host for direct gene expression have to be chosen with great care to ensure maximal chance of successful expression of the desired gene(s). In case the host has an expression system or a secretion system too different from the native donor strain (e.g. the simple issue of Gram type), no active compounds will be produced even though the genetic potential was actually there. Several commercial host organisms as well as broad-host-range

vectors are available, but there is no universal expression system available that covers all bacterial species yet (Aakvik et al. 2009; Lale et al. 2011).

Promoter trap or gene trap libraries have now become more commonly used since they rely on only small fragments of eDNA and can be easily screened for the presence of a desired gene or promoter by using a standardized reporter molecule like green fluorescent protein (GFP) or luciferase (Izallalen et al. 2002; Kondo et al. 1993; Rediers et al. 2005).

A variety of bioactive compounds including primarily enzymes but also therapeutics from the marine environment have in the last decade been discovered via metagenomic approaches (reviewed in Barone et al. 2014). In a study by Piel et al. (2004), DNA was extracted from the marine sponge *Theonella swinhoei*, cloned into cosmids and screened using primers specific for PKS and NRPS clusters. This resulted in the identification of two gene clusters originating from a bacterial endosymbiont encoding onnamides and theopederins, a class of polyketides with anti-tumor activity (Piel et al. 2004). Other examples of marine compounds found through metagenomic approaches can be seen in table 1.

6. SCREENING FOR BIOACTIVITY

When screening for bioactivity, two overall approaches are possible: sequence-based screening and function-based screening. In sequence-based screening, sequence analysis is performed to identify genes or gene clusters potentially encoding molecules or biosynthetic pathways of interest. In function-based screening, bioassays are performed to identify the desired bioactivity directly in the live microorganisms or the extracts. The two strategies should not be considered as independent and incompatible, instead they are often combined to accelerate and optimize the bioprospecting process (Mansson et al. submitted).

6.1 Sequence-based screening

In sequence-based screening, DNA sequences are analyzed to identify conserved regions deriving from known gene or protein families. This can be achieved *in vitro* by using e.g. DNA probes or degenerated primers (Ayuso-Sacido & Genilloud 2005; Ehrenreich et al. 2005) or phage display (Yin et al. 2007). However, with recent advances in DNA sequencing techniques, such analyses are most often done by *in silico* homology search based on whole genome sequencing of an isolated strain or sequencing of metagenomic libraries.

6.1.1 Genome mining

In silico sequence-based screening, often referred to as genome mining, is an attractive approach in the natural product discovery pipeline. Several tools have been developed for the identification of conserved domains that are likely to be part of biosynthetic gene clusters. Some of them are publicly available and include NaPDoS for the identification of keto-synthase and condensation domains, which are core enzymes in PKS and NRPS, respectively (Ziemert et al. 2012), BAGEL for the identification of bacteriocins (de Jong et al. 2006), and AntiSMASH for the identification of a wide range of biosynthetic clusters, ranging from PKS and NRPS to siderophores (Medema et al. 2011). For a complete review of the available *in silico* tools for the genome mining of natural products, see Weber (2014).

These sequence tools are based on homology searches, meaning that only previously characterized families of genes and gene clusters are detected, and that the discovery of novel classes of bioactive compounds is almost impossible. However, efforts are being made to overcome this problem, as demonstrated by the recently developed algorithm ClusterFinder (Cimermancic et al. 2014). This tool enables the identification of both known and unknown biosynthetic gene cluster by converting nucleotide sequences into protein family (Pfam) domains and calculating for each domain, the probability of it being part of a gene cluster. A comprehensive analysis of 1154 genome sequences using ClusterFinder has revealed the presence of large families of biosynthetic gene clusters of unknown function, indicating that the microbial biosynthetic potential is far from exhausted (Cimermancic et al. 2014).

Information obtained with genome mining can accelerate the natural product bioprospecting process by identifying isolates or clones that produce already known compounds, enabling the prediction of expected classes of compounds as well as structural predictions (Jensen et al. 2014). For example, mining of the genome of the marine bacterium *Salinispora pacifica* strain CNT-133 enabled the identification of a truncated gene cluster related to the salinosporamide A biosynthetic gene cluster in *Salinispora tropica* strain CNB-440 (Eustáquio et al. 2011). The anticancer compound salinosporamide A has a chloroethyl group on C-2 (Figure 2), and the comparison of the two gene clusters led to the hypothesis that, if a salinosporamide were to be produced by *S. pacifica*, it would not be halogenated. Indeed, the compound that later was isolated and structurally characterized, salinosporamide K, lacks the C-2 chloroethyl group (Figure 2).

Silent biosynthetic gene clusters

Genome mining has revealed that microorganisms often have the potential to produce other natural products than identified using extractions and bio-assay-guided fractionation (Gram 2015; Helfrich et al. 2014; Jensen et al. 2014; Machado et al. 2015). This has opened up a new branch in natural products research, where molecular biology, microbial ecology and physiology are merged to elucidate how the silent or cryptic biosynthetic gene clusters can be elicited. The "One Strain-MAny Compounds (OSMAC)" approach (Schiewe & Zeeck 1999) was successfully used in several studies, where the variation of culture parameters (e.g. media composition, culture vessel, aeration, addition of enzyme inhibitors or rare earth elements, or co-culturing with other microorganisms) elicited previously silent biosynthetic gene clusters (Bode et al. 2002). Culture parameters can be modified to simulate the environmental niches of microorganisms. For example, antibacterial activity was observed in the supernatant of cultures of two marine *Bacillus* strains, only when they were grown into an agar-coated roller bottle mimicking the intertidal environment of isolation and not when they were cultured in standard shaking flasks (Yan et al. 2002). Similar results have been obtained with "air-membrane surface" (Yan et al. 2003) and "rotating disc" bioreactors (Sarkar et al. 2008). The exposure to low concentrations of antibiotics and other small molecules produced by microorganisms elicited two cryptic biosynthetic gene clusters in the soil

bacterium *Burkholderia thailandensis* (Seyedsayamdost 2014), whilst co-culturing setups caused variations in the secondary metabolism profile of *Streptomyces coelicolor*, as captured by imaging mass spectrometry (Traxler et al. 2013). Access to a carbon source typical of the niche of isolation can influence the biosynthesis of a given compound. For instance, it has been observed that the addition of chitin to the growth medium can elicit a two-fold increase in the biosynthesis of the antibiotic compound andrimid in the marine bacterium *Vibrio coralliilyticus* S2052 as compared to glucose (Wietz et al. 2011).

An alternative way to access silent biosynthetic gene cluster relies on homologous and heterologous gene expression. In homologous gene expression, transcriptional, translational or metabolic elements are manipulated to elicit the expression of the targeted biosynthetic gene cluster. However, this is possible only when the host is easily culturable and not recalcitrant to genetic manipulation. The introduction of drug-resistance mutations in the ribosome and in RNA polymerase, so-called ribosomal engineering, greatly influences secondary metabolism in actinomycetes (Hosaka et al. 2009). Ribosomal engineering has also led to the isolation of the anti-tumor molecule fredericamycin A from the deep-sea derived *Streptomyces somaliensis* SCSIO ZH66 (Zhang et al. 2015) and to a 180-fold higher production of the antibiotic actinorhodin in *Streptomyces coelicor* A3(2) (Wang et al. 2008). Moreover, homologous overexpression of a regulatory gene controlling the biosynthesis of the precursor of the C-2 chloroethyl group in salinosporamide A selectively doubled the yield of the compound in the natural producer *Salinispora tropica* (Lechner et al. 2011).

In heterologous expression, single genes or gene clusters are expressed in a heterologous host. A prerequisite is the availability of well-developed genetic toolboxes that enable the introduction of gene clusters up to 100 kb in size. The native promoters of biosynthetic pathways are often not strong enough to trigger the expression in heterologous hosts, and it is necessary to place the biosynthetic cluster under control of strong, inducible promoters. For instance, it has been possible to express the gene cluster encoding biosynthesis of the antibiotic polyketide oxytetracycline from *Streptomyces rimosus* in *Escherichia coli* only when one of the heterologous host's native sigma factor was

overexpressed (Stevens et al. 2013). An example of heterologous expression of a large NRPS gene cluster from a marine *Micromonospora* in an industrial *Streptomyces* is described for the antitumoral peptide thiocoraline (Lombó et al. 2006). In general, several molecular tools have been used for heterologous expression of silent biosynthetic gene clusters in bacteria and fungi. Some examples to be mentioned here are the transformation associated recombination (TAR) strategy (Ross et al. 2015; Yamanaka et al. 2014), the TRansfer and EXpression of biosynthetic pathways (TREX) system (Loeschcke et al. 2013), the red/ET (Wenzel et al. 2005) and the RecE/RecT recombination systems (Fu et al. 2012). For further examples and strategies, the review from Ongley et al. (2013) exhaustively covers advances on the topic until 2013.

6.2 Function-based screening

In function-based screening, bioassays are performed to detect a desired bioactivity in collections of isolated microorganisms, on sequence-based sub-selections of such, on recombinant expression hosts, or on culture extracts. Strategies that are most commonly used in function-based screening campaigns can be sub-divided into phenotypic screens and target-based screens. By definition, phenotypic screens measure the effect, or phenotype, that the tested compounds induce in target cells or organisms, whereas target-based screens investigate the ability of a compound to bind or inhibit purified targets *in vitro* (Kotz 2012). Phenotypic screens can be cell-based (*in vitro* screens on single cells or tissues), or involve model organisms (*in vivo* screenings) such as *Saccharomyces cerevisiae* (yeast), *Drosophila melanogaster* (fruit fly), or *Caenorhabditis elegans* (nematode). In target-based screens, the target of interest is purified and *in vitro* biochemical assays are established to investigate the effects of a range of compounds on the target.

Traditionally, phenotypic screens were favored by the pharmaceutical industry. However, difficulties encountered in target identification as well as advancement in multiple disciplines such as molecular biology, flow cytometry, chemical proteomics and imaging techniques led to the establishment of target-based screens. The outcome of target-based screening campaigns was not as high as expected, though, and today there is a renewed interest in phenotypic

screens both in academia and in the pharmaceutical industry in companies such as Novartis AG and GlaxoSmithKline (Eggert 2013; Kell 2013; Kotz 2012; Swinney & Anthony 2011; Zheng et al. 2013). The two approaches are often combined: phenotypic screens are not completely target-agnostic and targets for target-based screens can be chosen based on results from phenotypic assays (Kell 2013; Moffat et al. 2014; Sams-Dodd 2005). Examples on how the two screening strategies can be used and combined can be seen in sections 6.2.1-6.2.4 on strategies for discovery of antibacterial, anti-viral, anti-tumor, anti-Alzheimer's, and anti-Parkinson's compounds.

6.2.1 Screens for antibacterial activity

Due to the increased antimicrobial drug resistance in many pathogenic microorganisms and the lack of therapeutic alternatives to the classical antibiotics, prospecting for novel antibacterial compounds has become of high priority. Classical function-based screening of live bacteria or extracts is often based on agar plate or broth based assays where for instance growth inhibition by an antagonistic compound or a colorimetric reaction indicative of a desired activity or molecule can be observed.

Live targets

A commonly used approach to detect antibacterial compounds is the overlay method developed by Waksman (Schatz et al. 1944). In brief, a potential producer strain is grown on a solid medium, which is then overlaid with soft agar seeded with a target bacterium. After a period of incubation, the presence of antibacterial compounds, to which the target is susceptible, is indicated by a clear halo in the top agar due to the lack of growth of the target strain. Alternatively, the potential producer strain can be on the seeded medium. The latter option can be used directly during the isolation procedure to select for strains displaying antibacterial activity or by replica plating a master plate on the seeded medium (Gram et al. 2010). The production of antagonizing compounds will be seen as a clear zone in the agar or the by lack of growth of the target strain, respectively (Figure 3).

BioMAP (antiBIOtic Mode of Action Profile) is an assay that can be used for the growth-independent function-based screening of crude extracts. The BioMAP assay consists of a panel of 15 clinically relevant bacterial pathogens and provides a function-based high-throughput platform for screening natural products in order to identify new lead compounds with unique biological profiles. The activity profile of a given extract can then be compared to profiles of known antibiotics and used to determine the structural class of the anti-bacterial compound in the extract (Higginbotham et al. 2014; Wong et al. 2012).

Biosensors

Another strategy to screen for novel antibiotic compounds is to apply the whole-cell antibiotic biosensors strategy described by Urban et al. (2007). Promoter regions selectively and strongly induced by bactericidal antibiotics were identified in *Bacillus subtilis* and used to construct five biosensors consisting of the *B. subtilis* promoters fused to the firefly luciferase reporter gene *lucFF*. This allowed for high-throughput detection of compounds interfering with DNA synthesis (*yorB* promoter), RNA synthesis (*yvgS* promoter), protein synthesis (*yheI* promoter), cell wall synthesis (*ypuA* promoter), and fatty acid synthesis (*fabHB* promoter). The biomarker-carrying strains of *B. subtilis* were then subjected to approximately 14,000 pure natural compounds, screened in a 384-well microtiter plate format using a luminescence detector, and the study led to the discovery of novel antibiotics in the form of DNA synthesis and translation inhibitors.

Since several bacterial virulence factors are under quorum sensing (QS) regulation, there is a great interest in compounds that can specifically block QS as potential novel classes of antibiotics (Rasmussen & Givskov 2006). Biosensors have been designed allowing for identification of such compounds. One example is the detection of the bacterial QS molecules *N*-acyl-homoserine lactones (AHLs). In *Agrobacterium tumefaciens lacZ::traG*, the *lacZ* reporter gene encoding a β-galactosidase is fused to the promoter of the QS-regulated *tra* operon. The AHL-induced expression of the operon and reporter gene is then visible as a blue precipitate due to β-galactosidase-mediated hydrolysis of X-gal present in the medium (Cha et al. 1998). High-throughput functional screening of single cells for AHL production is also possible as described by Williamson et

al. (2005), who prepared a soil metagenomic library using *E. coli* cells containing a green fluorescence protein (GFP)-based AHL biosensor. Clones producing QS inducers could then be detected by fluorescence microscopy due to GFP expression, while clones producing QS inhibitors were identified using fluorescence-activated cell sorting (FACS) on cells in which GFP expression was induced by an exogenous AHL.

Molecular targets

Novel antimicrobial compounds are also identified by screens that are aimed directly at the targets of the antimicrobial compounds such as components of cellular pathways. Typically, assays are performed to screen for inhibitors of macromolecular synthesis using radioactively labeled precursors of protein, RNA, DNA, lipid or peptidoglycan synthesis (Cotsonas King & Wu 2009; Nonejuie et al. 2013). However, these assays do not allow for determination of the mechanism of action (MOA), and often they do not distinguish between inhibitors that affect the same pathway.

Transcriptional profiling measures total gene expression to give an overall picture of the cellular functions. Even though transcriptional profiling sometimes can be used to identify the molecular targets of a bioactive compound, the method is slow and often fails to identify the target. Instead, Nonejuie et al. (2013) developed a bacterial cytological profiling (BCP) assay, which can distinguish between antibacterial compounds with different MOA and also predict the MOA of novel compounds. In practice, the bacterial cells were treated with the potential antibacterial compounds and after incubation stained with FM4-64 which stains the membrane (Pogliano et al. 1999), with DAPI which stains DNA (Kapuscinski 1979), and with SYTOX Green Nucleic Acid Stain, which functions as a live/dead stain, since it is only able to penetrate membranes that have been made permeable (Molecular Probes, Thermo Fisher Scientific Inc.). The cells were then subjected to fluorescence microscopy and the intensity of the stains was determined and used as a measure of the cell's cytological profile.

Several assays are available to screen for membrane-damaging compounds. However, such compounds have to be specific with respect to their

target, since compounds targeting the cytoplasmic membrane would potentially not limit their activity to bacteria and ultimately cause toxicity in the mammalian host. Therefore, a range of assays to detect membrane damaging compounds specific against e.g. *Staphylococcus aureus* have been developed. O'Neill et al. (2004) applied a β-galactosidase (BG) assay, where the *lacZ* gene from *E. coli* has been put under the control of a strong staphylococcal promoter, *cap1A*. Leakage of BG from the strain subjected to potential membrane damaging compounds could then be detected using a fluorescence assay. Other assays to detect membrane damage in bacteria includes ATP release (Johnston et al. 2003), leakage of nucleic acids (material absorbing at OD_{260}) (Carson et al. 2002), and the commercially available LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit from Molecular Probes (Thermo Fisher Scientific Inc.).

Other molecular targets used in screening for novel antibacterial compounds are components of the major signal transduction pathways such as histidine kinases (Bem et al. 2015) or cell wall components like lipopolysaccharides and transporter complexes (Nayar et al. 2015). Nayar et al. (2015) applied a high-throughput phenotypic screening assay based on a *Citrobacter freundii* AmpC β-lactamase reporter system to discover novel compounds inhibiting cell wall synthesis. Inhibitors of cell wall synthesis also induce expression of AmpC β-lactamase (Sun et al. 2002). Hence, when β-lactamase was expressed, degradation of nitrocefin (a chromogenic cephalosporin substrate added to the cultures) could be detected in a plate reader, allowing for a 384-well microtiter plate format. Using this strategy, Nayar et al. (2015) successfully discovered two novel antibacterial compounds, sulfonyl piperazine and pyrazole, which are not cross-reactive with any known antibiotic and with completely novel MOAs.

6.2.2 Antiviral drug screening with CPE inhibition test

From the screening perspective, most antiviral test systems are based on infection of a host cell line or an animal. Many animal virus models have been established but for high-throughput screening of natural products cell lines are the better choice also because of animal ethical reasons. In most cases, a virus is highly specific, shows organ tropism, and infects defined parts of the body. The

susceptibility for infection of the used viral type therefore regulates which type of host or cell line should be chosen for screening purposes.

Respiratory viruses like influenza, respiratory syncytial virus, para-influenza and especially human rhino virus, infect the upper respiratory tract including nose and throat and are some of the most common viruses in humans (Denny 1995). Being not highly virulent with low morbidity and weak symptoms, these types of viruses are in many cases precursors for more severe bacterial and fungal infections and can causes enormous financial damage (Gonzalez et al. 1987; Gonzales et al. 1997). With more than 100 different serotypes (Greve et al. 1989; Hofer et al. 1994) it is important to screen against as many viruses as is possible to check activity against the whole group, otherwise some serotypes will be preferred and might quickly become dominant (Andries et al. 1992).

The cytopathic effect (CPE) refers to the structural changes in a host caused by a viral infection. As screening method, the CPE inhibition assay is a fast and effective tool to verify if a natural product has antiviral potential (Schmidtke et al. 2001). Read-out from the assay is the inhibition of the CPE *i.e.* the survival of the targeted cells. In infected and non-treated cell cultures, an increasing cytopathic effect with characteristic cell granulation, shrinking cells, plaque production and spherical cell shapes can be observed. This cell morphology starts to change as the result of the reprogramming of the cell for viral production. At the end, most viruses replicates by destroying the host cell via lysis. In combination with plaque assay, the CPE inhibition assay could furthermore be used for definition of the detailed viral titer (Bachrach et al. 1957; Cooper 1961).

In general, there are two different types of screening in the CPE inhibition assay. In the prophylactic assay, the ability of a compound to affect the virus directly is tested. In this approach, the virus is mixed with the compound and incubated. This time allows the compound to target straight at the virus and block it or destroy it before it gets in touch with host cell line. Many antiviral blocking agents and biocides work with this mode of actions (Gonzalez et al. 1987). In contrast, in the therapeutic assay the compound

attacks the virus after cell infection, and, hence, mainly stays inside the host cell. Here the bioactive candidate is added to the cell culture media and the impact is on important switches in viral replication and packaging. Due to the fact that these approached require incubation time during the entire experiment, toxicity, solubility and stability are critical factors to be considered. Therefore, it is recommended to collect as much as possible pre-information about the compound to be tested.

6.2.3 Screening for antitumor activity

The fundamental goal in cancer drug discovery is to kill or re-program malignant cells while minimizing adverse effects on non-tumoral cells. Cancer encompasses a large number of molecularly and phenotypically distinct diseases, and hence it demands a much larger repertoire of drugs with distinct MOAs than most other diseases. For the past 50 years, phenotypic screenings in cancer drug discovery has been based on cytotoxicity assays using cancer cell lines that exhibit the phenotype of unrestrained fast growth. Such antiproliferative assays in cancer drug discovery have resulted in the development of a repertoire of chemotherapeutic agents (DeVita & Chu 2008) and are currently used in many oncology drug discovery programs. The National Cancer Institute 60 (NCI60) platform introduced the concept of high-throughput cell-based profiling using a panel of 60 different tumor cell lines.

The most frequently used cytotoxicity assay in a high throughput screening platform is usually a colorimetric method, for example sulforhodamine B (SRB), for quantitative measurement of cell growth and viability (Vichai & Kirtikara 2006). Cultured cell lines derived from many different types of human cancer are used in such assays. Cytotoxicity is typically estimated using the National Cancer Institute (NCI) algorithm (Boyd & Paull 1995) which gives three end-points that can be used to determine compound activity. These are GI_{50} (concentration required to inhibit 50% of cells), TGI (concentration required for total growth inhibition), and LC_{50} (concentration required to kill 50% of the cells) (Holbeck 2004). This screening technology requires the use of microplates, automated liquid handling and involves a high volume of data analysis.

Recent technologies that facilitate the parallel analysis of large panels of cell lines, together with genomic technologies that define their genetic constitution, have revitalized efforts to use cancer cell lines to assess the clinical utility of new investigational cancer drugs and to discover predictive biomarkers (Moffat et al. 2014). A human tumor cell line platform has been established to provide as broad a representation as possible of different cancers and includes 1,200 cell lines. This panel is referred to as the Centre for Molecular Therapeutics 1000 (CMT1000) and is being used to probe the genetic basis for sensitivity to approved and investigational anticancer agents (Sharma et al. 2010).

Several bioactive anti-cancer compounds have been isolated from marine invertebrates, but have later been proven to be of microbial origin. The structural similarity between safracin B and ecteinascidins (trabectedin, otherwise known as Yondelis®, which was the first European marine derived compound approved for some cancer treatments and developed by PharmaMar has been resolved to the genetic level by the identification of a NRPS gene cluster for the putative biosynthesis of ecteinascidins in a bacterial symbiont living in the ascidian cells (Rath et al. 2011). The biosynthetic gene cluster is closely related to the NRPS genes of safracin B produced by *Pseudomonas fluorescens* A2-2 (Velasco et al. 2005) which is the starting material for the current semisynthetic manufacture of Yondelis® (Cuevas et al. 2000). The common building blocks are two units of the unusual amino acid 3-hydroxy-5-methyl-O-methyltyrosine (Fu et al. 2009). Another interesting example of a widespread NRPS gene cluster is the case of the antitumor compounds didemnins, initially isolated from the marine Caribbean tunicate *Tridemnum solidum* and recently described by several authors as a bacterial compound produced by free living Alphaproteobacteria (Tsukimoto et al. 2011; Xu et al. 2012). This includes the isolation of several strains belonging to the *Tistrella* genus during PharmaMar's program for isolation of marine bacteria (PharmaMar, personal communication).

Technical and biological advances, especially with the sequencing of cancer genomes and analysis of tumor cell transcriptions, have provided new insights into the molecular basis and classification of tumor phenotypes. The

knowledge emerged from systematic cancer genome characterization during the last decades not only allows the discovery of new targets for target-based drug discovery programs, but also enables the definition of relevant and predictive phenotypic end points and cellular models for phenotypic screens (Moffat et al. 2014).

Some types of screening are in an intermediate category termed 'mechanism-informed phenotypic drug discovery' (MIPDD) where the mechanism of action can be detected by a specific phenotype. Moffat et al. (2014) have reviewed the screening origins of new small-molecule cancer drugs approved by the FDA between 1999 and 2013, as shown in Table 2.

For the 47 oncology NMEs (New Molecular Entities) approved by FDA between 1999 and 2013, the majority (30 compounds) originated from target-based drug discovery, seven originated from MIPDD and 10 originated from phenotypic screens. If the group of kinase inhibitors (21 compounds, highlighted in bold) is excluded, a higher number of drugs were discovered by mixed or phenotypic screening approaches.

Phenotypic assays have the advantage of identifying drug leads and clinical candidates that are more likely to possess therapeutically relevant molecular mechanisms of action (MMOAs) and clinical efficacy (Moffat et al. 2014; Swinney 2013). However, target-based approaches have also been prominent in the past two decades, particularly those directed against oncogenic kinases (Hoelder et al. 2012; Zhang et al. 2009) and have resulted in a new generation of anticancer agents with fewer side effects and impressive results in clinical trials.

6.2.4 Screens for novel drug candidates for Alzheimer's and Parkinson's disease.

Neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) cause serious public health problems due to the exponential increase in incidence of the diseases with age. The currently approved drugs for treatment only provide symptomatic relief to mild AD patients and do not stop progression of the disease. Therefore novel drug candidates are of great interest and besides the chemical synthesis of compounds natural compounds are again becoming of interest, including those of marine origin.

The difficulty in this area of drug research is the lack of validated therapeutic targets. In the absence of a target or in complex mechanisms like the human brain a drug screening method is almost impossible to set up. An alternative may come from phenotypic chemical screens using a whole organism. The mouse *Mus musculus* is the most commonly used animal model. But the costs limit its use in large-scale therapeutic screening. Instead, small invertebrate models like *Caenorhabditis elegans* or *Drosophila melanogaster* are suitable models combining genetic amenability, low cost and culture conditions used in large-scale screenings (Giacomotto & Ségalat 2010). The invertebrate models bridge the gap between traditional high-throughput screenings and the validation in mammalian models. They allow identifications of new active compounds, targets or molecular mechanisms, which can be further used in traditional screening assays.

