Response to phosphate limitation of *Pseudovibrio* sp. FO-BEG1, a versatile bacterium with the potential for a symbiotic lifestyle

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

–Dr. rer. nat.–

dem Fachbereich Biologie/Chemie

der Universität Bremen

vorgelegt von

Stefano Romano

aus Campobasso (Italien)

Bremen, March 2014

Diese Arbeit wurde von Sptember 2010 bis Februar 2014 im Rahmen des Graduiertenprogramms „The International Max Planck Research School of Marine Microbiology“ in der Abteilung Mikrobiologie (Arbeitsgruppe Ökophysiologie) am Max-Planck-Institut für Marine Mikrobiologie in Bremen angefertigt.

1. Gutachterin: Prof. Dr. Heide N. Schulz-Vogt
2. Gutachter: Prof. Dr. Ulrich Fischer

3. Prüfer: Prof. Dr. Jens Harder
4. Prüfer: Prof. Dr. Thorsten Dittmar

Tag des Promotionskolloquiums: 10.03.2014

To my parents

(ai miei genitori)

"I have had dreams and I have had nightmares, but I have conquered my nightmares because of my dreams"

Jonas Salk

Contents

Summary

Phosphorous is a fundamental macronutrient for all living organisms, since it plays essential metabolic, structural, and regulatory roles in the cell. The preferential phosphorous source for bacteria is phosphate (Pi), which is available only in nanomolar concentrations in many marine environments, limiting, therefore, bacterial growth. However, the response of heterotrophic marine bacteria to phosphate limitation has been poorly investigated. In the present thesis, I characterized the response of the marine heterotrophic alphaproteobacterium *Pseudovibrio* sp. FO-BEG1 to phosphate limitation, investigating multiple aspects of its physiology. Bacteria belonging to this genus are receiving progressively more scientific attention due to their versatile metabolism, their ability to produce bioactive compounds, and their frequent detection in association with marine invertebrates.

In the study presented in **Chapter 2** physiological experiments in combination with proteomic and bioinformatic analyses, showed that phosphate limitation does not only affect the phosphorus metabolism but also other important physiological traits such as cell morphology, carbon metabolism, and cellular lipid composition. Additionally, the data obtained indicated that phosphate limitation triggers the production and secretion of bioactive secondary metabolites and the expression of proteins potentially involved in the interaction with the eukaryotic host.

The physiological study conducted in **Chapter 2** suggested that phosphate limitation greatly influences the composition of the metabolites secreted into the medium by *Pseudovibrio* sp. FO-BEG1. The ability of bacteria to produce and release a large variety of compounds into the environment has long been suggested; however, no studies have yet been performed to quantify this diversity with high resolution methods. Phosphate limitation has been described

to have a drastic effect on these processes, affecting primary and secondary metabolisms.

In **Chapter 3** the secreted compounds, also referred to as exo-metabolome, were quantified and their composition was chemically characterized. We analyzed and compared the exo-metabolome of *Pseudovibrio* sp. FO-BEG1 under phosphate-limited and phosphate-surplus conditions using ultra-high resolution mass spectrometry. The data showed an unexpectedly large and diverse exo-metabolome, characterized by a dynamic recycling of compounds during bacterial growth, and drastically affected in composition by the physiological state of the strain induced by phosphate limitation.

Surprisingly, phosphate limitation also had a pronounced influence on the iron metabolism of *Pseudovibrio* sp. FO-BEG1. The concentration of total soluble iron decreased in both phosphate regimes during the exponential growth phase and only under phosphate-limited conditions it increased again during stationary phase. In **Chapter 4** physiological experiments and chemical analyses showed that *Pseudovibrio* sp. FO-BEG1 releases one or several chelating molecules, which were responsible for the solubilization of precipitated iron.

Environmental phosphate concentrations have been known to significantly influence bacterial metabolism and lifestyle, and, as shown by recent "omic" studies, also the bacterial distribution and evolution. In **Chapter 5** I contextualized the data obtained during my work in an ecological frame. Additionally, I proposed and discussed four hypotheses that can explain the puzzling production of chelating molecules by *Pseudovibrio* sp. FO-BEG1 when growing under phosphate-limited conditions.

Zusammenfassung

Phosphor ist ein essentieller Makronährstoff für alle Lebewesen, der entscheidende metabolische, strukturelle und regulatorische Funktionen erfüllt. Die wichtigste Phosphorquelle für Bakterien ist das Phosphat, welches in marinen Ökosystemen jedoch oft nur in geringen, wachstumslimitierenden Mengen verfügbar ist. Dennoch ist die Reaktion von marinen, heterotrophen Bakterien auf Phosphatlimitierung kaum erforscht. Gegenstand dieser Arbeit ist daher die Charakterisierung der Reaktion des marinen, heterotrophen Alphaproteobakteriums *Pseudovibrio* sp. FO-BEG1 auf Phosphatlimitierung, vorwiegendunter physiologischen Gesichtspunkten. Mitglieder dieser Gattung rücken zunehmend in den Fokus des wissenschaftlichen Interesses, da sie einen außerordentlich anpassungsfähigen Stoffwechsel besitzen, bioaktive Substanzen produzieren und häufig mit marinen, wirbellosen Tieren assoziiert sind.

In **Kapitel 2** wird anhand von physiologische Experimenten und Proteomanalysen gezeigt, dass Phosphatlimitierung nicht nur den Phosphorstoffwechsel sondern auch die Zellform, den Kohlenstoffstoffwechsel und die Zusammensetzung der Lipide beeinflusst. Ebenfalls lassen die erhobenen Daten vermuten, dass Phosphatlimitierung sich auf die Produktion von bioaktiven Sekundärmetaboliten und die Expression von Proteinen auswirkt, welche möglicherweise in einer Interaktion mit dem eukaryotischen Wirt involviert sind.

Die in **Kapitel 2** beschriebenenphysiologischen Beobachtungen legen nahe, dass die Zusammenstetzung der von *Pseudovibrio* sp. FO-BEG1 abgegebenen Stoffe stark von der Phosphatlimitierung beeinflusst wird. Obwohl seit Längerem angenommen wird, dass Bakterien die Fähigkeit besitzen, eine Vielzahl an Stoffen in die Umwelt abzugeben, wurden keinerlei Forschungsstudien durchgeführt, welche die Diversität und Quantität dieser Substanzen untersuchen. In **Kapitel 3** wird die Zusammensetzung dieses sogenannten Exo-Metaboloms von *Pseudovibrio* sp. FO-BEG1 unter

Phosphatüberschuss und Phosphatlimitation zu verschiedenen Zeitpunkten mittels hochauflösender Massenspektrometrie bestimmt. Das unerwartet große und diverse Exo-Metabolom ist durch eine dynamische Wiederverwertung von Stoffwechselprodukten charakterisiert und abhänging vom physiologischen Zustand des Stammes, der durch Phosphatlimitierung beeinflusst wird.

Überraschenderweise zeigte sich, dass Phosphatlimitierung auch den Eisenstoffwechsel von *Pseudovibrio* sp. FO-BEG1 drastisch beeinflusst. Unabhängig von der Phosphat-Verfügbarkeit sank die Konzentration des löslichen Eisens während der exponentiellen Wachstumsphase ab, stieg jedoch während der stationären Phase nur unter Phosphatlimitierung wieder an. In **Kapitel 4** wird anhand von physiologischen Experimenten und chemischen Analysen gezeigt, dass *Pseudovibrio* sp. FO-BEG1 ein oder mehrere komplexbildende Moleküle abgibt, welche präzipitiertes Eisen wieder in Lösung bringen. Bislang wurde eine Verbindung zwischen Phosphat- und Eisenstoffwechsel nur indirekt beschrieben und das hier beobachtete Phänomen wurde noch nie berichtet.

Es ist seit langem bekannt, dass Phosphatkonzentrationen den Stoffwechsel und die Lebensweise von Bakterien in der Umwelt signifikant beeinflussen und sich ebenfalls auf bakterielle Verbreitung und Evolution auswirken können. Aufgrund dieser großen Bedeutung des Phosphats werden daher in **Kapitel 5** die im Rahmen dieser Arbeit erlangten Ergebnisse in einem größeren ökologischen Zusammenhang betrachtet. Dabei werden vier verschiedene Hypothesen entwickelt und diskutiert, welche die Produktion komplexbildender Moleküle durch *Pseudovibrio* sp. FO-BEG1 unter Phosphatlimitierung erklären könnten.

List of publications

Publications included in the thesis:

1. **Romano, S.,** Schulz-Vogt, H.N., González, J.M., Bondarev, V., (2014) **Phosphate limitation induces drastic physiological changes, virulence related gene expression, and secondary metabolite production in strain *Pseudovibrio* sp. FO-BEG1**.
   Manuscript in preparation
2. **Romano, S.,** Dittmar, T., Bondarev, V., Weber, R.J.M., Viant, M.R., Schulz-Vogt, H.N., (2014) **Exo-metabolome of *Pseudovibrio* sp. FO-BEG1 analyzed by ultra high resolution mass spectrometry and the effect of phosphate limitation**.
   Manuscript in revision to *PLoS ONE*
3. **Romano, S.**, Bondarev, V., Dittmar, T., Schulz-Vogt, H.N., (2014) **Solubilization of precipitated iron by strain *Pseudovibrio* sp. FO-BEG1 growing under phosphate limitation.**
   Manuscript in preparation

Contributed works:

4. Bondarev, V., Richter, M., **Romano, S.**, Piel, J., Schwedt, A., Schulz-Vogt, H.N., (2013) **The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis**.
   Environmental microbiology, 15: 2095-2133

5. Schwedt, A., Seidel, M., Dittmar, T., Simon, M., Bondarev, V., **Romano, S.**, Lavik, G., Schulz-Vogt, H.N., (2014) **Substrate use of *Pseudovibrio* sp. growing in extremely oligotrophic seawater.** Manuscript in preparation

6. Schwedt, A.*, **Romano, S**.*, Bondarev, V., Seidel, M., Dittmar, T., Schulz-Vogt, H.N., (2014) **Isolation of facultatively oligotrophic bacteria.** Manuscript in preparation

* these authors contributed equally to this work

1

Introduction

1.1 The genus *Pseudovibrio*

Strain FO-BEG1 is an alphaproteobacterium belonging to the genus *Pseudovibrio* in the family *Rhodobacteraceae*, and shares 99.9% similarity at the 16S rDNA level with the type strain *Pseudovibrio denitrificans* (**Figure 1.1**). FO-BEG1 was isolated from an enrichment culture of *Beggiatoa* sp. 35Flor, a filamentous sulfide-oxidizing bacterium originally sampled from a black-band diseased coral in the coastal waters of Florida (Brock and Schulz-Vogt, 2011; Schwedt, 2011). The bacterium is a straight rod with an average cell length of 2-4 µm and cell width of 0.4-0.6 µm when cultivated under nutrient rich

conditions (**Figure 1.1**). To date the genus *Pseudovibrio* consists of four type strains: *P. denitrificans* and *P. japonicus*, isolated from coastal seawater (Shieh *et al.*, 2004; Hosoya and Yokota, 2007), *P. ascidiaceicola* isolated from a marine tunicate (Fukunaga *et al.*, 2006), and *P. axinellae* isolated from a marine sponge (O'Halloran *et al.*, 2013). All type strains share similar physiological traits, being described as marine, heterotrophic, and facultative anaerobic bacteria capable of denitrification and fermentation.

Bacteria belonging to the genus *Pseudovibrio* were isolated or detected via 16S rDNA analysis all over the world, in coastal waters (Agogué *et al.*, 2005), in oil reservoirs (Silva *et al.*, 2013), as members of biofilms or as epibionts of algae (Penesyan *et al.*, 2011; Pratheepa *et al.*, 2013), in the intestine of sea cucumbers (Zhang *et al.*, 2013), associated with marine polychaetes, tunicates, corals and bryozoa (Koren and Rosenberg, 2006; Sertan-de Guzman *et al.*, 2007; Riesenfeld *et al.*, 2008; Heindl *et al.*, 2010; Rypien *et al.*, 2010; Rizzo *et al.*, 2013), and in most cases associated with marine sponges (Hentschel *et al.*, 2001; Olson *et al.*, 2002; Thakur *et al.*, 2003; Thiel and Imhoff, 2003; Thoms *et al.*, 2003; Lafi *et al.*, 2005; Kennedy *et al.*, 2009; Santos *et al.*, 2010; Flemer *et al.*, 2012; Dupont *et al.*, 2013). These recurrent associations were considered as an indication for the existence of a symbiotic relationship between members of the *Pseudovibrio* genus and these marine invertebrates (Webster and Hill, 2001; Enticknap *et al.*, 2006; Taylor *et al.*, 2007). In support of this hypothesis bacteria belonging to the *Pseudovibrio* genus were found to be the most abundant prokaryotes associated with larvae of the sponge *Mycale laxissima*, indicating vertical transmission (direct transmission from the parental line to the progeny) of these bacteria within their hosts

(Enticknap *et al.*, 2006). Moreover, for some marine sponges the culturable fraction of their bacterial community was dominated by *Pseudovibrio* related strains (Webster and Hill, 2001; Muscholl-Silberhorn *et al.*, 2008).

In a recently published paper the genomes of two isolates belonging to the *Pseudovibrio* genus were analyzed and compared (Bondarev *et al.*, 2013; **Contributed works**). The analysis revealed their metabolic versatility and at the same time the presence of several genes encoding proteins that potentially play a role in prokaryote-eukaryote interaction. A high number of tripartite ATP-independent periplasmic (TRAP) and ATP-binding cassette (ABC) transporters for a variety of molecules together with the potential for utilizing different substrates to satisfy their carbon (C), nitrogen (N), and phosphorus (P) requirements were shared traits between the two isolates. Moreover, both bacteria were able to proliferate under extremely oligotrophic conditions (Bondarev *et al.*, 2013; for more information refer to the **Contributed works** section). These features make *Pseudovibrio* a bacterium well adapted to thrive in the open ocean, where often the nutrient availability is fluctuating in time and space. In addition, the presence of type III secretion systems (T3SSs) with three different types of effector molecules and type VI secretion systems (T6SSs), both described to be involved in virulence, together with the presence of several toxins and proteins potentially involved in the adhesion to the host cells (Bondarev *et al.*, 2013; **Contributed works**), underlined the potential of bacteria belonging to this genus to successfully establish and maintain symbiotic relationships with marine invertebrates.

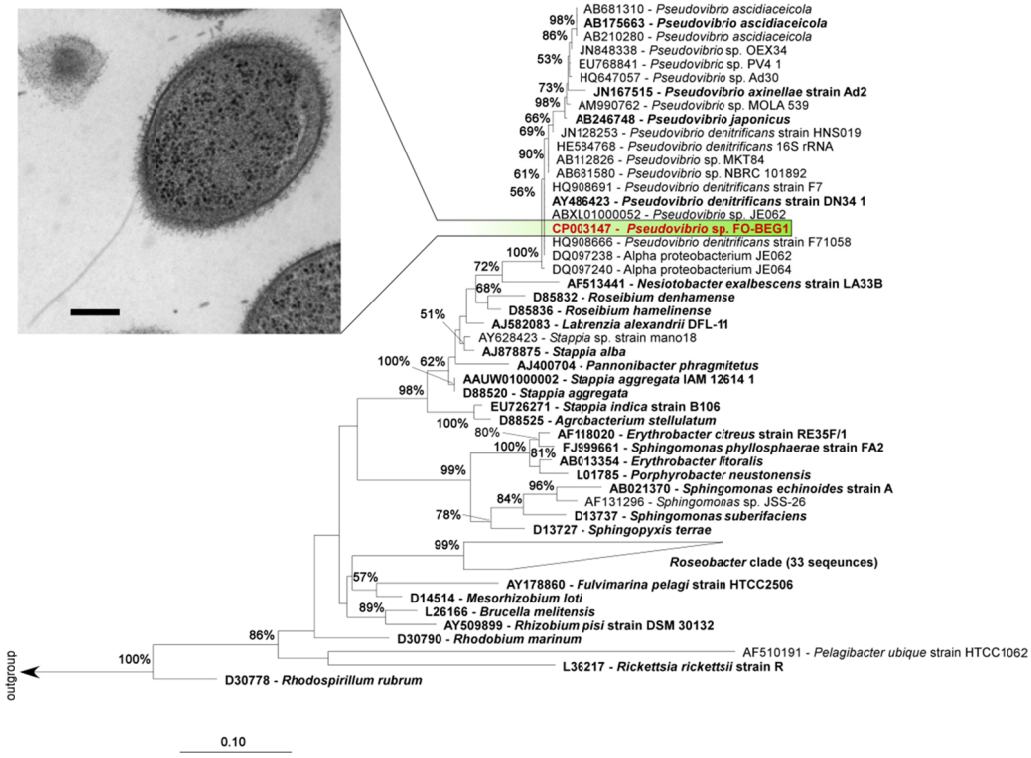


Figure 1.1 | Maximum likelihood (RAxML) phylogenetic tree of nearly full length 16S rRNA gene sequences constructed for the phylum *Alphaproteobacteria*. The tree was constructed using the GTRGAMMA model and a rapid bootstrap analysis algorithm with 100 repetitions. The ARB software package (Ludwig *et al.*, 2004) and release 111 of the SILVA SSU Ref database (Pruesse *et al.*, 2007) were used. Only bootstrap values higher than 50% are reported. For each sequence the accession number and the name of the attributed species are reported. Names in bold represent type strains. For the *Roseobacter* clade the following sequences were included: *Roseobacter denitrificans* (M96746), *R. litoralis* (X78312), *Sulfitobacter mediterraneus* (Y17387), *S. mediterraneus* (DQ915636), *S. brevis* (Y16425), *S. litoralis* (DQ097527), *S. pontiacus* (DQ915637), *S. delicates* (AY 180103), *S. dubius* (AY180102), *Oceanibulbus indolifex* (ABID01000001), *Roseivivax halodurans* (D85829), *R. halotolerans* (D85831), *Salipiger mucescens* (AY527274), *Oceanicola batsensis* (AAMoÓ01000005), *Silicibacter pomeroy* (DQ9156319), *S. pomeroyi* (AF098491), *Ruegeria lacuscaerulensis* (U77644), *Roseobacter gallaeciensis* (Y13244), *Roseovarius mucosus* (AJ534215), *R. tolerans* (Y11551), *R. nubinhibens* (AALY01000001), *R. halotolerans* (EU431217), *Loktanella fryxellensis* (AJ582225), *L. salsilacus* (AJ440997), *L. vestfoldensis* (AJ582226), *L. hongkongensis* (AY600300), *Oceanicola granulosus* (AAOT01000030), *Octadecabacter arcticus* (U73725), *Jannaschia helgolandensis* (AJ438157), *J. rubra* (AJ748748), *Rhodobacter capsulatus* (DQ342320), *R. sphaeroides* (X53854), *Fulvimarina pelagi* (AY178860).
The *Pseudovibrio* strain which is the object of the present thesis is highlighted in red.
The upper panel represents a transmission electron micrograph of *Pseudovibrio* sp. FO-BEG1 obtained in collaboration with Prof. Laura van Niftrik and Sara Neumann at the Radboud University in Nijmegen, the Netherlands. Scale bar = 200 nm.

To date, the nature of the symbiosis (mutualistic/commensalistic or pathogenic/parasitic) between *Pseudovibrio* and marine invertebrates is still unclear. However, bacteria related to *Pseudovibrio* have been isolated only from healthy sponges, which would indicate that they do not harm the host and might even be required for its living (Webster and Hill, 2001). Only recently *Pseudovibrio* related bacteria were isolated from bleached scleractinian corals (Moreira *et al.*, 2013), but so far this putative direct association with a diseased metazoan is the only reported occurrence.

