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**Marine Proteobacteria as a source of natural products:
Advances in molecular tools and strategies**

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Abstract

Humanity is in dire need for novel medicinal compounds with biological activities ranging from antibiotic to anticancer and anti-dementia effects. Recent developments in genome sequencing and mining have revealed an unappreciated potential for bioactive molecule production in marine Proteobacteria. Also, novel bioactive compounds have been discovered through molecular manipulations of either the original marine host bacteria or in heterologous hosts. Nevertheless, in contrast to the large repertoire of such molecules as predicted by *in silico* analysis, few marine bioactive compounds have been reported. This review summarizes the recent advances in the study of natural products from marine Proteobacteria. Here we present successful examples on genetic engineering of biosynthetic gene clusters of natural products from marine Proteobacteria. We also discuss the future prospects of discovering novel bioactive molecules via both heterologous production methodology and the development of marine Proteobacteria as new cell factories.

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1 INTRODUCTION

Humanity is in dire need for novel medicinal compounds with biological activities ranging from antibiotic to anticancer and anti-dementia effects. Infectious diseases that were easily cured by antibiotics have recently become challenging due to the rapid development and spread of antibiotic resistance in infectious microorganisms. Diseases that mostly occur in the elderly (dementia, cancer, arthritis) are increasing with the aging population and we are still lacking adequate treatments. Natural products from plants and microorganisms have been a significant source of medicinal compounds and almost two thirds of our antibiotics are produced by or derived from microorganisms.¹ Until recently, terrestrial microorganisms, especially Gram-positive Actinobacteria, have provided the majority of these bioactive compounds. However, our need for novel compounds has led to bioprospecting of microorganisms from hitherto unexplored or less explored environments, such as marine systems² and also to new strategies for biodiscovery, e.g. genome mining,³ heterologous expression from metagenomes⁴ and using synthetic biology.⁵

Covering more than two thirds of the Earth's surface, the oceans harbor many different marine-specific habitats that are defined by a broad range of temperature or hydrostatic pressure, fluctuations in salinity, eukaryotic predation or viral attacks.⁶ These unique ecological parameters drive the adaptation of marine organisms including their production of marine-specific molecular compounds.^{6,7} Bioactive medicinal compounds have been isolated from a range of marine organisms, including marine sponges, tunicates, bryozoans, and microorganisms.^{8–10}

It was anticipated that like on land, the Gram-positive Actinobacteria would be a prolific source of novel compounds and indeed many new promising compounds have been isolated from marine Actinobacteria.¹¹ A prime example is the discovery of the anti-cancer agent salinosporamide A produced by the obligate marine bacterium *Salinispora*.¹² Also, marine Cyanobacteria have provided many novel compounds such as curacin A, barbamide or the jamaicamides.^{13–15} However, it has also become evident that other marine Gram-negative bacteria are a rich source of secondary metabolites.^{16–19} For instance, more than 20 biosynthetic gene clusters (BGCs) can be found in a single genome of pigmented *Pseudoalteromonas* strains, which dedicate up to 15% of their genomes to production of secondary metabolites.²⁰

In general, mining genomes revealed a large number of BGCs likely encoding bioactive compounds.¹⁷ However, due to the *in vitro* conditions during culturing in the laboratory, many BGCs are likely cryptic and their products cannot be easily detected. This untapped potential can be unlocked by enhancing production in the native organisms, first addressing cultivation issues (carbon source, co-cultivation with other bacteria) and also by metabolic engineering, or using heterologous expression in robust, established cell factories.

Molecular manipulation in marine bacteria is challenging due to the lack of well-established universal methods for genetic modification, and, for instance the requirement for salts during growth can be a challenge for electroporation. Fortunately, both electroporation and conjugation strategies applicable in marine bacteria are being developed.^{21–23} Single gene products from marine Proteobacteria have since the early eighties been cloned and expressed

in heterologous hosts.²⁴ However, transferring and heterologous expression of larger, more complex biosynthetic pathways is challenging in part because of the size (10-100 kbp) in part because of nuances in regulatory elements or metabolic background between the native producers and the engineering expression hosts.²⁵ There are only very few successful examples, including cloning and expressing of alterobromide lipopeptides from *Pseudoalteromonas piscicida* in *Escherichia coli*.^{5,26,27}

A prerequisite for successful cell engineering is identifying relevant genes and gene clusters, as well as the sequential enzymatic steps in the biosynthesis of the target compound. This can be facilitated by knocking out/in genes in the native producers, hence – again – being able to molecularly manipulate the native producers is essential. The present paper outlines options and challenges in genetic editing of marine Proteobacteria as well as (the few) examples of heterologous expression of larger gene clusters and individual enzymes of the biosynthetic pathways involved in secondary metabolism. Finally, recent advances in employing marine Proteobacteria as chassis for synthetic biology and biotechnological production of bioactive compounds are outlined and a perspective on future potential cell factories is provided.

1.1 Bioactive compounds from marine Proteobacteria

Many bioactive compounds, notably antibiotics, have been isolated from Gram-positive Actinobacteria or filamentous fungi and Gram-negative bacteria have not been researched as extensively. However, recently genome mining has demonstrated that also Gram-negative bacteria of the phylum Proteobacteria harbor biosynthetic gene clusters likely encoding bioactive compounds.

Proteobacteria is the major phylum of Gram-negative bacteria and includes many well-known pathogens from Vibrionaceae, Enterobacteriaceae and Pseudomonadaceae. Although Proteobacteria is the most abundant phylum in marine pelagic systems,²⁸ only relatively few bioactive compounds have been isolated from this group of organisms. Most of these molecules, however, are not unique to the marine environment, and are also produced by terrestrial bacteria, algae, plants, or animals (Table 1). This is probably due to the horizontal gene transfer (HGT)-mediated spread of BGCs.³ For example, the dithiolopyrrolone group antibiotic, holomycin (**1**) (Table 1, Figure 1), initially isolated from cultures of the terrestrial Gram-positive bacteria *Streptomyces griseus*,²⁹ *Streptomyces* sp. P6621³⁰ and *Streptomyces clavuligerus*,³¹ was recently extracted from cultures of *Yersinia ruckeri*³² and *Photobacterium galathea* S2753.^{17,33} Comparison of the corresponding holomycin (**1**) BGCs of each strain reveals highly conserved core genes,^{32,34,35} and host specific genes involved in the holomycin (**1**) detoxification processes.^{32,35–37} This finding suggests that both HGT and host adaptation occurred during the evolution of holomycin (**1**) BGCs. Recently, along with the increasing number of genome sequences, novel BGCs have been uncovered in marine Proteobacteria and are being investigated.^{17,19,38–41} Despite this promising potential, genetic manipulations of these BGCs either in their native producers or in heterologous expression hosts are still challenging in the laboratory. An overview of selected marine Proteobacteria bioactive compounds, their native producers, the corresponding BGCs and their manipulation are summarized in Table 1 and discussed in the following sections.