Using live animals it is possible to screen bioactive compounds that are able to induce a certain phenotype (e.g. paralysis or fluorescence) or reverse an abnormal phenotype (e.g. ß-amyloid deposits, also known as plaques) to the wild-type phenotype (Arya et al. 2010). ß-amyloid and α-synuclein are aggregation-prone proteins typically associated with AD and PD, where the misfolding and accumulation of these proteins lead to neuronal cell death (Marsh & Blurton-Jones 2012). In order to discover novel drugs against AD and PD, marine derived extracts and compounds are screened for their effect against ß-amyloid and α-synuclein toxicity, with the proteins being transgenetically expressed in different strains of *C. elegans* in a high-throughput system (Sealife Pharma, Austria, personal communication). The transgenic

strains display different phenotypes allowing for direct detection of effect of the tested compound. For example, toxicity of soluble oligomers can be measured by a phenotypic read out because upshift of temperature leads to expression of ß-amyloid in muscle cells of the worms, which in turn get paralyzed. Alternatively, expression of toxic ß-amyloid or α-synuclein can be measured using a plate reader, because the proteins are coupled to fluorescent proteins like GFP or YFP. The ability of unknown compounds to prevent plaque building can be assessed because plaques can be stained with the fluorescent dye Thioflavin T and visualized using a fluorescence microscope. Fluorescence can also be used to detect expression of the reporter protein GFP in dopaminergic neurons. By using the neurotoxic molecule MPP+ (1-methyl-4-phenylpyridinium), which leads to neuronal cell death as it occurs in PD it is therefore possible to detect compounds, which are able to reverse neuronal cell death. Positive controls, which are compounds protective against ß-amyloid toxicity, include coffee extract (Dostal et al. 2010), thioflavin T (Alavez et al. 2011), and reserpine (Arya et al. 2009), which are all known to protect *C. elegans* from ß-amyloid peptide toxicity.

7. CHEMICAL DEREPLICATION, COMPOUND PURIFICATION AND STRUCTURE ELUCIDATION

When interesting lead activities have been detected in the bioassays described above, a key challenge is to isolate and identify the compound(s) responsible for the activity. As for the screening process, several strategies are possible, depending on the starting material and to which level the compound needs to be identified.

An example of a strategy to identify bioactive fractions followed by identification of the bioactive compound is the Explorative Solid-Phase (E-SPE) strategy, where microbial extracts are loaded into columns whose stationary phases display different functionalities; the eluted fractions are tested in a bioassay and results are organized in a bioactivity matrix. The pattern of the matrix gives indications about size and functional groups of the bioactive compounds, accelerating both the dereplication and isolation process, as

demonstrated for the extracts from *Pseudoalteromonas luteoviolacea* and *Penicillum roqueforti* (Mansson et al. 2010).

A key step to avoid rediscovery of already known compounds is dereplication. The identification of already known compounds occurs frequently, even in microorganisms not belonging to the same species (Egan et al. 2001; Ginolhac et al. 2005; Jin et al. 2006; Ziemert et al. 2014). Dereplication relies on analytical methods like LC-UV, LC-MS, LC-MS/MS, and LC-NMR, compound databases and, in recent years, metabolomics, molecular networking, and genome mining (El-Elimat et al. 2013; Helfrich et al. 2014; Tawfike et al. 2013; Vynne et al. 2012; Yang et al. 2013). Experimental data like UV profiles or fragmentation patterns are searched against databases such as PubChem, ChemSpider, AntiBase, or the Dictionary of Natural Products (170,000 entries as of July 2015). With increased focus on the marine environment as a reservoir of natural compounds, a number of databases containing only compounds of marine origin has become available, e.g. the Dictionary of Marine Natural Products (http://dmnp.chemnetbase.com) and MarinLit (http://pubs.rsc.org/marinlit/). However, research groups often developed their own dereplication strategies, databases and tools, see for example Klitgaard et al. (2014), Macintyre et al. (2014), and Kildgaard et al. (2014).

When a desired bioactivity is observed in an extract or a fraction thereof, the active compound should be purified in order to proceed to structure elucidation. As in the case of extraction of crude extracts, it is important to develop an isolation protocol that considers the physical and chemical nature of the compound, particularly its lipophilic and hydrophilic characters (Ebada et al. 2008). Qualitative tests like thin-layer chromatography (TLC) can be performed to gather information about its polarity, charge, size, solubility and acid-base properties, and such tests are able to indicate the most suitable chromatographic technique.

For low to medium polarity compounds, column chromatography (CC, normal or reverse phase) or high performance liquid chromatography (HPLC) is often preferred to produce a pure compound, whereas for high polarity

compounds, reverse phase CC with elution in H_2O/MeOH followed by another round of CC with a hydrophobic matrix, size-exclusion chromatography or HPLC is preferred (Ebada et al. 2008).

Once a pure compound has been produced, the compound is most often subjected to another round of bioactivity assaying to confirm its bioactivity, before continuing to structure elucidation. Structure elucidation can be carried out by either stereochemistry methods or by spectroscopic methods (Ebada et al. 2008). The latter includes mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), which is one of the most commonly used and versatile techniques for elucidation of the structure of organic compounds (Fuloria & Fuloria 2013; Kwan & Huang 2008). One of the possible approaches in natural products discovery from culturing of the microbial producer to structure elucidation by NMR of the bioactive compound is exemplified in Figure 4.

8. CONCLUDING REMARKS

Bioprospecting for natural products is in rapid development, especially within marine microbiology. The discovery of novel bioactive compounds continues to pose innovative and (bio)technological challenges. Recent advances in microbial cultivation techniques, genomics, molecular biology, and tools for chemical analyses and dereplication, mean that an ever expanding and diverse toolbox is becoming available for bioprospecting. These new tools, in combination with biological assays and the genetic analysis of organisms, means that marine bioprospecting is entering a new era. A key question in bioprospecting is "where to search?" for novel bioactive molecules and the marine environment is due to its chemical and physical uniqueness a promising source of novel chemistry. Whilst marine eukaryotic macro-organisms were the first to be analyzed and provided us with several compounds with therapeutic properties, we are now realizing that many of these metabolites are actually of bacterial origin and, hence, the marine microbial world is re-emerging as a promising source for bioprospecting.

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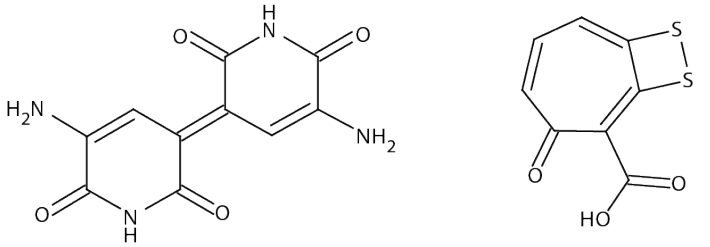
TABLES AND FIGURES

Table 1. Natural products discovers through metagenomic approaches. Modified from Barone et al. (2014).

Compound	Source	Type of screening
Onnamide A	*Theonella swinhoei*, bacterial symbiont	Sequence-based
Bryostatin	*Bugula neritina*, bacterial symbiont	Sequence-based
Minimide	*Didemnum molle*, microbiome	Sequence-based
Apratoxin A	*Lyngbya bouillonii*	Sequence-based
Patellamides	*Lissoclinum patella*	Function-based
Zn-coproporphyrin III	*Discodermia calyx*	Function-based

Table 2. Origin of new small-molecule cancer drugs approved by the FDA between 1999 and 2013. Kinase inhibitors are highlighted in bold. Information on the drugs to be analyzed was obtained from the (FDA) website. *First-in-class therapeutics. Modified from Moffat et al. (2014).

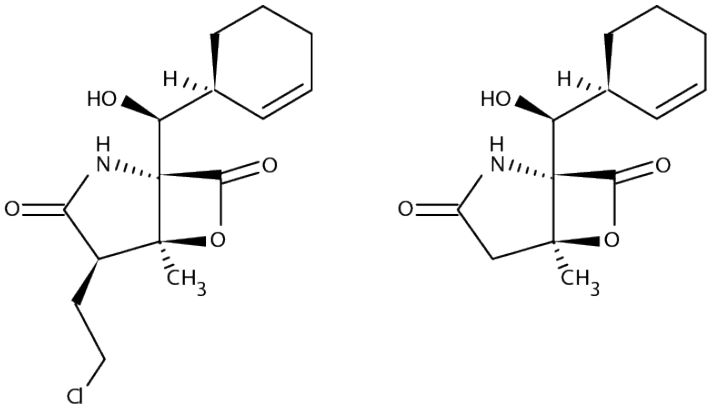
Lead Discovery			
Inhibition or modulation of target		**Mechanism-informed phenotypic screen**	***De novo* phenotypic screen**
Abiraterone*	Afatinib	Epirubicin	Carfilzomib
Bendamustine	Axitinib	Ixabepilone	Everolimus
Bexarotene*	Bosutinib	Nelarabine	Temsirolimus
Bortezomib*	Cabozantinib	Vismodegib*	Eribulin
Clofarabine	Crizotinib*	Cabazitaxel	Omacetaxine*
Decitabine	Dabrafenib	Pemetrexed	Lenalidomide
Exemestane	Dasatinib	Azacitidine*	Pomalidomide
Temozolomide	Erlotinib		Romidepsin
Enzalutamide	Ibrutinib*		Vorinostat*
Fulvestrant	Imatinib*		Trametinib*
Lapatinib	Ruxolitinib*		
Nilotinib	Sorafenib*		
Pazopanib	Sunitinib*		
Ponatinib	Vandetanib		
Regorafenib	Vemurafenib*		



Indigoidine

Tropodithietic acid
(TDA)

Figure 1. Structures of the antimicrobial compounds indigoidine and tropodithietic acid (TDA) derived from members of the marine *Roseobacter* clade.



Salinosporamide A

Salinosporamide K

Figure 2. Structures of the anti-tumor compounds salinosporamide A and salinosporamide K derived from the marine bacteria *Salinispora tropica* and *Salinispora pacifica,* respectively.

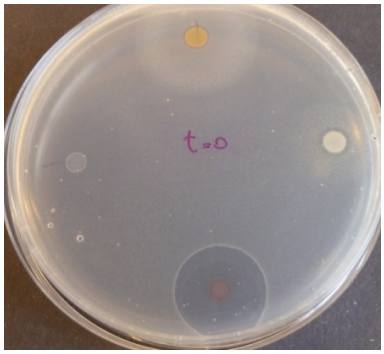


Figure 3. Agar plate assay for screening for antimicrobial compounds containing a solid medium seeded with a target strain (here, the fish pathogen *Vibrio anguillarum*) on which four potential producer strains have been spotted. The clear halo surrounding the red colony in the bottom part of the picture indicates production of an antibacterial compound.

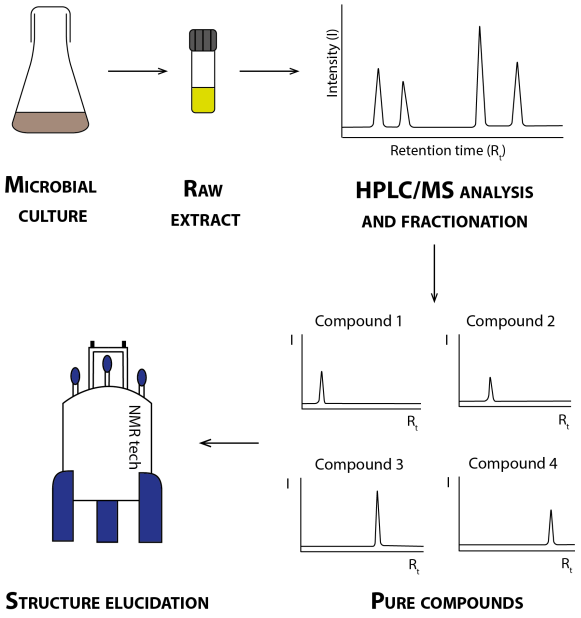


Figure 4. Simplified HPLC-MS approach used for the analysis and separation of the different compounds present in the crude extract of a bacterial culture. The pure compounds can be used in bioassays and undergo several analyses for structure elucidation.

ARTICLE 2

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

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

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Running title: Bioprospecting in *Vibrionaceae*.

Keywords: bioprospecting, chitin, *Vibrionaceae*, antibacterial compounds

List of abbreviations: GlcNAc: *N*-acetylglucosamine; ChiS: Chitin catabolic cascade Sensor histidine kinase; WGS: Whole Genome Sequence; SSBC Sea Salt Broth and Chitin medium WDA: Well Diffusion Assay; CAS assay: Chrome Azurol S assay; AHL: Acyl-Homoserine Lactones.

Abstract

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

249 words

Significance

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

Introduction

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

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

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

Following the increasing interest in natural products from the marine environment during the last decades of the 20th century, several groups are now pursuing methods for the identification and production of natural product in marine microorganisms (15, 16). Our group took part in the global marine research expedition Galathea 3 (http://www.galathea3.dk) with the aim of, on a global scale, isolating marine bacteria with bioactivity potential. We cultured microorganisms on marine agar and subsequently screened all colonies for antagonism against the fish pathogen Vibrio anguillarum, which is very sensitive to antibacterial compounds produced by marine bacteria. We isolated

approximately three hundred bioactive *Vibrionaceae* strains (17). During re-screening, only 39 strains retained their antagonistic activity (18). We isolated the potent antibiotics holomycin and andrimid from *V. coralliilyticus* and *Photobacterium galatheae*, respectively (9, 18), as well as modulators of virulence in *Staphylococcus aureus*, such as ngercheumicins F, G, H, I (19), nigribactin (20) and solonamide B (21). However, we were challenged by the marked reduction in bioactivity during re-screening.

We reasoned that one cause for this loss of activity could be that significant secondary metabolite production mostly occurs during the late exponential and in the stationary phase of microbial growth, and we hypothesized that the bio-discovery rate could be increased if the producing organisms were allowed more time to grow before being exposed to the target organism. In the initial screening and isolation, colonies were allowed to grow for 3-5 days before being tested (17), but this temporal advantage was not given during the re-screening (18). We also questioned whether the use of naturally co-occurring substrates such as mannose and chitin would restore bioactivity. Mannose is ubiquitous in the marine environment where it is commonly used by algae for protein glycosylation and production of extracellular polysaccharides (22, 23). Chitin is the most abundant organic molecule in the marine environment, being a component of the exoskeleton of crustacean and zooplankton (24). It is a polysaccharide composed of *N*-acetylglucosamine (GlcNAc) units. *Vibrionaceae* are considered among the major actors in marine chitin catabolism and the chitin utilization pathway is conserved within the family (25, 26). In *V. cholerae* chitin and derivatives can regulate the expression of genes involved in chitin metabolism (27) but also in biofilm formation and in virulence (28). In *V. coralliilyticus*, growth on chitin doubles the yield of the antibiotic andrimid in comparison to glucose (9).

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

Material and methods

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

Preparation of colloidal chitin. Colloidal chitin was prepared following a modified version of the method published by Hsu and Lockwood (30). Ten grams of practical grade shrimp shell chitin (Sigma C9213) was added to 400 mL of 37% HCl at 4°C and stirred at this temperature for 6 hours. The solution was poured into 4 L of cold H_2O and incubated overnight at 4°C, before it was neutralized with solid NaOH. After centrifugation (6000 g for 10 minutes), supernatant was discarded and the chitin pellet was suspended in 500 mL of H_2O and autoclaved. The concentration of colloidal chitin was calculated from the dry weight (100°C) of a subsample.

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

plate after 48 hours. Plates were incubated at 25°C and examined 24/48 hours after the target strain had been spotted. A biological replicate was performed for the isolates being bioactive in the first screening.

***In silico* analysis of the distribution of *chiS* and $(GlcNAc)_2$ operon**. The *chiS* (VC0622) gene and the $(GlcNAc)_2$ operon (VC0611-VC0620) of *Vibrio cholerae* were searched against a custom-built database using MultiGeneBlast (32). For the preparation of the database, genome sequences were downloaded from the GenBank database (Figures 2 and 3).

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

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

UHPLC-HRMS. Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA)

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

Influence of culture conditions on bioactivity and characterization of the antibacterial compound. Extracts from the cultures *V. furnissii* S0821 and *V. fluvialis* S1110 were analyzed by UHPLC-HRMS as described above. Extracts from the strains grown in SSBC supplemented with 0.1 g/L ferric citrate were also prepared and analyzed. For the bioassay-guided fractionation, fifty cultures of strain S0821 grown in 10 mL SSBC for 48 hours were extracted with equal volume of EtOAc, extracts were pooled together and evaporated until dryness under nitrogen. Portions of the pooled S0821 culture extracts were fractionated by Mixed-Mode Anion Exchange SPE on an Oasis MAX cartridge (Waters, Milford, MA, 30 μm, 30 mg, 1 mL). The sample was dissolved in 400 μL of 3:1 H_2O:MeOH containing 2% ammonium hydroxide then directly loaded onto a conditioned SPE column. The column was sequentially eluted with 2 mL of 3:1 H_2O:MeOH (F1), 2 mL of 1:1 H_2O:MeOH (F2), 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 of 1:1 H_2O:MeOH containing 1% formic acid (F5) and finally 2 mL of MeOH

containing 1% formic acid (F6). The fractions were dried under a stream of nitrogen before being resuspended in 200 μL MeOH. Fractions were tested for antibacterial activity in a WDA assay and for siderophore activity in a Chrome Azurol S (CAS) assay (38). Extracts were mixed with CAS solution in 1:1 ratio and the color change from blue to orange, indicating siderophore activity, was checked after 15 minutes and 24 hours.

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

Results

Screening of strains for antibacterial activity. Of the 295 *Vibrionaceae* strains, four isolates antagonized *V. anguillarum* when grown on the glucose medium, six when grown on the mannose medium and eleven when grown on the chitin medium using a procedure where the potential producers were not given any temporal advantage over the target strain. When the target strain was spotted twenty-four hours after the potential bioactive strains, six isolates were bioactive on glucose, nineteen on mannose and seventy-eight on chitin. Twenty-six, forty nine and ninety-one strains were bioactive on glucose, mannose and chitin, respectively, when the target strain was spotted with a forty-eight hour delay (Figure 1). Examples of one plate and of the behavior of one strain (*V. furnissii* S0821) over time on the mannose and the chitin-based media are shown in Figure S1.

Ethyl acetate extracts from the 91 antagonizing strains grown in chitin containing liquid medium for 48 hours were tested in a well-diffusion assay against *V. anguillarum*. Extracts from *V. coralliilyticus* (strains S2043, S2052, S2054, S2056 and S4053), *V. nigripulchritudo* (S2601, S2600 and S2604), *V. fluvialis* (S1110 and S1162), *V. furnissii* (S0821) and two *Vibrio* sp. (S1732 and S2150) inhibited the growth of *V. anguillarum* (Table 1). The strongest inhibition (i.e. the largest inhibition zone) was observed in extracts from the *V. coralliilyticus*

strains. The extracts from the *V. furnissii* and *V. fluvialis* strains were moderately growth inhibitory based on the size of the clearing zone. The remaining extracts exhibited a weak antibacterial activity.

Distribution of *chiS* and $(GlcNAc)_2$ operon. Given the pronounced increase in bioactivity when chitin was used as growth substrate, we speculated that this could be due to simple substrate change (e.g. catabolite repression) or to a direct involvement of chitin in the regulation. Since chitin is indeed involved in regulation of phenotypes in *Vibrio* species (27, 39–41), we addressed the possible chitin-dependent regulation of secondary metabolism in *Vibrionaceae*, possibly through the ChiS regulatory system (see discussion). Therefore, we investigated the distribution of the *chiS* gene and of the $(GlcNAc)_2$ operon in thirty-three genomes of vibrio species belonging to eight of the seventeen proposed *Vibrio* clades (42) and to three of the four proposed *Photobacterium* clades (42). In total, twenty-two *Vibrio* and eleven *Photobacterium* genomes were included in the analysis. This choice was driven by the quantity and the quality of the publicly available genome sequences. MultiGeneBlast-based analysis showed that the *chiS* gene and the complete $(GlcNAc)_2$ operon are widely distributed in both *Vibrio* and *Photobacterium* species, both being present in all analyzed species (Figure 2 and 3).

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

Both genomes harbored a biosynthetic gene cluster for the production of the quorum sensing auto-inducer molecules acyl-homoserine lactones (AHLs) and biosynthetic gene clusters with a relatively high gene similarity to those for the biosynthesis of ectoine, vibriobactin and aryl polyenes. A cluster for bacteriocin production was identified in both strains, but the bacteriocin prediction tool

BAGEL3 was not consistent with the antiSMASH results. Although BAGEL3 did predict the presence of one bacteriocin gene cluster, it differed from the one predicted by antiSMASH. A BLAST-based homology search using the bacteriocin amino acid sequences predicted in the two genomes (Table S1) as queries revealed a high similarity (E value=0, homology>98%) with endopeptidases from the M23 superfamily involved in cell wall biogenesis.

Investigation on the antibacterial compound produced by *Vibrio furnissii* and *Vibrio fluvialis*. There are no reports in the literature describing antibacterial compounds in *V. furnissii* and *V. fluvialis*. Given the importance of these two species as human pathogens (43, 44), we focused on these strains to determine the nature of the compound(s) responsible for the activity. Working under the hypothesis that these closely related species likely produced similar antimicrobial compounds, the bioactive extracts were dereplicated through a two phase approach: first, by comparison with extracts from cultures of related strains, which did not display bioactivity in the well diffusion assay. Compounds that were found in both the active and inactive strains were assumed to not be responsible for the observed antibacterial activity. The remaining unassigned compounds were further dereplicated by searching for all known compounds produced by *Vibrio* species found in AntiBase 2012, MarinLit 2012 and The Dictionary of Natural Products. Analysis of the dereplicated UHPLC-HRMS data revealed the presence of an abundant compound with ions at *m/z* 623.2342 $[M+H]^+$ and 645.2158 $[M+Na]^+$ in extracts from cultures of *V. furnissii* S0821 and *V. fluvialis* S1110 which was tentatively identified as the siderophore fluvibactin based on the accurate mass (mass deviation 0.96 ppm). Subsequent MS/MS analysis, comparison with the literature UV spectrum as well as isolation and NMR analysis confirmed this assignment (Figures S2-S5 and Tables S2-S3). The UHPLC-HRMS analysis also found another abundant ion with *m/z* 404.1818, which was assigned to the known compound 4 (*N*,*N*-bis-(2,3-dihydroxybenzoyl)-norspermidine (mass deviation 0.49 ppm) (Figure 4A and 4B).

These compounds (fluvibactin and compound 4) were not detected when extracts were prepared from *V. furnissii* S0821 and *V. fluvialis* S1110 grown in chitin medium supplemented with 0.1 g/L of ferric citrate (Figure S6). These extracts were not inhibitory against *V. anguillarum* 90-11-287 (Figure S7). The

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

Discussion

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

Different carbon sources can lead to significantly different profiles in microbial secondary metabolism (5, 9, 46). In our investigation, we used three molecules (glucose, mannose and chitin) that are abundant in the marine environment (22, 24) as substrate for marine *Vibrionaceae*. The number of bioactive (antibacterial) strains was nearly two and 3.5-fold higher when mannose and chitin were used as carbon-source, respectively, as compared to glucose.

The high efficacy of chitin in restoring (or inducing) the production of antibacterial compounds in the tested strains is in agreement with the ecology

and lifestyle of *Vibrionaceae* that are adapted to live in marine niches richer in this polysaccharide than in other carbohydrates (25, 26). Indeed, vibrios are well known for their association with chitin-rich biotic surfaces, such as zooplankton (24, 47). Chitinase genes and the chitin utilization pathway are conserved in *Vibrionaceae* (25, 26), and natural competence is induced by chitin in *V. vulnificus* (48) and *V. cholerae* (49). In the latter, chitin affects also chitin catabolism (27), biofilm formation and virulence (28, 50).

Chitin-dependent regulation of secondary metabolism mediated by the transcriptional regulator DasR occurs in the soil bacterium *Streptomyces coelicolor* A3(2) (51). In vibrios, one possible mechanism for a similar regulation could be through the two-component histidine kinase sensor ChiS, which has been characterized in *Vibrio cholerae* and is activated by chitin derived oligosaccharides (27). In the proposed model, a putative cognate receptor regulates the expression of target genes involved in the above-mentioned phenomena (27). Hunt and colleagues (25) suggested that genes with high homology to *chiS* (VC0622) and to some of the genes from the downstream $(GlcNAc)_2$ operon (VC0611-VC0613 and VC0616-VC0619) are widespread among *Vibrionaceae*. However, their genome analysis included a limited number (ten) of species, possibly due to low availability of genome sequences at the time the study was conducted. We performed a broader analysis and showed that, indeed, both *chiS* and the complete $(GlcNAc)_2$ operon (VC0611-VC0620), which were detected in all analyzed genomes, are very conserved and maintain their topological organization in *Vibrio* and *Photobacterium* species (Figures 2 and 3). The $(GlcNAc)_2$ operon includes the gene encoding for the periplasmic $(GlcNAc)_2$ binding protein which inactivates ChiS when chitin is not present in the environment (27). Given its importance in *V. cholerae*, such a degree of conservation of genes hypothesized to be involved in the ChiS regulatory system in *Vibrionaceae* indicates that chitin could serve a regulatory role in the whole family. Certainly, chitin-dependent regulation of phenomena such as biofilm formation and biosynthesis of antibacterial compounds would be advantageous during competition for nutrients with other microorganisms in the marine environment.

Although the use of chitin restored or induced the production of antibacterial compounds in approximately one third of the isolates, this approach was not

effective with the majority of the strains, even when they were allowed longer time before exposure to the target strain. Induction of silent/cryptic biosynthetic gene clusters has been achieved by exposing bacteria or fungi to small molecules produced by naturally co-occurring microorganisms (11, 52, 53). Similarly, it is likely that molecules that were present in the local seawater used to prepare the medium for the original screening/isolation procedure or that were produced by strains that were tested on the same plate ("co-cultivated") might have elicited the biosynthesis of antibacterial compounds. Co-cultivation could therefore also be a strategy to be used to induce the production of antibacterial compounds in our strain collection.