In general, obligate symbiotic bacteria undergo a progressive reduction of the genome size. Especially when they are vertically transmitted virulence genes such as T3SSs and their respective effectors are lost (McCutcheon and Moran, 2012). *Pseudovibrio* has a large genome for a heterotrophic marine bacterium, more than five million base pairs (bp) and more than five thousand protein-encoding genes (Bondarev *et al.*, 2013; **Contributed works**). In addition, it possesses several genes encoding secretion systems and effectors which would indicate that these bacteria could be acquired by the hosts from the surrounding environment. Therefore, it seems likely that bacteria belonging to the *Pseudovibrio* genus are mainly facultative symbionts of marine invertebrates, and still maintain a great metabolic versatility, which makes them well adapted to thrive in environments subject to nutrient fluctuations, such us the open ocean.

One characteristic shared among almost all isolates belonging to this genus is the ability to produce bioactive secondary metabolites. For example, the potent antibiotic tropodithietic acid (TDA) characteristically produced by members of the *Roseobacter* clade, was shown to be produced by different *Pseudovibrio* isolates (Penesyan *et al.*, 2011; Bondarev *et al.*, 2013; **Contributed**

works). In several other studies their ability to produce bioactive compounds was reported (Hentschel *et al.*, 2001; Thiel and Imhoff, 2003; Muscholl-Silberhorn *et al.*, 2008; Kennedy *et al.*, 2009; Heindl *et al.*, 2010; Rypien *et al.*, 2010; Santos *et al.*, 2010; O'Halloran *et al.*, 2011; Flemer *et al.*, 2012), but isolation and chemical characterization was successful only in one case (Sertan-de Guzman *et al.*, 2007). Sponges in general are seen as a source of new compounds of biotechnological interest, and recently it became more clear that parts of these compounds are produced by their symbionts (Piel, 2004; Thakur *et al.*, 2005; Hochmuth *et al.*, 2010; Roué *et al.*, 2012). Therefore, in the last number of years particular attention was directed to the isolation and characterization of new sponge symbionts.

1.2 Microbes and the phosphorus cycle

Phosphorus (P) is an essential element for life, being a structural and functional component of all organisms. It provides the phosphate ester backbone of DNA and RNA, and is crucial in the transmission of chemical energy through ATP molecules. In addition, phosphorus is a key constituent of many cell components such as phosphoproteins and phospholipids in the cell membranes. Moreover, most of the cellular processes are regulated by protein phosphorylation and de-phosphorylation. This underlines the importance of this element not only as constituent of cell biomass but also as a key component for the regulation of the overall cell metabolism (Stock *et al.*, 1989; Hubbard and Cohen, 1993).

Phosphorus availability can impact primary production rates in the ocean as well as species distribution and ecosystem structure (Smith, 1984; Karl *et al.*, 2001; Coleman and Chisholm, 2010). Moreover, there is evidence suggesting that the phosphorus availability can directly influence bacterial speciation and that it might have impacted both the expansion and decline of animal taxa in the history of life (Elser *et al.*, 2006; Souza *et al.*, 2008). Phosphorus has been traditionally considered to be the "ultimate" limiting macronutrient in marine systems, because unlike nitrogen it cannot be fixed from atmospheric gas (Tyrrell, 1999). This theory is based on the assumption that over prolonged time scales the phytoplankton nitrogen demand can be satisfied by N_2-fixation. Consequently, the standing stock of N_2-fixing organisms will increase as the N:P ratio in the ocean decreases. Since the reservoir of nitrogen in the atmosphere is so large, N_2-fixing organisms would eventually be limited by other nutrients. Given the long residence time of phosphorus in the ocean compared to other potentially bio-limiting nutrients and trace elements, such as silica (Si) and iron (Fe), phosphorus is considered limiting over long time scales (VanCappellen and Ingall, 1996; Tyrrell, 1999).

The main part of bio-available phosphorus-input in the ocean has continental origin and is transported by rivers (**Figure 1.2**). Only a small percentage of the total phosphorus-input (maximum 10%) was estimated to originate from soluble phosphorus present in atmospheric particles (**Figure 1.2**; Paytan and McLaughlin, 2007). Continental weathering of crustal materials, which contain on average 0.1% PO_4^{3-}, is the major source of riverine phosphorus-input, which can be transported in two main forms: particulate and

dissolved. In the particulate fraction phosphorus can be bound to organic matter, soil particles or soil oxyhydroxide and is thus regarded as biologically unavailable (Benitez-Nelson, 2000; Filippelli, 2008). It was estimated that only 10-30% of riverine phosphorus is available for biological uptake and one quarter of this can be trapped in estuaries and might never reach the open ocean (Paytan and McLaughlin, 2007; **Fig. 1.2**). Therefore, the dissolved phosphorus load which arrives in the marine systems may represent as little as 5-10% of total phosphorus transported by rivers to the ocean (Delaney, 1998).

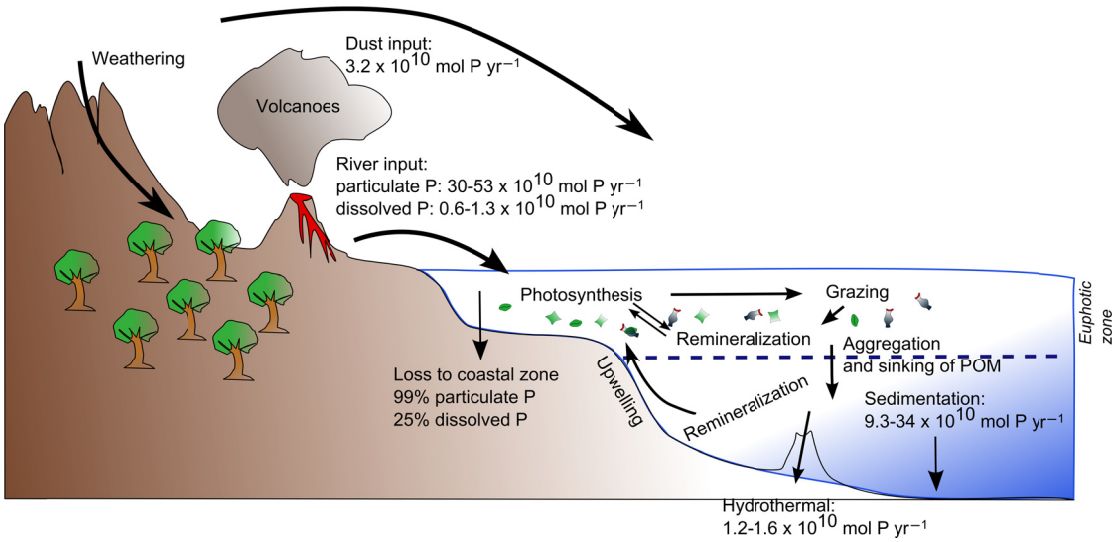


Figure 1.2 | Schematic overview of the phosphorus cycle. Different phosphorus sources are given in combination with their approximate amounts produced per year. The dimension of the arrows is not proportional to the amount of phosphorus involved in the processes. Data from Paytan and McLaughlin (2007).

Inorganic dissolved phosphorus in seawater is mainly present in form of PO_4^{3-} and HPO_4^{2-}, and in some regions they can represent almost 90% of the total dissolved fraction (Benitez-Nelson, 2000). Their depth profiles exhibit a

"nutrient trend" such that surface waters are depleted due to intense biological uptake in the euphotic zone, and concentrations increase with depth as a result of the conversion of sinking organic phosphorus into inorganic soluble forms (Benitez-Nelson, 2000). The primary oceanic sinks for phosphorus are believed to be the deposition of biogenic sediments (Froelich *et al.*, 1982) and the formation of apatite, which are minerals mainly composed of phosphorus and calcium (Ca). In coastal environments removal of phosphate (P_i) from the water column through adsorption onto sediment particles can be one of the dominant processes. Sediment composition greatly affects the adsorption of phosphate, and iron and aluminum (hydr)oxides have been reported to have the higher adsorption capacity, retaining in some cases almost 50% of the inorganic phosphorus in the superficial sediment (Lucotte and d'Anglejan, 1988; Brinkman, 1993; Jensen and Thamdrup, 1993). Dissolved phosphate reacts with hydrous iron oxides, in a process which could remove 5 to 40% of the total phosphorus input to the oceans, particularly in areas with high ridge crest volcanogenic iron oxide concentrations (Berner, 1973; Froelich *et al.*, 1977; Feely *et al.*, 1998)

Despite technical difficulties in the quantification of phosphorus turnover in the ocean, several researchers have successfully shown that phosphorus recycling rates in the dissolved and particulate pools in surface waters are more rapid than previously thought (less than a day to two weeks), suggesting that low phosphorus concentrations can support relatively high primary production (reviewed in Paytan and McLaughlin, 2007). Up to 95% of the bio-available phosphorus reaching the ocean surface waters is incorporated into particulate organic matter (POM) and exported from the euphotic zone to

deeper layers. At the sea-floor 99% of phosphorus derived from falling particles is then regenerated to the dissolved state (Delaney, 1998). The remaining 1% is removed from the ocean reservoir into the sediment. Here it interacts with calcium minerals and oxyhydroxides and takes part in re-mineralization and re-dissolution processes which depend, for intensity and typology, on the amount of oxygen (reviewed in Paytan and McLaughlin, 2007). In general these processes cause an increase in phosphorus concentration in the sediment-water interface and there are some indications that, in anoxic sediments, phosphorus can partially re-diffuse back into the water column (reviewed in Paytan and McLaughlin, 2007).

In certain areas of the ocean, particularly in surface waters, much of the dissolved and bio-available phosphorus for bacteria is in the form of dissolved organic phosphorus (DOP), but so far only a minor fraction of DOP has been characterized (Karl and Björkman, 2002; Young and Ingall, 2010). There are indications that in the dissolved organic matter phosphorus is mainly part of carbohydrates and amino acids, whereas in the particulate organic matter phosphorus is also present in lipids (Sannigrahi *et al.*, 2006). DOP can be divided into high molecular weight DOP (HMWDOP, >50kDa), which represents about a quarter of total DOP, and low molecular weight DOP (LMWDOP, <10kDa), which can reach up to 80% of total DOP. A recent study showed that LMWDOP could contain small phosphate esters such as nucleotides, phospho-sugars, phosphonates, and up to 13% of polyphosphate (Young and Ingall, 2010). The HMWDOP, instead, was shown to mainly consists of phosphate esters (75%) and phosphonates (25%) (Kolowith *et al.*, 2001).

There is growing evidence that DOP represents an important pool of phosphorus for bacteria (Björkman and Karl, 2003; Luo *et al.*, 2011). For example, pospho-ester bonds (C-O-P) can be cleaved by enzymes called alkaline phosphatases (AP). So far three AP have been described, and bioinformatic analyses revealed that, unlike previously thought, they can be localized in the cytoplasm, in the periplasm, and can also be secreted (Luo *et al.*, 2009). This underlines different strategies in the utilization of DOP, with the small phospho-ester compounds being imported into the cell and the bigger ones being digested extracellularly. Other components of DOP are phosphonates, which are characterized by the very stable C-P bound, which is resistant to chemical hydrolysis and thermal decomposition (Quinn *et al.*, 2007). They are found in glycolipids, glycoproteins, and phospholipids and they have been identified in different marine invertebrates (Quin, 1965; Kittredg and Roberts, 1969). In addition, recently it was shown that *Trichodesmium erythraeum* is able to produce phosphonates in amounts of up to 10% of its biomass (Dyhrman *et al.*, 2009). Accordingly, growing evidence suggests that these stable compounds can be also utilized as a source of phosphorus by marine bacteria (Dyhrman *et al.*, 2006; Gilbert *et al.*, 2009; Martinez *et al.*, 2010; Thomas *et al.*, 2010).

Not much is know about the phosphorus cycle in marine sponges, the principal potential host for bacteria belonging to the *Pseudovibrio* genus. The few data available report that some sponges have a positive phosphorus balance, meaning that they release more inorganic dissolved phosphorus than they take up (Yahel *et al.*, 2007; Ribes *et al.*, 2012). These data support the hypothesis that sufficient phosphorus is obtained by the sponges from their diet (Taylor *et al.*,

2007). Interestingly, a recent study reported that actinomycetes isolated from sponges were able to solubilize minerals containing phosphate. Therefore, it was speculated that, since sponges are filter feeders and pump a large volume of seawater every day, they can accumulate large quantities of inorganic insoluble phosphorus forms originating from close continental areas. Bacteria able to solubilize these compounds can provide an adjunctive phosphorus source for the microbial community and the host (Sabarathnam *et al.*, 2010).

1.3 The phosphate limitation response

Bacteria acquire phosphorus preferably as phosphate, which is often found in nanomolar concentrations in marine systems (Björkman and Karl, 1994; Thomson-Bulldis and Karl, 1998). Regions such as the Sargasso Sea in the North-West Atlantic Ocean, the Mediterranean Sea and the North Pacific subtropical gyre, have low phosphate concentrations with evidence suggesting that phosphate is limiting bacterial growth (Krom *et al.*, 1991; Fanning, 1992; Cotner *et al.*, 1997; Karl, 2000; Wu *et al.*, 2000; Thingstad *et al.*, 2005). Low phosphate concentrations have been reported to limit or co-limit the growth of phytoplankton species in the eastern tropical North Atlantic and central Atlantic Ocean (Sañudo-Wilhelmy *et al.*, 2001; Mills *et al.*, 2004). Moreover, Thingstad *et al.* (1998) reported evidence for bacterial phosphate limitation in the coastal region of the North-West Mediterranean Sea. Consistently, in the same regions seasonal changes in dissolved inorganic phosphorus concentrations were reported, and

this influenced the structure of the bacterial community during the year (Pinhassi *et al.*, 2006).

The differences in phosphate concentrations detected in different environments are reflected in specific adaptations observed in bacteria living in low phosphate regions. In a recent study Coleman and Chislohm (2010) performed a comparative genomic analysis quantifying the heterogeneity in gene contents of *Prochlorococcus* and *Pelagibacter* strains, within and between populations from the Atlantic and the Pacific Oceans. They showed that nearly all genes that differed significantly in abundance between the two biogeochemically distinct environments were related to phosphorus metabolism and were enriched in the Atlantic region, which is characterized by low phosphate concentrations. Their findings implicate that the phosphate availability was a driving force in the genetic divergence between the two populations. Moreover, exposure to long term phosphate limitation has selected specific traits that allow microorganisms to proliferate in such environments. For example *Prochlorococus* and *Synechoccoccus*, ubiquitous and abundant cyanobacteria in the photic zone of oligotrophic marine environments depleted in phosphate, have replaced their phospholipids with phosphorus-free lipids such as glycolipids and sulfur containing glycolipids. In this way they are able to decrease their cellular phosphorus demand by up to 43% (Van Mooy *et al.*, 2006; Van Mooy *et al.*, 2009).

In addition to long-term adaptations, bacteria evolved several mechanisms to sense phosphate concentrations and regulate their phosphorus metabolism accordingly. The global regulatory circuit involved in bacterial

phosphorus management is called phosphate(Pho)-regulon and it has been well studied in the model organism *Escherichia coli* (Wanner, 1996). The circuit is controlled by the two component regulatory system (TCRS) PhoR-PhoB (PhoR-PhoP in Gram-positive bacteria) and the ABC transporter system for phosphate Pst (Wanner, 1996; Hsieh and Wanner, 2010). PhoR is a transmembrane histidine kinase and is essential for three distinct processes that control PhoB activity as transcription factor: inhibition, via preventing the phosphorylation, activation via phosphorylation, and deactivation via dephosphorylation of phospho-PhoB. However, PhoR is unable to sense phosphate concentration and the current model suggests that this is mediated by the interaction with the Pst system, which works as transporter and as detector (**Figure 1.3**).

In conditions of high phosphate concentration in the environment (for *E. coli* >4 μmol L^{-1}) the Pst transport system is working as a transporter and it is in a conformational state that inhibits the activity of PhoR, via the interaction with the protein PhoU. When the environmental concentration of phosphate is below threshold the Pst system still works as transporter but is in a different conformational state and, not interacting with PhoU anymore, does not inhibit PhoR. The latter one becomes now active, it undergoes autophosphorylation and acts as a kinase phosphorylating the transcription regulator PhoB. PhoB is now in the active form, binds to a specific site in the promoter region of genes belonging to the Pho-regulon called Pho-box and regulates their transcription. When the concentration of phosphate in the environment increases again, the complex

Pst-PhoU is re-formed, PhoR is deactivated and can act to dephosphorylate PhoB, reducing gene regulation.

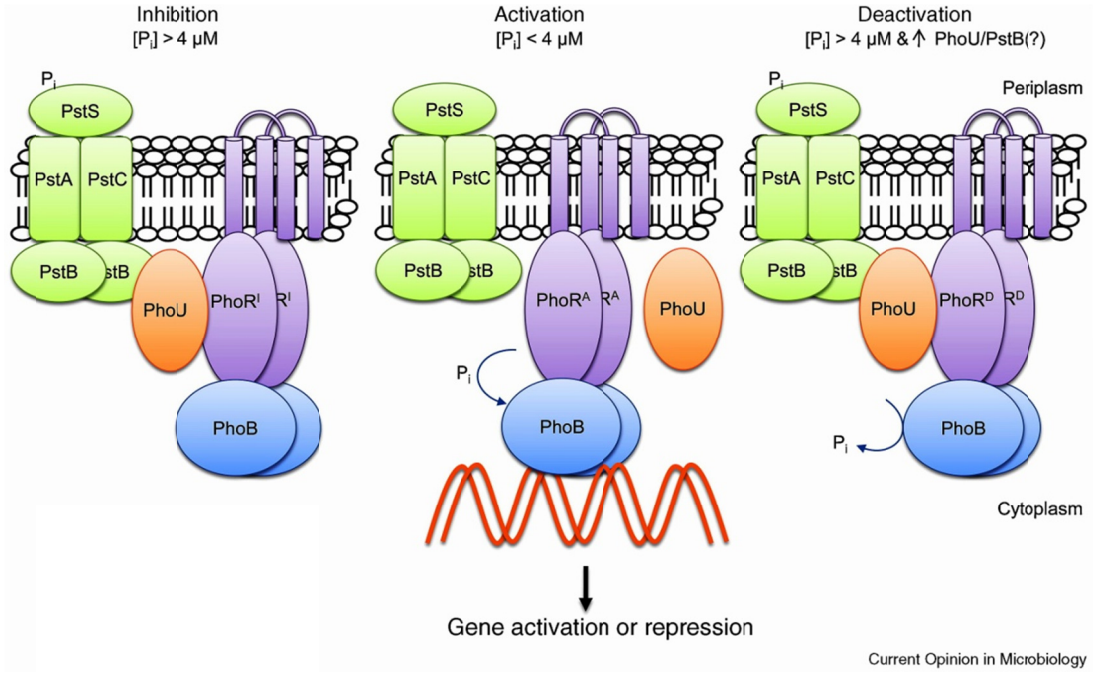


Figure 1.3 | Representation of the interaction between the high affinity transport system for phosphate, Pst, and the two component regulatory system PhoR-PhoB. The phosphate threshold at which the different processes take place refers to studies conducted on *Escherichia coli*. Picture taken from Hsieh and Wanner (2010).