Table 1 A selection of representative bioactive compounds isolated from marine Proteobacteria

Compounds	Bioactivity	Marine proteobacterial producers	Other producers	Biosynthetic gene cluster and its size	Bioengineering manipulations
Astaxanthin ^{42–44}	Food colorant; Dietary supplement; feed supplement	<i>Paracoccus haeundaensis</i> ; <i>Erythrobacter longus</i> ; <i>Haematococcus pluvialis</i>	Algae, shrimp, crab, fishes, bacteria etc.	<i>crtWZYIBE</i> : ~ 11 kb	Random chemical mutagenesis; Cloning entire BGC and overexpression in native strain; Heterologous expression in <i>Rhodovulum sulfidophilum</i>
Bromoalterochromide (7b) ⁴⁵	Antibiotic activity	<i>Pseudoalteromonas</i> species	-	<i>bmp</i> cluster and PPTase: ~ 34 kb	Heterologous expression and biosynthetic pathway characterized in <i>E. coli</i>
Bryostatins (10) ^{10,46}	Cytotoxic activity against carcinomas; Anti-Alzheimer's potential	<i>Candidatus Endobugula sertula</i> (uncultured)	-	<i>bry</i> cluster: 65-77 kb	Metagenomics cosmid libraries.
Ectoine (5) ^{47–51}	Maintain osmotic balance; superior moisturizer; anti-aging; against lung inflammation	<i>Halomonas elongate</i> ; <i>Halomonas titanicae</i> ; <i>Halomonas ventosae</i> ; <i>Halorhodospira halophila</i> .	<i>Brevibacterium linens</i> ; <i>Marinococcus halophilus</i> ; <i>Pseudomonas stutzeri</i> ;	<i>ect</i> cluster: 3.5-4.5 kb	Knockouts and overexpression.

Haliangicin (9) ⁵²	Antifungal antibiotic	<i>Haliangium ochraceum</i>	-	<i>hli</i> cluster: 47.8 kb	Heterologous expression in <i>Myxobacteria xanthus</i>
Holomycin (1) ^{32-34,53}	Antibiotic activity; anti- cancer	<i>Photobacterium galathea</i> ;	<i>Streptomyces</i> spp. <i>Yersinia ruckeri</i> ;	<i>hlm</i> cluster or <i>hom</i> cluster: ~ 11 kb	Knockouts and knockins
Pentabromo- pseudilin (6) ⁵⁴	Antibiotic activity	<i>Pseudomonas bromoutilis</i> ; <i>Pa. luteoviolacea</i>	-	<i>bmp</i> cluster: ~ 12.5 kb	Heterologous expression in <i>E. coli</i>
Poly-3- hydroxy- butyrate (PHB) ⁵⁵⁻⁵⁷	Bioplastics; Biomedical applications	<i>Halomonas</i> spp.	<i>Cupriavidus necator</i> ; <i>Bacillus megaterium</i> ; <i>Methylobacterium rhodesianum</i> ; etc.	<i>phb</i> gene cluster ~ 3.5 kb	Knockouts and overexpression
Prodigiosin (4) ^{58,59}	Red pigments; anti-cancer	<i>Pseudoalteromonas</i> strains	<i>Serratia marcescens</i> ; <i>Bacillus prodigiosus</i>	<i>cueR/pigA-N-copA</i> : ~25 kb	Knockouts and expression
Solonamide (11) ^{16,60,61}	Antivirulence of MRSA	<i>Pa. galathea</i>	-	-	-
Thiomarinol (3) ⁶²	Antibiotic activity	<i>Pseudoalteromonas</i> SANK 73390	-	Plasmid <i>pTML1</i> : ~87 kb	Knockouts

Tropodithietic acid, TDA (2) ⁶³⁻⁶⁵	Antibiotic activity	<i>Phaeobacter</i> ; <i>Roseobacter</i> spp.	-	<i>paa</i> and <i>tda</i> cluster: ~12.3 kb	Random transposon insertion mutagenesis
Vibriobactin ⁶⁶	Siderophore; iron chelator	<i>Vibrio cholerae</i>	-	<i>vib-vie</i> cluster: ~12 kb	Expression in native strain
Violacein (8) ^{67,68}	Pigment; antibiotic activity; anti-cancer	<i>Pseudoalteromonas</i> species	<i>Chromobacterium violaceum</i> ; <i>Collimonas</i> sp.; <i>Duganella</i> spp.; <i>Janthinobacterium</i> spp. <i>Microbulbifer</i> spp; etc.	<i>vioABCDE</i> : ~ 8 kb	Heterologous expression and biosynthetic pathway characterized in various hosts

2 GENETIC ENGINEERING OF BIOSYNTHETIC GENE CLUSTERS IN AND FROM MARINE PROTEOBACTERIA

2.1 Genetic engineering of BGCs in the native producers

Molecular engineering of natural product-producing microorganisms has become possible with the recent breakthroughs in genome sequencing and functional genomics.^{17,69–71} There are several advantages in developing such genetic manipulation tools. First, gene-targeting mutagenesis could link orphan BGCs to identified bioactive compounds,^{72,73} which is the foundation and prerequisite for refactoring BGCs in either the native producers or the surrogate hosts. In addition, the native producers encode all structural genes and regulatory elements for the expression of BGCs, such as promoters, genetic regulators, and host cell self-protection genes.^{37,68,74} Therefore, genetic manipulation in the native producer cells is essential to reveal the regulatory mechanism of BGC expression. This not only holds the potential to increase the yield of bioactive molecules,⁶⁸ but also, more importantly, reveals the physiological (and ecological) functions of these molecules.^{75,76} Genetic tools can also be used to activate presumed silent BGCs, facilitating the success rate of discovering more bioactive molecules in the native hosts.⁷⁷ In the following sections, we discuss the state-of-the-art of genetic manipulation tools in marine Proteobacteria, with a focus on strains that harbor BGCs.