Extracts of cultures from *V. furnissii* S0821 and *V. fluvialis* S1110 had antibacterial activity against *V. anguillarum*. The bioactivity was present in all tested media, however on chitin the antagonistic activity could be observed earlier than on the other media (data not shown). Genome analysis of the strains provided a list of four biosynthetic gene clusters potentially responsible for the biosynthesis of the antibacterial compound. Three of them (AHL, ectoine and arylpolyenes) could be excluded as the cause of bioactivity through testing of pure standards in WDA and analysis of UV/Vis spectra of the extracts (Figures SI8- SI9).

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

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

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

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*	+++

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

Algorithm	Type of cluster	# clusters		Similarity
		V. furnissii S0821	*V. fluvialis* 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	

* 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 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. fluvialis* S1110: one cluster with 14% gene similarity to the O-antigen BGC and one cluster with 36% gene similarity to the vibrioferrin BGC

Table 3. Siderophore (column "CAS assay") and antibacterial activity of the raw extract from a culture of *V. furnissii* S0821 and of the six derived fractions (F1-F6). The addition of a siderophore to the CAS solution causes a change in color from dark blue to orange-yellow. Activity against *V. anguillarum* is 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	-

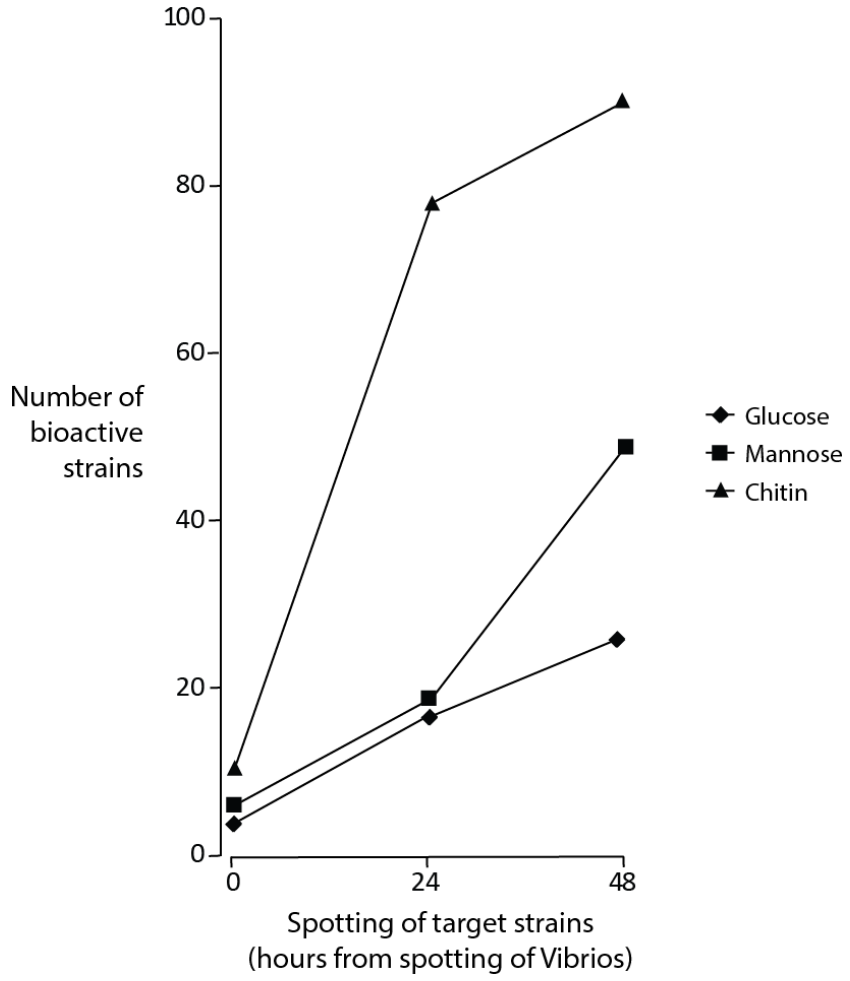


Figure 1. Number of bioactive *Vibrionaceae* strains (of 295 in total) on glucose (rhombus), mannose (square) and chitin (triangle) allowing 0, 24 and 48 hours pre-growth of the potential producer before exposing the target strain, *Vibrio anguillarum*.

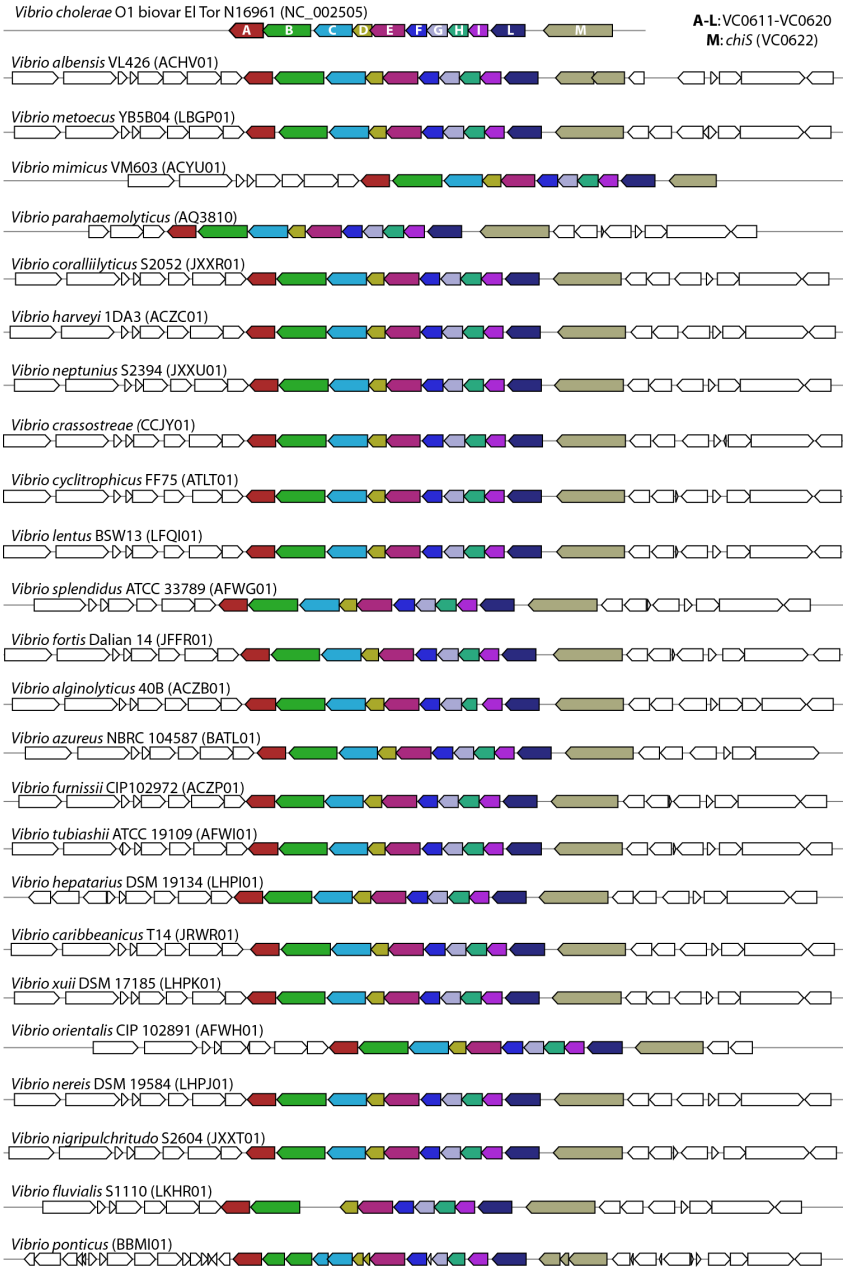


Figure 2. Distribution of the *chiS* gene and of the $(GlcNAc)_2$ operon among *Vibrio* spp.. GenBank/EMBL/DDBJ accession numbers of the used genomes are indicated in brackets.

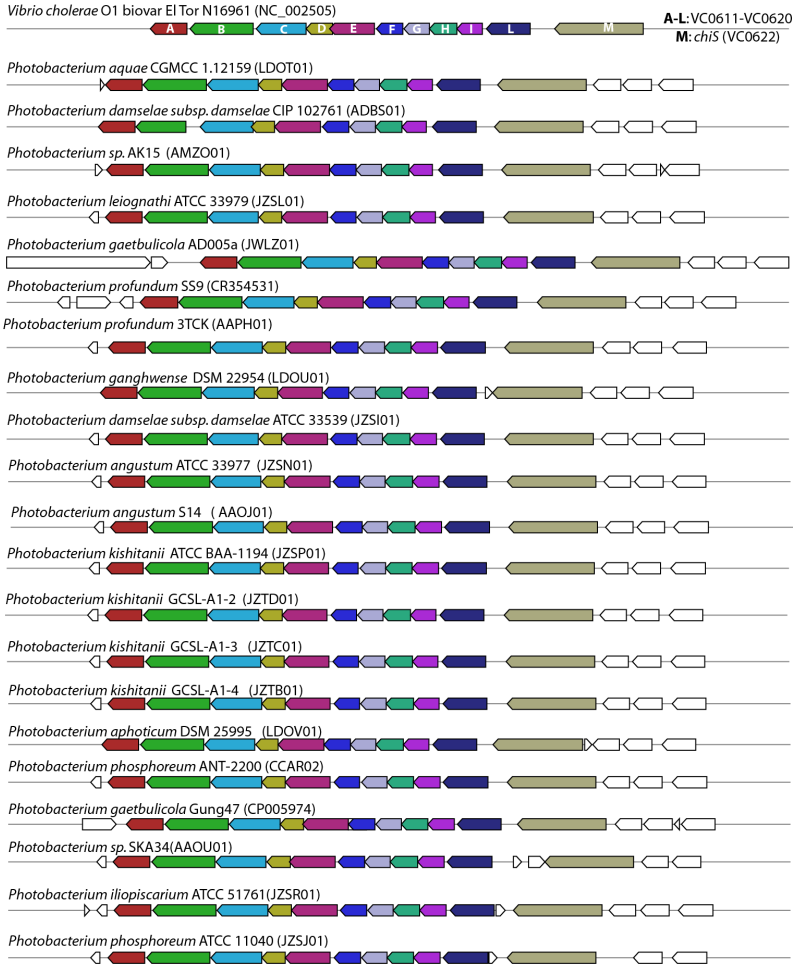


Figure 3. Distribution of the *chiS* gene and of the $(GlcNAc)_2$ operon (VC0611-VC0620) among *Photobacterium* spp.. GenBank/EMBL/DDBJ accession numbers of the used genomes are indicated in brackets.

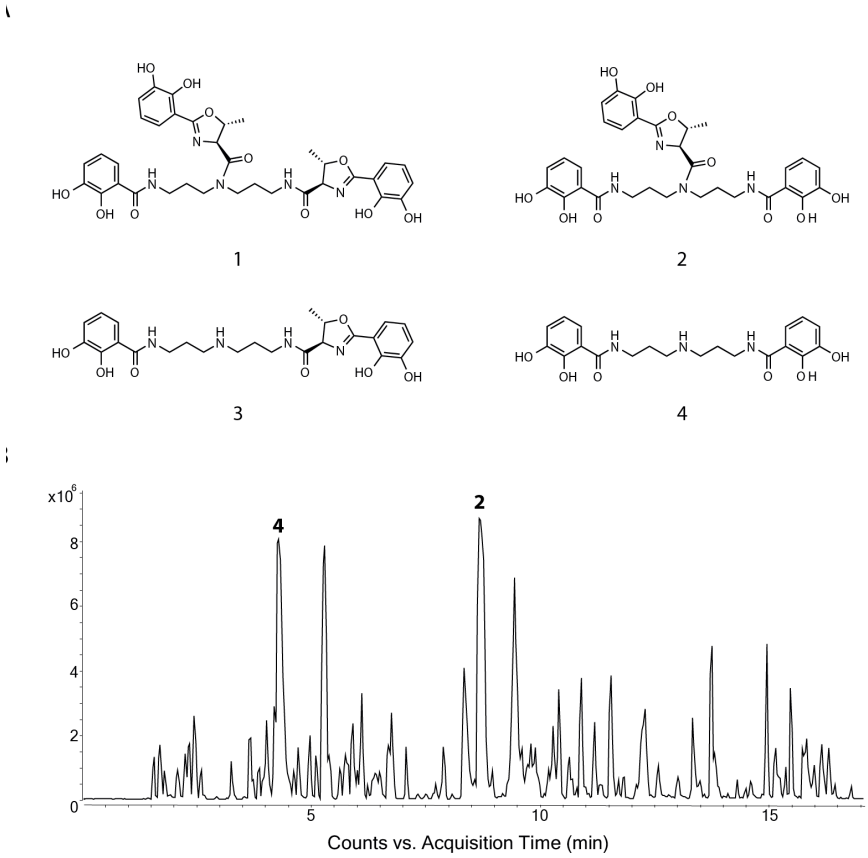


Figure 4 (A) Structures of vibriobactin (**1**) (54), fluvibactin (**2**) (55), **3** and **4**. **(B)** UHPLC-HRMS Total Ion Chromatogram (TIC) of the culture extract from *V. furnissii*. The peaks assigned to fluvibactin (**2**) and compound **4** are highlighted.

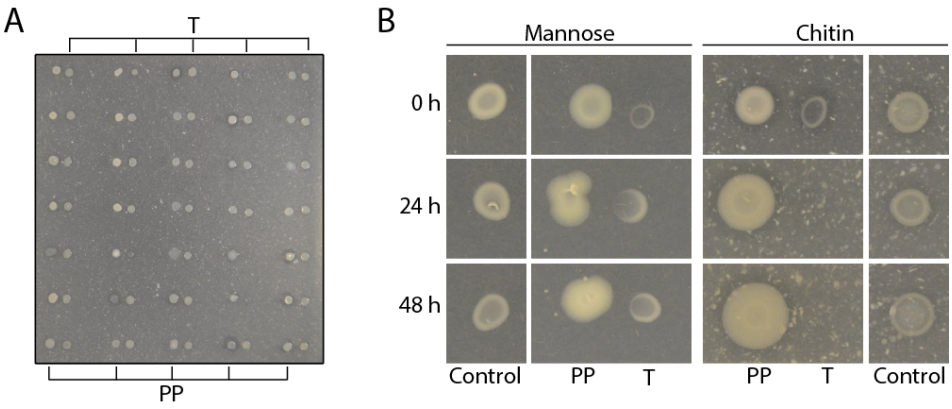


Figure S1. (A) Example of a plate prepared spotting the potential producers (PP) and the target strain *V. anguillarum* (T) on chitin medium. Clear haloes surrounding the colonies indicate chitinolytic activity. **(B)** Detailed behavior of *V. furnissii* S0821 over time and on the two media. "Control" is the target strain spotted alone on the same two media.

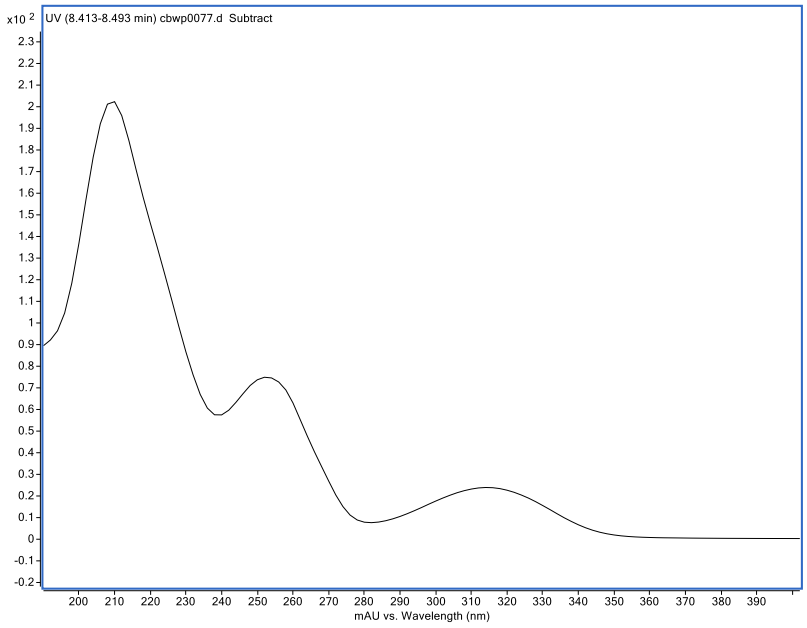


Figure S2 UV/Vis spectra of fluvibactin.

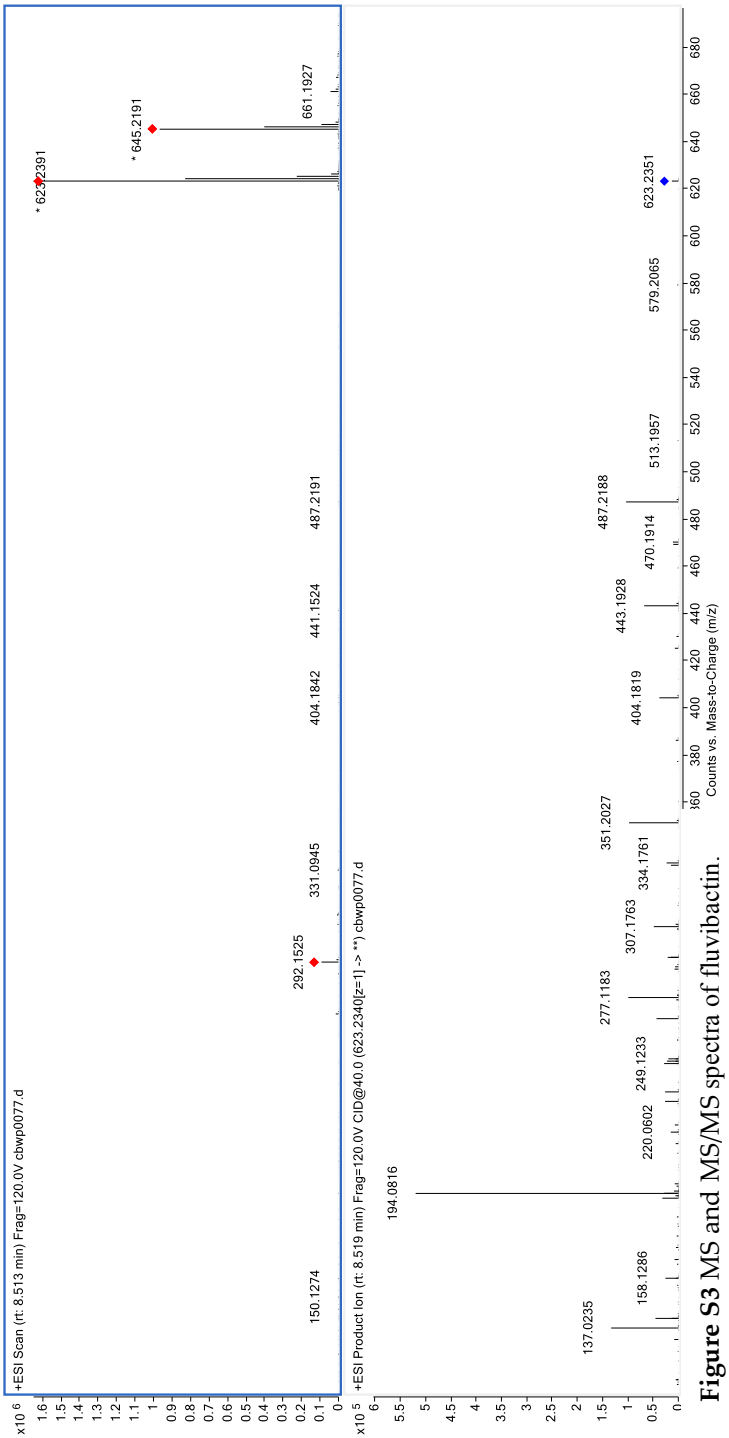


Figure S3 MS and MS/MS spectra of fluvibactin.

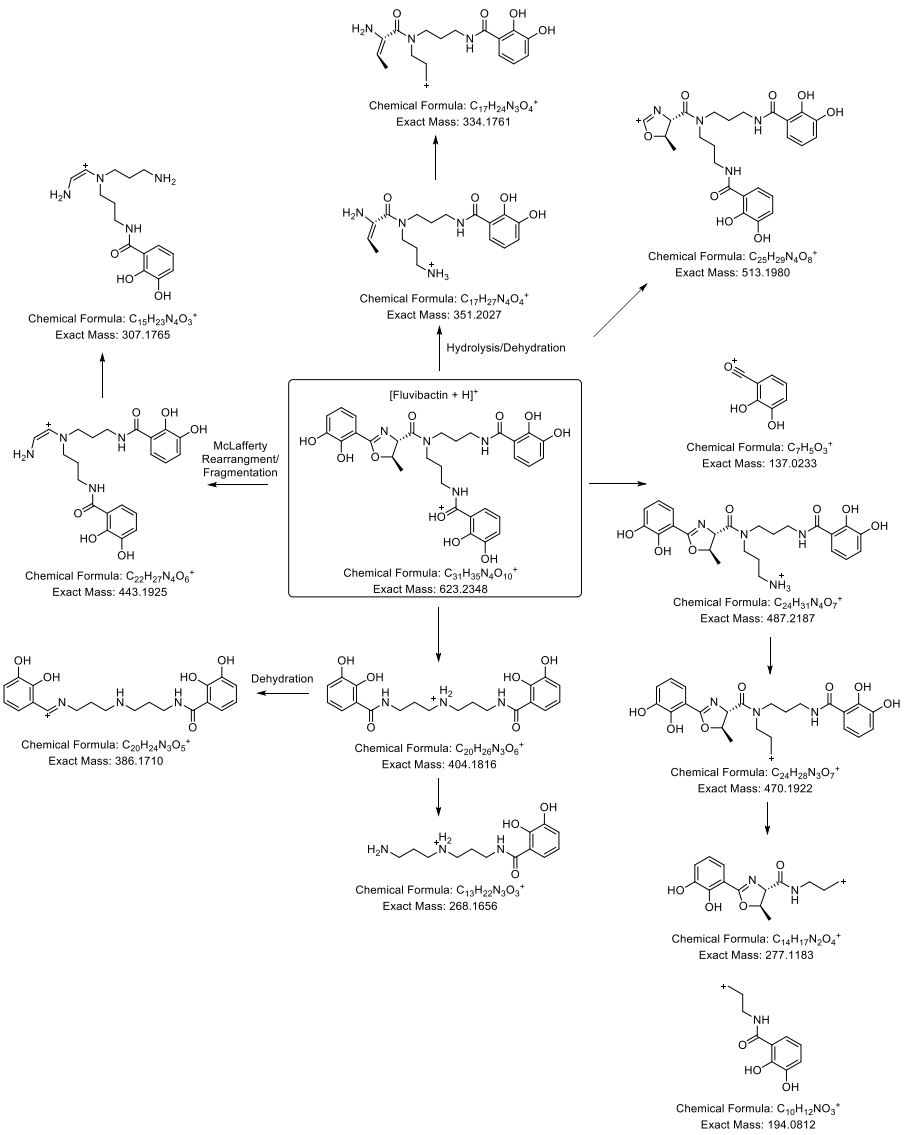


Figure S4 Proposed fragments of fluvibactin.

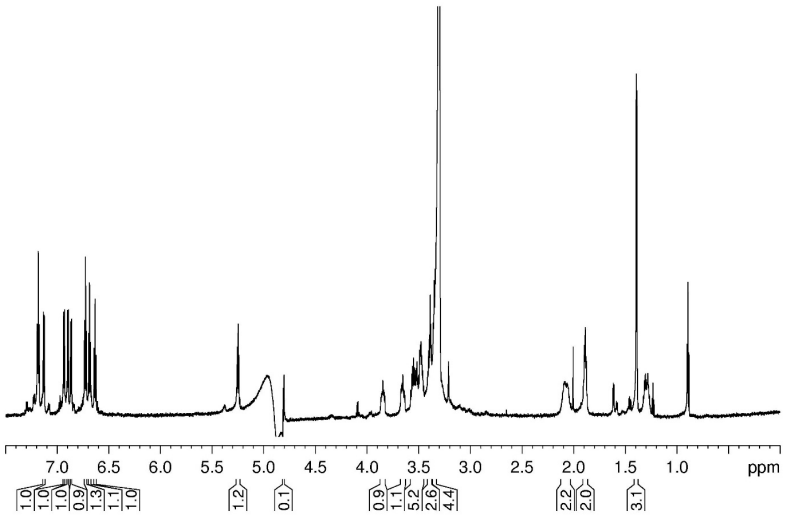


Figure S5 1D ^{1}H NMR spectrum of fluvibactin at 800 MHz.

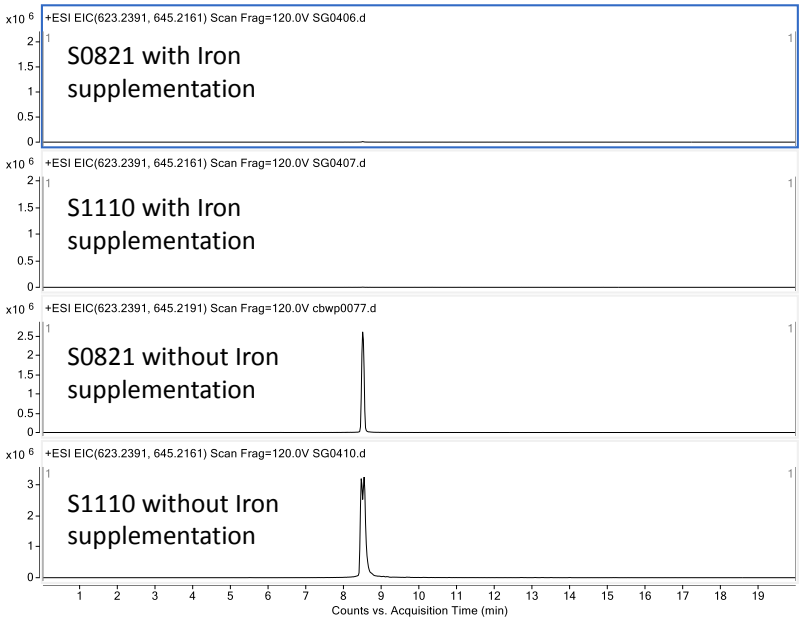


Figure S6 EIC of fluvibactin for S0821 and S1110 grown with and without iron supplementation.