The number of genes that is regulated and therefore belongs to the Pho-regulon vary widely. A proteomic study conducted on *E. coli* cells grown under phosphate excess and limitation showed that more than 400 proteins (almost 10% of the *E. coli* genome) had a differential expression under phosphate-limited conditions (VanBogelen *et al.*, 1996). This is in agreement with genomic predictions of potential PhoB binding sites in bacterial genomes which showed that in *E. coli* there are several hundreds of genes that are potential members of the Pho-regulon (Baek and Lee, 2006; Yuan *et al.*, 2006). Altogether these data

underline the importance of phosphate in the regulation of cellular physiology, including a large variety of metabolic processes not only related to phosphorus homeostasis.

1.4 Importance of environmental phosphate concentration for bacterial metabolism and lifestyle

It is constantly becoming more evident that the environmental concentration of phosphate affects primary metabolism, bacterial lifestyles as well as intra and interspecies relationships via modulating quorum sensing, secondary metabolite production, and virulence gene expression. Most of all, phosphate-limiting conditions trigger the expression of proteins involved in scavenging phosphate from the environment. At the same time, it can also affect nitrogen and carbon metabolism of the cells, leading, for example, to an accumulation of carbon storage compounds and to a direct control of the global nitrogen transcription regulator (Schembri *et al.*, 1995; Wanner, 1996; Rodríguez-García *et al.*, 2009; Levering *et al.*, 2012). Interestingly, a study conducted on *Streptococcus* sp. showed that the intracellular phosphate concentration had a direct effect on glycolysis by regulating the activity of the pyruvate kinase (Mason *et al.*, 1981). Moreover, the repression of secondary metabolite production by high phosphate concentrations is a well known phenomenon. This effect was especially investigated in *Actinobacteria* species, but recently it was shown that phosphate limitation was responsible for activating the production of antibiotics and quorum sensing molecules also in strains

belonging to the genera *Pseudomonas* and *Serratia* (Gunnarsson *et al.*, 2003; Slater *et al.*, 2003; Martín, 2004; Mendes *et al.*, 2007; Gristwood *et al.*, 2009; Zaborin *et al.*, 2009).

Bacteria live in a dynamic equilibrium with the environment, taking up and releasing specific compounds in a manner that is greatly influenced by environmental conditions. As described for phosphate, in most marine systems the nutrient availability is temporally and spatially variable, and this can have a great influence on bacterial proliferation and consequently on both the uptake and the release of specific molecules. Secretion of different kinds of compounds, for example involved in intraspecies signaling (e.g. acyl-homoserine lactone) or nutrient scavenging (e.g. siderophores), is tightly regulated by nutrient concentrations. The mentioned effect of phosphate limitation on the production of antibiotics in *Actinobacteria* is a classical example of these influences. However, bacteria can take up and release a huge variety of compounds from and into the environment, but only recently with the advent of high and ultra-high resolution analytical chemical techniques this diversity is starting to be investigated (Want *et al.*, 2007; Mapelli *et al.*, 2008; Kujawinski *et al.*, 2009). Therefore, it will be a future challenge to understand to which extent different nutrient regimes influence the exchange of molecules between the environment and bacteria, considering not only specific production or secretion, but also the overall ensemble of organic compounds involved in these processes. In this respect, metabolomic approaches could help to gain a systematic and comprehensive picture about the dynamics of these phenomena and about the effect of the environmental conditions on them.

The environmental phosphate concentration and the TCRS PhoR-PhoB can also have an effect on biofilm development and expression of genes involved in virulence (Lamarche *et al.*, 2008 and references therein). For example, in *Agrobacterium tumefaciens* it was shown that phosphate limitation significantly increases attachment and biofilm formation (Xu *et al.*, 2012). In addition, it was reported that PhoB directly binds and regulates the transcription of *A. tumefaciens* virulence genes (Aoyama *et al.*, 1991). Moreover, *phoB* mutants of *Vibrio cholerae* were shown to be less able to colonize the host intestine than the wild type (von Kruger *et al.*, 1999). Recently, several studies clearly showed that phosphate depletion increases the virulence of *Pseudmonas aeruginosa*, with lethal effect on the host in *in-vivo* experiments (Long *et al.*, 2008; Zaborin *et al.*, 2009; Zaborin *et al.*, 2012). In this specie phosphate limitation enhanced toxin production and biofilm formation, and operative injured mice with decreased intestinal phosphate content had 60% higher mortality due to *P. aeruginosa* infection. This was completely prevented when phosphate was supplemented in the diet and the intestinal phosphate concentration was restored (Long *et al.*, 2008). The same virulent phenotype was induced by phosphate limitation in strains of the genus *Candida* (Romanowski*et al.*, 2012).

However, the picture describing the interactions between the phosphate limitation response, the TCRS PhoR-PhoB, and bacterial virulence is far more complicated. For example, in uropathogenic *E. coli,* the deletion of the genes involved in the synthesis of the high affinity transport system for phosphate (Pst) was shown to constitutively activate the Pho-regulon and decrease fimbriae production and virulence (Crépin *et al.*, 2012). Accordingly,

different pathogenic *E. coli* strains showed a decrease in virulence combined with an increased sensitivity to the bactericidal effect of the host serum and antimicrobial peptides when the *pst* genes were inactivated and the Pho-regulon was constitutively active (reviewed in Lamarche *et al.*, 2008). Most of the studies concerning the effects of nutrient limitation, and in particular phosphate and the interaction between the PhoR-PhoB system with the regulation of virulent features, have been performed using human pathogens or bacteria of agricultural interest. It is imaginable that also in other bacteria, for example, facultative symbionts or pathogens of different metazoa, these aspects could play an important role in regulating mechanisms involved in their interaction with the host. However, to date studies addressing these questions are missing. This information would help to better understand how environmental nutrient stresses affect the lifestyle and the symbiotic/pathogenic behaviors of environmentally relevant bacterial species.

Aim of the work

Although phosphate limitation has a drastic effect on multiple aspects of bacterial physiology, and even though these conditions occur in several areas of the ocean, the response of heterotrophic marine bacteria to phosphate limitation has been poorly investigated. With the present thesis I seek to expand and deepen the knowledge on this topic, filling the gap concerning the response of heterotrophic marine bacteria to this environmentally important nutrient regime.

Applying different methodologies, I aimed to study the response to phosphate limitation of the versatile bacterium *Pseudovibrio* sp. FO-BEG1, investigating multiple aspects of its physiology. In the work presented in **Chapter 2** physiological experiments were combined with proteomic and bioinformatic analyses, with the intention to gain a comprehensive picture of the effects that phosphate limitation induced on the overall physiology of *Pseudovibrio* sp. FO-BEG1.

One of the most intriguing effects observed was the secretion by the bacterium of compounds that conferred a yellow-orange coloration to the cell-free supernatant. This observation suggested that phosphate-limited conditions also influence significantly the compounds released by FO-BEG1 during growth. In order to quantify and chemically characterize the observed diversity, ultra-high resolution mass spectrometry was used to analyze and compare the composition of the secreted metabolites under phosphate-limited and phosphate-surplus conditions (**Chapter 3**). In this study I aimed to elucidate the composition of the compounds that *Pseudovibrio* sp. FO-BEG1 secreted into the medium, considering and quantifying the effect of phosphate limitation.

While investigating the physiological response to phosphate limitation it was observed that these growth conditions also had a drastic effect on the iron metabolism of *Pseudovibrio* sp. FO-BEG1. During the exponential growth phase, the concentration of the total soluble iron decreased in both phosphate regimes and surprisingly, only under phosphate-limited conditions it increased again during stationary phase. There are no available studies which consider the direct interconnection between phosphate and iron metabolism and the observed

phenomenon has never been reported. Therefore, in **Chapter 4**, I investigated this response in more detail, with the idea that this could be a more common process among marine bacteria being so far overlooked. Initially, physiological experiments were carried out to understand the origin of the increasing iron. Subsequently, chemical analysis were performed to further characterize the molecules responsible for this process. Then the observed phenomenon was investigated in strains belonging to the *Roseobacter* clade. Finally, a bioinformatic study was performed in the attempt to identify possible genes involved in the production of molecules that own characteristics consistent with the experimental evidence.

References

References of **Chapter 1** are reported together with the references of **Chapter 5** starting from page 217.

Chapter 2

Phosphate limitation induces drastic physiological changes, virulence related gene expression, and secondary metabolite production in strain *Pseudovibrio* sp. FO-BEG1

Stefano Romano[1*], Heide N. Schulz-Vogt[2*], José M. González[3], Vladimir Bondarev[1]

[1] Max Planck Institute for Marine Microbiology, Germany.
[2] Leibniz-Institute for Baltic Sea Research Warnemuende (IOW), Section Biological Oceanography, Rostock, Germany.
[3] Department of Microbiology, University of La Laguna, La Laguna, Tenerife, Spain.

*Corresponding authors

Contributions:
The concept of this study was developed together with Vladimir Bondarev and Heide N. Schulz-Vogt. I performed all physiological experiments with the collaboration of Vladimir Bondarev. The proteomic data of this chapter were already part of Chapter V in the Ph.D. thesis of Vladimir Bondarev (University of Bremen, 2012). These data were newly analyzed by me and integrated with the additional physiological data I obtained. I wrote the manuscript considering the comments of all co-authors.

Manuscript in preparation

Abstract

Phosphorus is a vital nutrient for all living organisms, however, phosphate, the primary source of phosphorus for bacteria, is typically present in the ocean in concentrations limiting bacterial growth. The phosphate starvation response has been deeply investigated in the model organisms *Escherichia coli* and *Sinorhizobium meliloti*, but studies on heterotrophic marine bacteria are still scarce. In this study, we investigated the response to phosphate limitation of *Pseudovibrio* sp. FO-BEG1, a metabolically versatile alphaproteobacterium and potential symbiont of marine sponges, which has the genomic potential to produce bioactive compounds. We compared the physiology, the protein expression and the secondary metabolite production under phosphate-limited and phosphate-surplus conditions. Phosphate limitation triggered the expression of proteins involved in uptake and degradation of phospho-organic compounds, led to a severe cell elongation and to the exchange of membrane lipids in favor of phosphorus-free lipids, such as sulfoquinovosyl diacylglycerols. Moreover, we observed an increased expression of proteins that could play an important role in the interaction with the eukaryotic host. When phosphate-limited cultures entered stationary phase cells released compounds that conferred an intense yellow-orange coloration to the cell-free supernatant. One of these compounds was identified as the potent antibiotic tropodithietic acid (TDA). Our data suggest that strain FO-BEG1 has evolved a sophisticated response to phosphate limitation, which involves multiple physiological strategies affecting phosphorus, carbon and sulfur metabolisms, cell morphology, secondary metabolite production and expression of virulence related genes.

Introduction

Phosphorus (P) is an essential macronutrient for all living organisms, since it is an important component of bio-molecules and it is a fundamental element in cellular regulation processes. The preferential source of phosphorus for bacteria is phosphate, even though organic molecules containing phosphorus, such as phospho-esters (molecules with C-O-P bonds), and phosphonates (molecules with C-P bonds), which are components of the dissolved organic phosphorus pool (DOP), can also be used (Dyhrman *et al.*, 2007). In many marine environments, the concentration of phosphate is in the nanomolar range, and there is growing evidence that phosphorus is limiting bacterial growth and productivity in many areas of the ocean, at least during part of the year (Cotner *et al.*, 1997; Wu *et al.*, 2000; Thingstad *et al.*, 2005). In addition, unlike nitrogen, phosphorus cannot be fixed from the atmosphere, thus, over geological time scales it is considered to be the ultimate limiting macronutrient in marine ecosystems (Paytan and McLaughlin, 2007).

Due to its crucial role in cell metabolism and to its scarcity in natural environments, bacteria evolved several mechanisms to sense phosphate concentrations and regulate their phosphorus metabolism accordingly. Phosphate starvation induces the expression of the so-called phosphate starvation inducible (*psi*) genes which encode several proteins involved in phosphorus uptake and metabolism. Among these, there are genes coding for high affinity transporters (ABC transporters) for phosphate and enzymes involved in the uptake and degradation of organic molecules containing

phosphorus. All these genes are members of the Pho-regulon, a global regulatory circuit involved in bacterial phosphorus management (Hsieh and Wanner, 2010). This circuit is controlled by a two component regulatory system (TCRS), composed of a transmembrane histidine kinase PhoR and a response regulator PhoB. When the concentration of the environmental phosphate is low, PhoR undergoes autophosphorylation using ATP and acts as a kinase for the response regulator. PhoB then binds to a specific target sequence named Pho-box in the upstream region of the genes belonging to the Pho-regulon, regulating their transcription (Hsieh and Wanner, 2010). In *Escherichia coli* the phosphate limitation response can involve the regulation of up to 400 genes, which represent almost 10% of the *E. coli* genome (VanBogelen *et al.*, 1996). This high number of proteins reflects the importance of phosphate in the regulation of cellular physiology, including processes not directly correlated with phosphorus metabolism, such as the production of secondary metabolites (Martín, 2004). Moreover, it is well documented, that the PhoR-PhoB system is involved in the regulation of virulence related genes (Lamarche *et al.*, 2008).

The Pho-regulon and the phosphate limitation response have been deeply studied in the model organism *E. coli*, and in other important pathogenic bacteria, plant-symbionts, and bacteria of biotechnological interest (Wanner, 1996; Ishige *et al.*, 2003; Krol and Becker, 2004; von Kruger *et al.*, 2006; Rodríguez-García *et al.*, 2007). Despite the growing evidence suggesting that phosphate limitation is a common condition in many areas of the ocean, among marine microorganisms the phosphate limitation response was mainly investigated in phototrophs (Tetu *et al.*, 2009; Wurch *et al.*, 2011; Dyhrman *et al.*,

2012) and studies on the phosphate limitation response of heterotrophic marine bacteria are scarce. In this study, we investigated the response to phosphate limitation of the chemorganoheterotrophic strain FO-BEG1, an alphaproteobacterium closely related to *Pseudovibrio denitrificans*. Bacteria belonging to this genus have been isolated worldwide, often from sponges, and are, therefore, assumed to be their symbionts (Enticknap *et al.*, 2006; Taylor *et al.*, 2007).

In a previous study (Bondarev *et al.*, 2013; **Contributed works**) we analyzed the genome of strain FO-BEG1 and showed that it is metabolically versatile and possesses several genes that could play a role in prokaryote-eukaryote interaction, indicating that FO-BEG1 is well adapted to a free-living and a symbiotic lifestyle. Furthermore, we identified genes and gene clusters involved in the synthesis of bioactive secondary metabolites, in accordance with the numerous studies that showed a production of these compounds in many strains belonging to the *Pseudovibrio* genus (Sertan-de Guzman *et al.*, 2007; O'Halloran *et al.*, 2011).

In this study, we investigated the physiology, the protein expression, and the secondary metabolite production of strain FO-BEG1, comparing phosphate-limited ($-P_i$) and phosphate-surplus ($+P_i$) conditions. In addition, we performed a bioinformatic analysis for the identification of genes that present Pho-boxes in their upstream regions and are thus potential members of the Pho-regulon of strain FO-BEG1. We observed that phosphate limitation triggers a severe physiological reorganization, influencing not only the phosphorus metabolism but also the carbon and sulfur metabolism. Moreover, we gained a

first insight into the pronounced effect of phosphate limitation on secondary metabolite production and on the regulation of virulence related genes, which could play a crucial role in the establishment and maintenance of the host-symbiont interaction.

Materials and methods

Growth conditions

Strain FO-BEG1 was cultivated in the carbohydrate/mineral medium (CM) as described by Shieh *et al.* (2004) and modified by Bondarev *et al.* (2013; **Collaborated works**). For the $+P_i$ conditions phosphate was added to a final concentration of 1.4 mmol L^{-1}. For the $-P_i$ conditions the sole phosphate sources supplied was the buffer used for the preparation of vitamins, resulting in a final concentration of 0.1 mmol L^{-1}. Erlenmeyer flasks of 250 mL were filled with 100 mL of medium and inoculated with 100 µL of a pre-culture grown under $+P_i$ conditions. Cultures were incubated at 28 °C in the dark and shaken at 120 rpm. We monitored bacterial growth by means of Optical Density (OD) measured at 600 nm using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). The OD_{600} was then correlated with the cell number, determined using a Thoma counting chamber (Brand GmbH, Wertheim, Germany; data not shown). Micrographs were taken during bacterial growth using an Axioplan universal microscope (Carl Zeiss GmbH, Oberkochen, Germany), and at least 100 cells for each time point and each condition were measured using the program AxioVision Rel. 4.8 Imagining system (Carl Zeiss GmbH, Oberkochen, Germany).

During the entire growth period the UV-visible spectra of the cell-free supernatants were recorded using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Chemical analyses, determination of alkaline phosphatase activity, and detection of inclusions

Glucose and acetate concentrations were determined using a high performance liquid chromatography (HPLC) system (Sykam GmbH, Gilching, Germany) as described previously (Bondarev *et al.*, 2013; **Contributed works**). Phosphate concentrations were determined colorimetrically by the ascorbic acid method (Hansen and Karoleff, 1999), using a SpectroDirect Spectrophotometer (Aqualytic, Dortmund, Germany). The activity of alkaline phosphatase (AP) during bacterial growth was measured monitoring the degradation of 4-nitrophenyl phosphate bis(tris) salt (pNPP; ≥ 97.00%; Sigma Aldrich, St. Louis, MO, USA). Experiments were performed two times independently, always analyzing biological triplicates. Tropodithietic acid (TDA) was measured in collaboration with BioViotica Naturstoffe GmbH (Göttingen, Germany) by means of reverse phase HPLC and using pure TDA as reference standard (purity ≥ 98%; BioViotica Naturstoffe GmbH, Göttingen, Germany). Presence and quantification of polyphosphate (poly-P_i) was investigated by staining the cells with 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI) performed according to the method described by Kulakova *et al.* (2011). Nile Red was used for staining polyhydroxyalkanoate (PHA) inclusions. Polar lipid analysis was carried out by the identification service of the DSMZ and Dr. B. J. Tindall (DSMZ,

Braunschweig, Germany). Detailed information about materials and protocols used can be found in the Supplementary materials and methods section.

Proteome analysis

To investigate the effect of phosphate limitation on protein expression we chose an MS shotgun proteomic approach coupled with isotope coded protein labeling (ICPL) (Schmidt *et al.*, 2005). Two biological replicates were independently analyzed for each time point. Biomass from $-P_i$ cultures was harvested in the growth stage where we previously detected the first increase in AP activity, corresponding to the middle exponential growth phase ($OD_{600} \approx 0.55$). These samples were used as reference in the proteomic analysis, and they were compared with the protein profiles of $+P_i$ growing cells, collected in the middle exponential growth phase ($OD_{600} \approx 0.82$). Moreover, to evaluate the effect of prolonged phosphate starvation we also collected samples in the first half of the stationary phase of $-P_i$ conditions ($OD_{600} \approx 1.28$), and compared them with the samples collected in the exponential phase.