2.1.1 Natural transformation of marine Proteobacteria

Natural transformation has been widely used in marine Proteobacteria, especially the marine chitinolytic species of the Vibrionaceae family. In 2005, Meibom *et al.*⁷⁸ found that chitin induces natural competence in *Vibrio cholerae*. The molecular mechanism of chitin-induced competence was subsequently characterized in *V. cholerae* and several other *Vibrio* strains, such as *V. parahaemolyticus*,⁷⁹ *V. vulnificus*,⁸⁰ and *V. fischeri*.⁸¹ The form of chitin capable of inducing cell competence differs among species or even among strains. For example, competence of *V. fischeri*⁸¹ is only induced by oligosaccharidic chitin, whereas all forms of chitin induce competence in *V. cholerae* and *V. vulnificus*.^{78,80} Chen *et al.*⁷⁹ succeeded in generating a deletion mutant of *V. parahaemolyticus* O3:K6 with a chitin-induced natural transformation approach, while Chimalapati *et al.*²³ could not induce natural transformation in *V. parahaemolyticus* CAB2 using the same approach. Instead, the authors overexpressed the *V. cholerae* gene *tfoX*, a natural transformation regulator, and thereby induced the natural competence in *V. parahaemolyticus*.²³ Natural transformation is an efficient method for molecular manipulating of *Vibrio* species, however, it has seldom been used in genetic engineering of natural product BGCs in Vibrionaceae, mainly due to the fact that marine Proteobacteria have only recently been identified as a significant source of novel bioactive compounds.^{17,33,82}

2.1.2 Chemical transformation of marine Proteobacteria

Successful transformations by chemical procedures have only rarely been reported for marine Proteobacteria. Marcus *et al.*⁸³ used the osmotic shock protocol in an attempt to transform *V. cholerae* strains with the plasmid pBR322, however, without success. On the other hand, a similar procedure has been successfully applied in a DNase-negative mutant of *V. cholerae*,

suggesting that extracellular DNase is one of the major barriers for applying chemical transformation in *V. cholerae*. Piekarski *et al.*²¹ also attempted to transform 12 strains from the *Roseobacter* group using standard chemical transformation procedures, but these proved to be unsuccessful. These results highlight that more efforts should be directed toward the investigation of the transformability of marine Proteobacteria. In 2016, Weinstock *et al.*⁸⁴ deployed chemical transformation as well as electroporation to engineer the fast-growing marine species *Vibrio natriegens*. As discussed in section 3, this species has been proposed as a competitive host for biotech applications, including the production of bioactive molecules from marine Proteobacteria.

2.1.3 Electroporation in marine Proteobacteria

Electroporation is a well-established method, which has worked well for a broad range of bacterial species, however, failures have also been reported. The efficiency of electroporation is strain-dependent, and affected by many factors, such as growth conditions, temperature, restriction enzyme systems, field strength and type of exogenous DNA.^{22,85,86} Strain specific optimization of these parameters is therefore normally required. Electroporation has been used for several marine Proteobacterial species of the *Vibrio*, *Pseudoalteromonas*, *Roseobacter*, *Caulobacter* and *Halomonas* genera. In 2018, Delavat *et al.*⁸⁷ developed a rapid and efficient electroporation protocol for *Vibrio* and *Pseudoalteromonas* strains. However, electroporation was only successful in the stationary phase of *V. harveyi* cells grown at 37 °C, but not in cells grown at 28 °C or exponential phase cells grown at 37 °C. The same protocol was also successfully applied in *V. campbellii* BAA-1116, and in *Pseudoalteromonas* spp. MV21 and 3 J6.

The earliest example of the use of electroporation in marine Proteobacteria for secondary metabolite research is reported in 1997.⁶⁶ Wyckoff *et al.* studied the genetics of the biosynthesis of vibriobactin, a siderophore produced by the pathogen *V. cholerae*. Siderophores are iron-scavenging compounds and are commonly produced by marine bacteria, due to typically low iron levels in the marine environment.⁸⁸

Pre-dating the sequence revolution, Wyckoff and coworkers had to rely on classical methods such as library screening and complementation to identify the vibriobactin biosynthetic genes in *V. cholerae*. A set of well characterized *E. coli* mutants unable to produce enterobactin, a siderophore that is structurally similar to vibriobactin, were used in this study. Because the biosynthesis of enterobactin and vibriobactin share the same intermediate (2, 3-dihydroxybenzoic acid), the authors hypothesized that *E. coli* mutants unable to synthesize this molecule could be complemented by homologous genes from *V. cholerae*. A cosmid library of *V. cholerae* was screened for complementation of enterobactin biosynthesis, which led to the identification of the *vibABCDE* gene cluster.⁶⁶ To confirm the role of *vibA* in the biosynthesis of vibriobactin, the authors inactivated the *vibA* gene in *V. cholerae*. Transposon mutagenesis with the Tn5 transposon system was carried out in *E. coli* to obtain a plasmid carrying an inactivated *vibA* fragment, which was identified by loss of the ability to complement enterobactin biosynthesis.⁸⁹ A suicide vector was constructed by subcloning the *vibA*::Tn5 fragment and the *sacB* gene into vector pAT153. *V. cholerae* was subsequently transformed by electroporation according to the protocol described by

Henderson and Payne,⁹⁰ and a vibriobactin negative mutant was obtained by allelic exchange of *vibA* with *vibA::Tn5*.

Another more recent example of using electroporation is found in the genetic study of tropodithietic acid (TDA) (**2**) (Table 1 and Figure 1) production by bacteria from the *Roseobacter* group.⁶⁴ This compound has received a lot of interest due to its broad antibacterial activity, including its effect on fish and human pathogenic bacteria.⁹¹ Also, the TDA-producing *Phaeobacter* and *Ruegeria* strains occur naturally in aquaculture environments⁹² and several attempts to evolve TDA resistance have not resulted in TDA resistant mutants.^{93,94} For these reasons, natural TDA producers hold great promise as probiotic cultures in large-scale aquaculture industries.^{63,93}

Genetic interrogation of the TDA (**2**) biosynthetic genes in the Alphaproteobacterial roseobacters was first reported by Geng *et al.* in 2008.⁶⁴ Random transposon insertion mutagenesis in *Silicibacter* and *Phaeobacter* led to the finding that the *tda* genes reside on a previously unidentified 130 kb plasmid. Transfer of the commercially available EZ-Tn5 transposome into the cells was accomplished by electroporation, after preparation of electrocompetent cells as described by Miller and Belas.^{64,95} Kanamycin resistant transposon mutants were screened for loss of TDA (**2**) production by means of antibacterial assays: *Phaeobacter* and *Silicibacter* mutants unable to inhibit *V. anguillarum* growth were identified as TDA negative.

2.1.4 Conjugation in marine Proteobacteria

A more common approach of transforming marine Proteobacteria is using bacterial conjugation. Conjugation is the most efficient method to genetically manipulate the BGCs in marine Proteobacteria that are recalcitrant to electroporation and chemical transformation methods. However, conjugation is a time-consuming procedure that also requires a step to counter-select the donor cells from the transconjugant cells. Several methods have, therefore, been developed to address this issue. One of the most efficient strategies is employing the *E. coli* auxotrophic strains, such as B2163 and WM3064. Another concern in conjugation is the efficiency of the counter-selectable markers for allelic exchange.

Recently, Luo *et al.*⁹⁶ developed an efficient system for gene deletion in *Vibrio* spp. by constructing the suicide plasmids pLP11 and pLP12 (derived from the plasmid pSW23T), which carry the chloramphenicol antibiotic resistant gene and a toxin gene, *vmi480*, as the counter-selectable marker. The authors obtained knockout mutants by using *E. coli* B2163 as the donor. This conjugation-based genetic tool was successfully used in several *Vibrio* species including *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, and *Vibrio alginolyticus*. Consequently, *vmi480* is considered an effective counter-selectable gene for *Vibrio*.