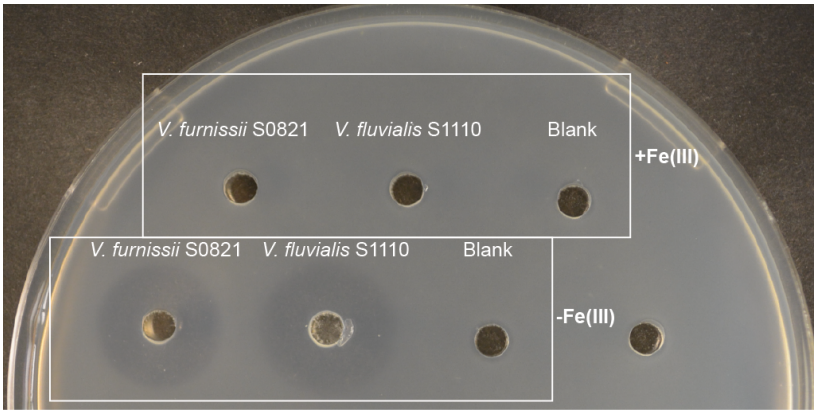


Figure S7 Test of the antibacterial activity of ethyl acetate extracts obtained from cultures of *V. furnissii* S0821 and *V. fluvialis* S1162 grown in presence (top panel) and in absence (lower panel) of Fe(III).

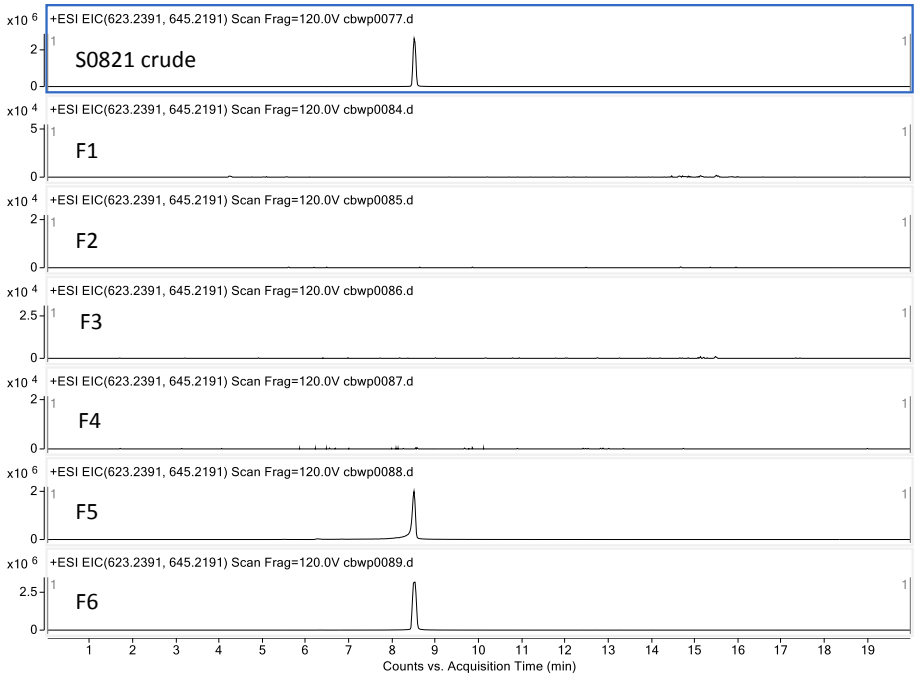


Figure S8 EIC of fluvibactin in SPE fractions from *V. furnissii* S0821 culture.

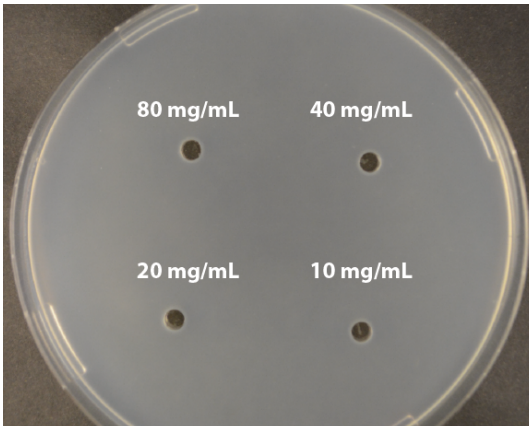


Figure SI 9 Assessment of antibacterial activity of ectoine (Fluka 81619, dissolved in sterile milliQ water). 50 µL of 10, 20, 40, 80 mg/mL solutions were transferred to wells punched in solid medium seeded with *V. anguillarum* 90-11-287. No growth inhibition of the pathogen was observed after 48 hours of incubation of the plate at 25 °C.

Table S1 Amino acid sequences of the putative bacteriocins predicted by Bagel3 based on the analysis of the genomic sequences of *V. furnissii* S0821 and *V. fluvialis* S1110.

Species	Strain	Pfam	Predicted amino acid sequence
V. furnissii	S0821	Peptidase_M23	FNQLGFSYQELMKIMETDLNYLALDTLKPGNVLRFWRSQDGRSLAKMELK FSLVERAVYVRTDDGSFEFKDVKIPGTWKEYPLIGEIQGSFSQSANQLGLGS SDIDQIVTLLKDKINFVRDVRAGDRFEVVLSRQFVGDQLTGNSEIQAIKIFSR SNDVTAYLYKDGQYYDKNGESLQRAFQRYPTTGKWRLSSGFDPNRRHPVT GRIAPHNGTDFAAPTGTPVVSTGDGVVVMTRNHPYAGNYVVIQHGSTYM TRYLHLSKILVSKGQKVSRGQRIGLSGATGRVTGPHIHYELIVRGRPVDAMK ANIPMANSVPKKDMANFTARRNELDRMLAHQEGLLASTNSQATPES
V. fluvialis	S1110	Peptidase_M23	TDLNYLALDTLKPGNILRFWRGQDGHSLAKMELEFSLVERAVYARTDDGSFE FKDVKIPGKWKEYPLIGEIQGSFSQSANQLGLGSSDIDQIVSLLKDKINFVR DIRAGDRFEVVLSRQFVGEKMTGNSEIQAIKIFSRSNEVTAYLYKDGQYYDK NGESLQRAFQRYPTTQKWRMSSGFDPNRHHPVTGRIAPHNGTDFAAPIG TPVVSTGDGVVVMTRNHPYAGNYVVIQHGSTYMTRYLHLSKILVRKGQKV SRGQRIGLSGATGRVTGPHIHYELIVRGRPVDAMKANIPMANSVPKKEMA SFVSRRNELDKMLAHQESLLASNSSPDNPES

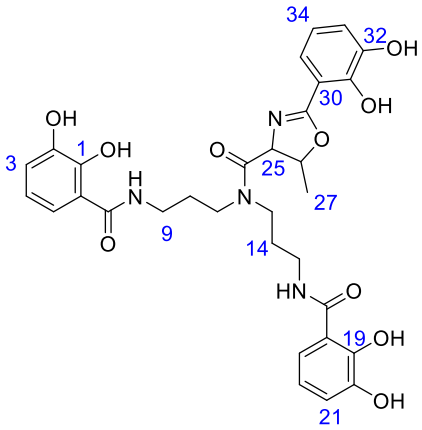
Table S2 MS/MS fragments of Fluvibactin

Observed Mass	Predicted Formula	Assignment	Predicted Mass	Error (ppm)
645.2158	$C_{31}H_{34}N_4O_{10}Na$	$[M+Na]^+$	645.2167	-1.394880201
623.2342	$C_{31}H_{35}N_4O_{10}$	$[M+H]^+$	623.2348	-0.962719027
513.1963	C25H29N4O8	See figure S4	513.198	-3.312561623
487.2186	C24H31N4O7	See figure S4	487.2187	-0.205246638
470.1918	C24H28N3O7	See figure S4	470.1922	-0.850715941
443.192	C22H27N4O6	See figure S4	443.1925	-1.128177936
404.1813	C20H26N3O6	See figure S4	404.1816	-0.742240617
386.1708	C20H24N3O5	See figure S4	386.171	-0.51790528
351.2023	C17H27N4O4	See figure S4	351.2027	-1.138943408
334.1758	C17H24N3O4	See figure S4	334.1761	-0.897730269
307.176	C15H23N4O3	See figure S4	307.1765	-1.627728684
277.1182	C14H17N2O4	See figure S4	277.1183	-0.360856717
268.1656	C13H22N3O3	See figure S4	268.1656	0
194.0812	C10H12NO3	See figure S4	194.0812	0
137.0234	C7H5O3	See figure S4	137.0233	0.729802888

Table SI3 NMR assignment for Fluvibactin in CD_3OD.

Atom assignment	^{13}C chemical shift [ppm]	^{1}H chemical shift [ppm], Integral,multiplicity, *J* [Hz]
1	150.2	-
2	147.3	-
3	119.7*	6.90, 1H, dd, 7.8, 1
4	119.6*	6.69, 1H, t, 8
5	118.6#	7.18, 1H, br. d, 8
6	116.7	-
7	171.5	
9a	37.8¤	3.39, 1H, m
9b	37.8¤	3.35, 1H, m
10	28.4	1.89, 2H, p, 7
11a	45	3.56, 1H, m
11b	45	3.49, 1H, m
13a	46.7	3.84, 1H, m
13b	46.7	3.65, 1H, m
14a	30.3	2,09, 1H, m
14b	30.3	2.05, 1H, m
15a	37.9¤	3,52, 1H, m
15b	37.9¤	3.47, 1H, m
17	171.8	-
18	116.7	-
19	150.2	-
20	147.3	-
21	119.6*	6.86, 1H, dd, 7.8,1
22	119.6*	6.63, 1H, t, 8
23	118.6#	7.19, 1H, br. d, 8
24	171.4	-
25	73	4.81, 1H, d, 6.4
26	79.8	5.25, 1H, p, 6.4
27	20.2	1.39, 3H, d, 6.4
28	167.8	-
30	111.8	-
31	149.4	-
32	146.7	-
33	120.2	6.93, 1H, dd, 8,1.4
34	119.9^	6,72, 1H, t, 8
35	119.9^	7,13, 1H, dd, 8,1.4

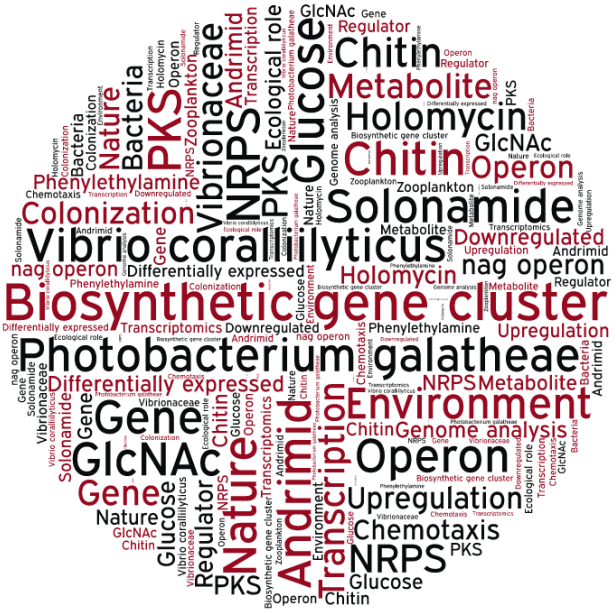
^,*,¤,# : indicates overlap and thereby specific assignment impossible.



All spectra were acquired on a Bruker Advance 800 MHz NMR spectrometer using standard pulse sequenced. Chemical shifts are reported in ppm relative to deuterated solvent peaks as internal standards (δH, CD_3OD 3.30 ppm; δC, CD_3OD 49 ppm). Coupling constants (*J*) are given in hertz (Hz). Multiplicities of ^1H NMR signals are reported as follows: d, doublet; br.d, broad doublet; t, triplet; p, pentet; m, multiplet.

ARTICLE 3

Growth on chitin impacts the transcriptome and metabolite profiles of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753.



Growth on chitin impacts the transcriptome and metabolite profiles of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753.

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Running title: Effects of chitin on *Vibrionaceae*

Keywords: chitin, *Vibrionaceae*, regulation, secondary metabolism

Summary

Members of the *Vibrionaceae* family are often found associated with chitin-containing organisms and they are thought to play a major role in chitin degradation. The purpose of the present study was to determine how chitin affected the transcriptome and metabolome of two bioactive *Vibrionaceae* strains, *Vibrio corallilyticus* and *Photobacterium galatheae*. We focused on chitin degradation genes and secondary metabolites based on the assumption that these molecules in Nature confer an advantage to the producer. Growth on chitin caused up-regulation of genes related to chitin metabolism and of genes potentially involved in host colonization and/or infection. The expression of genes involved in secondary metabolism was also significantly affected by growth on chitin, in one case being thirty-four folds upregulated. This was reflected in the metabolome, where the antibiotics andrimid and holomycin were produced in higher amounts on chitin. Interestingly, in cultures of *P. galatheae* grown on chitin we detected high amounts of the biogenic amine phenylethylamine. Overall, these results suggest that both *V. coralliilyticus* and *P. galatheae* have a specific lifestyle for growth on chitin, and that the secondary metabolites they produce are likely to play a crucial role during chitin colonization.

191 words (limit: 200 words)

Introduction

Chitin, a polysaccharide composed by *N*-acetyl-glucosamine (GlcNAc) units, is the most abundant molecule in the marine environment, where it is the primary component of the exoskeleton of zooplankton (Gooday, 1990). Members of the *Vibrionaceae* family (vibrios) are often associated with chitinous surfaces (Thompson et al., 2004) and, although the ability to metabolize this molecule has been suggested to be an ancestral feature of the whole family (Hunt et al., 2008), characterization of the chitin catabolic pathway has been performed only on a limited number of species from the *Vibrio* genus, mostly *V. cholerae* and *V. furnissii*.

The first steps of the establishment the bacteria-chitin association rely on a gradient of chitin-derived oligosaccharides released by chitin-containing organisms, which drives bacteria to the chitin surface by chemotaxis (Keyhani and Roseman, 1999). This is followed by adhesion of the bacteria to the surface (Keyhani and Roseman, 1999). In the chitin-utilization model proposed by Hunt and colleagues (Hunt et al., 2008) for *V. cholerae*, the next step is the secretion of chitinases, enzymes that hydrolyze chitin into GlcNAc oligosaccharides. These oligosaccharides are then transferred into the periplasmic space, where they are further cleaved and/or modified before being transported into the cytoplasm and converted to fructose-6-phosphate, which enters central metabolism (Hunt et al., 2008). Most of the genes required for the steps of this model occurring in the periplasmic and the cytoplasm are organized in the *nag* and in the $(GlcNAc)_2$ operons. The former has been well characterized in *Escherichia coli*, and is controlled by the transcriptional regulator NagC, which represses the operon when no GlcNAc is present in the environment (Plumbridge, 1991; Plumbridge and Kolb, 1993). The latter has been identified in *V. cholerae*, and its expression depends on ChiS, an hybrid sensor kinase that is active only when $(GlcNAc)_2$ is available (Li and Roseman, 2004; Meibom et al., 2004).

Genome mining has revealed that vibrios harbor the genetic potential for the production of numerous secondary metabolites (Machado, Sonnenschein, et al., 2015), and several bioactive molecules have been isolated from members of the *Vibrionaceae* family (Månsson et al., 2011). Microbial secondary metabolites are thought to have several ecological roles in Nature, including antagonism and

intercellular communication (Shank and Kolter, 2009; O'Brien and Wright, 2011). When grown on chitin, the coral pathogen *V. coralliilyticus* doubles the production of the antibiotic andrimid per cell and we hypothesized that its increased production may confer an advantage over competitors to the producer during chitin colonization (Wietz et al., 2011).

The purpose of this study was to investigate the influence of chitin on the metabolism of two species belonging to different genera of the *Vibrionaceae* family using a multi-omics approach. Analysis of the genomes of the two strains (*V. coralliilyticus* S2052 and *Photobacterium galatheae* S2753) revealed genetic potential for both chitin utilization and biosynthesis of secondary metabolites, and the transcriptomic and metabolite profiles of the two strains grown on chitin revealed insights about cellular components, processes and small molecules potentially involved in the colonization of chitinous surfaces in Nature.

Results

The genetic potential of *V. coralliilyticus* S2052 and *P. galatheae* S2753 for chitin degradation. We identified 15 and 7 genes in *V. coralliilyticus* S2052 and *P. galatheae* S2753, respectively, whose translated sequences contain one or more Pfam domains involved in the binding of chitin and/or cellulose (Pfam domains CBM_5_12, CBM_12_2, CHB_HEX, chiA_N term, chiC) and in the hydrolysis of chitin, chitin derived oligosaccharides or cellulose (Pfam domains GH3, GH18, GH19, GH20, LPMO_10) (Table SI1). Based on the presence of signal peptides in their amino acid sequence, most of these proteins are likely to be secreted in the extracellular environment, but putative outer membrane and periplasmic proteins were also predicted. In both genomes, one (*P. galatheae* S2753) or more (*V. coralliilyticus* S2052) putative cytoplasmic β-*N*-acetylhexosaminidase are present. Both genomes contain genes with high homology to *chiP*, which encodes for the chitoporin ChiP in *V. furnissii* (Keyhani et al., 2000). They also harbor both the *nag* and the $(GlcNAc)_2$ operons. The organization of the *nag* operon, however, is different in the two strains. In *P. galatheae* S2753 the four genes included in the operon are adjacent in the genome, whereas *nagB* is separated from the other genes in *V. coralliilyticus* S2052 (Figure 1). As for the

$(GlcNAc)_2$ operon (VC0611-VC0620 in *V. cholerae*), it is completely present in the genome of *V. coralliilyticus* S2052, whilst we did not detect any gene homolog of VC0611 and VC0612 in the genome of *P. galatheae* S2753 (Figure 1).

***V. coralliilyticus* S2052 and *P. galatheae* S2753 harbor genetic potential for the biosynthesis of secondary metabolites**. antiSMASH analysis of the genomes of *V. coralliilyticus* S2052 and *P. galatheae* S2753 found seven and thirteen putative biosynthetic gene clusters (BGC), respectively (Table SI2). Several of the predicted gene clusters included polyketide synthases and/or non-ribosomal peptide modules, which were present in five of the BGCs from *V. coralliilyticus* S2052 and in seven of the BGCs from *P. galatheae* S2753. Both genomes harbor a putative siderophore BGC and have the genetic potential for the production of the osmolyte ectoine. antiSMASH predicted one putative gene cluster for bacteriocin production in both genomes; however, BAGEL3 and Pfam domain analyses did not confirm these results (not shown).

Global transcription profile of *V. coralliilyticus* S2052 grown on chitin. We mapped 97.7% (±1.5%) and 83.40% (±3.5%) of the sequencing reads to the reference genomes of *V. coralliilyticus* S2052 and *P. galatheae* S2753, respectively. After statistical analysis, we evaluated the up and down regulation (abs. fold change >5) of the genes when the strains were grown on chitin as compared to growth on glucose (Figure 2). For *V. coralliilyticus* S2052, 231 genes were significantly upregulated and 42 were downregulated when cultures were harvested in the exponential phase, whereas 90 genes were upregulated and 96 were down regulated when RNA samples were prepared from cultures in the stationary phase. Genes encoding for proteins that are part of the respiratory chain and for components of the type III secretion system were downregulated at both samplings. Genes related to host colonization such as those encoding for proteins containing HCP effector domain or involved in the production of R-bodies were upregulated both in the exponential and the stationary phase. The same observation was made for genes potentially involved in natural competence (e.g. TW71_22895). Genes that were upregulated only in the exponential phase included genes encoding for components of transporters, including C4-dicarboxylate ABC transporters, for enzymes involved in fatty acid degradation and for proteins required for the synthesis of the storage compounds polyhydroxyalkanoates (PHA). Genes related to adhesion and

biofilm formation, including pilus assembly and production of cell capsule polysaccharides and exopolysaccharides, were upregulated on chitin in the stationary phase.

Global transcription profile of *P. galatheae* S2753 grown on chitin. For *P. galatheae* S2753, 23 genes were significantly upregulated and 21 were downregulated in the exponential phase, while 171 and 74 genes were up- and downregulated, respectively, when cultures reached stationary phase (Figure 2). Most of the genes predicted in the genome of this strain were annotated as "hypothetical protein", and unfortunately the use of alternative annotations and additional information as described in material and methods did not facilitate the analysis. However, genes related to the respiratory chain were downregulated and genes involved in histidine metabolism were upregulated in both exponential and stationary phase. Genes related to fatty acid degradation, phosphate uptake and biosynthesis of aromatic amino acids were up-regulated in stationary phase when grown on chitin as was genes encoding for proteins containing Pfam domains related to the biosynthesis of lipoproteins. While in *V. coralliilyticus* S2052 the most upregulated genes in the stationary phase were related to chitin utilization, in *P. galatheae* S2753 genes EA_20780 (encoding for a putative benzoylformate decarboxylase) and EA_20785 (encoding for a hypothetical protein including a flavin containing amine oxidoreductase Pfam domain) were the most significantly differentially expressed genes (Tables SI5).

Chitin-utilization related genes are significantly upregulated in exponential and stationary phase when *V. coralliilyticus* S2052 is grown on chitin. In *V. coralliilyticus* S2052, eight of the fourteen genes that do not belong to the $(GlcNAc)_2$ or to the *nag* operons but that we predicted to be required for chitin utilization were upregulated on chitin during exponential and stationary phase (Figure 3, Table SI3). Gene TW71_20615, encoding for a putative porin, was upregulated in the exponential but not in the stationary phase. In contrast, gene TW71_13355, encoding for a putative β-*N*-acetylhexosaminidase, was significantly upregulated on chitin only in the stationary phase. The $(GlcNAc)_2$ operon was upregulated both in the late exponential and in the stationary phase. With respect to the *nag* operon, the homolog of the NagC transcriptional regulator encoding gene (TW71_13375) was positively differentially expressed

at both time points, while the rest of the operon was upregulated only in the stationary phase.

Chitin-utilization related genes are upregulated mostly in the late stages of growth on chitin in *P. galatheae* 2753. All the genes predicted to be related to chitin utilization beside the $(GlcNAc)_2$ and the *nag* operons were positively differentially expressed in the stationary phase except EA58_19965, which was not differentially expressed at any time point. The $(GlcNAc)_2$ operon was upregulated both in exponential and stationary phase (Figure 3, Tables SI4). With respect to the *nag* operon, the homolog of the NagC transcriptional regulator encoding gene (TW71_13375) was positively differentially expressed at both time points, while the rest of the operon was upregulated only in the stationary phase. As for the same operon in *P. galatheae* S2753, *nagA*, *nagB* and the homolog of *nagE* were upregulated only in the stationary phase, while the gene encoding for the transcriptional regulator (EA58_03240) was not significantly differentially expressed in the exponential nor in the stationary phase.

The BGCs of *V. coralliilyticus* S2052 undergo both up and downregulation on chitin as compared to glucose. When we looked at the fold change in the expression levels of the putative biosynthetic genes that are part of the BGCs predicted by antiSMASH in the genomes of *V. coralliilyticus* S2052, we found that the biosynthetic genes of one of the predicted type 3 polyketide synthase (T3PKS) BGC was not differentially expressed when grown on chitin, while those from the remaining five predicted BGC were either up- or down-regulated (Table 1). In all cases but the andrimid BGC (see below), the change in gene expression was observed in the stationary phase. For one of the two predicted hybrid non ribosomal peptide-type 1 PKS (NRPS-T1PKS) and the siderophore and ectoine BGCs, some of the biosynthetic genes were upregulated while others were downregulated. The biosynthetic genes of the second predicted NRPS-T1PKS clusters were downregulated, while those from the putative NRPS and the arylpolyene-NRPS BGC were upregulated. The latter encodes for the machinery required for the biosynthesis of the acetyl-CoA carboxylase inhibitor andrimid, as suggested by the antiSMASH results and confirmed by BLAST search against the genome of *V. coralliilyticus* S2052 using the nucleotide sequence of the andrimid BGC from *Pantoea agglomerans* (Jin et

al., 2006) as query (not shown). While the andrimid biosynthetic genes were significantly upregulated only in the exponential phase (Table 1), gene TW71_08085, encoding for a putative acetyl-CoA carboxylase carrying the single amino acid mutation M203L required for andrimid resistance (Liu et al., 2008) (Figure SI2), was significantly upregulated in both exponential and stationary phase . The gene encoding for a putative LysR family transcriptional regulator (TW71_08080) and located downstream of the andrimid biosynthetic genes was also slightly upregulated (fold change: 1.61).

The majority of BGCs of *P. galatheae* S2753 are upregulated on chitin as compared to glucose. In *P. galatheae* S2753, the expression of biosynthetic genes from five of the putative BGCs predicted by antiSMASH was not different when grown on chitin as compared to glucose. Five BGCs (one NRPS-T1PKS, two NRPSs, one "other", one ectoine BGCs) were significantly upregulated on chitin, and no BGC was downregulated. As in the case of *V. coralliilyticus* S2052, one BGC was upregulated in the exponential phase, whilst four BGCs were up-regulated only in the stationary phase (Table 1). By homology search, we identified the biosynthetic genes from one of the most upregulated putative NRPS BGC as those required for the production of the antibiotic holomycin (not shown). The gene encoding for the ArsR family transcriptional regulator (EA58_20500) that is located upstream of the holomycin biosynthetic genes was slightly downregulated (-1.45 fold change).