Proteins from different samples were labeled at the free amino groups with one of the four isotopic forms of nicotinoyloxysuccinamide. The labeled extracts were combined resulting in one mix for each replicate, and their complexity was reduced via SDS-PAGE. Proteins were then digested into peptides and analyzed via LC-ESI-MS/MS. Since peptides with identical amino acid sequences derived from the differentially labeled samples differ in mass, they appeared in the acquired MS spectra as sister peaks (multiplets; complete multiplets if the peptides were detected in all samples; incomplete if not), with a

mass shift corresponding to the difference between the masses of the labels. The abundance ratio of each protein was then calculated using the ratios of the ion intensities of the sister peptides. An abundance change of ≤−2.0-fold or ≥2.0-fold was regarded as significant regulation. The regulated proteins were then divided into 15 arbitrary categories: [A] carbon metabolism; [B] phosphorus metabolism; [C] amino acid and nucleotide metabolism; [D] protein and peptide metabolism; [E] transport and secretion; [F] fatty acid and lipid metabolism; [G] cell wall and membrane biogenesis; [H] translation, transcription and DNA metabolism; [I] regulation of transcription; [J] oxidative stress response and detoxification; [K] cofactor and vitamin synthesis; [L] chemotaxis and motility; [M] secondary metabolite production; [N] only general function predicted; [O] unknown function. Detailed description of the experimental procedure and the data analysis is given in the Supplementary materials and methods section

Screening of the genome of FO-BEG1 for the presence of Pho-boxes

The *in-silico* screening for the identification of Pho-boxes in the genome of strain FO-BEG1 was based on the method described by Yuan *et al.* (2006). The Pho-box sequences they propose were used to build a position-weight matrix (Staden, 1984; Stormo, 2000) utilized to scan the intergenic regions in the genome of FO-BEG1. This resulted in a first list from which we selected a total of 28 genes with the best alignment scores against the position-weight matrix (scores calculated according to Staden, 1984; **Table S3**) and which encode for proteins detected to be up-regulated in the proteomic approach we performed. These genes were used to build a new position-weight matrix specific

for *Pseudovibrio* sp. FO-BEG1. The latter one was then used to obtain the final list of genes, which presented a potential Pho-box in their upstream regions. Detailed description of the method used is provided in the Supplementary materials and methods section.

Results

Effect of phosphate limitation on bacterial growth, cell morphology, and nutrient uptake

Phosphate limitation significantly repressed bacterial growth, leading to a final cell density 2.5-3.5 times lower than the one observed under $+P_i$ conditions (**Fig. 2.1**). Cultures growing under $+P_i$ conditions were characterized by a doubling time (t_d) of 4.33 h and a growth rate (μ) of 0.16 gen h^{-1}. $-P_i$ cultures had a similar growth during the first half of the exponential phase (t_d of 4.77 h, μ 0.15 gen h^{-1}), but in the second half, when phosphate was completely taken up by the cells (**Fig. 2.1**), the t_d increased to 7.68 h and the μ dropped to 0.09 gen h^{-1}. Cells growing under $+P_i$ conditions did not consume all phosphate provided, indicating that they were not phosphate-limited, whereas phosphate was completely taken up under $-P_i$ conditions during the first 30 h of growth (**Fig. 2.1**). Exactly after this point we detected a considerable increase in alkaline phosphatase (AP) activity (**Fig. S2.1**), which was negligible during the whole growth period under $+P_i$ conditions (data not shown).

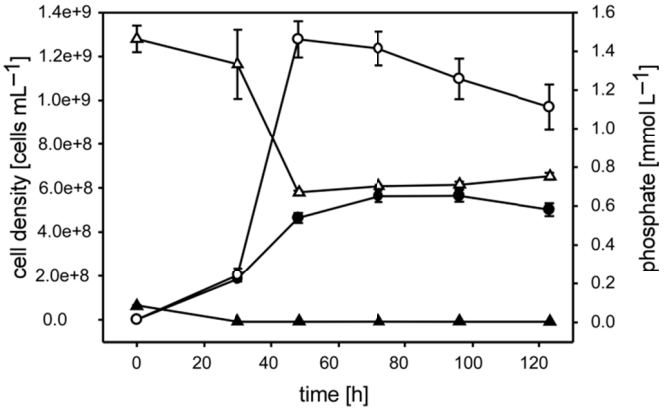


Figure 2.1 | Cell density (circles) and phosphate concentration (triangles) over time of cultures grown under +Pi (empty symbols) and −Pi (filled symbols) conditions. The error bars represent the standard deviation of biological triplicates.

Regardless of the difference in growth, glucose was taken up completely under both conditions (**Fig. 2.2**). Under $-P_i$ conditions, the uptake continued also during the non-proliferating period, indicating a possible accumulation of storage compounds like polyhydroxyalkanoates (PHA), which we showed using Nile Red staining (**Fig. 2.2**). Moreover, under phosphate limitation cells almost doubled their length, leading to a pronounced increase in cell surface (from 8.95 ± 1.59 μm^2 to 15.34 ± 3.49 μm^2; **Fig. S2.2**).

General results of the proteome analysis and the *in-silico* screening for the identification of Pho-boxes

During the proteomic analysis we identified 704 proteins with an overall false discovery rate of 0.22%. The regulated proteins detected during the exponential phase of $-P_i$ conditions were 140, and the highest number of up-regulation occurred in category [C] ("amino acid and nucleotide metabolism") and [E] ("transport and secretion"), whereas most of the down-regulated

proteins were detected in category [H] ("translation, transcription and DNA metabolism"; **Table S2.1**; **Fig. 2.3**). These represented 45.5% of all down-regulated proteins detected in the exponential phase under $-P_i$ conditions and reflected the repressed growth. The difference in protein expression between the exponential and the stationary phase under $-P_i$ conditions was mainly due to the arrest of cell proliferation. In fact, 84.5% of the 102 regulated proteins were down-regulated and belonged mainly to category [C] and [H] (**Table S2.2**; **Fig. 2.3**).

The *in-silico* analysis for the identification of PhoB binding sites revealed that strain FO-BEG1 possesses an 18 nucleotides long Pho-box, characterized by two repetitions 5´-CTGTCAT-3´ separated by a region of four nucleotides (**Fig. S2.3**). In the first repetition the first position does not show a high degree of conservation, however, due to the absence of molecular data that could elucidate the Pho-box structure of this bacterium, we assumed that it resembles the well conserved 18 nucleotides sequence known from *Escherichia coli* and *Sinorhizobium meliloti* (Makino *et al.*, 1998; Yuan *et al.*, 2006). The list of all genes (1004) which presented a potential Pho-box in their upstream regions is reported in **Table S2.4**. Of these, 61 were detected to be regulated during the proteomic analysis (**Table S2.1** and **S2.2**).

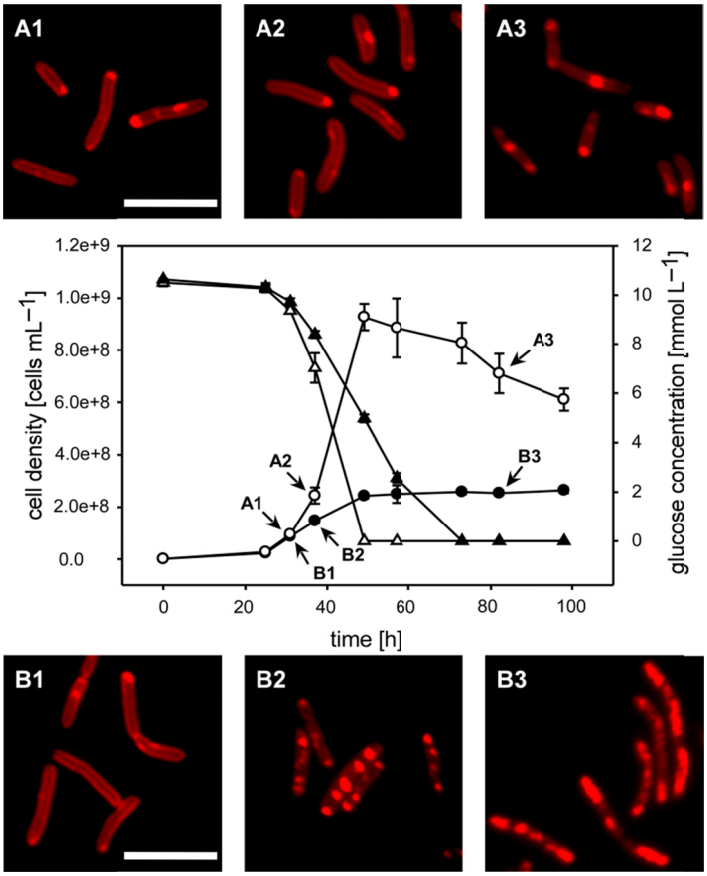


Figure 2.2 | Cell density (circles) and glucose (triangles) over time of cultures grown under +P_i (empty symbols) and −P_i (filled symbols) conditions. Error bars represent the standard deviation of biological triplicates. The upper and lower panels show micrographs of the Nile red staining performed for the detection of polyhydroxyalkanoates under +P_i (A1-A3) and −P_i (B1-B3) conditions. The micrographs were taken after 24 (A1, B1), 37 (A2, B2), and 81 (A3, B3) hours of growth. Scale bar = 5 μm.

Phosphate limitation leads to a profound reorganization of the phosphorus metabolism

As reported for other bacteria (Krol and Becker, 2004; Hsieh and Wanner, 2010), in strain FO-BEG1 phosphate limitation triggered the up-regulation of several genes involved in phosphorus uptake and metabolism

(category [B] "phosphorus metabolism" and [E] "transport and secretion"; **Fig. 2.3**; **Table S2.1**). Among these, we detected three out of four protein sub-units (PSE_1688; PSE_1690; PSE_1691; **Table S2.1**) of the Pst high affinity ABC transport system for phosphate. In proximity of its gene cluster, there is a gene (PSE_1692) encoding for a protein homologous to PhoU, expressed only under $-P_i$ conditions. Its gene belongs to the *pst* operon in *E. coli* and it seems to play a role in signal transduction (Hsieh and Wanner, 2010). As in *E. coli* in strain FO-BEG1 the Pst system is likely controlled directly by PhoB, due to the presence of a potential Pho-Box in the upstream region of the first gene (*pstS*) of the *pst* cluster.

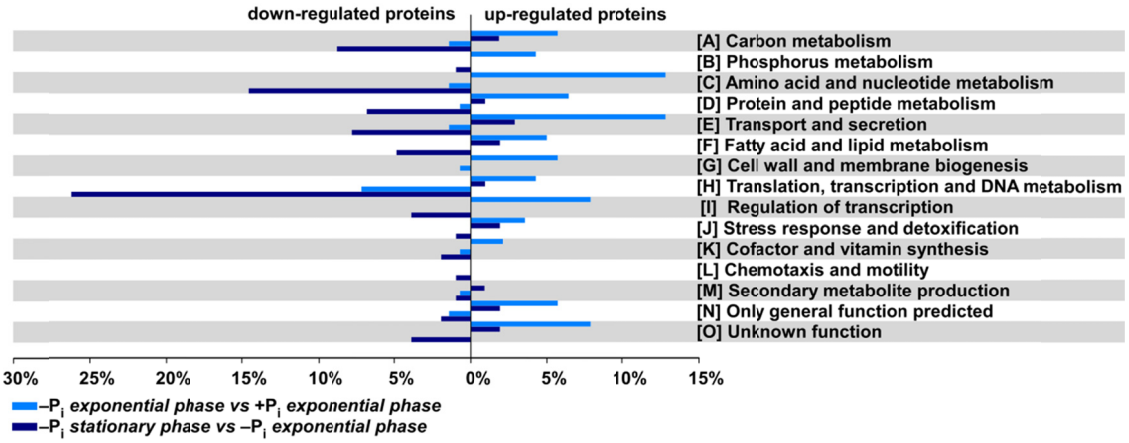


Figure 2.3 | Percentages of proteins detected to be up- or down-regulated during growth under phosphate limitation. The shotgun proteomic analysis was performed comparing the protein expression of cells in the exponential growth phase of $-P_i$ and $+P_i$ conditions (light blue), and the protein expression of cells under $-P_i$ exponential and stationary phase (dark blue).

After the complete consumption of the provided phosphate in $-P_i$ cultures, and the consequent increase in AP activity, we still observed cell proliferation (**Fig. 2.1**; **Fig. S2.1**). Since no other source of phosphorus was provided, we quantified the stored polyphosphate (poly-P_i), previously detected in the cells, in order to verify their use as a phosphorus source. Indeed, under $-P_i$ conditions we detected a poly-P_i decrease from 87.3 ± 3.4 fg cell^{-1} to 30.2 ± 2.4 fg cell^{-1} occurring right after that the provided phosphate was completely taken up by the cells (**Fig. S2.4**). Under phosphate-limited conditions, we also detected an up-regulation of a protein homologous to poly-P_i kinase 1 (Ppk1, PSE_2769; **Table S1**), which catalyzes the reversible synthesis of poly-P_i from ATP (Rao *et al.*, 2009).

In consistence with the enzymatic assay performed to detect AP activity, a protein homologous to the AP PhoX (PSE_1012) was found to be two-fold up-regulated, and a potential Pho-box was identified in the upstream region of its gene (**Table S2.1**). Moreover, we detected two up-regulated proteins, PhnD (PSE_3629) and PhnC (PSE_3630), of the ABC transport system for phosphonates, three proteins involved in their degradation (PhnI PSE_4851; PhnJ PSE_4852; PhnM PSE_4857), and three subunits of the ABC transport system for *sn*-glycerol-3-phosphate (UgpB PSE_0472; PSE_0680; UgpC PSE_0683; **Table S2.1**). Potential Pho-boxes were identified in the upstream regions of PSE_0680 and PSE_3630 genes. Finally, we detected an up-regulation of three nucleosidases (PSE_1783; PSE_1587; PSE_2573; **Table S2.1**), and for two of them we identified a potential Pho-box in the promoter region of their genes.

Effect of phosphate limitation on cellular lipid composition

The thin layer chromatography (TLC) analysis performed to characterize the cellular polar lipids revealed that under $+P_i$ conditions most of the detected lipids contained phosphorus, whereas under $-P_i$ conditions amino lipids (AL) and glycolipids (GL) were dominant (**Fig. 2.4**). Consistently, under phosphate-limited conditions several proteins involved in lipid metabolism were up-regulated (category [F] "fatty acid and lipid metabolism" and [G] "cell wall and membrane biogenesis"; **Fig. 2.3**; **Table S2.1**). For instance, we detected up-regulated a protein containing a phospholipase D/transphosphatidylase domain (PSE_0624) and a protein homologous to a phosphatidic acid phosphatase (PA-phosphatase, PSE_2249; **Table S2.1**), both involved in the catabolism of phospholipids. The gene for the PA-phosphatase presented a potential Pho-box in its upstream region. In addition, we detected an up-regulated protein homologous to acyl-CoA dehydrogenase (PSE_ 0035) and two proteins homologous to enoyl-CoA hydratase (PSE_0406; PSE_3595; **Table S2.1**), which are involved in the oxidation of fatty acids and could be required for the degradation of the exchanged lipids.

Under phosphate limitation, four different types of GL were detected (**Fig. 2.4**). One (GL4) was consistent with being the sulfur containing lipid sulfoquinovosyl diacylglycerol (SQDG). Consistently, under $-P_i$ conditions we detected a 22-fold up-regulated protein homologous to SqdB (PSEp_0373; **Table S2.1**), an enzyme which catalyzes the first step in the SQDG synthesis (Benning, 1998). A similar enzyme (PSE_2321) was up-regulated as well, but it

was not considered due to the low statistical significance. Intriguingly, the latter gene had a potential Pho-box in its upstream region (**Table S2.4**).

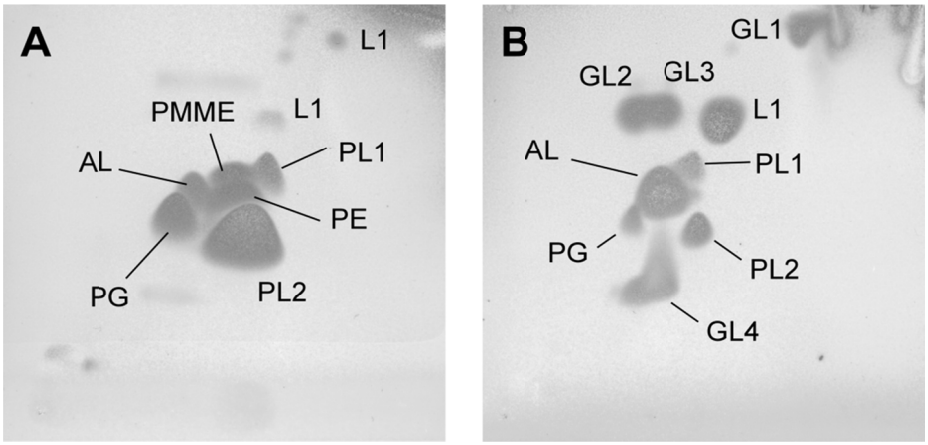


Figure 2.4 | Thin layer chromatography (TLC) analysis of the polar lipids of cells growing under +P_i (A) and −P_i (B) conditions. Spots represent: phospholipids (PL), phosphatidylglycerol (PG), phosphatidylmonomethylethanolamine (PMME), phosphatidylethanolamine (PE), aminolipids (AL), glycolipids (GL), general lipids (L).

Effect of phosphate limitation on the carbon metabolism

In accordance with the detection of PHA, under −P_i conditions two phasin-like proteins (PSE_1257; PSE_3807; **Table S2.1**) were up-regulated. These are associated with PHA granules and have been shown to affect polymer synthesis (de Almeida *et al.*, 2007). We identified a Pho-box in the upstream region of gene PSE_3807, indicating that its transcription is subject to the direct control of PhoB. Consistently, PHA accumulation started after 37 h of growth, when phosphate in the medium was completely taken up by the cells (**Fig. 2.1** and **Fig. 2.2**). Under −P_i conditions, we detected two up-regulated enzymes (PSE_4767; PSE_3294) involved in the conversion of phosphoenolpyruvate into acetyl-CoA. Nevertheless, the citrate synthase (PSE_3391), the first enzyme in the

tricarboxylic acid cycle (TCA), was down-regulated (**Table S2.1**). In addition, we detected two up-regulated enzymes involved in the acetate kinase-phosphotransacetylase (Pta-AckA) pathway (PSE_1087; PSE_1086), which converts acetyl-CoA into acetate via acetyl phosphate. In agreement with this, we observed acetate production, starting during the second half of the exponential phase (0.15 mmol L^{-1} ± 0.02) and reaching the maximum (0.73 mmol L^{-1} ± 0.09) at the beginning of the stationary phase. The acetate was then taken up by the cells during the rest of the growth period (data not shown).

Control of oxidative stress

Under $-P_i$ conditions we identified several proteins, such as a protein homologous to an alkyl hydroperoxidase (AhpD, PSE_0181; **Table S2.1**), potentially involved in the oxidative stress response. Ahps are enzymes responsible for detoxification of peroxides and were shown to be specifically required for viability of *E. coli* cells starved for phosphate (Moreau *et al.*, 2001). Also up-regulated were two proteins homologous to bacterioferritin (PSE_1030; PSE_3844; **Table S2.1**), which is responsible for iron storage and protection against oxidative stress via iron detoxification (Carrondo, 2003).

Under $-P_i$ conditions we detected two highly up-regulated proteins homologous to PotD and PotA (PSE_1679; PSE_1681; **Table S2.1**), which belong to the Pot ABC transporter system for putrescine and spermidine. These molecules are polyamines, and they are described to play an important role in the protection of macromolecules against radicals (Wortham *et al.*, 2007). In addition, the *potD* gene presented a Pho-box in its upstream region (**Table S2.1**),

which is consistent with previous studies conducted on *S. meliloti* (Jensen *et al.*, 2006; Yuan *et al.*, 2006). Up-regulated was also a protein responsible for the synthesis of glutathione (PSE_0381), a molecule that can help cells to protect macromolecules via scavenging reactive oxygen species (ROS). Protection against oxidative stress also requires a secondary line of defense consisting of repairing enzymes (Cabiscol *et al.*, 2000). Indeed, in strain FO-BEG1, phosphate limitation triggered up-regulation of several proteases, peptidases, chaperon-like proteins and proteins involved in DNA repairing (category [D] "protein and peptide metabolism" and [H] "translation, transcription and DNA metabolism"; **Fig. 2.3**; **Table S2.1**).