The well-known *sacB* counter-selection system (pDPM-derived plasmids) has also been widely used in marine Proteobacteria. Although in *Vibrio* strains the toxicity of sucrose-SacB is affected by saline media, many *Pseudoalteromonas* strains are susceptible to the toxicity of SacB.⁵⁹ In short, the combination of auxotrophic donor strains and effective counter-selection markers makes conjugation a reliable technique for engineering BGCs in marine Proteobacteria.

Investigation of the genetics of thiomarinol (**3**) (Table 1 and Figure 1) production by the Gammaproteobacterium *Pseudoalteromonas* SANK 73390 is also relevant.⁶² Three separate gene knockouts were constructed by using the suicide vector pAKE604, which contains the *sacB* gene for counterselection to facilitate allelic exchange.⁹⁷ Transfer of the conjugational plasmid was accomplished via the donor strain *E. coli* S17-1. This work demonstrated that two distinct biosynthetic pathways are responsible for thiomarinol (**3**) production in *Pseudoalteromonas* SANK 73390, each of them synthesizing its own moiety. Mupirocin, a polyketide antibiotic produced by *Pseudomonas fluorescens*, and holomycin (**1**), a pyrrothine antibiotic first discovered from *S. clavuligerus* are both produced and joined together to form thiomarinol (**3**) in this *Pseudoalteromonas* strain. Furthermore, the authors discovered that all the biosynthetic genes reside on a 97 kb plasmid. These results raise interesting questions concerning the evolution and acquisition of the clusters and the role that HGT events have played herein. However, the authors reported a high degree of sequence divergence between the mupirocin biosynthetic genes found in *P. fluorescens* and their homologues in *Pseudoalteromonas* SANK 73390.⁶²

Other studies have explored and developed genetic tools for *Pseudoalteromonas* engineering.⁵⁹ A gene knockout protocol was established by cloning homologous fragments upstream and downstream of the target gene in the pK18mobsacB-based suicide vector.⁹⁸ Conjugational transfer of the plasmid was accomplished via the *E. coli* WM3064 strain, which is auxotrophic for diaminopimelic acid (DAP), facilitating an easy way to counter select the donor cells from transconjugant cells. Two homologous recombination crossover events then ultimately lead to either the wild type genotype or the desired knock out mutant. Using this system, a knockout of the *pigM-K* genes was confirmed by the lack of production of the red pigment prodigiosin (**4**) (Table 1, Figure 1), thus showing the feasibility of the method.⁵⁹ Importantly, the authors demonstrated the compatibility of this system in nine *Pseudoalteromonas* strains from at least six differently classified species.

Conjugational transfer of DNA is more versatile than solely constructing gene knockout mutants. An example is the metabolic engineering of the Alphaproteobacterium *Paracoccus* for improved production of the secondary metabolite astaxanthin.⁴⁴ The native BGC responsible for astaxanthin production was cloned into the broad-host-range expression vector pBBR1-MCS2.⁹⁹ The authors subsequently transformed the recombinant plasmid into *Paracoccus* by mating with donor *E. coli* S17-1. In this case, conjugative broad-host-range plasmid was used to increase the copy number of BGC in the native *Paracoccus* strain. Overexpression of a gene was also accomplished in the halophilic Gammaproteobacterium *Halomonas elongata*. Tanimura *et al.* overexpressed the *lysC* gene (encoding aspartate kinase) in order to improve production of ectoine (**5**). In this case, the broad-host expression vector pH515N was used and conjugation was achieved by *E. coli* HB101 carrying the mobilization helper plasmid pRK2013.¹⁰⁰

Finally, we highlight a case, which combines conjugation with other genetic engineering tools. Fu *et al.* genetically manipulated a *Halomonas* strain to improve production of the storage polymer and bioplastic poly-3-hydroxybutyrate (PHB).⁵⁵ To this end, a markerless knock out strategy was developed based on the rare I-SceI restriction site.¹⁰¹ Six I-SceI

restriction sites were cloned into the suicide vector pRE112, and the resulting plasmid was introduced into *Halomonas* TD01 by conjugation using *E. coli* S17-1. Chromosomal integration of the suicide vector by homologous recombination was achieved under selective pressure of chloramphenicol resistance. Subsequently, a second plasmid based on the broad-host-range vector pBBR1-MCS1 was introduced in order to express the I-SceI endonuclease. *In vivo* digestion by I-SceI endonuclease would result in lethal double strand breaks, providing the selective pressure for a second homologous crossover event and deletion of the gene target. Although the selective pressure by lethal restriction may increase transformation efficiency compared to conventional counter-selection genes, the use of an I-SceI endonuclease expression vector necessitates a second time-costly conjugation step.

2.1.5 CRISPR-Cas9 systems in marine Proteobacteria

More recently, Tao *et al* from the same group reported the use of the CRISPRi system¹⁰² for controlled transcriptional repression in the same strain.⁵⁶ This tool makes use of a catalytically dead Cas9 protein (dCas9), which is lead to a target sequence by a designed small guide RNA (sgRNA). Instead of making a double strand cut, dCas9 is able to suppress gene transcription, presumably by sterically blocking RNA polymerase. Tao *et al.* constructed the conjugational plasmid pli-dCas9-sgRNA and used this as vector for the introduction of sgRNA sequences into *Halomonas* TD01.⁵⁶ The IPTG-inducible P_{trc} promoter was used for the transcription of the sgRNA and the dCas9 protein. After conjugation with *E. coli* S17-1, the expression of *prpC* (2-methylcitrate synthase) was reduced by adding IPTG into the culture of *Halomonas* conjugants carrying pli-dCas9-sgRNA plasmid. This resulted in a higher content of 3-hydroxyvalerate in the poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) polymer.⁵⁶ In parallel, Yin *et al.*¹⁰³ developed a conjugative plasmid carrying the CRISPRi system¹⁰² to circumvent the issue of a counter-selective marker. The CRISPRi system was successfully transferred into marine vibrio strain *Vibrio fluvialis* QY27 by the donor *E. coli* WM3064 and the expression of trimethylamine N-oxide (TMAO) reductase gene was knocked down in QY27.