Influence of chitin on the metabolite profiles of *V. coralliilyticus* S2052 and *P. galatheae* S2753. The levels of andrimid and of the related compound moiramide detected in extracts from 24 hour old cultures of *V. coralliilyticus* S2052 grown on chitin were much higher than those detected in extracts from cultures grown on glucose, which is in agreement with the upregulation of the andrimid biosynthetic genes observed during exponential phase. Solonamides and ngercheumicins were present in similar levels in extracts from cultures of *P. galatheae* S2753 grown on the two substrates. We identified two new members of the solonamide family (solonamide C and D) based on their accurate mass and retention time. These analogues differed from the known solonamides and ngercheumicins in the composition and order of the constituent amino acids. The amino acid sequence of solonamide C and D could be tentatively assigned based on analysis of the MS/MS fragmentation pattern and by analogy with the

observed fragments in the known solonamides (Fig SI3-SI6). Extracts from cultures grown in chitin contained higher levels of holomycin than those from cultures grown in glucose, which reflects the different expression levels of the related biosynthetic genes observed in the stationary phase. Interestingly, these extracted contained also approximately twice the amount of 2-phenylethylamine (identified by HRMS/MS and comparison to a standard) (Figure SI7) found in extracts from cultures grown on glucose.

Discussion

Studying the dynamics and evolution of the interactions between microorganisms and the surrounding environment is crucial for understanding their role in ecological systems. We studied the genetic potential of two members of the *Vibrionaceae* family for the utilization of chitin, the most abundant molecule in the marine environment (Gooday, 1990), and analyzed at the transcriptional and metabolome levels their response to the presence of this polysaccharide. We found that both strains possess the genetic information to produce a range of enzymes for chitin degradation and utilization, and that their metabolite repertoire greatly varies when grown on chitin as compared to growth on glucose, suggesting a role of the secondary metabolites during chitin colonization and utilization.

Most work on chitin utilization in *Vibrionaceae* has been done on *Vibrio* species, although the core gene set has been shown to be widespread in the whole family (Hunt et al., 2008). Therefore, besides a *Vibrio* species (*V. coralliilyticus*), we also studied a species from the genus *Photobacterium* (*P. galatheae*). The former is a coral pathogen (Ben-Haim et al., 2003), while the latter is a newly described species (Machado, Giubergia, et al., 2015) and, although it was isolated from a mussel (Gram et al., 2010), its preferred niche of colonization is unknown.

Both *V. coralliilyticus* S2052 and *P. galatheae* S2753 have the potential to produce a broad range of enzymes capable of binding and/or hydrolyzing chitin, chitin derived molecules and/or cellulose. Some of these enzymes contain the LPMO_10 domain, which is also present in GbpA (Loose et al., 2014), a

colonization factor contributing to *V. cholerae* adhesion to chitinous surfaces (Kirn et al., 2005). The putative cytoplasmic localization for all of the GH3 domain containing proteins indicates that they may actually be involved in other functions rather than chitin degradation, like in the case of the GH3 hydrolase NagZ from *Salmonella typhimurium* and *Bacillus subtilis*, which participate in peptidoglycan recycling (Bacik et al., 2012). The organization of the *nag* operon in *V. coralliilyticus* S2052 in which, unlike *Escherichia coli* (Plumbridge, 1991; Plumbridge and Kolb, 1993) and *P. galatheae*, the *nagB* gene is separated from the rest of the operon, resembles the organization of the same operon in *V. cholerae* (Yamano et al., 1997; Ghosh et al., 2011). Notably, the two different organizations of this operon in *V. coralliilyticus* S2052 and *P. galatheae* S2753 reflect those occurring in a number of other *Vibrio* and *Photobacterium* species, respectively (Figures SI8 and SI9). In contrast, the lack of homologues of genes VC0611 and VC0612 in the $(GlcNAc)_2$ operon of *P. galatheae* seems to be a peculiarity only of this strain and of the very closely related species *P. halotolerans* (Figure SI10).

With respect to the dynamics of chitin colonization and utilization program, the models currently available in literature (see introduction) would cover the species used in this study, although for *P. galatheae* S2753 the poor annotation of the genome and the lack of complementary information in literature and databases did not allow a thorough analysis. During exponential growth *V. coralliilyticus* S2052 upregulated a number of genes encoding for chemotaxis proteins (Table SI6) and transporters. These were transporters involved in the uptake of chitin and chitin derived oligosaccharides, but also C4-dicarboxylate ABC transporters, and this upregulation was observed also in *P. galatheae* S2753 in the stationary phase. C4-dicarboxylate ABC transporters have different substrates, including sialic acids. Sialic acids are GlcNAc derivative molecules used by mammals to glycosylate proteins on cell surfaces, where they act as determinants in bacterial adhesion events (Tanner, 2005). Indeed, the ability to catabolize sialic acid is thought to be an important feature in *V. cholerae* during intestinal colonization (Almagro-Moreno and Boyd, 2009). Some prokaryotes can produce sialic acids, and *V. coralliilyticus* BAA450 harbors the genes for their biosynthesis (Lewis et al., 2011). However, the homologs of such genes in *V. coralliilyticus* S2052 were not differentially expressed in our experimental setup. Therefore, the upregulation of these transporters indicates that they may also

use substrates such as chitin-derived oligosaccharides, or that *V. coralliilyticus* S2052 was deceived by the presence of GlcNAc and activated a response for the colonization of a potential host. The latter hypothesis would be in agreement with the upregulation of genes encoding for proteins containing one Hcp effector domain. In *V. cholerae*, Hcp proteins in are involved in its virulence towards eukaryotic (Pukatzki et al., 2006) and prokaryotic cells, conferring a competitive advantage to the producer (MacIntyre et al., 2010). Furthermore, in *V. coralliilyticus* S2052, a set of genes (TW71_18705 to TW71_18720) annotated as "glycerol-3-phosphate dehydrogenase" were upregulated in both the exponential phase and in the stationary phase. However, we believe these genes were misannotated since their encoded products contain a RebB domain, necessary for the production of R-bodies. These are cytoplasmic inclusions occurring widely in Proteobacteria whose exact function is not known, but have been suggested to play a role during host infection (Raymann et al., 2013).

Growth on chitin resulted in changes of expression of biosynthetic genes for the production of secondary metabolites. Expression changes were seen in both strains and were most pronounced in stationary phase. This strongly indicates that the molecules produced by the biosynthetic machinery encoded by those genes may have ecological functions, and may confer an advantage to the producers during chitin colonization, as it was suggested for andrimid (Wietz et al., 2011). The upregulation of the andrimid biosynthetic genes and of the LysR family transcriptional regulator located just downstream of them supports the recently suggested hypothesis that such regulator may have an important regulatory role in andrimid biosynthesis (Matilla et al., 2016). Similarly, the downregulation of the ArsR family transcriptional regulator gene located upstream of the holomycin biosynthetic genes may indicate a role of the encoded regulator as repressor of holomycin production. For most of the remaining differentially expressed biosynthetic genes, however, we do not know the metabolite produced and could not be revealed by comparing transcriptomics and metabolomics data. An interesting observation was the detection of high amounts of 2-phenylethylamine when *P. galathae* S2753 was grown on chitin. The biogenic amine phenylethylamine is produced by decarboxylation of phenyalanine. Genes related to the biosynthesis of aromatic amino acids were upregulated during stationary phase in *P. galatheae* S2753 grown on chitin, and the one of most upregulated gene encodes for a putative

benzoylformate decarboxylase. Given the structural similarity between benzoylformate and phenyalanine, we suggest that the substrate of the decarboxylase may actually be phenylalanine. The product of the gene next to the one encoding for the benzoylformate decarboxylase, which was also highly upregulated, is a hypothetical protein containing an amide oxidoreductase domain. We propose that this enzyme removes the amine group from phenyethylamine with the production of ammonia and phenylacetaldehyde (Figure SI6), although we did not detect this molecule in culture extracts. The production of biogenic amines has recently been reported in other *Photobacterium* species (Torido et al., 2014; Bjornsdottir-Butler et al., 2016), but their role in Nature remains unclear.

In conclusion, the results of this study show that growth on chitin triggers a comprehensive response at the transcriptional and biosynthetic levels in vibrios, providing insights into the dynamics of colonization of chitinous surfaces in Nature. We showed that the up-regulation of genes related to the production of secondary metabolites is reflected into the metabolite profile, suggesting a role of these molecules during chitin colonization. We showed that, when grown on chitin, *P. galatheae* produces high amounts of the biogenic amine phenylethylamine. Additional work, possibly in experimental setups with live chitin containing zooplankton, may help to identify its function in natural settings.

Experimental procedures

Genomes analyses. A list of genes possibly involved in chitin utilization was compiled based on the analysis of the genomes of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753 (accession numbers: JXXR01 and JMIB01, respectively). The choice of the genes to be included in the list was made based on: i) the NCBI gene annotation list associated with each genome, ii) homology searches using genes known to be related to chitin metabolism as queries and iii) the presence of Pfam and/or InterPro domains related to chitin metabolism in the amino acid sequences encoded by the genes. Pfam and InterPro domains were obtained by running through Blast2GO (Conesa et al., 2005) the amino acid sequences encoded by all predicted genes in each genome. Amino acid

sequences of the identified proteins were also submitted to the bacterial protein subcellular localization prediction tool (PSORTb 3.0) (Yu et al., 2010) to identify signal peptides linked to specific cellular compartments. Furthermore, genomes were submitted to antiSMASH 3.0 (Weber et al., 2015) for the prediction of putative biosynthetic gene cluster involved in the production of secondary metabolites.

Bacterial strains and media composition. *Photobacterium galatheae* S2753 and *Vibrio coralliilyticus* S2052 were isolated during the Galathea 3 global research expedition (Gram et al., 2010). The composition of the media used in this work was: 2% Sigma Sea Salts (Sigma S9883) solution with 0.3% casamino acids (BD 223050) supplemented with either 40 mM MOPS pH 7.5 and 0.2% glucose (SSBG) or 0.2% colloidal chitin (SSBC). Colloidal chitin was prepared as described previously (Giubergia et al, 2016).

Growth conditions. Unless stated otherwise, all cultures in liquid medium were grown aerated (200 rpm) at 25°C in four biological replicates. Single colonies of *P. galatheae* S2753 or *V. coralliilyticus* S2052 were grown in 10 mL of half-strength YTSS medium (½ YTSS) (González et al., 1996) for 24 hours. 100 µL of each culture were then used to inoculate 10 mL of fresh ½ YTSS medium. After 24 hours, each culture was used to inoculate 50 mL of SSBG or SSBC in 250 mL Erlenmeyer flasks at approximately 10^3 CFU/mL. When cultures reached late exponential and early stationary phase (Figure SI1), a subsample was taken and mixed with 0.2 volumes of ice-cold STOP solution (95% [v/v] ethanol, 5% [v/v] phenol], incubated on ice for 5 minutes and pelleted by centrifugation. Supernatants were removed and cell pellets were stored at -80°C until RNA extraction.

RNA isolation and sequencing. RNA was extracted using the RNAeasy kit (Qiagen, 74104) following manufacturer's instructions. DNA was removed on-column with the RNase-free DNase set (Qiagen 79254). Integrity and quality of total RNA were assessed with a NanoDrop Spectrometer (Saveen Werner, Sweden) and an Agilent 2100 Bioanalyzer (Agilent Technologies). For each strain in each condition, the three best total RNA samples were sent to the Beijing Genome Institute (BGI, Hong Kong), where rRNA was removed using the Ribo-Zero rRNA removal kit (Illumina). Libraries were then constructed

with the TruSeq RNA Library Preparation kit (Illumina) and 100 bp paired-end sequenced on a HiSeq 2000. Sequencing data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE80783.

Data analysis. RNA-seq data were analyzed using CLC Genomics Workbench version 8 (CLC Bio, Aarhus, Denmark). Quality control of the reads was done based on GC%, PHRED-score, nucleotide contribution and enriched 5mers sequences. Reads were trimmed removing the first 15 nucleotides from the 5' end when nucleotide contribution was not normally distributed. Subsequently, reads were mapped to the reference genomes and expression values were calculated as Reads Per Kilobase per Million mapped reads (RPKM). Gene expression profiles of biological replicates were merged and, for each strain at each time point, the profiles deriving from the two media were compared. The quality of the transcriptomic data was evaluated using hierarchal clustering and principal component analysis. Datasets that did not pass the quality control were discarded, however no less than two biological replicates per stain per condition per data point were used. Statistically significant gene expression differences were assessed through a Baggerly's test (Baggerly et al., 2003) using p-value < 0.05 and false discovery rate (FDR) q-value < 0.05. For the analysis of the global transcription profiles, only genes with an absolute fold change > 5 were considered. For other analyses (chitin utilization related genes and secondary metabolism biosynthetic genes), no fold change limit was set.

Extraction of liquid cultures for chemical analysis. Sub-samples (2 mL) of the 24 hours old cultures (harvest point in the stationary phase) were collected to be used for secondary metabolite analysis. Cultures were extracted sequentially with an equal volume of HPLC-grade ethyl acetate (EtOAc) (neutral extract), EtOAc containing 1% formic acid (acidic extract) and then EtOAc containing 2% ammonia (basic extract). The organic phases were transferred to fresh vials and evaporated until dryness under a stream of nitrogen. Extracts were dissolved in 250 μL methanol (MeOH) and stored at -20°C. The neutral, acidic and basic extracts were kept separate and analyzed separately. For each species three biological replicates and two technical replicates were analyzed.

UHPLC-HRMS. Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) was performed on an Agilent

Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 μm) with a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and remaining for 3 min (0.35 mL/min, 60 °C). An injection volume of 1 μL was used. MS detection was performed in both positive and negative detection on an Agilent 6540 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 L/min, sheath gas temperature of 300 °C and flow of 12 L/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Mass spectra were recorded at 10, 20 and 40 eV as centroid data for *m/z* 85–1700 in MS mode and *m/z* 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Lock mass solution in 70:30 methanol:water was infused in the second sprayer using an extra LC pump at a flow of 15 μL/min using a 1:100 splitter. The solution contained 1 μM tributylamine (Sigma-Aldrich) and 10 μM Hexakis(2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The $[M + H]^+$ ions (*m/z* 186.2216 and 922.0098 respectively) of both compounds was used.

Dereplication. The extracts were dereplicated by searching by formula for all compounds known to be produced by *Vibrio* and *Photobacterium* species found in AntiBase 2012, MarinLit 2012 and The Dictionary of Natural Products. The chromatograms were then examined for peaks of intensity which correlated to the change in expression levels revealed by the transcriptomic data. Unknown compounds of potential interest were analyzed by examination of the MS/MS data to assist with identification of the compound class and derive structural information.

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Table 1 Range of the expression fold change (FC) of the biosynthetic genes contained in the biosynthetic gene clusters predicted by antiSMASH in the genomes of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753. The time point at which the up- or downregulation was observed is indicated. Exp: time point in the exponential growth phase; Stat: time point in the stationary growth phase; X: not differentially expressed.

	Cluster	**Range FC biosynthetic genes**	**Time point**
V. coralliilyticus S2052	Cluster 1 - T3PKS	X	X
	Cluster 2 - NRPS	2/3.8	Stat
	Cluster 3 - Arylpolyene - NRPS (Andrimid)	2.6/6.8	Exp
	Cluster 4 - NRPS-T1PKS	-3/-18	Stat
	Cluster 5 - NRPS-T1PKS	-4.8/-7.6	Stat
	Cluster 6 - Siderophore-ectoine	-2.2/2.5	Stat
P. galatheae S2753	Cluster 1 - Other	X	X
	Cluster 2 - NRPS	X	X
	Cluster 3 - Other	X	X
	Cluster 5 - NRPS-T1PKS	2.2/3	Exp
	Cluster 6 - NRPS	34.6	Stat
	Cluster 7 - Siderophore	X	X
	Cluster 8 - T1PKS	X	X
	Cluster 9 - Other	2.3/3.4	Stat
	Cluster 10 - Ectoine	9	Stat
	Cluster 11 - NRPS (Holomycin)	5/10	Stat

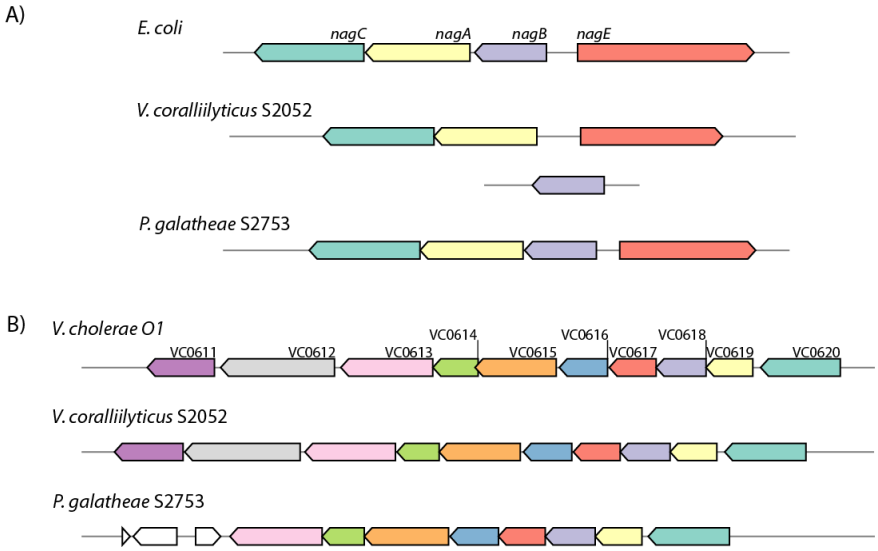


Figure 1 A) *nag* operon in *E. coli*, in which the operon was originally characterized, *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753 **B)** $(GlcNAc)_2$ operon in *V. cholerae* O1, in which the operon was identified first, *V. coralliilyticus* S2052 and *P. galatheae* S2753. The analysis was done using MultiGeneBlast (Medema et al., 2013).

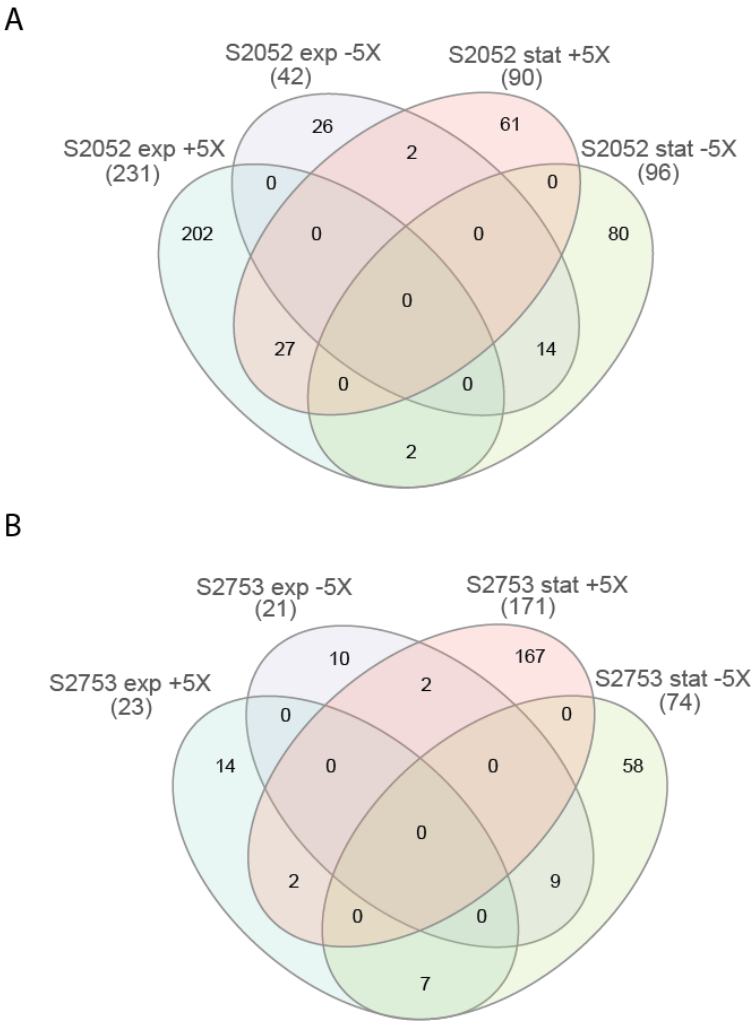


Figure 2 Venn diagrams representing the mRNAs which are up- (+5X) or down- (-5X) regulated (at least fivefold change, *p*-value < 0.05, *q*-value < 0.05) during exponential (exp) or stationary phase (stat) when *Vibrio coralliilyticus* S2052 **A)** and *Photobacterium galatheae* S2753 **B)** are grown on chitin compared to growth on glucose. The analysis was done using the InteractiVenn web-tool (Heberle et al., 2015).

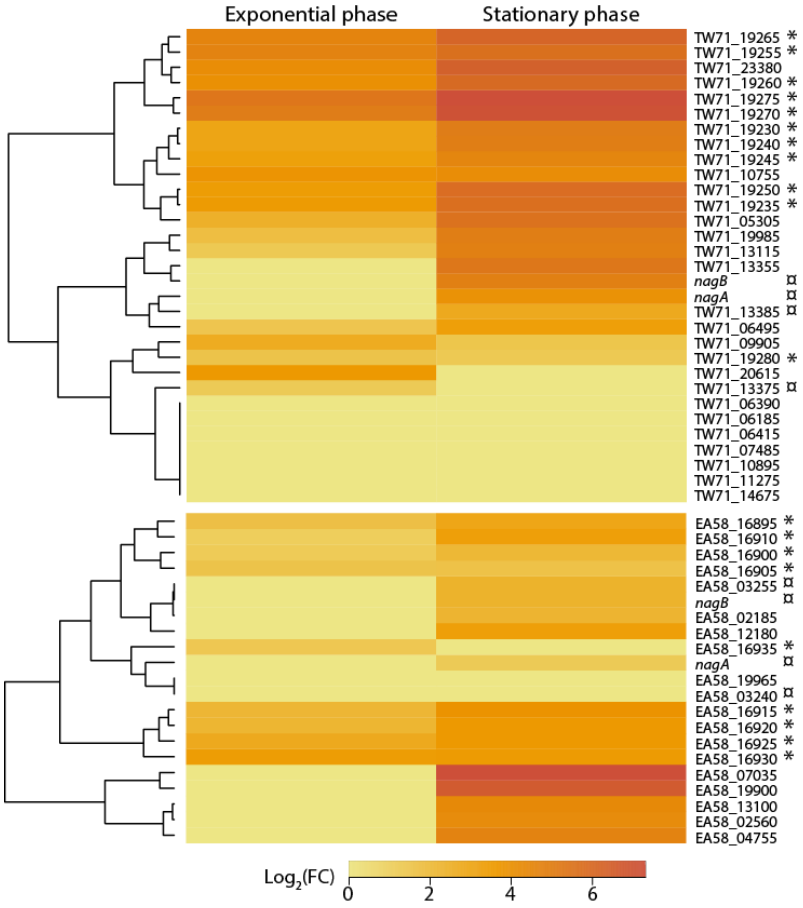


Figure 3 Heat map and hierarchical cluster analysis of the fold change of genes related to chitin utilization identified in the genome of *Vibrio corallilyticus* S2052 (top) and *Photobacterium galatheae* S2753 (bottom) at the two time points analyzed in this study (exponential and stationary phases). FC: fold change *: genes from the $(GlcNAc)_2$ operon plus *chiS* homolog; ¤: genes from the *nag* operon.

Table SI1 List of genes identified in the genomes of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753 and encoding for proteins containing Pfam domains related to the binding or the hydrolysis of chitin and cellulose. The predicted cellular localization is indicated as well. DUF: domain of unknown function.

	Gene	PGAP annotation	Predicted domains												PSORTb cellular localization (probability)
			Substrate binding					Hydrolysis							
			CBM_5_12	CBM_5_12_2	CHB_HEX	ChiA_N term	ChiC	GH3	GH18	GH19	GH20	LPMO_10	DUF		
Vibrio coralliilyticus S2052	TW71_05305	chitodextrinase	■	■											Unknown (not cytoplasmic)
	TW71_06185	chitinase	■						■						Outer membrane (95.2%)
	TW71_06390	beta-hexosaminidase						■							Cytoplasmic (99.7%)
	TW71_06415	chitin-binding protein	no Pfam match												Extracellular (97.1%)
	TW71_06495	Spindolin	■									■			Extracellular (97.2%)
	TW71_07485	beta-hexosaminidase						■							Cytoplasmic (99.7%)
	TW71_09905	chitinase		■					■					5011	Extracellular (100%)
	TW71_10755	chitinase	■			■			■						Extracellular (96.5%)
	TW71_10895	chitin-binding protein										■			Extracellular (97.1%)
	TW71_11275	chitin-binding protein	■									■			Extracellular (100%)
	TW71_13115	carbohydrate-binding protein								■					Unknown (not cytoplasmic)
	TW71_13355	beta-N-acetylhexosaminidase		■							■				Outer membrane (99.2%)
	TW71_14675	beta-N-acetylhexosaminidase									■				Periplasmic (97.6%)
	TW71_19985	chitinase	■						■						Extracellular (97.2%)
	TW71_23380	chitinase	■			■			■						Unknown (not cytoplasmic)
Photobacterium galatheae S2753	EA58_02560	chitinase	■			■			■						Unknown (not cytoplasmic)
	EA58_04755	hypothetical protein	■									■			Extracellular (98.4%)
	EA58_07035	chitodextrinase		■			■		■						Periplasmic (59.1%) Extracellular (40.8%)
	EA58_12180	hypothetical protein	■							■					Unknown (may have multiple localization site
	EA58_13100	hypothetical protein										■			Extracellular (100%)
	EA58_19900	chitinase	■						■						Extracellular (100%)
	EA58_19965	beta-hexosaminidase						■							Cytoplasmic (99.7%)

Table SI2 antiSMASH prediction of the genetic potential of *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753 for the biosynthesis of secondary metabolites. The table lists the type of putative biosynthetic gene cluster (BGC), the locus tags of the genes predicted to be part of them and, when known, the most similar known biosynthetic gene cluster. T3PKS: type 3 polyketide synthase; NRPS: non ribosomal peptide synthase; T1PKS: type 1 polyketide synthase.