Phosphate limitation affects cell-envelope stability and secondary metabolite production

Under $-P_i$ conditions, we detected an up-regulated protein homologous to TolB (PSE_4678), belonging to the Tol-Pal complex and two bacterial outer membrane proteins containing an OmpA domain (PSE_1259; PSE_4677; **Table S2.1**) described to interact with the Tol-Pal system. Different functions were purposed for this system, but it seems to be mainly involved in the maintenance of cell-envelope stability (Lloubès *et al.*, 2001). The gene PSE_4677 presented a potential Pho-box in its promoter region and it is located in the same genomic region of the *tolB* gene, suggesting that the transcription of the complete region increased under phosphate limitation. Also, two proteins homologous to the phage shock proteins PspA (PSE_4192; PSE_4592; **Table S2.1**) were detected to be up-regulated. They are members of the phage shock regulon,

which is induced by impairment of inner membrane integrity, and they regulate the transcription of enzymes involved in the maintenance of membrane stability, proton motive force and protein secretion (Joly *et al.*, 2010).

Phosphate limitation triggered the secretion of compounds that gave a yellow-orange coloration to the cultures and to the cell-free supernatant. This showed a characteristic UV-visible absorption spectra, with a maximum absorbance around 320 nm and a broad shoulder at 400 nm (**Fig. S2.5**). The color appeared when cells entered stationary phase, and its intensity increased during the remaining incubation time. The high performance liquid chromatography (HPLC) analysis of the $-P_i$ cell-free supernatants revealed the presence of a compound with retention time and UV-visible spectra consistent with the ones of tropodithietic acid (TDA). This is an antibiotic compound usually produced together with a yellow pigment by members of the *Roseobacter* clade (Bruhn *et al.*, 2005). Its production was detected only under $-P_i$ conditions (1.55 mg L^{-1}) and only during stationary phase. Consistently, TdaD (PSE_2260), an enzyme involved in TDA production, was up-regulated during $-P_i$ stationary phase and down regulated in the exponential phase (**Table S2.1** and **S2.2**). Moreover, a potential Pho-box was identified in the promoter region of two genes directly upstream *tdaD*, *tdaC* (PSE_2261) and *paaK* (PSE_1790; **Table S2.4**), both potentially involved in TDA production (Bondarev *et al.*, 2013; **Contributed works**).

Effect of phosphate limitation on proteins potentially involved in host-symbiont interaction

Under $-P_i$ conditions, we detected the up-regulation of proteins, such as TolB, OmpA and PspA, which can be involved in host-symbiont interactions (Bowe *et al.*, 1998; Smith *et al.*, 2007; Godlewska *et al.*, 2009; Karlinsey *et al.*, 2010). Moreover, we detected an up-regulated protein homologous to the transcriptional regulator PhoP (PSE_2475; **Table S2.1**). This is a member of the two component regulatory system PhoP-PhoQ, which controls the expression of virulence genes. The expression of a protein homologous to TolC was increased more than four-fold under phosphate limitation (PSE_3720; **Table S2.1**). This is a versatile outer membrane protein utilized for efflux of noxious molecules and secretion of antibiotics or virulence factors (Federici *et al.*, 2004; Holland *et al.*, 2005). The *tolC* gene is located in a genomic region encoding proteins for a type I secretion system (T1SS). Adjacent to this region, there is the largest gene of the whole genome (PSE_3716). It encodes a protein of 4,159 amino acids containing five T1SS-143 repeat domains usually found in proteins of the genus *Vibrio* and *Legionella*, which share properties with RTX (repeats in toxin) proteins. These proteins are secreted by Gram-negative bacteria via the T1SS and represent virulence factors with cytotoxic functions, proteolytic and lipolytic activities, or can have a role in adhesion and biofilm formation (Linhartová *et al.*, 2010; Satchell, 2011). The RTX-like protein was up-regulated in one replicate under $-P_i$ conditions and, in agreement with Yoshida *et al.* (Yoshida *et al.*, 2010), its gene and the *tolC* gene had a potential Pho-box in their upstream regions (**Table S2.1**).

Discussion

Reduced growth and rearrangement of phosphorus metabolism

Phosphate has essential metabolic, structural and regulatory roles in all bacteria (Stock *et al.*, 1989; Wanner, 1996). Environmental phosphate concentrations influence not only bacterial phosphorus metabolism but also bacterial life-style and inter-species and inter-kingdom relations via affecting secondary metabolite production and expression of virulence factors (Martín, 2004; Lamarche *et al.*, 2008). Much attention has been paid to the effect of phosphate limitation on the antibiotic production of *Streptomyces* species, and on the physiology of human pathogens and plant-symbiotic bacteria of agricultural interest (Krol and Becker, 2004; Martín, 2004; Yuan *et al.*, 2006; Crépin *et al.*, 2011). In contrast, despite growing evidence suggesting that this condition is common in many areas of the ocean (Wu *et al.*, 2000; Thingstad *et al.*, 2005), the response of heterotrophic marine bacteria to phosphate limitation has been rarely investigated. In the present work, we studied in detail the response to phosphate limitation of a heterotrophic marine bacterium belonging to the genus *Pseudovibrio*. This genus is receiving progressively more scientific attention owing to its ability to produce bioactive secondary metabolites and to its frequent detection in association with marine invertebrates all over the world (Sertan-de Guzman *et al.*, 2007; O'Halloran *et al.*, 2011; Bondarev *et al.*, 2013; **Collaborated works**).

Fig. 2.5 represents a schematic overview of the physiological adaptation observed in *Pseudovibrio* sp. FO-BEG1 during growth under

phosphate limitation. As described in other strains (VanBogelen *et al.*, 1996; Ishige *et al.*, 2003; Krol and Becker, 2004) phosphate limitation significantly repressed bacterial growth and this was reflected in the down-regulation of proteins involved in DNA replication and protein synthesis (category [C] and [E]; **Fig. 2.1**; **Table S1**). The μ dropped concomitantly with the complete consumption of phosphate (**Fig. 2.1**) and a consistent increase in alkaline phosphatase (AP) activity was observed immediately afterwards. Therefore, it is reasonable to assume that in FO-BEG1 the phosphate limitation response is activated at this growth stage, with an environmental phosphate concentration below 10 µmol L^{-1} (**Fig. 2.1**; **Fig. S2.1**). APs are enzymes which cleave the phosphate groups in organic molecules containing phospho-ester bonds, and their expression has been widely used as an indicator of phosphorus limitation in bacteria (Torriani, 1960; Hoppe, 2003). As described for other bacteria (Wanner, 1996; Krol and Becker, 2004; von Kruger *et al.*, 2006), the strategy of FO-BEG1 to scavenge phosphate from the environment is characterized by the up-regulation of ABC transporters for phosphate and proteins involved in the uptake and degradation of organic molecules containing phosphorus. These genes are typically present and expressed in bacteria adapted to thrive in environments limited in phosphate (Martiny *et al.*, 2011). Therefore, these data underline the versatility of *Pseudovibrio* and indicate that it is well adapted to thrive as a free-living organism in the open ocean, where phosphate has often been described as limiting nutrient and DOP can represent the only bio-available phosphorus pool (Dyhrman *et al.*, 2007). This is consistent with recent evidence, which suggest that DOP is an important phosphorus source for marine bacteria (Gilbert *et al.*, 2009).

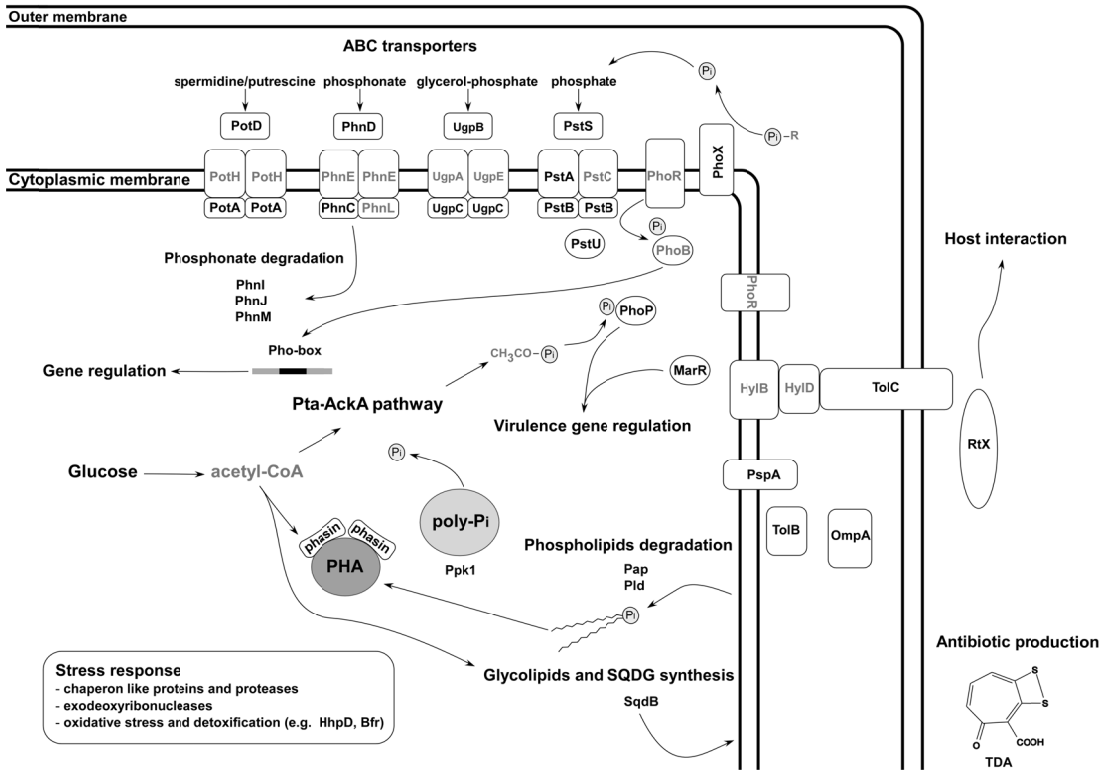


Figure 2.5 | Schematic overview of the major physiological adaptation of strain FO-BEG1 to phosphate limitation. The proposed model was inferred considering the proteins and the physiological traits induced under $-P_i$ condition (reported in black). Proteins and metabolite not directly detected during the experiments are reported in grey.

The cell proliferation observed after the consumption of the provided phosphate was likely sustained by the degradation of the stored poly-P_i, known to be an intra-cellular phosphorus source (Kulaev and Kulakovskaya, 2000). However, cell proliferation stopped even though poly-P_i was not completely degraded (**Fig. S2.4**). Several studies showed the importance of poly-P_i in cell survival under stress condition, in translation efficiency, and in stationary phase gene transcription (Tsutsumi *et al.*, 2000; McInerney *et al.*, 2006; Rao *et al.*, 2009). Therefore, it is reasonable to assume that, even under phosphate limitation,

Pseudovibrio sp. FO-BEG1 did not degrade poly-P_i completely due to its important role in the regulation of the overall cell machinery.

Even though in the genome of strain FO-BEG1 several enzymes involved in poly-P_i degradation are present (e.g. exopolyphosphatase, PSE_2770) we did not detect any of them during the proteomic analysis. Instead, we detected an up-regulated protein homologous to Ppk1, which is an enzyme involved in the reversible synthesis of poly-P_i (Rao *et al.*, 2009), and as described in other species (Geißdörfer *et al.*, 1998; Ghorbel *et al.*, 2006) it can also be responsible for their degradation. Interestingly, in *Escherichia coli* and *Pseudomonas* sp. strains it was shown that mutations in the *ppk1* gene affected motility, tolerance to stress, quorum sensing, biofilm formation, and virulence (Fraley *et al.*, 2007; Peng *et al.*, 2012). Thus, we cannot exclude that the up-regulation of this protein in FO-BEG1 is related to its role in cellular processes other then poly-P_i metabolism.

In the model organisms *E. coli* and *Sinorhizobium meliloti* the phosphate limitation response is controlled by the two component regulatory system (TCRS) PhoR-PhoB. Both were detected in the genome of FO-BEG1 (PSE_1693 and PSE_1687), but not in the proteomic analysis. However, their genes are in the same genomic region as the Pst system which was greatly up-regulated under –P_i conditions. This indicates that the whole genomic region was likely up-regulated under phosphate-limited conditions. Moreover, we detected many similarities to other organisms concerning the regulated proteins and the genes which presented a potential Pho-box in their promoter regions. Therefore, we assume

that also in *Pseudovibrio* the response to phosphate limitation is controlled by the PhoR-PhoB system.

Adjustment of the carbon metabolism, membrane lipids rearrangement, and protection against oxidative stress

Confirming the importance of phosphorus in the regulation of the overall cell physiology we showed that phosphate limitation greatly affects also the carbon metabolism of *Pseudovibrio* sp. FO-BEG1. Phosphate-limited cells took up all glucose provided and, due to a lower cell density, the consumption of carbon per cells was consequently higher than in $+P_i$ cultures. Some carbon might have been used for the synthesis of new lipids, during the membrane rearrangement, or for the accumulation of polyhydroxyalkanoates (PHA). PHA accumulation may represent a strategy which bacteria adopt, under conditions of nutrient limitation and carbon surplus, to survive in future periods of carbon or energy shortage (Malmcronafriberg *et al.*, 1986; Anderson and Dawes, 1990; Løvdal *et al.*, 2008). In consistency with the PHA accumulation we detected up-regulated two phasin-like proteins and in the promoter region of one of their genes a Pho-box was identified (**Table S2.1**). Altogether these data are in agreement with previous reports, which showed that PHA synthesis can be a process part of the Pho-regulon (Schembri *et al.*, 1995). PHA could be produced via condensation of acetyl-CoA, since, as suggested by the down-regulation of the citrate synthase, its flow through the TCA cycle was repressed. Another possibility could be that PHA were synthesized starting from the degradation of fatty acids, as it was described that this pathway would require a

PHA polymerase, enoyl-CoA hydratase, and phasin-like proteins (reviewed in Madison and Huisman, 1999), of which the latter two were detected up-regulated in FO-BEG1 under phosphate limitation. Finally, the acetate production and the up-regulation of the two proteins responsible for the kinase-phosphotransacetylase pathway (Pta-AckA) indicated acetogenesis, a process required for the regeneration of NAD^+ and CoA whenever the full TCA cycle does not operate properly or when the carbon flux into cells exceeds its capacity (reviewed in Wolfe, 2005).

Pseudovibrio sp. FO-BEG1 undergoes a pronounced cell elongation under phosphate-limited conditions. This has been frequently described in phosphate-starved cells, and can be due to the activation of the SOS stress response (Løvdal *et al.*, 2008; van der Veen *et al.*, 2010; Goclaw-Binder *et al.*, 2012). Longer cells would result in an increase of the cellular phosphorus demand for phospholipid synthesis. However, we were able to show a rearrangement in the composition of the cellular lipids in favor of phosphorus-free lipids, such as AL and GL (**Fig. 2.4**). The latter are more common in plants, algae, and Gram-positive bacteria (Holzl and Dormann, 2007), but can also be important in the restructuration of the membranes of Gram-negative bacteria during phosphate-limited growth (Minnikin *et al.*, 1974; Benning *et al.*, 1995; Geske *et al.*, 2013). Consistently, we detected an up-regulation of several proteins involved in lipid metabolism. The phospholipase D and the PA-phosphatase were likely responsible for the phospholipid degradation, which would allow FO-BEG1 to access an "internal" phosphorus source while facing phosphate limitation. As suggested by previous studies performed on *S. meliloti* (Krol and Becker, 2004;

Yuan *et al.*, 2006; Schlüter *et al.*, 2013) this process seems to be directly controlled by the environmental phosphate concentration, as Pho-boxes were identified in the upstream region of the genes encoding PA-phosphatase. In addition, as in *S. meliloti*, our data indicate that in *Pseudovibrio* sp. FO-BEG1 the production of SQDGs is directly controlled by phosphate concentrations, as their synthesis and the expression of a key enzyme involved in their synthesis were enhanced under $-P_i$ conditions.

Changes of the membrane phospholipids were suggested to activate the *psp* response, which control enzymes involved in membrane stability and maintenance of proton motive force (reviewed in Joly *et al.*, 2010). Therefore, the up-regulation of PspA and other proteins involved in the maintenance of cell-envelope stability supports the hypothesis that *Pseudovibrio* sp. FO-BEG1 experiences membrane stress during growth under phosphate limitation, likely due to the lipid rearrangement.

In oceanic surface waters phosphorus uptake for phospholipids synthesis can represent an important contribution to the total bacterial phosphate incorporation (Van Mooy *et al.*, 2008), since phosphate presents in phospholipids can constitute up to 36% of the total cellular phosphorus demand (Geider and La Roche, 2002). During phosphate limitation phospholipids can decrease by 97% (Minnikin *et al.*, 1972; Benning *et al.*, 1995), therefore, membrane lipid rearrangement provides *Pseudovibrio* with an additional phosphorus source, and decreases its cellular phosphorus demand resulting in a competitive advantage in environments depleted in this element. SQDGs can play an important role in the membrane restructuration under phosphate-limited conditions (Benning *et al.*,

1993; Geiger *et al.*, 1999; Wurch *et al.*, 2011; Dyhrman *et al.*, 2012), but to the best of our knowledge this is the first study where their production was observed as a direct response to phosphate limitation in a heterotrophic marine bacterium. Conversely, it was shown that members of the SAR11 clade and other heterotrophic bacteria of the Sargasso Sea were not able to decrease their phosphorus demand via lipid exchange, despite being well adapted to thrive under oligotrophic conditions (Van Mooy *et al.*, 2009). Our data support recent molecular evidence, which suggested that many heterotrophic marine bacteria have the genomic potential to produce SQDGs (Villanueva *et al.*, 2013).

As observed in other strains (Moreau *et al.*, 2001; Ishige *et al.*, 2003; Yuan *et al.*, 2005), under $-P_i$ conditions we detected several proteins potentially involved in protection against oxidative stress. So far it has not been clarified why cells growing under phosphate limitation experience increased oxidative stress. Yuan *et al.* (2005) suggested that the physiological changes induced by phosphate limitation, such as membrane lipid rearrangement, could make cells more sensitive to ROS. Accordingly, the membranes rearrangement observed in *Pseudovibrio* sp FO-BEG1 could generate a more intense lipid metabolism making these molecules more exposed to radicals. Lipids can be easily oxidized by radicals, generating a chain reaction that damage proteins and DNA (Cabiscol *et al.*, 2000). Even though mono-unsaturated lipids, commonly present in Bacteria, were shown to be less reactive to ROS (Bielski *et al.*, 1983), there is evidence suggesting that bacteria are affected by oxidative lipid damage (Yoon *et al.*, 2002; Becerra *et al.*, 2006; Pradenas *et al.*, 2012), supporting the hypothesis that the lipid

rearrangement, occurring under phosphate limitation, increases the cells sensitivity to oxidative stress.