2.2 Heterologous expression of BGCs from marine Proteobacteria

A large number of marine proteobacterial enzymes have been cloned and expressed in different bacterial expression systems and have a broad potential in industrial applications.¹⁰⁴ Cold-active enzymes, such as the α -amylase from the Antarctic psychrophile, *Alteromonas haloplanctis* has been studied extensively with applications in baking, textile and detergent industries.¹⁰⁵ Recently, fucoidanases from marine Proteobacteria that are able to degrade brown algae have gained increasingly attention due to the beneficial biomedical properties of fucoidan derived oligosaccharides.^{106,107}

Heterologous expression has been a powerful tool to identify novel gene functions, but it becomes more cumbersome when gene fragments exceed 10 kb.²⁵ However, most biosynthetic pathways involve dozens of proteins that catalyze and regulate the production of secondary metabolites and thus are often larger than 10 kb and some may even exceed 100 kb. Yet, a number of BGCs, mainly encoding natural products from Actinomycetes,¹⁰⁸ have been cloned and expressed using different bacterial expression systems, while very few examples have been reported for BGCs of the marine Proteobacteria derived natural products.

2.2.1 Heterologous expression of BGCs from cultivable marine Proteobacteria

Many marine natural products are halogenated, reflecting the availability of chloride and bromide ions in seawater. Thus, marine organisms including the Proteobacteria have developed a range of halogen-acting enzymes that activate and transfer chloride and bromide to organic molecules. Interestingly, organobromines are the most commonly found halogenated natural products produced by marine organisms, even though the concentration of the bromide anion is lower than that of chlorine.¹⁰⁹ Pentabromopseudilin (**6**) (Table 1, Figure 1) produced by the marine Gammaproteobacterium *Pseudomonas bromoutilis*¹¹⁰ was the first marine antibiotic to be discovered in 1966. Since then, several polybrominated aromatic organic compounds have been isolated from marine Proteobacteria.^{111,112} Agarwal *et al.*¹¹³ reconstructed *in vitro* all the enzymatic steps in the marine brominated pyrroles/phenols biosynthesis from *P. luteoviolaceae* and *P. phenolica* and furthermore identified two novel flavin-dependent brominases. The entire BGC (~12.5 kb) responsible for the production of the widespread polybrominated compounds (Figure 2) was expressed in *E. coli* using transformation-associated recombination (TAR).

The TAR strategy¹¹⁴ enables specific capture of target biosynthetic pathways, avoiding the typical size limitations of traditionally used cloning techniques such as Gibson assembly and USER-cloning. With this technique, large fragments of DNA (up to 100 kb) are captured without the need for intermediate PCR steps using homologous recombination in yeast (*Saccharomyces cerevisiae*) and subsequently transferred to and expressed in *E. coli*.¹¹³ Thus, cloning of large DNA fragments has become more affordable.

A great advantage of heterologous expression is that it reduces the need to develop new genetic tools for the manipulation of each genus or species of interest. Heterologous expression of enzymes has been performed in *E. coli*, *Bacillus subtilis*, *Streptomyces*, lactic acid bacteria, yeast and filamentous fungi for decades. Due to the well-characterized genome of *E. coli*, the variety of genetic tools available and its taxonomic relation to marine Proteobacteria, this organism is a good candidate for recombinant production of marine Proteobacteria derived natural products. In addition, *E. coli* is able to grow rapidly to high densities on inexpensive substrates and there are an increasing number of cloning vectors and mutant strains.¹¹⁵

The antibacterial and cytotoxic lipopeptides, alterochromides (**7**) (Figure 1) are produced by multiple species of the marine genus *Pseudoalteromonas*, including *P. piscicida* that can synthesize alterochromides (**7**) with bromination of the lipid moiety.^{45,116} Again, the TAR strategy was used to capture the ~34 kb pathway for production of the bromoalterochromide (**7b**) (Table 1, Figure 1), of which the biosynthetic pathway included a non-ribosomal peptide synthetase (NRPS), fatty acid synthase (FAS), flavin-dependent halogenase and transporter proteins.²⁶ Furthermore, to compensate for the lack of specific thiolation machinery in *E. coli*, a recombinant PPTase from the related Gammaproteobacterium *P. luteoviolacea* was used.^{26,117} By this, the full alterochromide biosynthetic pathway was constructed and confirmed to be functional in *E. coli*. However, the production titer was 60-fold lower than that of one of the native producers. Further replacement of the native promoter with the inducible T7-promoter improved the yield of bromoalterochromide A (**7b**) by 20-fold.

The trend of using either native or T7 promoters to achieve substantial expression of large gene clusters extends to BGCs of other compounds.^{27,118,119} In some cases, production titers using T7-driven expression in *E. coli* are lower than those from the native producers despite the closer phylogenetic relationship of *E. coli* and marine Gammaproteobacteria. One reason could be that the production of natural products involves specific chaperones or other post-translational maturations of the synthetic enzymes which are not encoded within the BGC or the *E. coli* host. Additionally, lowering the temperature can improve the folding of the recombinant proteins from some BGCs.⁶⁸

Zhang *et al.*⁶⁸ challenged the reasoning that *E. coli* should be the obvious candidate for recombinant expression of marine Proteobacteria-derived natural products. One broad-host-range expression vector was constructed in order to validate the recombinant production titer of the antibacterial pigment violacein (**8**) (*vio2ta16*; Table 1, Figure 1) from *P. luteoviolacea* 2ta16 in three Proteobacterial hosts, the Alphaproteobacterium *Agrobacterium tumefaciens* and two Gammaproteobacteria, *Pseudomonas putida* and *E. coli*. Both *A. tumefaciens* and *P. putida* are isolated from soil; and the former one is often used for plant transformation. *P. putida* is a metabolically versatile and biosafety-certified host that is often used for heterologous expression.¹²⁰ Some *P. putida* strains possess a phosphopantetheinyl transferase that post-translationally activates the carrier protein (CP) domains of various polyketide synthases (PKSs), NRPs and FASs, which are required for heterologous expression of CP-utilizing BGCs from myxobacteria and *Streptomyces*.¹²¹ Despite that both *E. coli* and *P. luteoviolacea* belong to the Gammaproteobacteria, little or no production was observed in *E. coli* host strains. However, violacein (**8**) production was readily detectable from both *A. tumefaciens* and *P. putida*.