		Type BGC	**Locus_tags**	**Most similar known BGC (% genes showing similarity)**
***V. coralliilyticus* S2052**	1	T3PKS	TW71_00340-TW71_00545	-
	2	NRPS	TW71_01010-TW71_01220	Cupriachelin BGC (11%)
	3	Arylpolyene-NRPS	TW71_07885-TW71_08150	Andrimid BGC (71%)
	4	NRPS-T1PKS	TW71_09375-TW71_09590	-
	5	NRPS-T1PKS	TW71_15090-TW71_15305	-
	6	Siderophore-Ectoine	TW71_15330-TW71_15480	Aerobactin BGC (88%)
	7	Bacteriocin	TW71_23595-TW71_23645	-
***P. galatheae* S2753**	1	Other	EA58_01380-EA58_01585	Indigoidine BGC (80%)
	2	NRPS	EA58_06305-EA58_06400	-
	3	Other	EA58_06305-EA58_06400	-
	4	Bacteriocin	EA58_06515-EA58_06820	-
	5	NRPS-T1PKS	EA58_09560-EA58_09700	-
	6	NRPS	EA58_10530-EA58_10790	-
	7	Siderophore	EA58_10780-EA58_10830	Aerobactin BGC (88%)
	8	T1PKS	EA58_15485-EA58_15615	-
	9	Other	EA58_15310-EA58_15400	-
	10	Ectoine	EA58_16435-EA58_16480	Ectoine BGC (66%)
	11	NRPS	EA58_20400-EA58_20585	Thiomarinol BGC (16%)
	12	NRPS		-
	13	NRPS	EA58_21730	-

Table SI3 Fold change values of the $(GlcNAc)_2$ operon, *chiS* homolog (TW71_19280), *nag* operon and other putative chitin utilization-related genes present in the genome of *Vibrio coralliilyticus* S2052.

			Fold change	
	Gene	PGAP annotation (NCBI)	Exp phase	Stat phase
$(GlcNAc)_2$ operon + *chiS*	TW71_19230	phosphomutase	8.2	34.3
	TW71_19235	N'-diacetylchitobiose phosphorylase	11.6	52.5
	TW71_19240	beta-hexosaminidase	8.3	31.8
	TW71_19245	N-acetylglucosamine kinase	9.7	23.7
	TW71_19250	chitobiase	11.4	56.6
	TW71_19255	chemotaxis protein	27.2	50.3
	TW71_19260	sugar ABC transporter ATP-binding protein	16.7	62.9
	TW71_19265	peptide ABC transporter permease	24.7	75.4
	TW71_19270	peptide ABC transporter permease	34.4	140.1
	TW71_19275	peptide ABC transporter substrate-binding protein	41.8	158.7
	TW71_19280	histidine kinase	3.0	2.6
nag operon	TW71_13385	PTS N-acetylmuramic acid transporter subunit IIBC	1	7.2
	nagB (TW71_15705)	glucosamine-6-phosphate deaminase	1	28.8
	nagA (TW71_13380)	N-acetylglucosamine-6-phosphate deacetylase	1	15.6
	TW71_13375	transcriptional regulator	2.5	6.7
Other chitin related genes	TW71_20615	porin (PGAP)	12.7	1
	TW71_05305	chitodextrinase	5.8	47.8
	TW71_06185	chitinase	1	1
	TW71_06390	beta-hexosaminidase	1	1
	TW71_06415	chitin-binding protein	1	1
	TW71_06495	Spindolin	2.8	10.1
	TW71_07485	beta-hexosaminidase	1	1
	TW71_09905	chitinase	6.6	2.9
	TW71_10755	chitinase	14.4	18.3
	TW71_10895	chitin-binding protein	1	1
	TW71_11275	chitin-binding protein	1	1
	TW71_13115	carbohydrate-binding protein	2.6	28.3
	TW71_13355	beta-N-acetylhexosaminidase	1	39.7
	TW71_14675	beta-N-acetylhexosaminidase	1	1
	TW71_19985	chitinase	3.6	31.9
	TW71_23380	chitinase	18.4	85.1

Table SI4 Fold change values of the $(GlcNAc)_2$ operon, *chiS*, *nag* operon and other putative chitin utilization-related genes present in the genome of *Photobacterium galatheae* S753.

			Fold change	
	Gene	**PGAP annotation (NCBI)**	**Exp phase**	**Stat phase**
$(GlcNAc)_2$ operon + *chiS*	EA58_16895	beta-hexosaminidase	2.9	6.3
	EA58_16900	N-acetylglucosamine kinase	2.1	3.8
	EA58_16905	chitobiase	2.7	2.8
	EA58_16910	chemotaxis protein	2.0	7.9
	EA58_1691½5	sugar ABC transporter ATP-binding protein	3.8	11.0
	EA58_16920	peptide ABC transporter permease	4	9.5
	EA58_16925	peptide ABC transporter permease	5.6	9.5
	EA58_16930	peptide ABC transporter substrate-binding protein	8.6	9.1
	EA58_16935	ChiS	2.4	1
***nag* operon**	EA58_03255	PTS glucose transporter subunit IIBC	1	4.4
	nagB (EA58_03250)	glucosamine-6-phosphate deaminase	1	4.4
	nagA (EA58_03245)	N-acetylglucosamine-6-phosphate deacetylase	1	2.2
	EA58_03240	transcriptional regulator	1	1
Other chitin related genes	EA58_02185	porin (PGAP)	1	4.2
	EA58_02560	chitinase	1	13.9
	EA58_04755	hypothetical protein	1	18.6
	EA58_07035	chitodextrinase	1	89.8
	EA58_12180	hypothetical protein	1	8
	EA58_13100	hypothetical protein	1	14.5
	EA58_19900	chitinase	1	60.8
	EA58_19965	beta-hexosaminidase	1	1

Table SI5 The ten most up and down regulated genes for each strain in each condition at the two sampling points (late exponential and stationary phase). Values refer to chitin compared to glucose. Up: upregulated; down: downregulated; FC: fold change

Sample	Gene	FC	PGAP annotation
***V. coralliilyticus* S2052 exponential phase up**	TW71_04295	196	aldehyde dehydrogenase
	TW71_05770	89	C4-dicarboxylate ABC transporter
	TW71_07295	81	acetyl-CoA synthetase
	TW71_07940	79	hypothetical protein
	TW71_19770	70	hypothetical protein
	TW71_00320	68	hypothetical protein
	TW71_07870	63	hypothetical protein
	TW71_00325	57	dehydrogenase
	TW71_07945	51	hypothetical protein
	TW71_18840	50	hypothetical protein
***V. coralliilyticus* S2052 stationary phase up**	TW71_19275	159	peptide ABC transporter substrate-binding protein
	TW71_19270	140	peptide ABC transporter permease
	TW71_18580	140	Trp operon leader peptide
	TW71_14655	103	amino acid deaminase
	TW71_23380	85	chitinase
	TW71_16830	83	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
	TW71_19265	75	peptide ABC transporter substrate-binding protein
	TW71_15905	68	hypothetical protein
	TW71_19260	63	sugar ABC transporter ATP-binding protein
	TW71_19250	57	chitobiase
***P. galatheae* S2753 exponential phase up**	EA58_07425	39	dethiobiotin synthetase
	EA58_12415	21	formimidoylglutamase
	EA58_12420	17	urocanate hydratase
	EA58_12425	17	histidine ammonia-lyase
	EA58_20235	12	hypothetical protein
	EA58_07075	12	hypothetical protein
	EA58_10775	12	glyceraldehyde-3-phosphate dehydrogenase
	EA58_18255	12	thioesterase
	EA58_12410	11	imidazolonepropionase
	EA58_16300	9	hypothetical protein
***P. galatheae* S2753 stationary phase up**	EA58_20785	799	hypothetical protein
	EA58_20780	648	benzoylformate decarboxylase
	EA58_06000	239	glycosyltransferase
	EA58_06005	150	hypothetical protein
	EA58_21470	137	lactonizing lipase
	EA58_15365	102	hemolysin
	EA58_16750	94	hypothetical protein
	EA58_07035	90	chitodextrinase
	EA58_12040	73	phosphonate ABC transporter substrate-binding protein
	EA58_19900	61	chitinase

Sample	Gene	FC	PGAP annotation
V. coralliilyticus S2052 exponential phase down	TW71_11480	-50	maltoporin
	TW71_00570	-34	hypothetical protein
	TW71_14985	-33	maltodextrin phosphorylase
	TW71_14690	-28	cytochrome O ubiquinol oxidase
	TW71_14695	-24	cytochrome o ubiquinol oxidase subunit I
	TW71_04930	-23	oxalate:formate antiporter
	TW71_14990	-22	4-alpha-glucanotransferase
	TW71_14700	-19	cytochrome o ubiquinol oxidase subunit III
	TW71_14705	-17	cytochrome O ubiquinol oxidase
	TW71_14095	-16	glyoxalase
V. coralliilyticus S2052 stationary phase down	TW71_00500	-2273	secretion protein
	TW71_00505	-2235	type III secretion protein
	TW71_00510	-2115	secretion protein EspA
	TW71_00495	-1833	pathogenicity island effector protein
	TW71_00490	-1197	type III secretion protein
	TW71_00515	-990	secretion protein EspA
	TW71_00485	-693	hypothetical protein
	TW71_00345	-551	hypothetical protein
	TW71_00355	-275	hypothetical protein
	TW71_00430	-249	type III secretion system needle protein SsaG
P. galatheae S2753 exponential phase down	EA58_17070	-17	glutamate synthase
	EA58_02025	-15	hypothetical protein
	gltD	-15	
	EA58_16650	-12	cytochrome D ubiquinol oxidase subunit I
	EA58_07025	-11	hypothetical protein
	EA58_19405	-10	acetate kinase
	EA58_07020	-9	cytochrome o ubiquinol oxidase subunit I
	EA58_07015	-8	cytochrome o ubiquinol oxidase subunit III
	EA58_17025	-8	lactoylglutathione lyase
	EA58_07010	-7	cytochrome O ubiquinol oxidase
P. galatheae S2753 stationary phase down	EA58_06945	-47	flagellar basal body rod protein FlgC
	EA58_07025	-47	hypothetical protein
	EA58_00065	-37	hypothetical protein
	EA58_12415	-30	formimidoylglutamase
	EA58_07020	-26	cytochrome o ubiquinol oxidase subunit I
	flgB_2	-24	
	EA58_12410	-23	imidazolonepropionase
	EA58_20875	-23	hypothetical protein
	EA58_01880	-21	acetate kinase
	EA58_11270	-21	hypothetical protein

Table SI6 Fold changes (FC) of genes related to chemotaxis in *Vibrio coralliilyticus* S2052 and *Photobacterium galatheae* S2753 when the organisms are grown on chitin as compared to glucose.

	Gene	FC	PGAP annotation
***V. coralliilyticus* S2052 exponential phase**	TW71_01215	2,4	chemotaxis protein CheY
	TW71_01445	-2,1	chemotaxis protein
	TW71_05740	3,7	chemotaxis protein CheY
	TW71_06500	-5,9	chemotaxis protein
	TW71_07880	2,5	chemotaxis protein CheY
	TW71_09420	2,3	chemotaxis protein CheY
	TW71_11490	21,1	chemotaxis protein
	TW71_11670	3,5	chemotaxis protein
	TW71_13190	2,2	chemotaxis protein
	TW71_14260	2,2	chemotaxis protein CheY
	TW71_14270	2,6	chemotaxis protein
	TW71_14275	4,2	chemotaxis protein
	TW71_14290	2,9	chemotaxis protein
	TW71_14295	3,9	chemotaxis protein CheW
	TW71_14305	5,9	chemotaxis protein CheA
	TW71_14320	4,2	chemotaxis protein
	TW71_14940	9,5	chemotaxis protein
	TW71_15635	-4,8	chemotaxis protein
	TW71_16580	2,7	chemotaxis protein CheX
	TW71_18425	2,2	chemotaxis protein
	TW71_18985	7,8	chemotaxis protein CheY
	TW71_19075	8,1	chemotaxis protein
	TW71_19255	27,2	chemotaxis protein
	TW71_24705	4,5	chemotaxis protein
***V. coralliilyticus* S2052 stationary phase**	TW71_07720	2,2	chemotaxis protein
	TW71_11490	-2,1	chemotaxis protein
	TW71_15165	2,0	chemotaxis protein
	TW71_19255	50,3	chemotaxis protein
	TW71_24705	-2,1	chemotaxis protein
***P. galatheae* S2753 exponential phase**	EA58_05570	2,2	chemotaxis protein CheY
	EA58_15700	-2,9	chemotaxis protein
***P. galatheae* S2753 stationary phase**	EA58_01610	-5,1	chemotaxis protein CheY
	EA58_02020	4,1	chemotaxis protein
	EA58_02250	-2,2	chemotaxis protein CheW
	EA58_02255	-2,2	chemotaxis protein CheR
	EA58_02470	2,4	chemotaxis protein CheA
	EA58_05760	2,3	chemotaxis protein
	EA58_07330	2,9	chemotaxis protein
	EA58_07600	-4,2	chemotaxis protein
	EA58_10710	4,6	chemotaxis protein
	EA58_16910	7,9	chemotaxis protein
	EA58_18325	3,7	chemotaxis protein

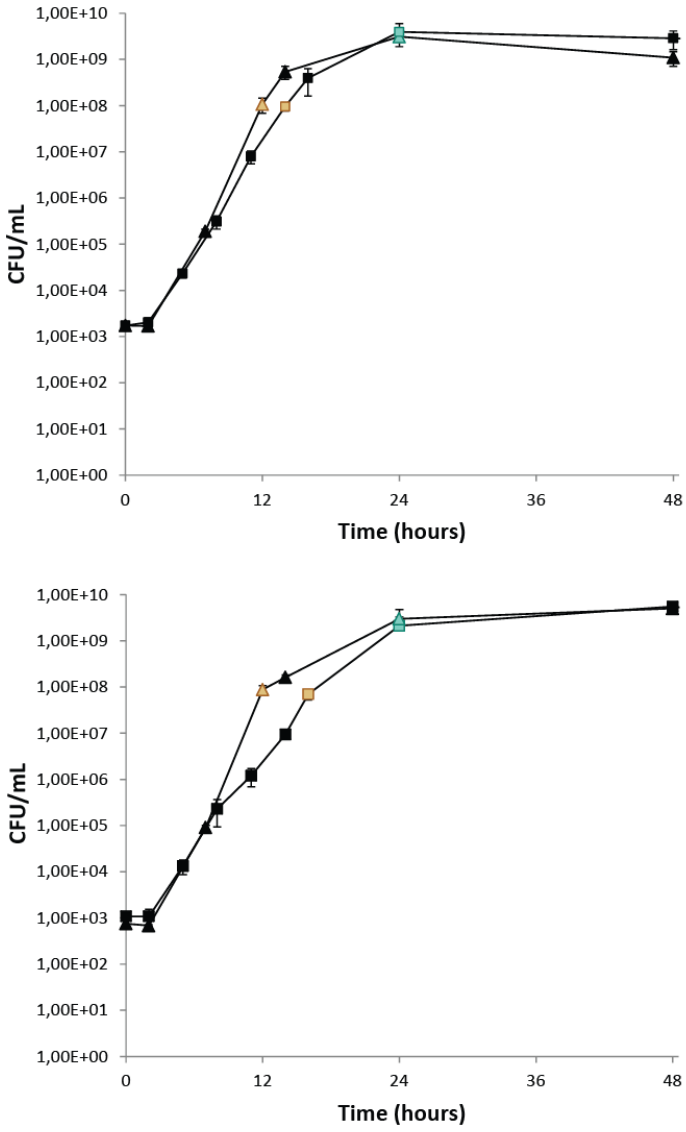


Figure SI1 Growth curves of *Vibrio coralliilyticus* S2052 (top) and *Photobacterium galatheae* S2753 (bottom) in glucose (SSBG triangles) and chitin (SSBC squares). The beige and light green time points indicate the harvest points in the late exponential and in the stationary phase, respectively.

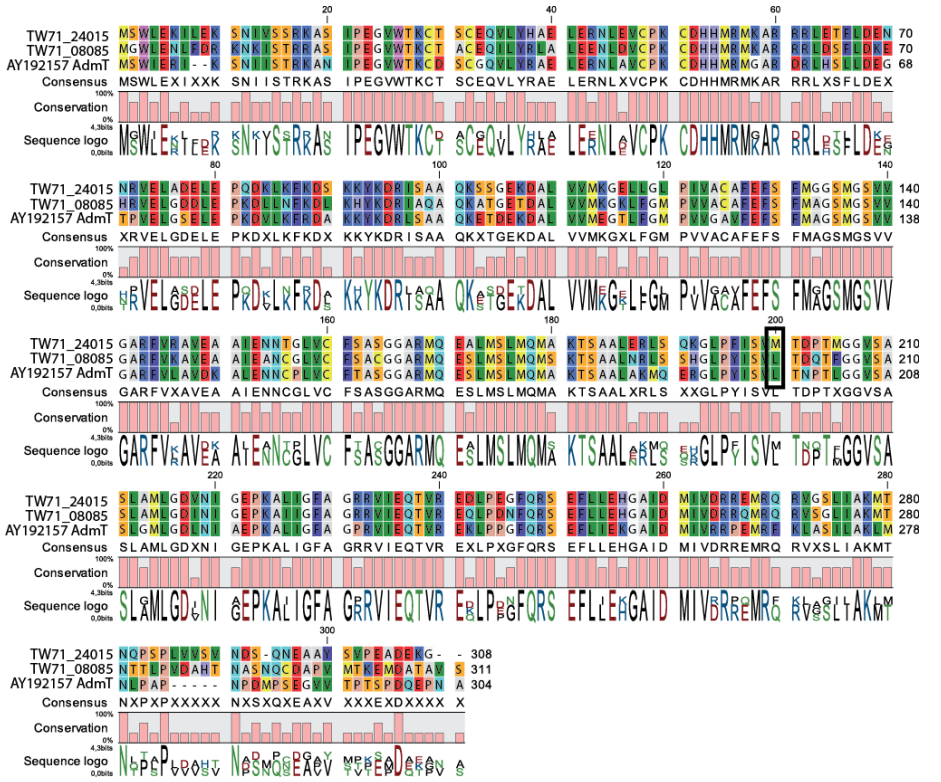
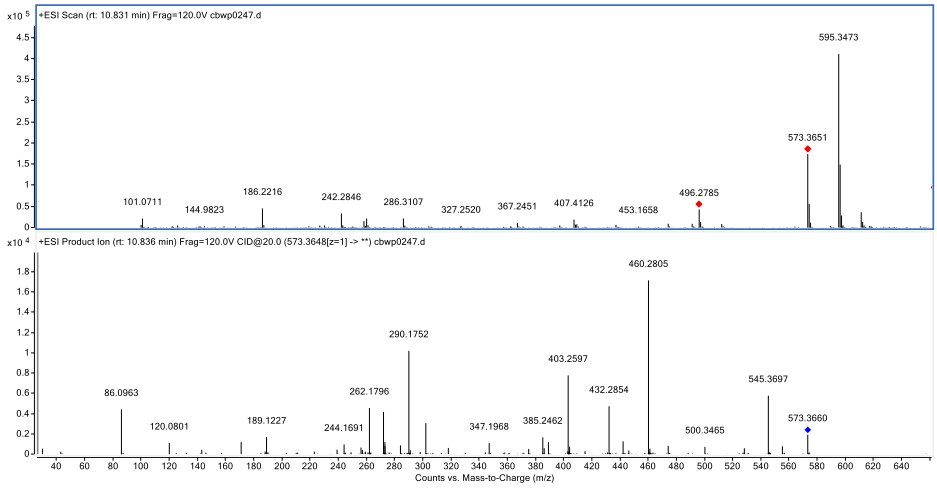
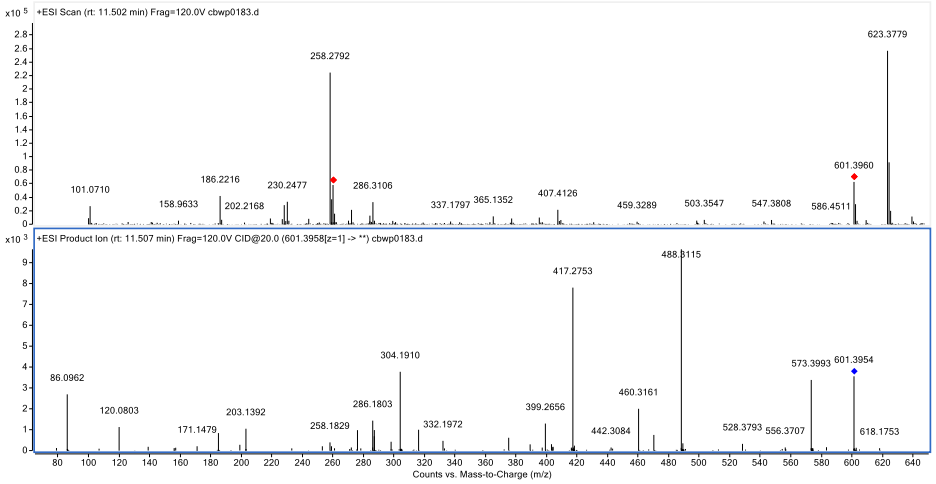


Figure SI2 Alignment of the amino acid sequences of the acetyl-CoA carboxylases encoded by TW71_24015 and TW71_08085 in *Vibrio coralliilyticus* S2052, and by *admT* in *Pantoea agglomerans*. The one encoded by TW71_08085, which is part of the andrimid BGC by antiSMASH in *V. coralliilyticus* S2052, carries the same single amino acid mutation (M→L) present in AdmT (highlighted by the black frame) that confers andrimid resistance to the carrier (Liu *et al.*, 2008).



Name	Observed mass	Predicted formula	Predicted Mass	Error (ppm)
Solonamide C		$C_{31}H_{48}N_4O_6$	572.3574	
M+Na	595.3473	$C_{31}H_{48}N_4O_6Na^+$	595.3466	1.175784
M+H	573.366	$C_{31}H_{49}N_4O_6^+$	573.3647	2.267313
	545.3697	$C_{30}H_{49}N_4O_5^+$	545.3697	0
	460.2805	$C_{25}H_{38}N_3O_5^+$	460.2806	-0.21726
	432.2854	$C_{24}H_{38}N_3O_4^+$	432.2857	-0.69399
	403.2597	$C_{23}H_{35}N_2O_4^+$	403.2591	1.487875
	385.2462	$C_{18}H_{33}N_4O_5^+$	385.2445	4.412763
	347.1968	$C_{19}H_{27}N_2O_4^+$	347.1965	0.864063
	290.1752	$C_{17}H_{24}NO_3^+$	290.1751	0.344619
	262.1796	$C_{16}H_{24}NO_2^+$	262.1802	-2.28851
	120.0801	$C_8H_{10}N^+$	120.0808	-5.82944
	86.0963	$C_5H_{12}N^+$	86.0964	-1.16149

Figure SI3 HRMS/MS spectra of solonamide C.



Name	Observed Mass	Predicted Formula	Predicted Mass	Error (ppm)
Solonamide D		$C_{33}H_{52}N_4O_6$	600.3887	
M+Na	623.3779	$C_{33}H_{52}N_4O_6Na^+$	623.3779	0
M+H	601.396	$C_{33}H_{53}N_4O_6^+$	601.396	0
	573.3993	$C_{32}H_{53}N_4O_5^+$	573.401	-2.964775157
	488.3115	$C_{27}H_{42}N_3O_5^+$	488.3119	-0.819149252
	460.3161	$C_{26}H_{42}N_3O_4^+$	460.317	-1.955178192
	417.2743	$C_{24}H_{37}N_2O_4^+$	417.2748	-1.198252564
	399.2656	$C_{24}H_{35}N_2O_3^+$	399.2642	3.50643782
	304.191	$C_{18}H_{26}NO_3^+$	304.1907	0.986222472
	286.1803	$C_{18}H_{24}NO_2^+$	286.1802	0.349430062
	185.128	$C_9H_{17}N_2O_2^+$	185.1285	-2.700834018
	120.0803	$C_8H_{10}N^+$	120.0808	-4.163880337
	86.0962	$C_5H_{12}N^+$	86.0964	-2.322982896

Figure SI4 HRMS/MS spectra of solonamide D.

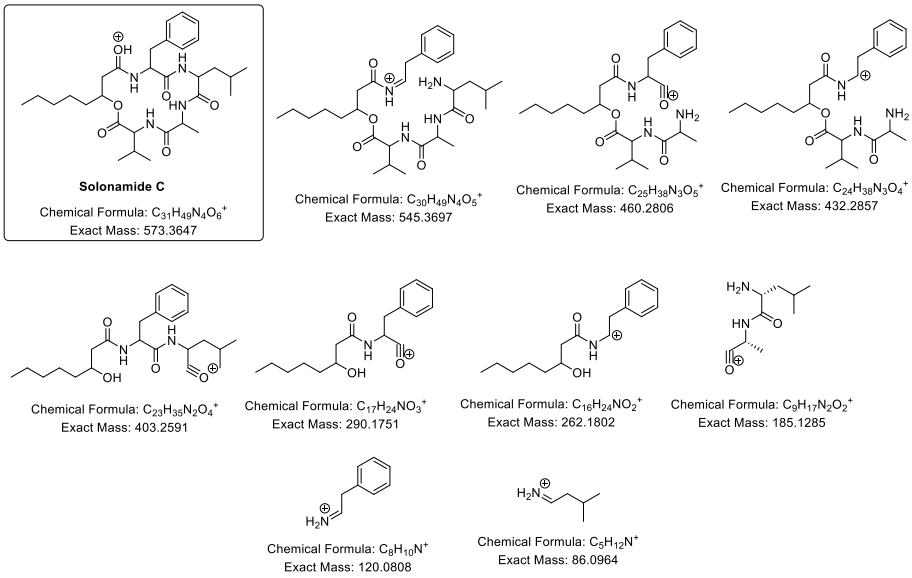


Figure SI5 Proposed major MS/MS fragments of solonamide C.