Secretion of secondary metabolites and expression of virulence related genes

The production of compounds that conferred the yellow-orange coloration and the characteristic UV-visible spectra to the cell-free supernatant (**Fig. S2.5**) suggest that phosphate limitation triggered the production of secondary metabolites in *Pseudovibrio* sp. FO-BEG1. Among those we identified the potent antibiotic tropodithietic acid (TDA) for which stably resistant strains could not yet be obtained (Porsby *et al.*, 2011). Consequently, its production confers to *Pseudovibrio* a great ecological advantage when competing for limiting resources. Moreover, the production of the antibiotic can also be beneficial for the hosts, providing protection against potentially pathogenic bacteria. The production of TDA, which has two atoms of sulfur per molecule, together with the synthesis of sulfur containing lipids shows how phosphate limitation can also affect the sulfur metabolism of *Pseudovibrio* sp. FO-BEG1, increasing its cellular demand.

So far only static growth conditions were described to stimulate TDA production (Bruhn *et al.*, 2007) and its synthesis was never observed under of phosphate-limited conditions. There is evidence that TDA production is regulated by acyl-homoserine lactone (AHL) quorum sensing (QS) (Berger *et al.*, 2011) and in *Pseudomonas* it was shown that phosphate limitation can induce QS (Zaborin *et al.*, 2009). This information would explain the up-regulation of TdaD

and the production of TDA under $-P_i$ stationary phase, when a higher cell density was reached. However, genes for the synthesis of known autoinducers were not identified in the genome of strain FO-BEG1 (Bondarev *et al.*, 2013; **Contributed works**). This observation is consistent with recent evidence that showed how in *Phaeobacter* TDA production can occur independently from AHL QS (Prol Garcia *et al.*, 2013), suggesting multiple regulation processes. Altogether these data do not clarify whether TDA production in FO-BEG1 is a response to phosphate limitation, to QS or to a combination of both processes, and further molecular work is required to elucidate the regulatory network behind its synthesis.

The PhoR-PhoB system and the Pho-regulon were repeatedly shown to influence microbial colonization of the host and to directly control the expression of virulence related genes (Lamarche *et al.*, 2008; Long *et al.*, 2008; Zaborin *et al.*, 2009). Accordingly, we detected several up-regulated protein homologues which have been described to be involved in prokaryote-eukaryote interaction. For example, TolB, OmpA, and PspA were described to induce the immune response in macrophages of damaged plant tissue, to be involved in adhesion and invasion of host cells, to interact with the hosts defense mechanism, and to be up-regulated during macrophage infection and biofilm formation (Bowe *et al.*, 1998; Smith *et al.*, 2007; Godlewska *et al.*, 2009; Karlinsey *et al.*, 2010). Several virulence factors are regulated by the TCRS PhoQ-PhoP, which has been well studied in *Salmonella*. It is activated by Mg^{2+}-limitation, low pH, and acetyl phosphate (Chamnongpol and Groisman, 2000; Groisman, 2001). We detected an up-regulation of PhoP, but in our system, both the Mg^{2+} concentration and the

pH (final pH was 7.8) can be ruled out as possible inducing factors. One potential source of activation is acetyl phosphate, deriving from the up-regulated Pta-AckA pathway (**Table S2.1**). Acetyl phosphate can activate the regulator proteins of TCRSs independently from the respective histidine kinase (Mccleary and Stock, 1994; Wanner, 1996). Therefore, it was suggested that over-expression of the Pta–AckA pathway is a way to connect phosphate limitation with the general induction of a large number of genes (Summers *et al.*, 1998; Klein *et al.*, 2007).

The up-regulation of TolC and an RTX-like protein, and the presence of Pho-boxes in the promoter region of their genes are strong indications that the whole genomic region, which contains also two subunits required for a functional T1SS (*hlyD*, PSE_3718; *hlyB*, PSE_3719), is under the direct control of PhoB and it was up-regulated under phosphate-limited conditions. The RTX-like protein possesses a serralysin-like metalloprotease domain and two galactose-binding domains, which could interact with the glycosylated groups on the eukaryotic cell surface, suggesting that, as described in other bacteria (Morova *et al.*, 2008; Linhartová *et al.*, 2010; Satchell, 2011), FO-BEG1 could use this protein to adhere to the host cells surface and penetrate via disruption of the host tissues.

Interestingly, the *in-silico* analysis for the identification of Pho-boxes revealed that several genes encoding proteins potentially involved in prokaryote-eukaryote interaction (e.g. hemolysin) are likely controlled by PhoB (**Table S2.4**). Altogether these data suggest that phosphate limitation or the general stress response induced by phosphate limitation could enhance the

expression of proteins involved in the establishment and maintenance of a symbiotic relationship with marine invertebrates such as sponges. These are filter-feeding organisms and are able to pump hundreds of liters of seawater per day (Vogel, 1977). Consequently, the colonization of such organisms might represent a strategy adopted by *Pseudovibrio* to overcome nutrient limitation encountered as free-living organism in the surrounding water, since the association with the sponge will expose the bacterium to a continuous flow of water and nutrients.

Acknowledgments

We are very grateful to M. Meyer, S. Menger, C. Probian, and R. Appel for technical support. We are indebted to R. Marmulla for critical reading of the manuscript and L. Wöhlbrand for suggestions on technical aspect of the proteomic analysis. This study was funded by the European Research Council, Grant No. 203364, and the Max Planck Society. J.M.G. was supported by the grant MarineGems (CTM2010-20361) from the Spanish Ministry of Science and Innovation. The authors have no conflict of interest to declare.

References

Anderson, A.J., and Dawes, E.A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews* **54**: 450-472.

Becerra, M.C., Paez, P.L., Larovere, L.E., and Albesa, E. (2006) Lipids and DNA oxidation in *Staphylococcus aureus* as a consequence of oxidative stress generated by ciprofloxacin. *Molecular and Cellular Biochemistry* **285**: 29-34.

Benning, C. (1998) Biosynthesis and function of the sulfolipid sulfoquinovosyl diacylglycerol. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**: 53-75.

Benning, C., Huang, Z.H., and Gage, D.A. (1995) Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. *Archives of Biochemistry and Biophysics* **317**: 103-111.

Benning, C., Beatty, J.T., Prince, R.C., and Somerville, C.R. (1993) The Sulfolipid Sulfoquinovosyldiacylglycerol Is Not Required for Photosynthetic Electron-Transport in *Rhodobacter sphaeroides* but Enhances Growth under Phosphate Limitation. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 1561-1565.

Berger, M., Neumann, A., Schulz, S., Simon, M., and Brinkhoff, T. (2011) Tropodithietic acid production in *Phaeobacter gallaeciensis* Is regulated by N-acyl homoserine lactone-mediated quorum qensing. *Journal of Bacteriology* **193**: 6576-6585.

Bielski, B.H.J., Arudi, R.L., and Sutherland, M.W. (1983) A Study of the reactivity of HO2/O2- with unsaturated fatty-acids. *Journal of Biological Chemistry* **258**: 4759-4761.

Bondarev, V., Richter, M., Romano, S., Piel, J., Schwedt, A., and Schulz-Vogt, H.N. (2013) The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. *Environmental Microbiology* **15**: 2095-2113.

Bowe, F., Lipps, C.J., Tsolis, R.M., Groisman, E., Heffron, F., and Kusters, J.G. (1998) At least four percent of the *Salmonella typhimurium* genome is required for fatal infection of mice. *Infection and Immunity* **66**: 3372-3377.

Bruhn, J.B., Gram, L., and Belas, R. (2007) Production of antibacterial compounds and biofilm formation by *Roseobacter* species are influenced by culture conditions. *Applied and Environmental Microbiology* **73**: 442-450.

Bruhn, J.B., Nielsen, K.F., Hjelm, M., Hansen, M., Bresciani, J., Schulz, S., and Gram, L. (2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacte*r clade. *Applied and Environmental Microbiology* **71**: 7263-7270.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E., and Ros, J. (2000) Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **275**: 27393-27398.

Carrondo, M.A. (2003) Ferritins, iron uptake and storage from the bacterioferritin viewpoint. *The EMBO Journal* **22**: 1959-1968.

Chamnongpol, S., and Groisman, E.A. (2000) Acetyl phosphate-dependent activation of a mutant PhoP response regulator that functions independently of its cognate sensor kinase. *Journal of Molecular Biology* **300**: 291–305.

Cotner, J.B., Ammerman, J.W., Peele, E.R., and Bentzen, E. (1997) Phosphorus-limited bacterioplankton growth in the Sargasso Sea. *Aquatic Microbial Ecology* **13**: 141-149.

Crépin, S., Chekabab, S.M., Le Bihan, G., Bertrand, N., Dozois, C.M., and Harel, J. (2011) The Pho regulon and the pathogenesis of *Escherichia coli*. *Veterinary Microbiology* **153**: 82-88.

de Almeida, A., Nikel, P.I., Giordano, A.M., and Pettinari, M.J. (2007) Effects of granule-associated protein PhaP on glycerol-dependent growth and polymer production in Poly(3-Hydroxybutyrate)-producing *Escherichia coli*. *Applied and Environmental Microbiology* **73**: 7912-7916.

Dyhrman, S.T., Ammerman, J.W., and Van Mooy, B.A.S. (2007) Microbes and the marine phosphorus cycle. *Oceanography* **20**: 110-116.

Dyhrman, S.T., Jenkins, B.D., Rynearson, T.A., Saito, M.A., Mercier, M.L., Alexander, H., Whitney, L.P., Drzewianowski, A., Bulygin, V.V., Bertrand, E.M., Wu, Z.J., Benitez-Nelson, C., and Heithoff, A. (2012) The transcriptome and proteome of the diatom *Thalassiosira pseudonana* reveal a diverse phosphorus stress response. *Plos One* **7:** e33768. doi:10.1371/journal.pone.0033768

Enticknap, J.J., Kelly, M., Peraud, O., and Hill, R.T. (2006) Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied and Environmental Microbiology* **72**: 3724-3732.

Federici, L., Walas, F., and Luisi, B. (2004) The structure and mechanism of the TolC outer membrane transport protein. *Current Science* **87**: 190–196.

Fraley, C.D., Rashid, M.H., Lee, S.S.K., Gottschalk, R., Harrison, J., Wood, P.J., Brown, M.R.W., and Kornberg, A. (2007) A polyphosphate kinase 1 (ppk1) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 3526-3531.

Geider, R.J., and La Roche, J. (2002) Redfield revisited: variability of C : N : P in marine microalgae and its biochemical basis. *European Journal of Phycology* **37**: 1-17.

Geiger, O., Rohrs, V., Weissenmayer, B., Finan, T.M., and Thomas-Oates, J.E. (1999) The regulator gene phoB mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-N,N,N-trimethylhomoserine in *Rhizobium* (*Sinorhizobium*) *meliloti*. *Molecular Microbiology* **32**: 63-73.

Geißdörfer, W., Ratajczak, A., and Hillen, W. (1998) Transcription of ppk from *Acinetobacter* sp. strain ADP1, encoding a putative polyphosphate kinase, is induced by phosphate starvation. *Applied and Environmental Microbiology* **64**: 896-901.

Gérard, F., Dri, A.M., and Moreau, P.L. (1999) Role of *Escherichia coli* RpoS, LexA and H-NS global regulators in metabolism and survival under aerobic, phosphate-starvation conditions. *Microbiology* **145**: 1547-1562.

Geske, T., vom Dorp, K., Dormann, P., and Hölzl, G. (2013) Accumulation of glycolipids and other non-phosphorous lipids in *Agrobacterium tumefaciens* grown under phosphate deprivation. *Glycobiology* **23**: 69-80.

Ghorbel, S., Smirnov, A., Chouayekh, H., Sperandio, B., Esnault, C., Kormanec, J., and Virolle, M.J. (2006) Regulation of ppk expression and in vivo function of Ppk in *Streptomyces lividans* TK24. *Journal of Bacteriology* **188**: 6269-6276.

Gilbert, J.A., Thomas, S., Cooley, N.A., Kulakova, A., Field, D., Booth, T., McGrath, J.W., Quinn, J.P., and Joint, I. (2009) Potential for phosphonoacetate utilization by marine bacteria in temperate coastal waters. *Environmental Microbiology* **11**: 111-125.

Goclaw-Binder, H., Sendersky, E., Shimoni, E., Kiss, V., Reich, Z., Perelman, A., and Schwarz, R. (2012) Nutrient-associated elongation and asymmetric division of the cyanobacterium *Synechococcus* PCC 7942. *Environmental Microbiology* **14**: 680-690.

Godlewska, R., Wiśniewska, K., Pietras, Z., and Jagusztyn-Krynicka, E.K. (2009) Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis. *FEMS Microbiology Letters* **298**: 1–11.

Groisman, E.A. (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *Journal of Bacteriology* **183**: 1835-1842.

Hansen, H.P., and Karoleff, F. (1999) Determination of nutrients. In *Methods of Seawater Analysis*. Grasshoff, K., Kremling, K., and Ehrhardt, M. (eds). Weinheim: Wiley-VCH, pp. 159–226.

Holland, I.B., Schmitt, L., and Young, J. (2005) Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway. *Molecular Membrane Biology* **22**: 29–39.

Holzl, G., and Dormann, P. (2007) Structure and function of glycoglycerolipids in plants and bacteria. *Progress in Lipid Research* **46**: 225-243.

Hoppe, H.G. (2003) Phosphatase activity in the sea. *Hydrobiologia* **493**: 187-200.

Hsieh, Y.J., and Wanner, B.L. (2010) Global regulation by the seven-component P_i signaling system. *Current Opinion in Microbiology* **13**: 198-203.

Ishige, T., Krause, M., Bott, M., Wendisch, V.F., and Sahm, H. (2003) The phosphate starvation stimulon of *Corynebacterium glutamicum* determined by DNA microarray analyses. *Journal of Bacteriology* **185**: 4519-4529.

Jensen, V., Lons, D., Zaoui, C., Bredenbruch, F., Meissner, A., Dieterich, G., Munch, R., and Haussler, S. (2006) RhlR expression in *Pseudomonas aeruginosa* is modulated by the *Pseudomonas* quinolone signal via PhoB-dependent and -independent pathways. *Journal of Bacteriology* **188**: 8601-8606.

Joly, N., Engl, C., Jovanovic, G., Huvet, M., Toni, T., Sheng, X., Stumpf, M.P.H., and Buck, M. (2010) Managing membrane stress: the phage shock protein (Psp) response, from molecular mechanisms to physiology. *FEMS microbiology reviews* **34**: 797-827.

Karlinsey, J.E., Maguire, M.E., Becker, L.A., Crouch, M.L.V., and Fang, F.C. (2010) The phage shock protein PspA facilitates divalent metal transport and is required for virulence of *Salmonella enterica* sv. Typhimurium. *Molecular Microbiology* **78**: 669-685.

Klein, A.H., Shulla, A., Reimann, S.A., Keating, D.H., and Wolfe, A.J. (2007) The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators. *Journal of Bacteriology* **189**: 5574-5581.

Krol, E., and Becker, A. (2004) Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Molecular Genetics and Genomics* **272**: 1-17.

Kulaev, I., and Kulakovskaya, T. (2000) Polyphosphate and phosphate pump. *Annual Review of Microbiology* **54**: 709-734.

Kulakova, A.N., Hobbs, D., Smithen, M., Pavlov, E., Gilbert, J.A., Quinn, J.P., and McGrath, J.W. (2011) Direct quantification of inorganic polyphosphate in microbial cells using 4'-6-diamidino-2-phenylindole (DAPI). *Environmental science & technology* **45**: 7799-7803.

Lamarche, M.G., Wanner, B.L., Crepin, S., and Harel, J. (2008) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS microbiology reviews* **32**: 461-473.

Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., Procházková, K., Adkins, I., Hejnová-Holubová, J., Sadílková, L., Morová, J., and Šebo, P. (2010) RTX proteins: a highly diverse family secreted by a common mechanism. *FEMS microbiology reviews* **34**: 1076-1112.

Lloubès, R., Cascales, E., Walburger, A., Bouveret, E., Lazdunski, C., Bernadac, A., and Journet, L. (2001) The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity? *Research in Microbiology* **152**: 523-529.

Long, J., Zaborina, O., Holbrook, C., Zaborin, A., and Alverdy, J. (2008) Depletion of intestinal phosphate after operative injury activates the virulence of *P. aeruginosa* causing lethal gut-derived sepsis. *Surgery* **144**: 189-197.

Løvdal, T., Skjoldal, E.F., Heldal, M., Norland, S., and Thingstad, T.F. (2008) Changes in morphology and elemental composition of *Vibrio splendidus* along a gradient from carbon-limited to phosphate-limited growth. *Microbial Ecology* **55**: 152-161.

Madison, L.L., and Huisman, G.W. (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiology and Molecular Biology Reviews* **63**: 21-53.

Makino, K., Amemura, M., Kim, S.K., Yokoyama, K., and Kimura, S. (1998) Mechanism of transcriptional activation of the phosphate regulon in *Escherichia coli*. *Journal of Microbiology* **36**: 231-238.

Malmcronafriberg, K., Tunlid, A., Mårdén, P., Kjelleberg, S., and Odham, G. (1986) Chemical changes in cell envelope and poly-β-hydroxybutyrate during short-term starvation of a marine bacterial Isolate. *Archives of Microbiology* **144**: 340-345.

Martín, J.F. (2004) Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *Journal of Bacteriology* **186**: 5197-5201.

Martiny, A.C., Huang, Y., and Li, W. (2011) Adaptation to nutrient availability in marine microorganisms by gene gain and loss. In *Handbook of Molecular Microbial Ecology II: Metagenomics in Different Habitats* de Bruijn, F.J. (ed). Hoboken, NJ, USA: John Wiley & Sons, Inc., pp. 269-276.

Mccleary, W.R., and Stock, J.B. (1994) Acetyl phosphate and the activation of two-component response regulators. *Journal of Biological Chemistry* **269**: 31567-31572.

McInerney, P., Mizutani, T., and Shiba, T. (2006) Inorganic polyphosphate interacts with ribosomes and promotes translation fidelity in vitro and in vivo. *Molecular Microbiology* **60**: 438-447.

Minnikin, D.E., Baddiley, J., and Abdolrah.H (1972) Variation of Polar Lipid Composition of Bacillus-Subtilis (Marburg) with Different Growth Conditions. *FEBS letters* **27**: 16-&.

Minnikin, D.E., Abdolrah.H, and Baddiley, J. (1974) Replacement of Acidic Phospholipids by Acidic Glycolipids in Pseudomonas-Diminuta. *Nature* **249**: 268-269.

Moreau, P.L. (2004) Diversion of the metabolic flux from pyruvate dehydrogenase to pyruvate oxidase decreases oxidative stress during glucose metabolism in nongrowing *Escherichia coli* cells incubated under aerobic, phosphate starvation conditions. *Journal of Bacteriology* **186**: 7364-7368.

Moreau, P.L., Gerard, F., Lutz, N.W., and Cozzone, P. (2001) Non-growing *Escherichia coli* cells starved for glucose or phosphate use different mechanisms to survive oxidative stress. *Molecular microbiology* **39**: 1048-1060.

Morova, J., Osicka, R., Masin, J., and Sebo, P. (2008) RTX cytotoxins recognize β2 integrin receptors through N-linked oligosaccharides. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 5355-5360.

O'Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O'Gara, F., and Dobson, A.D.W. (2011) Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *Journal of Applied Microbiology* **110**: 1495-1508.

Paytan, A., and McLaughlin, K. (2007) The oceanic phosphorus cycle. *Chemical Reviews* **107**: 563-576.