Regulatory elements, such as the LuxR-type regulators are often located within the BGCs or just outside the clusters.¹²² If the native promoter is to be included in the recombinant BGC, and the transcription factor that activates the promoter is not captured or co-expressed along with the target BGC, production cannot be achieved in the expression host. In order to understand why the *vio2ta16* native promoter was recognized by *A. tumefaciens* and *P. putida* and not *E. coli*, Zhang *et al.*⁶⁸ identified transcriptional factors in the two foreign expression hosts which are homologous to the Pvi regulator (PviR) found in *P. luteoviolacea* 2ta16. Even though *E. coli* harbors PviR homologs with significant sequence similarity, none of the regulators were able to increase violacein (**8**) production when overexpressed. This study suggests that neither similar phylogeny of the host organism nor the presence of homologous individual protein components are directly predictive with the capability to express the target BGC. Instead, one may consider the distribution of homologous BGCs as well. An example is the regulator PviR, which is conserved among all *Pseudoalteromonas* that harbor the antibacterial pigment violacein (**8**) and thus it is possible to develop *Pseudoalteromonas* hosts that contain the full regulatory machinery for activation of the BGC.⁶⁸

Myxobacteria are a group of Deltaproteobacteria, which commonly produce antifungal compounds such as myxothiazols that inhibit the mitochondrial electron transport chain.¹²³ They also produce metabolites with antibacterial activity including several RNA polymerase inhibitors such as corallopironin, etnangien, myxopyronin or pipostatin.¹²³ Marine

myxobacteria have emerged as one of the richest sources in terms of secondary metabolites.^{40,124} Haliangicin (**9**) (Table 1, Figure 1) is naturally produced by the *Haliangium ochaceum* SMP-2, a slow-growing marine myxobacterium, which is difficult to manipulate genetically. Structural analysis of the haliangicin (**9**) backbone suggested a biosynthetic pathway purely composed by PKs.⁵² Several modification genes are essential for its biosynthesis. Thanks to the analysis of haliangicin precursors using ¹³C-labelled substrates, it was possible to determine the biosynthetic pathway. The authors reconstructed the pathway from a cosmid library using λ -Red recombineering and integrated it into the *Myxococcus xanthus* genome. The engineered strain was capable of producing 10-fold higher amounts of haliangicin than the wild-type *H. ochaceum*.⁵²

As new cloning tools become available and the repertoire of expression hosts expands,²⁵ heterologous expression offers a promising alternative to unravel new natural products from the cryptic or “silent” BGCs of marine Proteobacteria.

2.2.2 Expression of BGCs from uncultivable marine Proteobacteria

For decades, microbiologists have recognized the fact that the vast majority of environmental microbes are recalcitrant to cultivation.¹²⁵ Deep amplicon sequencing analyses suggest substantial biosynthetic potential of the uncultivable bacteria, and even frequently sampled microbial communities, such as those present in soil, give a diverse collection of novel genes involved in the biosynthesis of potential natural products such as NRPs and PKs.^{126,127} As a substantial fraction of this biosynthetic potential likely lies beyond the reach of conventional culture-based approaches to natural product discovery,^{128,129} cultivation-independent strategies, i.e. mining of environmental DNA (eDNA), are necessary in order to exploit this resource. Through rigorous efforts to construct and screen cosmid libraries containing environmental DNA from soil, and subsequent heterologous expression of candidate clusters, such metagenomics-based approaches have led to the recovery of an array of interesting bioactive natural products including violacein (**8**),¹³⁰ turbomycine,¹³¹ and the malacidins.¹²⁹

Metagenomic data suggest that Proteobacteria comprise more than half of the marine pelagic microbial communities.²⁸ Considering the biosynthetic potential of cultivable representatives of this phylum, marine metagenomes are likely a trove of potential novel proteobacterial natural products and comparisons of chemoinformatic data has shown that >70 % of molecular scaffolds in the Dictionary of Marine Natural Products are unique for marine organisms.^{132a,b}

Mining marine metagenomes through sequence-based screening of cosmid libraries containing marine eDNA have revealed that bioactive compounds initially believed to be produced by marine animals and algae are in fact, in some cases, bacterial metabolites produced by the associated microbiome.^{133,134} Furthermore, such efforts have aided in the elucidation of the biosynthetic pathways of bioactive metabolites from marine Proteobacteria. One example is the currently uncultivable marine Gammaproteobacterium ‘*Candidatus* Endobugula sertula’, which is responsible for the production of bryostatins (**10**) (Table 1, Figure 1). Bryostatins (**10**) comprise a group of bioactive type I PK compounds, some of which presented cytotoxic activity against carcinomas as well as anti-Alzheimer’s potential.¹³⁵ Initially, the bryozoan *Bugula neritina* was believed to be responsible for the

synthesis of the compound. Mining metagenomes of *B. neritina*-associated microbiome ultimately proved that bryostatin is synthesized by an unusual modular polyketide synthase encoded by the 65 kb *bry* gene cluster in a proteobacterial symbiont.^{133,134} Similar metagenome mining approaches has led to the identification of biosynthetic pathways of several natural products in uncultivable marine Proteobacteria,¹³⁵ however such BGCs have yet to be cloned and expressed in their entirety.

Expression of BGCs from uncultivable marine Proteobacteria is still in its infancy. Several challenges hamper the successful expression and subsequent functional screening of metagenomic libraries. In general, natural products like NRPs and PKs are notoriously difficult to obtain from metagenomes compared to ordinary hydrolytic enzymes. Metagenomics-based approaches for mining such compounds have not yet proved to be as efficient as originally anticipated.¹²⁸ This is due to the immense diversity and complexity of natural microbial communities. In addition, on average less than 2 % of bacterial genomes are devoted to natural product biosynthesis.¹³⁶ Hence, assembling and recovering entire natural product gene clusters demand a deep level of sequencing, which is currently not cost-effective.¹²⁸

Functional screening of cosmid and fosmid libraries limits redundant sequencing, yet such an approach is restricted by the dependence on successful heterologous expression of BGCs, which is limited by host compatibility and the significant size of most BGCs. Despite these obstacles, Schmidt *et al.*¹³⁷ expressed the relatively small (12.5 kb) *pat* BGC from a cyanobacterial symbiont of the ascidian *Lissoclinum patella* in *E. coli*, resulting in the successful production of patellamide A and C. Hence, expression of BGCs from uncultivable marine bacteria represents a means of tapping into the vast biosynthesis potential of marine microbial communities. Despite the fact that entire BGCs from uncultivable marine Proteobacteria have yet to be expressed heterologously, improvements in DNA sequencing depth and continued development of appropriate marine cell factories will likely facilitate such findings within the near future.

3 MARINE PROTEOBACTERIA AS CELL FACTORIES

A major challenge of translating the vast chemical space of Nature into application is the development of efficient production systems to exploit these molecules at a commercial scale. Whilst some natural products have been synthesized chemically,¹³⁸ biological production often offers the most feasible way to achieve a sustainable large-scale supply. As described in section 2.2, there are only a few examples of marine-derived natural products heterologously produced in the pioneering host organisms. Therefore, bacterial hosts other than these “old”, well-studied workhorses are drawing attention as novel cell factories because of their metabolic diversity and biosynthetic potential.^{103,139,140} Expressing certain genes heterologously in the work horse of heterologous expression, *E. coli*, may result in poor recognition of regulatory sequences, misfolding of the translated product and low protein secretion. The latter is often due to an impaired recognition of the *E. coli* SRP/SEC-dependent pathway elements. Another detrimental effect in protein solubility is commonly experienced under mesophilic conditions, during heterologous expression in *E. coli*.¹⁴¹

3.1 Marine Proteobacterial cell factory platforms

Marine Proteobacteria harbour great potential as future cell factories. At the current state of knowledge, Proteobacteria represent by far the largest group in the pelagic ocean microbiome, representing 55% of all bacteria.²⁸ Within the Proteobacteria phylum, the classes of Alpha-, Gamma-, Beta-, Delta- and Epsilonproteobacteria have been shown to comprise 32, 13, 2, 2, and 0.4% of all bacteria in the water column, respectively (Figure 3). From a different angle, bacteria of these classes might have become the dominant group in the ocean, because they have evolved diverse metabolic and biosynthetic strategies in order to adapt to the extremely diverse environments, which makes them attractive and reasonable candidates for new cell factories. Considering successful heterologous expression of natural products as a starting point for novel cell factories, current knowledge points to the following species having potential for future development.