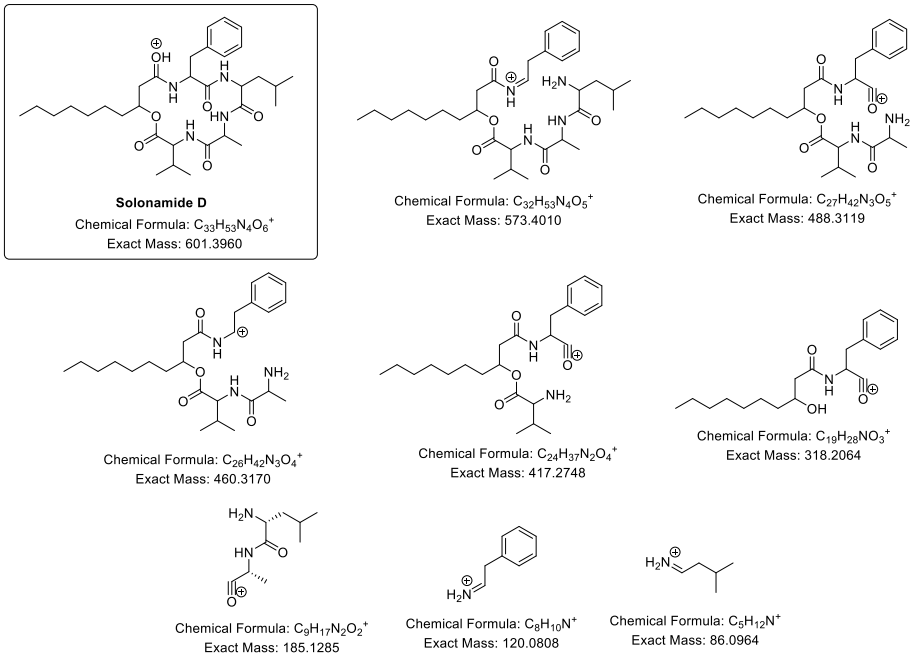


Figure SI6 Proposed major MS/MS fragments of solonamide D.

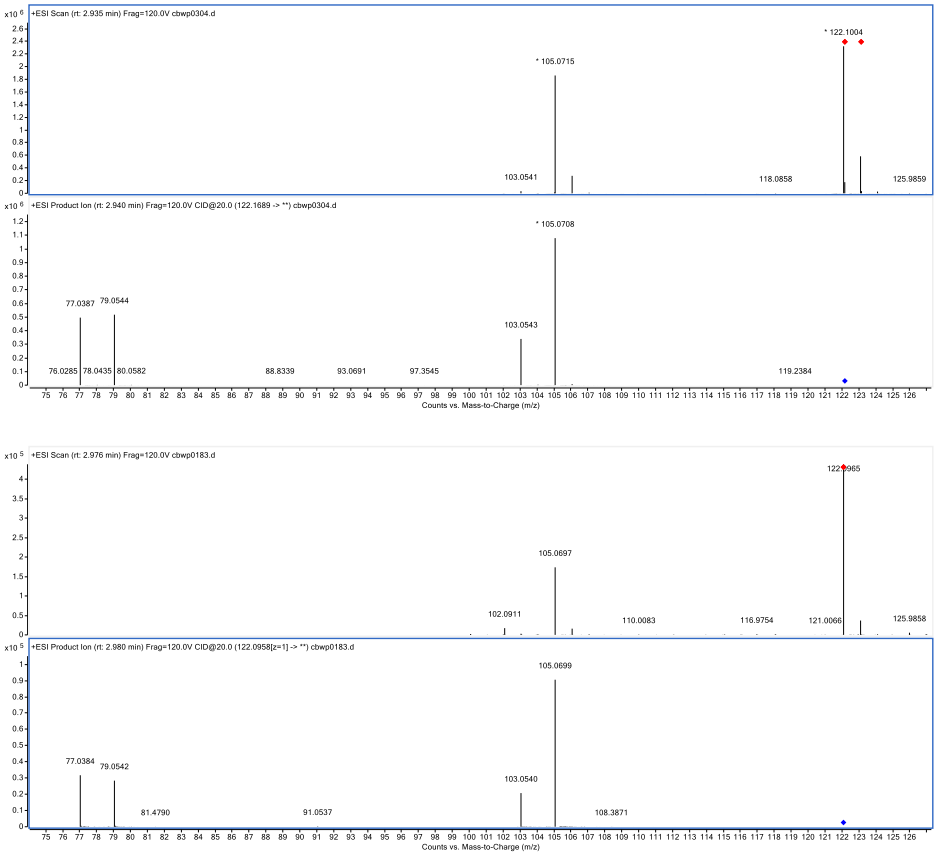


Figure SI7 MS/MS spectra of 2-phenylethylamine standard (top) and of the compound present in the extracts from cultures of *Photobacterium galatheae*.

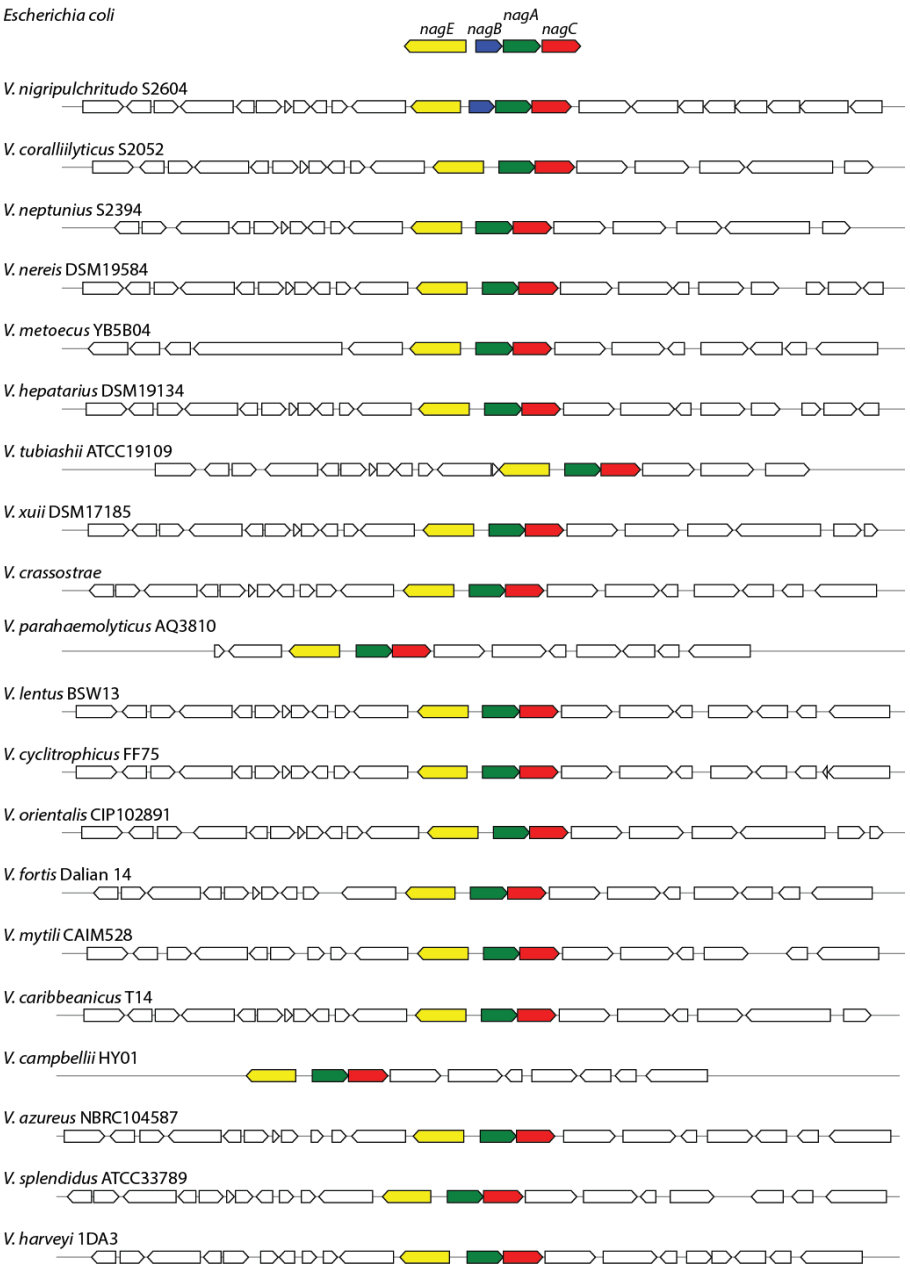


Figure SI8 Distribution of the *nag* operon in *Vibrio* species. The homology search was done by MultiGeneBlast (Medema *et al.,* 2013) with the well characterized *nagE-BAC* operon from *Escherichia coli* as search query.

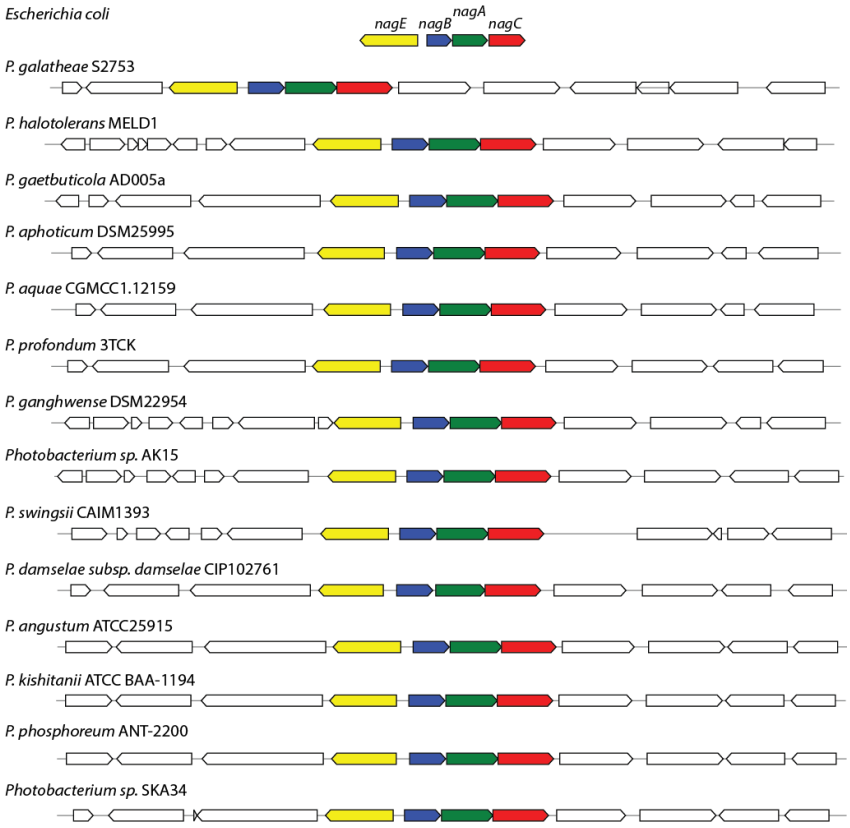


Figure SI9 Distribution of the *nag* operon in *Photobacterium* species. The homology search was done by MultiGeneBlast (Medema *et al.,* 2013) with the well characterized *nagE-BAC* operon from *Escherichia coli* as search query.

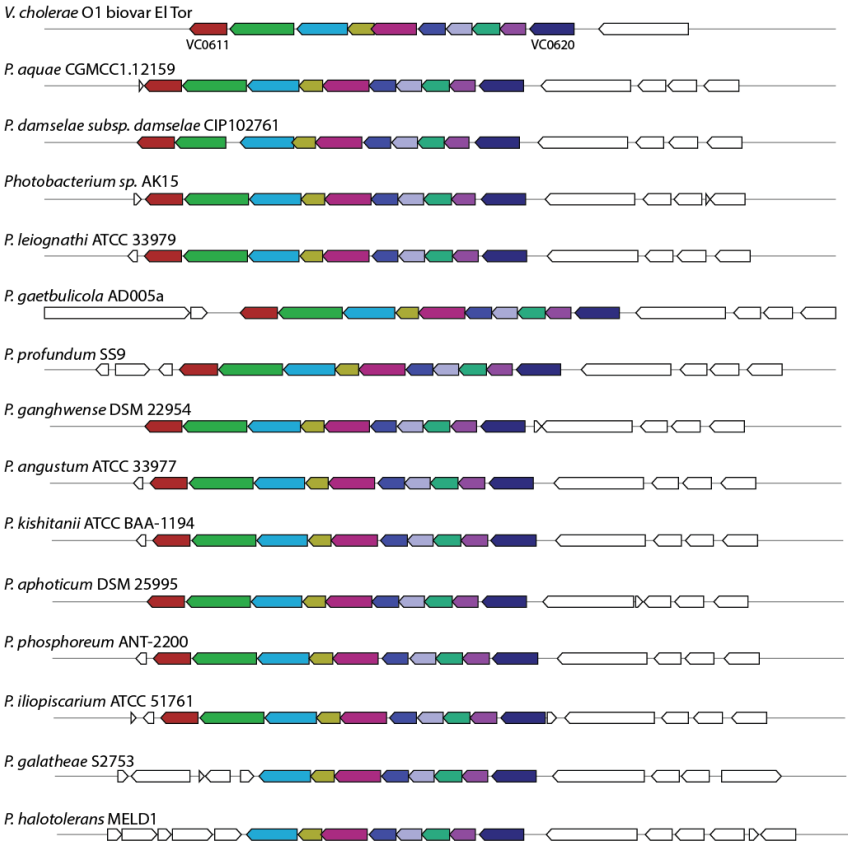


Figure SI10 Distribution of the $(GlcNAc)_2$ operon in *Photobacterium* species. The homology search was done by MultiGeneBlast (Medema *et al.,* 2013) with the $(GlcNAc)_2$ operon from *V. cholerae* (VC0611-VC0620) as search query.

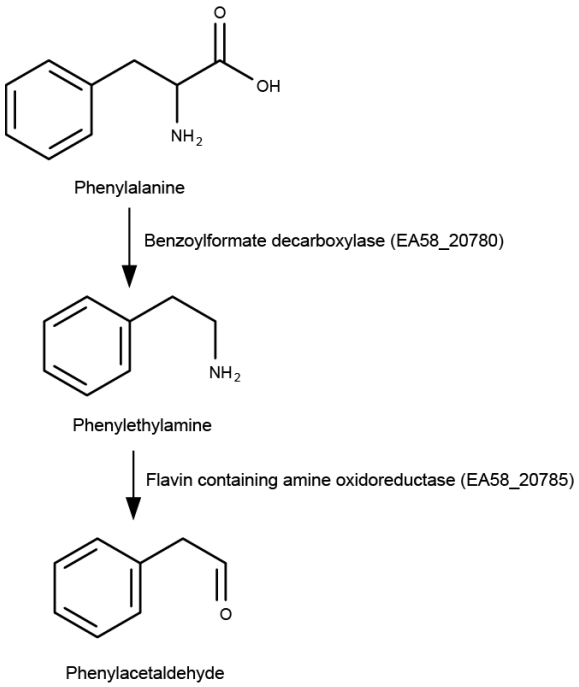


Figure SI11 Proposed phenylalanine degradation pathway occurring in *Photobacterium galatheae* S2753.

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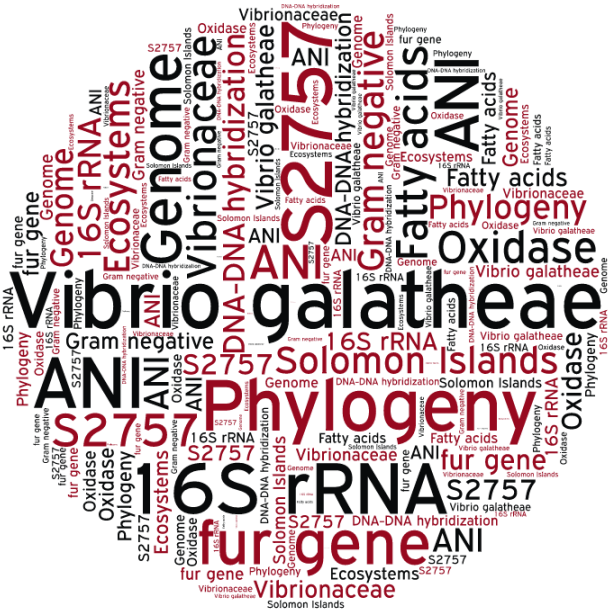
ARTICLE 4

Vibrio galatheae sp. nov., a member of the family *Vibrionaceae* isolated from a mussel.



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Vibrio galatheae sp. nov., a member of the family *Vibrionaceae* isolated from a mussel

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Based on genetic, chemotaxonomic and phenotypic characteristics, a novel species belonging to the genus *Vibrio* is described. The facultatively anaerobic strain S2757^T was isolated from a mussel collected in the Solomon Sea (Solomon Islands). Phylogenetic analyses based on sequences of 16S rRNA and *fur* genes indicated affiliation of the strain to a novel species. This observation was supported by a multilocus sequence analysis including sequences of the housekeeping genes 16S rRNA, *gyrB*, *pyrH*, *recA* and *topA*. *In silico* DNA–DNA hybridization and average nucleotide identity values comparing the genomic sequence of strain S2757^T with those of closely related type strains were lower than 23 and 82 %, respectively. The DNA G+C content of the strain was 45.3 mol%. Phenotypic and chemotaxonomic analyses clearly differentiated the strain from other *Vibrio* species. Hence, strain S2757^T should be considered to represent a novel species of the genus *Vibrio*, for which the name *Vibrio galatheae* sp. nov. is proposed. The type strain is S2757^T (=DSM 100497^T=LMG 28895^T).

Members of the family *Vibrionaceae* are Gram-negative bacteria that are widespread in aquatic environments (Thompson *et al.*, 2004). Vibrios have been isolated as both planktonic and surface-associated organisms from several ecosystems, including seawater, marine sediments and animals (Thompson *et al.*, 2004). The number of vibrios colonizing different environmental niches can vary over orders of magnitude, depending on factors such as availability of nutrients, temperature and salinity (Takemura *et al.*, 2014). For instance, *Vibrio* species were shown to account for more than 50 % of the total microbiota during a bacterial bloom that was possibly due to an increase in the concentration of available nutrients (Gilbert *et al.*, 2012).

A number of vibrios have been intensively studied because of their role as pathogens (Ben-Haim *et al.*, 2003; Faruque *et al.*, 1998; Jones & Oliver, 2009; Ramamurthy *et al.*, 2014) and symbionts (Nyholm *et al.*, 2000). In recent years, *Vibrionaceae* have also emerged as a reservoir of secondary metabolites with therapeutic applications, including antibacterial, anticancer and antifungal activities (Månsson *et al.*, 2011). Here, we report the taxonomic characterization of a strain belonging to the genus *Vibrio*. Strain S2757^T was isolated in 2007 from a mussel collected in the Solomon Sea (Solomon Islands) during the Galathea 3 global research expedition (http://www.galathea3.dk/uk) and was affiliated to the family *Vibrionaceae* based on its 16S rRNA gene sequence, as previously described (Gram *et al.*, 2010).

The reference type strains included in this study, namely *Vibrio brasiliensis* DSM 17184^T (Thompson *et al.*, 2003a), *V. orientalis* DSM 19136^T (Yang *et al.*, 1983), *V. hepatarius* DSM 19134^T (Thompson *et al.*, 2003b), *V. tubiashii* DSM 19142^T (Hada *et al.*, 1984), *V. sinaloensis* DSM 21326^T (Gomez-Gil *et al.*, 2008), *V. xuii* DSM 17185^T (Thompson *et al.*, 2003a) and *V. nereis* DSM 19584^T (Baumann *et al.*, 1980), were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). All strains were routinely cultivated on marine agar (MA) (212185; Difco) or in marine broth (MB) (279110; Difco) at 25 °C. Strain S2757^T

Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession number for the *fur* gene sequence of *Vibrio sinaloensis* DSM 21326^T is KT380049. GenBank accession numbers for the whole genome sequences of *Vibrio hepatarius* DSM 19134^T, *Vibrio xuii* DSM 17185^T and *Vibrio nereis* DSM 19584^T are LHPI01, LHPK01 and LHPJ01, respectively. Accession numbers of all nucleotide sequences used in this work, including those previously publicly available, are listed in Table S1 (available in the online Supplementary Material).

One supplementary table and two supplementary tables are available with the online Supplementary Material.

grew as small (2–4 mm), round, beige colonies after 48 h on MA at 25 °C.

Cell morphology of strain S2757^T was observed by means of phase-contrast microscopy (1000× magnification; Olympus BX51) and scanning electron microscopy (FEI Quanta 200 FEG ESEM) after growth in filtered MB for 24 h at 25 °C. Gram testing and catalase activity were assessed with the 3 % KOH (Gregersen, 1978) and the 3 % H_2O_2 (Cowan, 1974) methods, respectively. Oxidase activity was determined on a BBL DrySlide Oxidase kit (231746; BD Diagnostics) following the manufacturer's instructions. A test of susceptibility to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropyl pteridine, 10 and 150 µg per disc) was performed on Iso-sensitest agar (CM04741B; Oxoid) supplemented with 1.5 % (w/v) NaCl and incubation at 25 °C for 48 h. Salinity requirements of strain S2757^T were determined in synthetic ZoBell medium (5 g Bacto peptone l^{-1}, 1 g yeast extract l^{-1}, 0.1 g ferric citrate l^{-1}) (ZoBell, 1941) with different NaCl concentrations (0–9 %, w/v) at 28 °C. Growth was assessed using a microplate reader (Spectra Max i3; Molecular Devices). The temperature range for growth was determined on MA. The ability of strain S2757^T to grow under anaerobic conditions was tested on MA at 25 °C using an anaerobic jar and anaerobic atmosphere generation bags (68061; Fluka).

Physiological and biochemical characterization using API 20 NE strips (20050; bioMérieux), API ZYM strips (25200; bioMérieux) and Biolog GN2 plates (Biolog) was done on strain S2757^T and on the closely related *V. tubiashii* DSM 19142^T, *V. brasiliensis* DSM 17184^T, *V. orientalis* DSM 19136^T and *V. hepatarius* DSM 19134^T. Bacterial suspensions were prepared in 1.5 % (w/v) NaCl using biomass grown overnight on MA at 25 °C. Inoculation of strips and plates was done in agreement with the manufacturers' instructions. The cellular fatty acids of strain S2757^T and related species were analysed as methyl esters by GC. The analysis was performed in duplicate by the DSMZ using biomass grown for 24 h on MA at 25 °C and according to the instructions of the Microbial Identification System (MIDI). Cell morphology of strain S2757^T and related species was observed on thiosulfate-citrate-bile-sucrose (TCBS, CM0333; Oxoid) agar plates. Detailed morphological, physiological and biochemical features distinguishing strain S2757^T from related species are summarized in Table 1 and in the species description.

Table 1. Features differentiating strain S2757^T from closely related *Vibrio* species

Strains: 1, S2757^T; 2, *V. brasiliensis* DSM 17184^T; 3, *V. orientalis* DSM 19136^T; 4, *V. hepatarius* DSM 19134^T; 5, *V. tubiashii* DSM 19142^T. G, Green; Y, yellow; +, positive; −, negative. All data were generated in this work in biological duplicates.

Characteristic	1	2	3	4	5
Citrate*	+	+	−	−	−
Malic acid*	+	+	−	−	+
Growth in/on:					
8 % NaCl	+	−	+	−	−
TCBS (colour)	G	Y	Y	Y	Y
Production of:					
Lipase†	−	+	+	+	+
α-Glucosidase†	+	−	−	−	+
Acid phosphatase†	−	+	+	−	−
N-Acetyl-β-glucosaminidase†	−	−	+	+	+
Utilization of:					
D-Glucose 6-phosphate	+	+	+	−	+
L-Threonine	−	+	−	+	−
L-Proline	−	+	+	−	−
D-Alanine	+	+	−	−	−
Sucrose	−	+	−	+	+
Fatty acid methyl esters (%):					
$C_{16:0}$	19.5±0.2	24.8±0.3	23.3±0.4	22.6±0.2	22.2±0.2
iso-$C_{15:0}$	2.1	0.3	0.2	0.1	0.1
iso-$C_{16:0}$	3.7	0.5	0.6	1.1	0.4
iso-$C_{17:0}$	4.0	0.3	−	0.1	0.2
Summed feature 3‡	34.7±0.4	35.9±0.4	44.7±0.4	39±0.4	35.8±0.3
Summed feature 8‡	15.8±0.2	19.5±0.2	10.6±0.2	18.3±0.2	23.0±0.2

*API 20 NE results.
†API ZYM results.
‡Summed feature 3: one or more of $C_{16:1}\omega7c$, $C_{16:1}\omega6c$ and/or iso-$C_{15:0}$. Summed feature 8: $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

The complete list of the results of the performed tests and analyses is available in Table S2.

Cells of strain S2757^T were Gram-negative, slightly curved rods (1.5±0.4 μm in length) that were motile by means of one polar flagellum (0.7±0.2 μm in length) (Fig. S1). Strain S2757^T was catalase- and oxidase-positive, and sensitive to the vibriostatic agent O/129. NaCl was required for growth and was tolerated up to a concentration of 8 % (w/v). Strain S2757^T grew as green, small (2–3 mm) colonies on TCBS agar. Strain S2757^T produced α-glucosidase but not acid phosphatase, *N*-acetyl-β-glucosaminidase or lipase. The strain could utilize D-glucose 6-phosphate and D-alanine, but not L-threonine, L-proline or sucrose. The major cellular fatty acids of strain S2757^T were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ and/or iso-$C_{15:0}$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). These values were comparable to those of the closely related species; however, the fatty acid pattern of strain S2757^T was distinct due to the presence of a relatively high amount (combined 9.8 % of the total) of the fatty acids iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$ compared with the patterns of the other analysed species, for which values were lower than 1.2 %.

For *V. hepatarius* DSM 19134^T, *V. xuii* DSM 17185^T and *V. nereis* DSM 19584^T no whole genome sequence was publicly available at the time this study was started. Therefore, high-purity genomic DNA was obtained as described previously (Sambrook & Russel, 2001) by repeated phenol/chloroform/isoamyl alcohol purification steps followed by RNase treatment and DNA precipitation. Quantification was performed on a NanoDrop Spectrometer (Saveen Werner) and a Qubit 2.0 Analyser (Invitrogen).