Peng, L., Luo, W.Y., Zhao, T., Wan, C.S., Jiang, Y., Chi, F., Zhao, W., Cao, H., and Huang, S.H. (2012) Polyphosphate kinase 1 is required for the pathogenesis process of meningitic *Escherichia coli* K1 (RS218). *Future Microbiology* **7**: 411-423.

Porsby, C.H., Webber, M.A., Nielsen, K.F., Piddock, L.J.V., and Gram, L. (2011) Resistance and tolerance to tropodithietic acid, an antimicrobial in aquaculture, is hard to select. *Antimicrobial Agents and Chemotherapy* **55**: 1332-1337.

Pradenas, G.A., Paillavil, B.A., Reyes-Cerpa, S., Pérez-Donoso, J.M., and Vásquez, C.C. (2012) Reduction of the monounsaturated fatty acid content of *Escherichia coli* results in increased resistance to oxidative damage. *Microbiology* **158**: 1279-1283.

Prol Garcia, M.J., D'Alvise, P.W., and Gram, L. (2013) Disruption of cell-to-cell signaling does not abolish the antagonism of *Phaeobacter gallaeciensis* toward the Fish pathogen *Vibrio anguillarum* in algal systems. *Applied and Environmental Microbiology* **79**: 5414-5417.

Rao, N.N., Gómez-García, M.R., and Kornberg, A. (2009) Inorganic polyphosphate: essential for growth and survival. *Annual Review of Biochemistry* **78**: 605-647.

Rodríguez-García, A., Barreiro, C., Santos-Beneit, F., Sola-Landa, A., and Martín, J.F. (2007) Genome-wide transcriptomic and proteomic analysis of the primary response to phosphate limitation in *Streptomyces coelicolor* M145 and in a ΔphoP mutant. *Proteomics* **7**: 2410-2429.

Satchell, K.J.F. (2011) Structure and function of MARTX toxins and other large repetitive RTX proteins
Annual Review of Microbiology **65**: 71-90.

Schembri, M.A., Bayly, R.C., and Davies, J.K. (1995) Phosphate concentration regulates transcription of the *Acinetobacter* polyhydroxyalkanoic acid biosynthetic genes. *Journal of Bacteriology* **177**: 4501-4507.

Schlüter, J.P., Reinkensmeier, J., Barnett, M.J., Lang, C., Krol, E., Giegerich, R., Long, S.R., and Becker, A. (2013) Global mapping of transcription start sites and promoter motifs in the symbiotic α-proteobacterium *Sinorhizobium meliloti* 1021. *BMC Genomics* **14**.

Schmidt, A., Kellermann, J., and Lottspeich, F. (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. *Proteomics* **5**: 4-15.

Sertan-de Guzman, A.A., Predicala, R.Z., Bernardo, E.B., Neilan, B.A., Elardo, S.P., Mangalindan, G.C., Tasdemir, D., Ireland, C.M., Barraquio, W.L., and Concepcion, G.P. (2007) *Pseudovibrio denitrificans* strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate. *FEMS Microbiology Letters* **277**: 188-196.

Shieh, W.Y., Lin, Y.T., and Jean, W.D. (2004) *Pseudovibrio denitrificans* gen. nov., sp. nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2307-2312.

Smith, S.G.J., Mahon, V., Lambert, M.A., and Fagan, R.P. (2007) A molecular Swiss army knife: OmpA structure, function and expression. *FEMS microbiology letters* **273**: 1-11.

Staden, R. (1984) Computer methods to locate signals in nucleic acid sequences. *Nucleic Acids Research* **12**: 505-519.

Stock, J.B., Ninfa, A.J., and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiological Reviews* **53**: 450-490.

Stormo, G.D. (2000) DNA binding sites: representation and discovery. *Bioinformatics* **16**: 16-23.

Summers, M.L., Elkins, J.G., Elliott, B.A., and McDermott, T.R. (1998) Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. *Molecular Plant-Microbe Interactions* **11**: 1094-1101.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews* **71**: 295-347.

Tetu, S.G., Brahamsha, B., Johnson, D.A., Tai, V., Phillippy, K., Palenik, B., and Paulsen, I.T. (2009) Microarray analysis of phosphate regulation in the marine cyanobacterium *Synechococcus* sp WH8102. *The ISME Journal* **3**: 835-849.

Thingstad, T.F., Krom, M.D., Mantoura, R.F.C., Flaten, G.A.F., Groom, S., Herut, B., Kress, N., Law, C.S., Pasternak, A., Pitta, P., Psarra, S., Rassoulzadegan, F., Tanaka, T., Tselepides, A., Wassmann, P., Woodward, E.M.S., Riser, C.W., Zodiatis, G., and Zohary, T. (2005) Nature of phosphorus limitation in the ultraoligotrophic eastern Mediterranean. *Science* **309**: 1068-1071.

Torriani, A. (1960) Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **38**: 460-469.

Tsutsumi, K., Munekata, M., and Shiba, T. (2000) Involvement of inorganic polyphosphate in expression of SOS genes. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1493**: 73-81.

van der Veen, S., van Schalkwijk, S., Molenaar, D., de Vos, W.M., Abee, T., and Wells-Bennik, M.H.J. (2010) The SOS response of Listeria monocytogenes is involved in stress resistance and mutagenesis. *Microbiology* **156**: 374-384.

Van Mooy, B.A.S., Moutin, T., Duhamel, S., Rimmelin, P., and Van Wambeke, F. (2008) Phospholipid synthesis rates in the eastern subtropical South Pacific Ocean. *Biogeosciences* **5**: 133-139.

Van Mooy, B.A.S., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblížek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappé, M.S., and Webb, E.A. (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature* **458**: 69-72.

VanBogelen, R.A., Olson, E.R., Wanner, B.L., and Neidhardt, F.C. (1996) Global analysis of proteins synthesized during phosphorus restriction in *Escherichia coli*. *Journal of Bacteriology* **178**: 4344-4366.

Villanueva, L., Bale, N., Hopmans, E.C., Schouten, S., and Damsté, J.S. (2013) Diversity and distribution of a key sulpholipid biosynthetic gene in marine microbial assemblages. *Environmental Microbiology*. **Article first published online**: DOI: 10.1111/1462-2920.12202.

Vogel, S. (1977) Current-induced flow through living sponges in nature. *Proceedings of the National Academy of Sciences of the United States of America* **74**: 2069-2071.

von Kruger, W.M.A., Lery, L.M.S., Soares, M.R., de Neves-Manta, F.S., Silva, C.M.B.E., Neves-Ferreira, A.G.D., Perales, J., and Bisch, P.M. (2006) The phosphate-starvation response in *Vibrio cholerae* O1 and phoB mutant under proteomic analysis: disclosing functions involved in adaptation, survival and virulence. *Proteomics* **6**: 1495-1511.

Wanner, B.L. (1996) Phosphorus assimilation and control of the phosphate regulon. In *Escherichia coli and Salmonella. Cellular and molecular biology*. Neidhardt, F.C., Curtis III, R., Ingraham, J.L., Lin, E.C.C., Brooks low, K., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M., and Umbarger, H.E. (eds). Washington, D.C.: ASM press, pp. 1357-1381.

Wolfe, A.J. (2005) The acetate switch. *Microbiology and Molecular Biology Reviews* **69**: 12-50.

Wortham, B.W., Oliveira, M.A., and Patel, C.N. (2007) Polyamines in bacteria: pleiotropic effects yet specific mechanisms. *Genus Yersinia: From Genomics to Function* **603**: 106-115.

Wu, J.F., Sunda, W., Boyle, E.A., and Karl, D.M. (2000) Phosphate depletion in the western North Atlantic Ocean. *Science* **289**: 759-762.

Wurch, L.L., Bertrand, E.M., Saito, M.A., Van Mooy, B.A.S., and Dyhrman, S.T. (2011) Proteome changes driven by phosphorus deficiency and recovery in the brown tide-forming alga *Aureococcus anophagefferens*. *Plos One* **6**: e28949. doi:10.1371/journal.pone.0028949

Yoon, S.J., Park, J.E., Yang, J.H., and Park, J.W. (2002) OxyR regulon controls lipid peroxidation-mediated oxidative stress in *Escherichia coli*. *Journal of Biochemistry and Molecular Biology* **35**: 297-301.

Yoshida, Y., Sugiyama, S., Oyamada, T., Yokoyama, K., and Makino, K. (2010) Identification and characterization of novel phosphate regulon genes, ecs0540-ecs0544, in *Escherichia coli* O157:H7. *Molecular Genetics and Genomics* **284**: 197-205.

Yuan, Z.C., Zaheer, R., and Finan, T.M. (2005) Phosphate limitation induces catalase expression in *Sinorhizobium meliloti*, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. *Molecular Microbiology* **58**: 877-894.

Yuan, Z.C., Zaheer, R., Morton, R., and Finan, T.M. (2006) Genome prediction of PhoB regulated promoters in *Sinorhizobium meliloti* and twelve proteobacteria. *Nucleic Acids Research* **34**: 2686-2697.

Zaborin, A., Romanowski, K., Gerdes, S., Holbrook, C., Lepine, F., Long, J., Poroyko, V., Diggle, S.P., Wilke, A., Righetti, K., Morozova, I., Babrowski, T., Liu, D.C., Zaborina, O., and Alverdy, J.C. (2009) Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 6327-6332.

Supplementary materials and methods

Chemical analyses, determination of alkaline phosphatase activity, and detection of inclusions

Alkaline phosphatase **(**AP) activity was detected by adding to 1 mL of culture 4-nitrophenyl phosphate bis(tris) salt (pNPP; ≥ 97.00%; Sigma Aldrich, St. Louis, MO, USA) to a final concentration of 540 µmol L^{-1}. Samples were incubated at 28 ^{o}C and absorbance at 420 and 550 nm was monitored using a Beckman DU 640 spectrophotometer. Absorbance was recorded every 30 min for a time period sufficient for the detection of activity (2 h). AP activity was expressed in Miller unit (Miller, 1972), which were calculated using the formula: 10,000 x [A420 – (1.75 x A550)] / t x log(cells ml^{-1}) x V, where A420 and A550 are the absorbances of the reactions at the respective wavelengths after a defined incubation time t (min) and V is the volume in ml used in the assay.

Detection of tropodithietic acid (TDA) in the cell-free supernatant of $-P_i$ and $+P_i$ cultures was performed in collaboration with BioViotica Naturstoffe GmbH (Göttingen, Germany). Samples were collected during the exponential and stationary phase from two parallel flasks under $-P_i$ and $+P_i$ conditions. Cells were removed by centrifugation at 11,000 x *g* for 15 min at 5 °C. Cell-free supernatant was acidified to pH 3 with 2 mol L^{-1} HCl and 20 mL of each sample were extracted twice with an equal volume of ethyl acetate. Solvent was evaporated, and samples were resuspended in 1 mL acetonitrile. Analysis was performed via reverse phase high performance liquid chromatography (RP-HPLC) using a Nucleodur 100-5 C18 ec (250 x 3 mm) column. The mobile

phase consisted of A: deionized water with 0.1% trifluoroacetic acid (TFA) and B: acetonitrile with 0.1% TFA. The method was: 0–25 min: 20% B to 100% B; 25–30 min: 100% B and the flow rate 0.5 mL min^{-1}.

Polyphosphate (poly-P_i) was quantified using the method described by Kulakova *et al.* (2011). In short, cell pellets were washed with 50 mmol L^{-1} HEPES buffer (pH 7.5), snap-frozen in liquid nitrogen and thawed at room temperature. Cells were resuspended in 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI) assay buffer (150 mmol L^{-1} KCl, 20 mmol L^{-1} HEPES-KOH, pH 7.0) containing 25 µmol L^{-1} DAPI at a dilution appropriate for the used calibration. After 30 min of incubation the DAPI fluorescence was measured using a luminescence spectrometer LS 50 B (Perkin Elmer, Germany) with an excitation filter of 420 nm and an emission filter of 550 nm. The calibration was performed using a poly-P_i standard (sodium phosphate glass type 45, Sigma-Aldrich, Germany) between 0.5 and 6 µg mL^{-1}. The background fluorescence of the cells and the fluorescence of the HEPES buffer (without DAPI) were subtracted from the fluorescent signal for normalization. Presence of poly-P_i was confirmed microscopically by staining cells with 0.1 g L^{-1} DAPI for 1 h at room temperature. Inclusions were observed by fluorescence microscopy using an Axioplan universal microscope (Zeiss, Oberkochen, Germany) with an HBO 50 mercury lamp (Osram, Germany) for excitation with UV light and a UV-G 365 filter set (G 365 exciter filter, FT 395 chromatic beam splitter and an LP 420 barrier filter, Zeiss, Oberkochen, Germany; data not shown).

In order to verify the presence of polyhydroxyalkanoates (PHA) cells were stained for 15 min in the dark with Nile red in dimethyl sulfoxide

(Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 2.5 µg L^{-1}. Inclusions were observed using the same microscope as described above with filter set 15 (Zeiss, Oberkochen, Germany), with excitation at 546 nm and emission at 590 nm.

Polar lipids of cells collected at the beginning of the stationary phase in both $-P_i$ and $+P_i$ cultures were extracted using a chloroform:methanol:0.3% aqueous NaCl mixture 1:2:0.8 (v/v/v) (modified after Bligh and Dyer, 1959). The extraction solvent was stirred overnight, and the polar lipids were recovered into the chloroform phase by adjusting the chloroform:methanol:0.3% aqueous NaCl mixture to a ratio of 1:1:0.9 (v/v/v). Separation was achieved by two dimensional silica gel thin layer chromatography (Macherey-Nagel Art. No. 818 135), according to Tindall *et al.* (2007), with the first direction developed in chloroform:methanol:water (65:25:4, v/v/v), and the second in chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), Dragendorff (quaternary nitrogen), and α-naphthol-sulphuric acid (glycolipids). Plates were stained with 5% molybdophosphoric acid to show all lipids.

Proteome analysis

Protein extraction, labeling, and separation

Cultures were centrifuged at 11,000 x *g* for 10 min at 5 °C, using a J-26XP Beckmann centrifuge (Beckman Instruments, Inc., CA, USA), supernatant

was discarded and cells were frozen immediately and stored at –80 °C until further processing. The proteomic analysis was performed by TopLab GmbH (Martinsried, Germany). Cells were lysed on ice by means of sonication (tip, Benedelin Sonoplus; Bandelin Electronics, Berlin, Germany) 6 times for 10 seconds with power amplitude of 25%. Samples were then shaken for 20 min, to improve protein solubilization. After centrifugation at 20,000 x *g* for 30 min and removal of the cellular debris, the supernatant was transferred into a low binding tube for subsequent ICPL labeling and an aliquot was used to determine the protein concentration by Bradford assay (Bradford, 1976).

100 µg total protein was used for each ICPL labeling reaction performed using SERVA ICPL™ Quadruplex-Kit (SERVA Electrophoresis GmbH, Heidelberg, Germany) according to the manufacturer's instruction. The labeling was performed independently for the two biological replicates. ICPL0, not containing isotope, was used for the sample $-P_i$ exponential phase; ICPL4, containing four D, was used for the sample $-P_i$ stationary phase; ICPL6, containing six ^{13}C, was used for the sample $+P_i$ exponential phase; ICPL10, containing four D and six ^{13}C, was used for labeling a sample obtained pooling all six protein extracts. The acetone precipitated ICPL labeled proteins were dissolved in sodium dodecyl sulfate (SDS) sample buffer. Complexity of the samples was reduced via SDS-PAGE, performed according to Laemmli (1970) using a 4-20% gradient gel for separation (SERVA*Gel*™, SERVA Electrophoresis GmbH, Heidelberg, Germany). For each biological replicate the samples labeled with ICPL0, ICPL4, ICPL6, and ICPL10 were pooled, and these mixtures were run in two different lines (run 1 and run 2), one for each replicate. In addition, all

three protein extracts of both biological replicates were combined, stained with the four ICPL compounds, and run in a third line (run 3). In this run all detected peptides will appear in the MS analysis as complete multiplets (quadruplets), increasing the probability of a correct identification. Run 3 was used as a reference run for the identification of proteins not present in all biological sample runs. After staining with colloidal Coommassie Brilliant Blue, each line in the SDS-PAGE gel was sliced in five pieces. De-staining was then performed using 50 mmol L^{-1} NH_4HCO_3 in 30% acetonitrile. Reduction and alkylation were performed during the ICPL labeling. In-gel digestion was carried out overnight with trypsin sequencing grade (SERVA Electrophoresis GmbH, Germany) using a protein to enzyme ratio of 200:1 in 10 mmol L^{-1} NH_4HCO_3. Then Glu-C MS grade (Protea™ Chemicals, South Africa) was added using a protein to enzyme ratio of 50:1. The cleavage was performed for approximately 8 h at room temperature. Due to the complete labeling of lysine residues the tryptic digest will result only in an Arg-C cleavage, producing peptides that show the tendency to be outside the working range of the MS. Therefore, the second cleavage with Glu-C generates smaller peptides that lie within the working range of the MS. The peptides were extracted and acidified to 1% formic acid for subsequent MS analysis.

LC-ESI-MS/MS and protein identification

For nanoLC-ESI-MS/MS approximately a quarter of the digested proteins were used. 1D-nano-LC separation was performed on a multi-dimensional liquid chromatography system (Ettan MDLC, GE Healthcare,

Munich, Germany). Peptides were separated with an analytical column (C18 PepMap 100, 3 μm bead size, 75 μm i.d., 15 cm length LC Packings) with a three step 120 min linear gradient (A: 0.1% formic acid, B: 84% Acetonitrile and 0.1% formic acid) at a flow rate of 260 nL per min. The gradient used was: 0-30% B for 80 min, 30-60% B for 30 min and 60-100% B for 10 min.

Mass spectrometry was performed on a linear ion trap mass spectrometer (Thermo LTQ, Thermo Scientific, USA) operating in positive polarity mode online coupled to the nano-LC system. For electrospray ionization a distal coated SilicaTip (FS-360-50-15-D-20) and a needle voltage of 1.4 kV were used. The MS method consisted of a cycle combining one full MS scan (Mass range: 300-2,000 m/z) with data dependent MS/MS events (CID; 35% collision energy). The dynamic exclusion was set to 30 secs. The raw data were converted to mzXML format using the software Trans-Proteomic Pipeline. Then the peak detection, deconvolution, deisotoping and quantification were done using ICPL-ESIQuant. Quadruplets detection was first performed for each run separately. Subsequently, the detected quadruplets of the reference run (run 3) were used to search for incomplete quadruplets in the biological sample (run 1 and run 2). In this way complete and incomplete quadruplets were identified. Only peptides with a Mascot score above 20, defined as the 95% confidence level, were considered. The Glu-C cleavage specificity was set for Glu. A specific database was built using the genome of the strain (available at DDBJ/EMBL/GenBank under the accession number CP003147 for the chromosome and CP003148 for the plasmid). For each LC-ESI-MS/MS run four separate database queries were always done using one of the ICPL labels as

second fixed modification. For database searches a mass tolerance of 50 ppm in the MS mode and 0.5 Da in MS/MS mode with two missed cleavages were set.