3.1.1 *Vibrio natriegens* as the new powerhouse

The marine Gammaproteobacterium *Vibrio natriegens* (Figure 4), initially isolated from salt marshes, has been studied since 1960.¹⁴² A distinctive feature of *V. natriegens* is its short doubling time and its high substrate uptake rates.¹⁴³ *V. natriegens* grows both on solid and liquid media faster than the commercial strain *E. coli* NEB Turbo, with generation times of 15 min vs 27 min, respectively.⁸⁴ This has sparked the interest of the synthetic biology community to develop future applications. Standard DNA transformation and conjugation protocols are available for *V. natriegens* with similar efficiencies as for *E. coli*. Expression vectors with p15A, pMB1 and pMB1-derived origins of replication have been developed to facilitate heterologous expression, and four inducible promoters could be used in this strain: IPTG inducible *lacUV5* and *trc* promoters, arabinose inducible *araBAD* promoter and the λ phage *pR* promoter.⁸⁴ Furthermore, Weinstock *et al.*⁸⁴ attempted to improve the characteristics of *V. natriegens* for use in molecular biology by genomic reduction. A total of 192 kb of genomic DNA was removed, including nucleases and restriction-modification systems. Further developments of this strain, called Vmax, are pursued with the aim to commercialize this *Vibrio* as superior host for use in molecular biology.¹⁴⁴

The laboratory of George Church recently developed recombineering techniques for multiplexed cell factory development in *V. natriegens*.¹⁴⁵ The λ -Red homologs SXT-beta, SXT-Exo and λ -Gam present in *V. natriegens* achieve up to 10,000-fold higher recombination rates of single and double stranded DNA oligonucleotides. In parallel, Dalia *et al.* designed an engineering tool by exploiting natural transformation in *V. natriegens*.¹⁴⁶ Multiplexed genome editing by natural transformation or MuGENT, as the method is called, facilitates rapid and scarless genome editing. The technique is based on overexpression of *TfoX*, which renders *V. natriegens* natural competent. MuGENT allows for multiple scarless genome edits in one single step, but can also be employed sequentially to generate a heterogeneous population of mutants. As a proof of concept, the authors used the sequential transformation approach in order to increase production of the bioplastic PHB by a 100 fold.

Molecular tools in *V. natriegens* are relatively new, however, the outstanding characteristics of this bacterium have captured the attention of the community for further synthetic biology and cell factory applications. Furthermore, this promising cell factory belongs to a dominant group in the ocean surface waters: Vibrionaceae represent 1% of

Proteobacteria. Marine vibrios have been demonstrated to natively produce numerous bioactives^{147,148} and *V. natrigens* could serve as a suitable host for those compounds.

3.1.2 An upcoming evident marine Proteobacteria expression host: *Pseudoalteromonas*

Pseudoalteromonas haloplanktis is an Antarctic Gram-negative bacterium able to circumvent the above-mentioned limitations and has been intensively investigated as expression host, particularly for recombinant proteins.¹⁴⁹ Expression vectors with signal peptides have been reported to facilitate expression of α -amylases of *P. haloplanktis*.¹⁴¹ The plasmid pFFamy containing the secreted α -amylase gene was tested with three passenger proteins produced intracellularly from different hosts; indole-3-glycerol-phosphate synthase (SsIGPS) from *Sulfolobus solfataricus*, DsbA (PhDsbA) from *P. haloplanktis*, and the β -lactamase (EcBlaM) from *E. coli*. Plasmids were introduced by intergeneric conjugation. With these approaches, the authors achieve secreted expression in *P. haloplanktis* under psychrophilic conditions (4 °C) facilitating slow protein folding. Additionally, *P. haloplanktis* has recently been demonstrated to natively produce anticancer-active 4-hydroxybenzoic acid.¹⁵⁰ In a similar study, Yu *et al.*⁷⁴ developed a cold-adapted expression system in *Pseudoalteromonas* sp. BSi20429 and successfully expressed the active form of pseudoalterin, a cold-adapted enzyme. This was not possible in *E. coli* because of the lack of some enzymes in protein secretion systems. In this case, a new expression vector (pEV) was constructed with the native xylanase promoter and signal peptide from *Pseudoalteromonas* sp. SM20429. Pseudoalterin was successfully produced in expression cultures at 15 °C for 72 h and purified from the culture. These examples suggest that *Pseudoalteromonas* could become an attractive expression host for certain intractable proteins of marine origin and due to the vast bioactive potential of *Pseudoalteromonas*¹⁵¹ could be employed for the production of native or foreign secondary metabolites.

3.1.3 Other future marine cell factories

Today, marine Proteobacteria have been exploited for biotech production. Knowledge on them is growing fast to reach a level necessary for employment. The existing knowledge on terrestrial relatives can contribute to this aim. Marine Alphaproteobacteria have received little attention as heterologous production hosts and while being a rich source of novel natural products,^{148,152} the molecular tools for their modification are generally limited to knockouts.^{153,154} Their terrestrial relatives have been successfully assessed for the heterologous expression of natural products, e.g. the lasso peptide brevunisin by *Sphingomonas subterranea*¹⁵⁵ or a triterpene in *Rhodococcus capsulatus*.¹⁵⁶ Terrestrial Beta-, Gamma- and Deltaproteobacteria including the genera *Burkholderia*,^{157,158} *Pseudomonas*,^{68,120,159,160} and *Myxococcus*¹⁶¹ have been shown to be promising cell factories. Therefore, marine representatives of Proteobacteria could also be the candidates for future marine cell factories. Furthermore, it should also be considered if existing workhorses could be modulated to flourish at higher salt concentrations. While not prominent in the marine environment, *E. coli* can be found particularly close to harbors and wastewater outlets,¹⁶² which could push for a natural evolution of the species to high salt tolerance, and has also been demonstrated to be engineerable to an increased salt tolerance up to 1.54 M.¹⁶³

In order to establish future cell factories, the criteria for the specific production process need to be carefully reviewed and accordingly, high throughput screening combining laboratory and computational research¹⁶⁴ need to be applied to develop a dedicated cell factory for a natural product.