Genome sequencing was carried out at the NovoNordisk Foundation Center for Biosustainability. Libraries of 300–400 bp were prepared and used for 151 bp paired-end sequencing by Illumina sequencing technology on a MiSeq sequencer. Data were assembled to contigs using the *de novo* assembly algorithm of CLC Genomic Workbench, version 7 (CLC Bio). The list of the GenBank/EBI accession numbers of the nucleotide sequences used in this study, including those generated herein, is available in Table S1. For the *in silico* phylogenetic analysis, sequences of the single genes were obtained directly from the GenBank database or extracted from whole genome sequences based on their PGAP (NCBI Prokaryotic Genome Annotation Pipeline) annotation (Tatusova *et al.*, 2013) or by BLAST search using CLC Main Workbench, version 7.6.2 (CLC Bio).

Comparison of the 1487 bp long 16S rRNA gene sequence obtained from the complete genome sequence of the new isolate with those from type strains available in the GenBank database using the BLASTN algorithm (https://blast.ncbi.nlm.nih.gov) and the Ez-Taxon-e service (http://www.ezbiocloud.net/eztaxon) confirmed that strain S2757^T belongs to the genus *Vibrio*, as previously established (Gram *et al.*, 2010). Pairwise alignment of the almost-complete 16S rRNA gene sequences was carried out using CLC Main Workbench. A phylogenetic tree was reconstructed in MEGA6 (Tamura *et al.*, 2013) using the neighbour-joining method. The robustness of the tree topology was tested with 1000 bootstrap iterations (Fig. 1). Based on the 16S rRNA

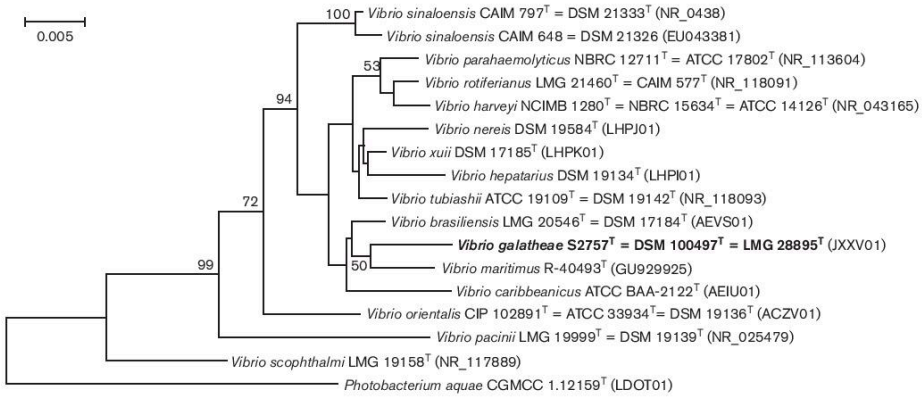

Fig. 1. Phylogenetic tree based on partial 16S rRNA gene sequences, obtained using the neighbour-joining method. Numbers at nodes indicate the level of bootstrap support based on 1000 replicates; only values >50 % are shown. *Photobacterium aquae* CGMCC 1.12159^T was used as outgroup. Bar, 0.5 % estimated sequence divergence.

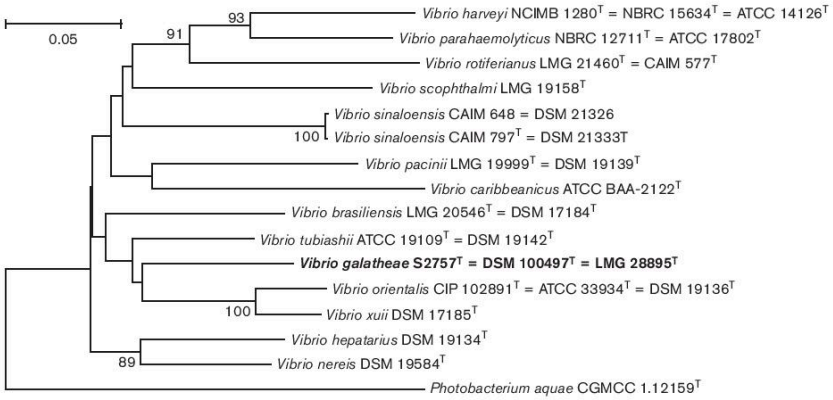

Fig. 2. Phylogenetic tree based on complete *fur* gene sequences, obtained using the neighbour-joining method. Numbers at nodes indicate the level of bootstrap support based on 1000 replicates; only values >50 % are shown. *Photobacterium aquae* CGMCC 1.12159^T was used as outgroup. Bar, 5 % estimated sequence divergence.

gene sequences, strain S2757^T was phylogenetically closely related to *V. hepatarius* DSM 19134^T, *V. brasiliensis* LMG 20546^T, *V. maritimus* R-40493^T and *V. tubiashii* ATCC 19109^T, sharing 98.5, 98.3, 98.2 and 97.8 % similarity, respectively. However, due to the low interspecies resolution that can be obtained in *Vibrionaceae* by using the 16S rRNA gene sequence (Sawabe *et al.*, 2007), two phylogenetic trees based on complete sequences of the recently proposed *Vibrionaceae* phylogenetic marker *fur* gene (Machado & Gram, 2015) (Fig. 2) and on the concatenated sequences of five housekeeping genes (Fig. 3) were reconstructed. These phylogenetic trees were obtained as described above and elsewhere (Machado & Gram, 2015; Sawabe *et al.*, 2013; Thompson *et al.*, 2005). For the *fur* gene phylogenetic tree, gene sequences were obtained either by PCR-based gene amplification followed by sequencing as described previously (Machado & Gram, 2015), or from whole genome sequences as described above. For the multilocus sequence analysis, sequences of the 16S rRNA, DNA gyrase subunit B (*gyrB*), uridylate kinase (*pyrH*), recombinant protein RecA (*recA*) and DNA topoisomerase I (*topA*) genes were retrieved from the GenBank database or from whole genome sequences, as described above. Sequences were trimmed to a common length and concatenated to a final length of 3800 bp. Both phylogenetic trees showed that strain S2757^T was clearly separated from the other analysed *Vibrio* species.

Whole genome sequences of strain S2757^T and closely related species were compared by DNA–DNA hybridization (DDH) and average nucleotide identity (ANI) values obtained *in silico* using the Genome-to-Genome Distance calculator 2.0 (provided by the DSMZ; http://ggdc.dsmz.de/) (Meier-Kolthoff *et al.*, 2013) and the ANI calculator (http://enve-omics.ce.gatech.edu/ani/) developed by the Kostas Lab (Goris *et al.*, 2007). All DDH and ANI values were below the thresholds used for species definition (70 % for DDH and 95 % for ANI) and identified *V. tubiashii* ATCC 19109^T as the closest relative of strain S2757^T, with DDH of 22.50 % and ANI of 81.13 % (Table 2). The DNA G+C content of S2757^T calculated *in silico* using CLC Main Workbench was 45.3 mol%, which is in agreement with values reported in the literature for *Vibrio* species.

The results presented indicate that strain S2757^T should be classified as representing a novel species of the genus *Vibrio*, for which the name *Vibrio galatheae* sp. nov. is proposed.

Description of *Vibrio galatheae* sp. nov.

Vibrio galatheae (ga.la.the′ae. N.L. gen. n. *galatheae* referring to the name of the Danish research expedition Galathea 3 during which the type strain was isolated).

Cells are slightly curved rods, Gram-negative and motile by means of one polar flagellum. Colonies are circular, beige and 2–4 mm in size after 48 h at 25 °C on MA and round, green and 2–4 mm in size after 48 h at 25 °C on TCBS. Growth occurs in the presence of 0.5–8 % (w/v) NaCl in synthetic ZoBell medium, with optimal growth at 2–5 %. Grows at 15–40 °C, with optimal growth at 25–30 °C. Growth is observed under anaerobic conditions. Positive for catalase and oxidase and sensitive to the vibriostatic agent O/129. Reduces nitrates to nitrites, produces indole and hydrolyses aesculin. Positive for alkaline

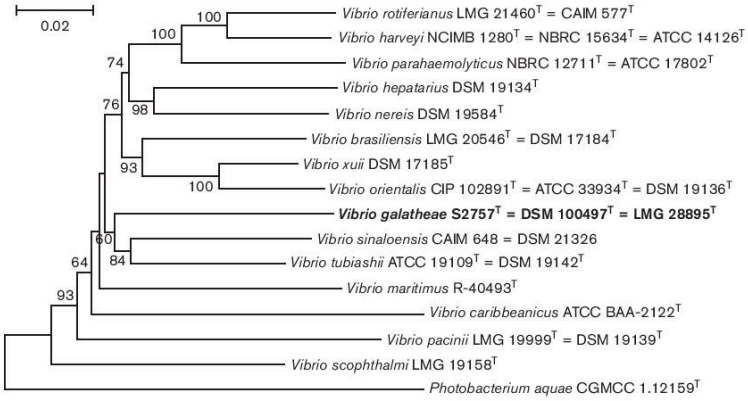

Fig. 3. Phylogenetic tree based on concatenated sequences of five genes (16S rRNA, *gyrB*, *pyrH*, *recA* and *topA*; approximately 3800 bp) obtained using the neighbour-joining method. The sizes of the gene sequences were: 16S RNA, 1439 bp; *gyrB*, 738 bp; *pyrH*, 530 bp; *recA*, 554 bp; *topA*, 552 bp. Numbers at nodes indicate the level of bootstrap support based on 1000 replicates; only values >50 % are shown. *Photobacterium aquae* CGMCC 1.12159^T was used as outgroup. Bar, 2 % estimated sequence divergence.

phosphatase, esterase lipase, leucine arylamidase, valine arylamidase and cysteine arylamidase but not for lipase or acid phosphatase. Can utilize as sole carbon sources: *N*-acetyl-D-glucosamine, cellobiose, D-fructose, α-D-glucose, maltose, D-mannitol, D-mannose, trehalose, D-gluconic acid, DL-lactic acid, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, inosine, uridine, thymidine, α-D-glucose 1-phosphate and D-glucose 6-phosphate. Cannot utilize: *N*-acetyl-D-galactosamine, sucrose, succinic acid, glycyl L-aspartic acid, L-proline, L-threonine or glycerol. The most abundant fatty acids are summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ and/or iso-$C_{15:0}$), $C_{16:0}$ and summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$).

The type strain, S2757^T (=DSM 100497^T=LMG 28895^T), was isolated from a mussel collected in the Solomon Sea, Solomon Islands. The DNA G+C content of the type strain is 45.3 mol%.

Table 2. Comparison of the genomic sequences of S2757^T and related species based on DDH and two-way ANI values obtained with *in silico* methods

Strains: 1, S2757^T; 2, *V. hepatarius* DSM 19134^T; 3, *V. xuii* DSM 17185^T; 4, *V. nereis* DSM 19584^T; 5, *V. brasiliensis* LMG 20546^T (=DSM 17184^T); 6, *V. orientalis* DSM 19136^T; 7, *V. tubiashii* ATCC 19109^T (=DSM 19142^T). Values are percentages shown as means±SD.

		ANI						
		1	2	3	4	5	6	7
DDH	1		80.22±6.17	80.51±5.90	79.81±5.89	80.32±5.73	80.56±5.81	81.13±5.48
	2	20.00±2.31		80.35±5.76	82.56±6.48	80.45±6.00	80.24±5.79	80.53±6.16
	3	20.80±2.33	20.90±2.33		80.41±6.06	81.29±6.06	88.74±4.55	81.61±6.13
	4	19.70±2.30	22.30±2.36	35.50±2.48		80.06±5.91	80.06±5.72	80.26±5.90
	5	20.60±2.32	20.30±2.32	21.20±2.34	19.90±2.30		81.28±6.01	81.14±5.90
	6	20.70±2.33	20.30±2.31	38.00±2.49	20.30±2.32	21.40±2.34		81.83±6.99
	7	22.50±2.36	20.60±2.32	21.80±2.35	20.30±2.31	21.60±2.34	21.80±2.35	

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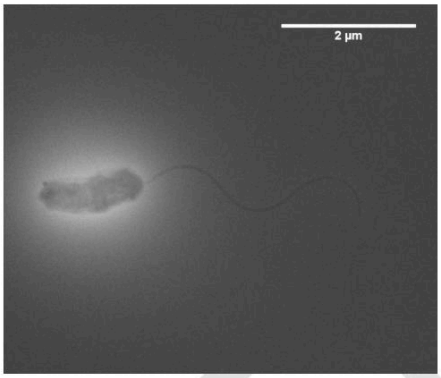

Figure S1. Wet scanning transmission electron Micrograph in Scanning Electron Microscope (wet-STEM SEM) image of uranyl acetate stained strain S2757^{T} grown in MB for 24 hours at 25 °C.

Table S1. GenBank/EBI Accession numbers of the nucleotide sequences used in this study. For gene sequences that were extracted from whole genome sequences, the WGS accession number is listed.

Species	Strain	GenBank/EBI accession number						
		WGS	*16S rRNA*	*fur*	*gyrB*	*pyrH*	*recA*	*topA*
V. galatheae	S2757^T = DSM 100497^T = LMG 28895^T	JXXV01	JXXV01	JXXV01	JXXV01	JXXV01	JXXV01	JXXV01
V. brasiliensis	LMG 20546^T = DSM 17184^T	AEVS01	AEVS01	AEVS01	AEVS01	AEVS01	AEVS01	AEVS01
V. orientalis	CIP 102297^T = ATCC 33934^T = DSM 19136^T	ACZV01	ACZV01	ACZV01	ACZV01	ACZV01	ACZV01	ACZV01
V. hepatarius	DSM 19134^T	LHPI01*	LHPI01*	LHPI01*	LHPI01*	LHPI01*	LHPI01*	LHPI01*
V. tubiashii	ATCC 19109^T = DSM 19142^T	AFWI01	NR_118093	AFWI01	AFWI01	AFWI01	AFWI01	AFWI01
V. caribbeanicus	ATCC BAA-2122^T	AEIU01	AEIU01	AEIU01	AEIU01	AEIU01	AEIU01	AEIU01
V. xuii	DSM 17185^T	LHPK01*	LHPK01*	LHPK01*	LHPK01*	LHPK01*	LHPK01*	LHPK01*
V. nereis	DSM 19584^T	LHPJ01*	LHPJ01*	LHPJ01*	LHPJ01*	LHPJ01*	LHPJ01*	LHPJ01*
V. harveyi	NCIMB 1280^T = NBRC 15634^T = ATCC 14126^T	BAOD01	NR_043165	BAOD01	BAOD01	BAOD01	BAOD01	BAOD01
V. pacinii	LMG 19999^T = DSM 19139^T	JONH01	NR_025479	JONH01	JONH01	JONH01	JONH01	JONH01
V. parahaemolyticus	NBRC 12711^T = ATCC 17802^T	LATW01	NR_113604	LATW01	LATW01	LATW01	LATW01	LATW01
V. rotiferianus	LMG 21460^T = CAIM 577^T	BAOI01	NR_118091	BAOI01	BAOI01	BAOI01	BAOI01	BAOI01
V. scophthalmi	LMG 19158^T	AFWE01	NR_117889	AFWE01	AFWE01	AFWE01	AFWE01	AFWE01
V. sinaloensis	CAIM 797^T=DSM 21333^T		NR_043858	KT380049*				
V. sinaloensis	CAIM 648 = DSM 21326	AEVT01	EU043381	AEVT01	AEVT01	AEVT01	AEVT01	AEVT01
V.maritimus	R 40493^T		GU929925		GU929929	GU929933	GU929935	GU929939
P. aquae	CGMCC 1.12159^T	LDOT01	LDOT01	LDOT01	LDOT01	LDOT01	LDOT01	LDOT01

* Sequences generated in this study.

Table S2: Results from the phenotypic and chemotaxonomic analyses. +, positive; -, negative, ND, not determined; w, weak reaction.

		Strains				
	Characteristic	***V. galathea***	***V. brasiliensis***	***V. orientalis***	***V. hepatarius***	***V. tabiashii***
Biolog GN2	water	-	-	-	-	-
	a-cyclodextrin	-	-	-	-	-
	dextrin	+	+	+	+	+
	glycogen	+	+	+	+	+
	Tween 40	*w*	+	+	*w*	+
	Tween 80	*w*	+	+	w	+
	N-acetyl-D-galactosamine	-	+	-	-	-
	N-acetyl-D-glucosamine	+	+	+	+	+
	adonitol	-	-	-	-	-
	L-arabinose	-	+	-	-	-
	D-arabitol	-	-	-	-	-
	D-cellobiose	+	+	-	+	+
	i-erythritol	-	-	-	+	-
	D-fructose	+	+	-	+	+
	L-fucose	-	+	-	-	-
	D-galactose	-	-	-	w	-
	Gentibiose	-	-	-	-	-
	a-D-glucose	+	+	+	+	+
	m-inositol	-	-	-	-	-
	a-D-lactose	-	-	-	-	-
	lactulose	-	-	-	-	-
	maltose	+	+	+	+	+
	D-mannitol	+	+	+	+	+
	D-mannose	+	+	-	+	+
	D-melibiose	-	-	-	-	-
	b-methyl-D-glucoside	w	-	-	-	-
	D-psicose	w	+	-	+	+
	D-raffinose	-	-	-	-	-
	L-rhamnose	-	-	-	-	-
	D-sorbitol	-	+	-	-	-
	sucrose	-	+	-	+	+
	D-trehalose	+	+	+	+	+
	turanose	-	-	-	-	-
	xylitol	-	-	-	-	-
	pyruvic acid methyl ester	w	+	-	w	-
	succinic acid mono-methyl ester	-	+	-	-	-
	acetic acid	-	+	-	-	w
	cis-aconitic acid	-	-	-	-	-
	citric acid	-	-	-	-	-
	formic acid	-	-	-	-	-
	D-galactonic acid lactone	-	-	-	-	-
	D-galacturonic acid	-	-	-	-	-
	D-gluconic acid	+	+	-	+	+
	D-glucosaminine acid	-	-	-	-	-
	D-glucuronic acid	-	-	-	-	+
	a-hydroxybutyric acid	-	-	-	-	-
	b-hydroxybutyric acid	-	+	-	-	w
	g-hydroxybutyric acid	-	-	-	-	-
	p-hydroxyphenylacetic acid	-	-	-	-	-
	itaconic acid	-	-	-	-	-
	a-ketobutyric acid	-	-	-	-	-

	Characteristic	Strains				
		V. galathea	*V. brasiliensis*	*V. orientalis*	*V. hepatarius*	*V. tubiashii*
	a-ketoglutaric acid	-	+	-	-	-
	a-ketovaleric acid	-	-	-	-	-
	D,L-lactic acid	+	+	-	+	+
	malonic acid	-	-	-	-	-
	proprionic acid	-	-	-	-	-
	quinic acid	-	-	-	-	-
	D-saccharic acid	-	-	-	-	-
	sebacic acid	-	-	-	-	-
	succinic acid	-	+	+	+	+
	bromosuccinic acid	-	+	-	-	-
	succinamic acid	-	-	-	-	-
	glucuronamide	-	-	-	-	w
	L-alaninamide	-	-	-	-	-
	D-alanine	+	+	-	-	-
	L-alanine	+	+	+	-	+
	L-alanyl-glycine	+	+	+	+	+
	L-asparagine	+	+	+	+	+
	L-aspartic acid	+	+	+	+	+
	L-glutamic acid	+	+	+	+	+
	glycil-L-aspartic acid	-	+	-	+	+
	glycil-L-glutamic acid	+	+	-	+	+
	L-histidine	-	-	-	-	-
	hydroxy-L-proline	-	-	-	-	-
	L-leucine	-	-	-	-	-
	L-ornithine	-	+	-	-	-
	L-phenylalanine	-	-	-	-	-
	L-proline	-	+	+	-	-
	L-pyroglutamic acid	-	-	+	-	-
	D-serine	w	-	-	-	-
	L-serine	w	+	-	+	+
	L-threonine	-	+	-	+	-
	D,L-carnitine	-	-	-	-	-
	g-aminobutyric acid	-	-	-	-	-
	urocanic acid	-	-	-	-	-
	inosine	+	+	+	+	+
	uridine	+	+	+	+	+
	thymidine	+	+	+	+	+
	phenylethylamine	-	-	-	-	-
	putrescine	-	-	-	-	-
	2-aminoethanol	-	-	-	-	-
	2,3-butanediol	-	-	-	-	-
	glycerol	-	+	+	+	+
	D,L,a-glycerol phosphate	-	+	+	-	-
	a-D-glucose-1-phosphate	+	-	-	-	-
	D-glucose-6-phosphate	+	+	+	-	+
API ZYM	alkaline phosphatase	+	+	+	+	+
	esterase	+	+	+	+	+
	esterase lipase	+	+	+	+	+
	lipase	-	+	+	+	+
	leucine arylamidase	+	+	+	+	+
	valine arylamidase	+	+	-	+	-
	cystine arylamidase	+	+	-	+	-
	trypsin	-	-	-	-	-
	a-chymotripsin	-	-	-	-	-
	acid phosphatase	-	+	+	-	-
	naphtol-AS-BI-phosphohydrolase	+	+	+	+	+
	a-galactosidase	-	-	-	-	-

	Characteristic	Strains				
		V. galathea	*V. brasiliensis*	*V. orientalis*	*V. hepatarius*	*V. tabiashii*
	b-galactosidase	-	-	-	-	-
	b-glucuronidase	-	-	-	-	-
	a-glucosidase	+	-	-	-	+
	b-glucosidase	-	-	-	-	-
	N-acetyl-b-glucosaminidase	-	-	+	+	+
	a-mannosidase	-	-	+	-	-
	a-fucosidase	-	-	-	-	-
FAME analysis	Sum in Feature 2 12:0 aldehyde					
	10:0 3OH		0,06			0,06
	unknown 11.799					
	12:0 iso	0,1				
	12:0	3,4	3,2	3,6	3,6	3,4
	unknown 12,484	0,4		0,5	0,3	
	13:0 ISO	0,7	0,3	0,1	0,1	0,2
	13:1 at 12-13		0,3		0,2	0,2
	13:0		0,1	0,2	0,1	
	13:0 iso 3 OH	0,3				
	12:0 2OH	0,1	0,1	0,1	0,1	0,0
	12:0 3OH	1,8	2,0	1,9	2,1	2,6
	14:0 ISO	0,4	0,1	0,2	0,1	
	14:0	6,5	6,4	9,1	6,3	6,5
	unknown 14,502		0,1			
	14:1 w5c			0,1		
	15:0 ISO	2,1	0,3	0,2	0,1	0,1
	15:0 ANTEISO	0,3	0,1	0,1	0,1	0,1
	15:0	0,4	0,3	0,7	0,4	0,3
	15:1 w8c					0,2
	15:0 iso 3 OH	0,3				
	14:0 ISO 3 OH	0,4	0,1		0,2	
	Sum in feature 2 14:0 3OH/16:1 ISO I					
	16:0 ISO	3,7	0,5	0,6	1,1	0,4
	16:1 w9c		0,5	0,5	0,5	
	16:1 w7c alcohol	0,6		0,3		
	Sum in feature 3 16:1 w7c/16:1w6c					
	Sum in feature 3 16:1 w7c/15 iso 2OH					
	sum in feature 3 15:0 ISO 2OH/16:1w7c					
	sum in feature 3 16:1 w6c/16:1 w7c					
	16:1 w5c	0,2	0,2	0,3	0,3	0,3
	16:0	19,5	24,8	23,3	22,6	22,2
	17:0 ISO	4,0	0,3		0,1	0,2
	17:0 anteiso	0,6			0,1	0,1
	17:1 w8c	0,3	0,2	0,2	0,2	0,2
	17:1 w9c	0,3				
	17:0	0,4	0,2	0,4	0,3	0,3
	16:0 3OH		0,3		0,3	0,4
	Sum in feature 8 18:1 w7c	15,9			18,5	
	Sum in feature 8 18:1 w6c					
	18:1 w9c		0,3		0,3	0,3
	18:1 w7c 11-methyl					0,2
	18:1 w7c		19,5			
	18:1 w5c				0,1	
	18:0	0,3	0,4	0,3	0,4	0,4
	18:0 iso	0,4				
	sum in feature 7 un 18.846/19:1 w6c		0,4			
	20:1 w7c				0,2	
	Summed feature 2 *	2,1	2,6	2,0	2,2	2,4
	Summed feature 3 *	34,7	35,9	44,7	39,0	35,8

	Characteristic	Strains				
		V. galathea	*V. brasiliensis*	*V. orientalis*	*V. hepatarius*	*V. tubiashii*
	Summed feature 7 *		0,4		0,4	
	Summed feature 8 *	15,8		10,6	18,3	23,0
API 20 NE	Nitrates--> nitrites	+	+	+	+	+
	L-tryptophane	+	+	+	+	+
	D-glucose	-	+	-	-	+
	L-arginine	-	-	-	-	-
	urea	-	-	-	-	-
	esculin ferric citrate (b-glucosidase)	+	+	-	-	-
	gelatin (protease)	-	-	-	+	-
	b-galactosidase	+	+	-	ND	+
	D-glucose	-	-	-	-	-
	L-arabinose	-	-	-	-	-
	D-mannose	-	-	-	-	-
	D-mannitol	-	-	-	-	-
	N-acetylglucosamine	-	-	-	-	-
	D-maltose	-	-	-	-	-
	potassium gluconatecapric acid	-	-	-	-	-
	capric acid	-	-	-	-	-
	adipic acid	-	-	-	-	-
	malic acid	+	+	-	-	+
	trisodium citrate	-	-	-	-	-
	phenylacetic acid	-	-	-	-	-
	oxidase	-	-	-	-	-

*Summed feature 2: one or more among 12:0 aldehyde, 14:0 3OH and/or 16:1 iso; summed feature 3: one or more among 16:1 w7c, 16:1w6c and/or 15:0 iso 2OH; summed feature 7: one or more among unknown 18.846 and/or 19:1 w6c; summed feature 8: one or more among 18:1 w7c and/or 18:1 w6c.