3.2 Marine Proteobacteria in industrial biotech production

The transition of a lab-based cell factory to a robust industrial strain is not a trivial one, but crucial for the development of a marine strain for large-scale production of natural products. Process economics become crucial, and saving on the production costs is a never-ending target. Marine Proteobacteria, and especially halophiles, possess a couple of characteristics that make them interesting to use in the biotech industry (see reference⁵⁷ for a comprehensive review). Here, we focus on halophilic Proteobacteria for use in industrial biotech processes, and highlight a couple of success examples on secondary metabolite production.

Bio-based production processes suffer from high expenses caused by, among others, energy-intensive fermentations, a relative low productivity and large consumption of fresh water.¹⁶⁵ Halophilic marine Proteobacteria can tolerate and thrive at high salt concentrations, which allows for replacing valuable fresh water with low-cost seawater in the fermentation broth. Usage of fresh water might currently not be a significant cost-driver in water-rich countries; however, some geographical locations already struggle with fresh water supply, a problem that will aggravate as the worldwide water consumption keeps swelling.¹⁶⁶ However, currently there are still limitations regarding the use of seawater in fermentations, e.g. due to corrosion of stainless steel.¹⁶⁷ A technical advancement or change in equipment and infrastructure seems to be necessary to support large-scale seawater-based processes.

A second benefit of using halophiles as production strains in fermentations is the reduced risk of contamination^{55,168}, as non-halophiles do not proliferate in high-salt media. Contaminations can interfere with the stability and health of a production process and thus cause economic insecurity. Risk of contaminations can be lowered even further if the halophile is, for example, also able to grow at high pH (alkaliphilic) or a non-conventional substrate is used in the process. Moreover, the genetic background of halophilic Proteobacteria might be better suited for the biosynthesis of marine derived compounds. For example, the majority of halogenated natural products are marine derived, and many of these compounds possess biological activity.¹⁶⁹ However, some marine derived brominated compounds were successfully produced in *E. coli*.²⁶ Although this provides evidence that heterologous expression in non-marine bacteria can be achieved, it is difficult to extrapolate these results, as failures of similar approaches are generally not published.

Demonstration of the advantages of halophile-based fermentation processes were given by using *Halomonas* strains.^{170,171} In the most recent example, an alkaliphilic *Halomonas campaniensis* strain was used in an unsterile, continuous fermentation process for the production of PHB.¹⁷¹ Operating under high salt concentration and a pH of 10, no significant contamination was detected for up to 65 days. Yield of PHB was improved by constructing a recombinant strain with the PHB BGC put under control of a strong promoter of a native porin. Synthetic biology efforts to control the morphology of the same *H. campaniensis* strain

in a temperature-controlled manner showed a further improvement in PHB yield and productivity.¹⁷²

Other examples of marine Proteobacteria studied to be used as production strains in industrial biotechnology setups involve the production of ectoine (**5**). Ectoine (**5**) is an osmoprotectant used by halophilic bacteria to counter the high osmolarity and is commercially used in the pharmaceutical industry. *Halomonas elongata* was engineered to produce ectoine (**5**) from mixed sugar substrates, simulating a lignocellulose-based bioprocess.⁴⁷ Furthermore, it was demonstrated that rice straw hydrolysate could be used as substrate rendering the process economics much more favorable.¹⁷³ The use of another low-cost substrate for ectoine (**5**) production was explored in a study with *Methylobacterium alcaliphilum*. Methane, a potent greenhouse gas, was converted to ectoine (**5**) in an attempt to reduce greenhouse gas emission and simultaneously produce this valuable compound.⁴⁸

Astaxanthin is a natural compound that belongs to the carotenoid family. Due to its potent antioxidant activity, astaxanthin has many applications in food, feed, and pharmaceuticals.⁴³ A marine isolate of the Alphaproteobacterium *Paracoccus* was engineered for production of this high-value compound.⁴⁴ A combination of random mutagenesis and overexpression of the native astaxanthin BGC from a broad-host-range vector resulted in a mutant with over 50 times higher astaxanthin production compared to the wild-type. The authors reported an astaxanthin titer of 480 mg/L and subsequently filed a patent.¹⁷⁴ The concentration achieved with this *Paracoccus* strain is higher than the recently published astaxanthin titer from a heavily engineered *E. coli* strain.⁴² This example shows that it could be more advantageous to choose a native producer strain over a traditional industrial workhorse organism.

These examples demonstrate the usefulness and potential of marine Proteobacteria as production strains in industrial biotechnology processes. Although most of these examples already involve some degree of genetic engineering, the continuous development of molecular tools for this group of bacteria will ensure a significant role of marine Proteobacteria on the biotechnology scene.

4 CONCLUSION

With up to 15 % of their genomes dedicated to biosynthesis of secondary metabolites, marine Proteobacteria remain an attractive source of new drug leads. Also, the uncultivated “microbial dark matter” hidden in the ocean potentially increases the natural chemistry space even further. A crucial bottleneck in this research field is the lack of efficient genetic tractability in most marine Proteobacteria. Additionally, examples of producing marine bacterial BGCs in hosts such as *E. coli* are scarce, as heterologous expression of large clusters remains challenging. Recent improvements in genetic tools in marine proteobacterial cell factories are demonstrated by the emergence of *V. natriegens* in the field of molecular biology. These efforts will ultimately lead to a significant role of marine Proteobacteria in natural product discovery and the development of specialized hosts and cell factories for production of marine natural compounds.

5 CONFLICTS OF INTEREST

There are no conflicts to declare.

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Figures:

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Figure 1. Selected bioactive compounds from marine Proteobacteria.

a

b

Figure 2. Biosynthesis of polybrominated aromatic compounds from the marine Gammaproteobacteria *P. luteoviolacea* 2ta16. (a) Polybrominated diphenyl esters produced by *P. luteoviolacea* 2ta16 and (b) the biosynthesis of the natural products by the *bmp* pathway. Figure modified from Agarwal *et al.*¹¹⁷ Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Nature Chemical Biology, Biosynthesis of polybrominated aromatic organic compounds by marine bacteria*,¹¹⁷ Vinayak Agarwal, Abraham A El Gamal, Kazuya Yamanaka, Dennis Poth, Roland D Kersten, Michelle Schorn, Eric E Allen and Bradley S Moore, Copyright (2014).

Figure 3. Abundances of marine Proteobacteria in marine surface waters as identified in the TARA Ocean dataset. ²⁸

Figure 4. *V. natriegens* as the new proteobacterial powerhouse. Figure modified from ^{84,143–146,175,176} Fast Growth & High Cell Density: Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Nature Methods*, *Vibrio natriegens* as a fast-growing host for molecular biology,⁸⁴ Matthew T Weinstock, Eric D Hesk, Christopher M Wilson, Daniel G Gibson, Copyright (2016).

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