Data analysis

The fold of change of each protein, identified via Mascot, was determined by calculating the median of all respective peptides intensity ratios, but only if at least two quadruplets per protein were present. Proteins represented by incomplete quadruplets in the MS data cannot be quantified and, therefore, were only valued as turned ON or OFF at the respective condition. The false discovery rate (FDR) was calculated at the peptides level for all experimental runs using a randomized Decoy database (Peng *et al.*, 2003), applying the same restriction as used for protein identification. The results of the calculation of the protein fold of change are values from 1 to infinity for up-regulated and from 1 to 0, for down-regulated proteins. This generates an asymmetry, which could lead to an incorrect interpretation during the evaluation process. For this reason, we multiplied the inverse of the down-regulated protein by –1. In this way, all the down-regulated proteins under the condition tested will have a negative but symmetric value to the up-regulated ones. As a consequence, in the comparison between $+P_i$ and $-P_i$, if a protein is up-regulated in the reference condition ($-P_i$ exponential phase), we will have a negative value, due to the normalization described above. However, the main aim of the present paper is to describe the variation occurring exactly at this growth stage. For this reason, we decided that it will be more intuitive to refer to the proteins up-regulated in the reference condition with positive value. To achieve this, we

multiplied all values for –1. This correction was not performed for the values obtained in the comparison between $-P_i$ exponential and stationary phase.

Subsequently, the average values were newly calculated and a new dataset was created with all regulated proteins defined by a fold of change equal or below −2, for down-regulated proteins, and equal or above 2, for up-regulated proteins. The latter dataset was further filtered according to the following criteria: for each protein we considered the standard deviation (STDV) from the median intensity ratio of the peptides. If the value of the STDV was lower than 50% of the protein regulatory ratio, or if it was higher but not affecting the definition of the regulation of the protein (e.g. from up-regulated to down-regulated), the protein was kept in the database otherwise, we investigated manually the peptide intensity ratios. If all peptide intensity ratios were below the threshold of 0.75 for down-regulated proteins and above 1.33 for up-regulated proteins, the proteins were kept; otherwise they were removed due to the low statistical significance of their regulation. The proteins were then sorted in 15 arbitrary categories as described in the Materials and Methods section.

Screening of the genome of strain FO-BEG1 for the presence of Pho-boxes

The prediction of potential PhoB binding sites within the genome of *Pseudovibrio* sp. FO-BEG1 was based on a position-weight matrix constructed using the Pho-box described for *Sinorhizobium meliloti* and *Escherichia coli* by Yuan *et al.* (2006). As a first approach, an 18 nucleotides long position-weight matrix based on ten *E. coli* and five *S. meliloti* PhoB binding sites was constructed. This

matrix was then used to scan each of the intergenic regions of the FO-BEG1 genome for the highest score (log-odds), using an in-house Python script. To verify the length and the position of the Pho-box in strain FO-BEG1, 18 nucleotides of the potential Pho-box, plus 10 nucleotides upstream and downstream of the target were retrieved. These sequences were then used in the successive analysis for the identification of the conserved Pho-box. From the first gene list we selected all those genes that encode for proteins that were found to be up-regulated in the proteomic approach. The result was a list of 67 genes which possessed a potential Pho-box with a score (log-odds) ranging from 0.27 to 17.06. For strain FO-BEG1 there are no molecular studies available which describe the Pho-box sequences or genes directly regulated by PhoB; therefore we did not have any reference that could have been used to set a threshold that defines a good "hit" in our bioinformatic screening. Consequently, we manually screened the list of candidates to find genes directly controlled by PhoB in other strains. The last gene for which we could find a correspondence in literature was PSE_3720, which encodes an outer membrane protein TolC, described in Yoshida *et al.* (2010). The score for this gene was 4.86, which was taken as the lowest value which defines a reliable hit in the Pho-box search.

Subsequently, to construct a Pho-box position-weight matrix specific for *Pseudovibrio* sp. FO-BEG1, we considered all genes that encode up-regulated proteins under phosphate-limited conditions and having a sequence score between 17.06 and 4.86 for the Pho-box. This resulted in 32 sequences (**Table S2.3**) that were aligned and used to create a logo using WebLogo 3.0 (Schneider and Stephens, 1990; Crooks *et al.*, 2004). This analysis showed (**Figure S2.1**) that the

inner region of the 38 nucleotides, between nucleotide 11 and 28, possesses the typical conserved sequence known for *E. coli* and *S. meliloti* (Makino *et al*., 1998; Yuan *et al*., 2006). Thus, for the successive analysis we only considered for all selected sequences this region of 18 nucleotides. In the last step, we created a new position-weight matrix using the 32 selected genes of *Pseudovibrio* sp. FO-BEG1 and scanned the intergenic regions of its genome once again. The scan resulted in 1004 sequences, which can be found in **Table S2.4**. As a threshold for the lowest score that defines a good "hit" we took the lowest score of the proteins which we found up-regulated under phosphate-limited conditions in strain FO-BEG1 and which was part of the second matrix (PSE_3720, encoding an outer membrane protein TolC; score 6.66).

References

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911-917.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72**: 248-254.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome research* **14**: 1188-1190.

Kulakova, A.N., Hobbs, D., Smithen, M., Pavlov, E., Gilbert, J.A., Quinn, J.P., and McGrath, J.W. (2011) Direct quantification of inorganic polyphosphate in microbial cells using 4'-6-diamidino-2-phenylindole (DAPI). *Environmental science & technology* **45**: 7799-7803.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Makino, K., Amemura, M., Kim, S.K., Yokoyama, K., and Kimura, S. (1998) Mechanism of transcriptional activation of the phosphate regulon in *Escherichia coli*. *Journal of Microbiology* **36**: 231-238.

Miller, J.H. (1972) *Experiments in molecular genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Peng, J.M., Elias, J.E., Thoreen, C.C., Licklider, L.J., and Gygi, S.P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *Journal of Proteome Research* **2**: 43-50.

Schneider, T.D., and Stephens, R.M. (1990) Sequence logos: a new way to display consensus sequences. *Nucleic Acids Research* **18**: 6097-6100.

Tindall, B.J., Sikorski, J., Smibert , R.M., and Kreig, N.R. (2007) Phenotypic characterization and the principles of comparative systematics. In *Methods for General and Molecular Microbiology 3rd edn*. Reddy, C.A., Beveridge, T.J., Breznak, J.A., Marzluf, G., Schmidt, T.M., and Snyder, L.R. (eds). Washington DC: ASM Press, pp. 330-393.

Yoshida, Y., Sugiyama, S., Oyamada, T., Yokoyama, K., and Makino, K. (2010) Identification and characterization of novel phosphate regulon genes, ecs0540-ecs0544, in *Escherichia coli* O157:H7. *Molecular Genetics and Genomics* **284**: 197-205.

Yuan, Z.C., Zaheer, R., Morton, R., and Finan, T.M. (2006) Genome prediction of PhoB regulated promoters in *Sinorhizobium meliloti* and twelve proteobacteria. *Nucleic Acids Research* **34**: 2686-2697.

Supplementary figures

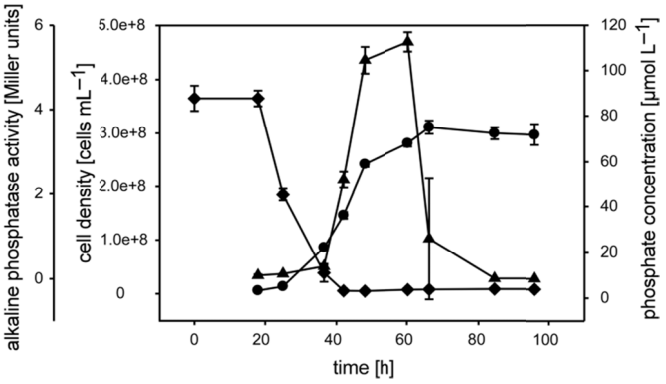


Figure S2.1 | Cell density (circles), phosphate concentration (diamonds) and alkaline phosphatase activity (triangles) over time in cultures grown under −P_i conditions. Error bars represent the standard deviation of biological triplicates.

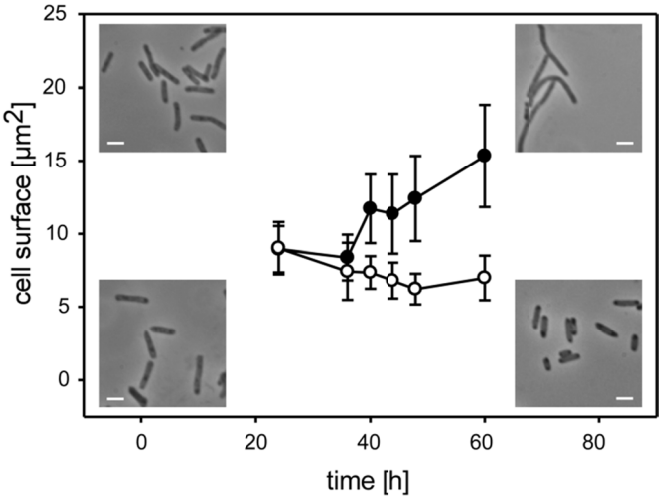


Figure S2.2 | Cellular surface measured in +P_i (empty circles) and –P_i (filled circles) cultures during bacterial growth. The upper panels show micrographs of cells growing under –P_i conditions and the lower panels micrographs of cells growing under +P_i conditions. Micrographs were taken after 24 h (left) and 60 h (right) of growth. Scale bar is 2 µm. Error bars represent the standard deviation of at least 100 cell surfaces measured.

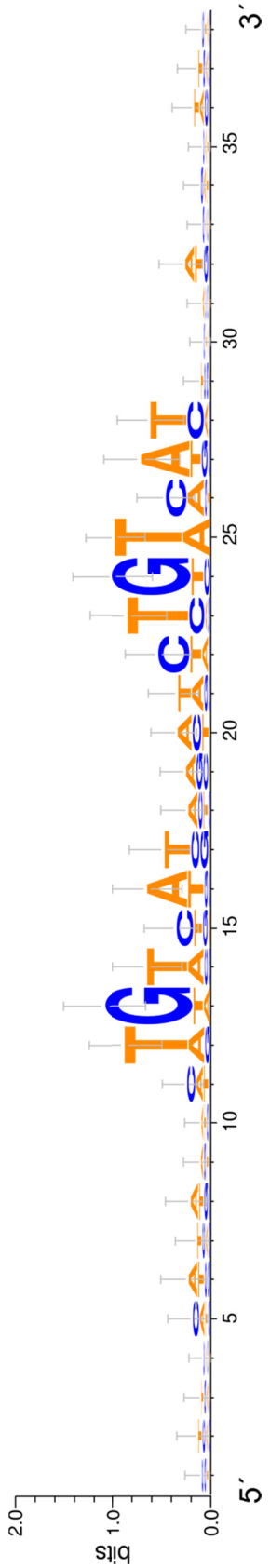


Figure S2.3 | Sequence logo of the Pho-box of FO-BEG1. A window of 38 nucleotides was considered to verify the position of the conserved region.

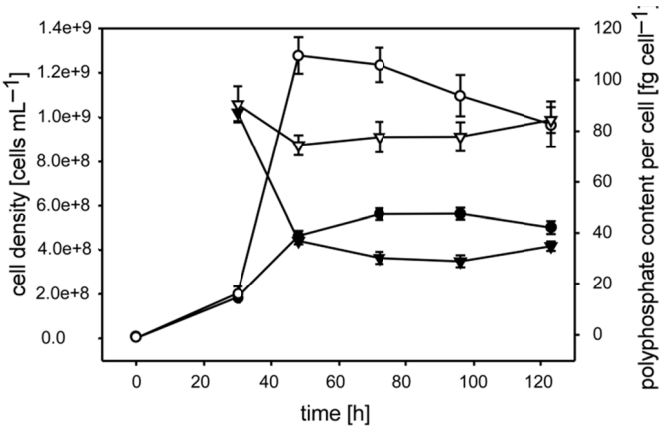


Figure S2.4 | Cell density (circles) and polyphosphate content per cell (triangles) over time measured during bacterial growth under +P_i (empty symbols) and −P_i (filled symbols) conditions. The phosphate concentration measured during bacterial growth is reported in Fig. 2.1. Error bars represent standard deviation of biological triplicates.

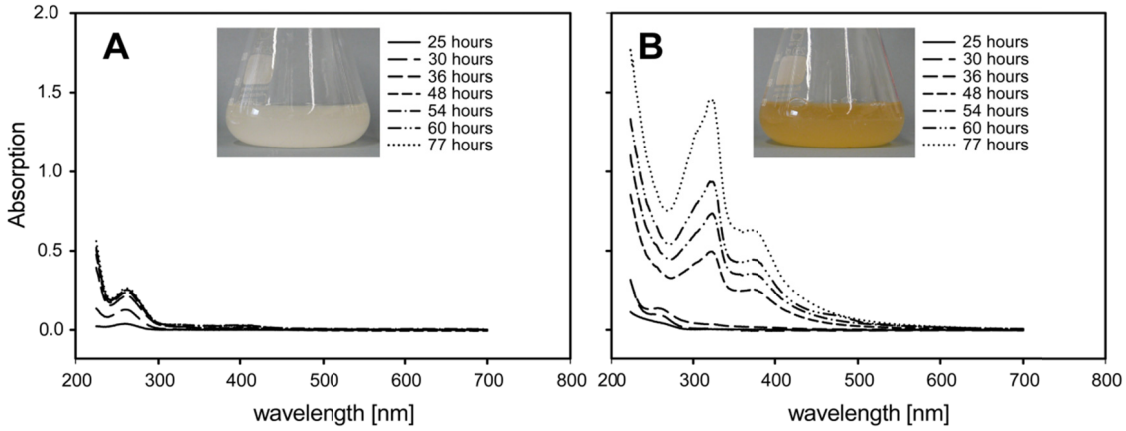


Figure S2.5 | Absorption spectra of ultraviolet and visible light of the cell free supernatant of cultures grown under +P_i (A) and −P_i (B) conditions. Photographs demonstrate the visual appearance of the respective cultures after 77 hours of growth. Figure modified from the Ph.D. thesis of Vladimir Bondarev (Univeristy of Bremen, 2012).

Supplementary tables are provided as digital material

Table S2.1 | List of proteins regulated during phosphate limitation. The fold of change (Fc) was calculated comparing the protein expression of cells in the exponential phase of $-P_i$ and $+P_i$ cultures.

Table S2.2 | List of proteins regulated during prolonged phosphate limitation. The fold of change (Fc) was calculated comparing the protein expression of cells in the exponential and stationary phase of $-P_i$ cultures.

Table S2.3 | List of genes used for the construction of a specific Pho-box position-weight matrix for strain FO-BEG1.

Table S2.4 | List of genes that present a potential Pho-box in their upstream regions.

Chapter 3

Exo-metabolome of *Pseudovibrio* sp. FO-BEG1 analyzed by ultra high resolution mass spectrometry and the effect of phosphate limitation

Stefano Romano[1*], Thorsten Dittmar[2], Vladimir Bondarev[1], Ralf J. M. Weber[3], Mark R. Viant[3], Heide N. Schulz-Vogt[4]

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany.
[2] Research Group for Marine Geochemistry, Institute for Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Oldenburg, Germany.
[3] School of Biosciences, University of Birmingham, Birmingham, UK.
[4] Leibniz-Institute for Baltic Sea Research Warnemuende (IOW), Section Biological Oceanography, Rostock, Germany.

* corresponding author

Contributions:
The concept of this work was developed by me with the help of Heide N. Schulz-Vogt and Thorsten Dittmar. I performed all experiments and analyzed all data. I wrote the manuscript including comments from all co-authors

Manuscript in revision to *PLoS ONE*. The original manuscript was adapted to the format of the present thesis.

Abstract

Dissolved organic matter (DOM) in the ocean is an assemblage of reduced carbon compounds and it is the result of biotic and abiotic processes. The biotic processes consist of either release or uptake of specific molecules by marine organisms. Heterotrophic bacteria have been mostly considered to influence the DOM composition by preferential uptake of certain compounds. However, they also secrete a variety of molecules depending on physiological state, environmental and growth conditions. In this study we analyzed the exo-metabolome, metabolites secreted into the environment, of the heterotrophic marine bacterium *Pseudovibrio* sp. FO-BEG1 via ultra-high resolution mass spectrometry, comparing phosphate-limited with phosphate-surplus growth conditions. Bacteria belonging to the *Pseudovibrio* genus have been isolated worldwide, mainly from marine invertebrates. They are metabolically versatile *Alphaproteobacteria* and have the potential to produce bioactive secondary metabolites. We show that the exo-metabolome is unexpectedly large and diverse, it is characterized by a dynamic recycling of compounds, and it is drastically affected by the physiological state of the strain. Moreover, we show that phosphate limitation greatly influences both the amount and the composition of the secreted molecules. By assigning the detected masses to general chemical categories we observed that under phosphate-surplus conditions peptides and highly unsaturated compounds were the dominant constituent of the exo-metabolome. In contrast, under phosphate limitation the composition of the molecules released into the medium changed during bacterial growth, showing an increase in highly unsaturated, phenolic, and polyphenolic

compounds. Finally, we annotated the detected masses using the KEGG database as reference. This revealed a big discrepancy between the number of detected masses and the number of uniquely annotated metabolites (less than 3%), which underlines the current gap in knowledge concerning the biosynthetic ability of marine heterotrophic bacteria.

Introduction

Microorganisms dynamically interact with their environment, they are influenced by its composition and, in turn, they influence its composition. This reciprocity has an effect on bacterial gene expression, protein synthesis and metabolite uptake and production. In the ocean the dissolved organic matter (DOM), which consists of a collection of reduced carbon compounds often containing heteroatoms (e.g. N, P, S), is the result of these interconnected processes. Photosynthetic and non-photosynthetic bacteria can release metabolites into the environment according to their physiological state (Carlson, 2002). Examples are compounds secreted for nutrient acquisition (e.g. siderophores), for communication (e.g. acyl-homoserine lactones), and for interspecies competition (e.g. antibiotics). Several studies have investigated the effect of photosynthetic bacteria on DOM composition (reviewed in Carlson, 2002 and Kujawinski, 2011), but the composition of the DOM produced by heterotrophic bacteria is almost unknown. Special attention has been paid to metabolites of biotechnological application, but little is known about the full suite of compounds produced by bacteria under different nutrient regimes and growth phases, resulting in a general lack of information on the effect of marine heterotrophic bacteria on DOM composition in the ocean (Kujawinski, 2011).

Metabolomics is the field of science that aims to characterize and quantify metabolites, or low molecular weight molecules, originating from cellular activity under a given set of physiological conditions. This collection of metabolites is termed the metabolome (Oliver *et al.*, 1998), which can be partitioned into the so called endo-metabolome (all intracellular metabolites) and

exo-metabolome (all extracellular metabolites; Fiehn, 2001; Allen *et al.*, 2003; Mapelli *et al.*, 2008). The investigation of the metabolome has many benefits for understanding the effect of environmental changes on the cell phenotype, and it complements the associated techniques of proteomics and transcriptomics. Metabolomics is a "downstream" approach and reflects the final response of cells to specific environmental conditions (Oliver *et al.*, 1998). Microbial metabolomic studies have already been performed for different purposes, e.g. to elucidate metabolic pathways, to investigate the response of bacterial metabolism to environmental stresses, to support bacterial identification, and to diagnose bacterial infections (Carlier, 1990; Cundy *et al.*, 1991; Rinas *et al.*, 1995; Boersma *et al.*, 2001; Shnayderman *et al.*, 2005; Brauer *et al.*, 2006; Coucheney *et al.*, 2008). Such studies have the potential to provide new insights into the composition of the metabolites secreted by marine heterotrophic bacteria and into their influence on the oceanic DOM composition.

Among the different analytical techniques, high resolution accurate mass (HRAM) mass spectrometry has acquired a predominant position in metabolic studies (Want *et al.*, 2007). Among these techniques, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is emerging as the most promising technology since it provides accurate mass measurement with ppm or sub-ppm error. It allows to obtain ultra-high resolved profiles with thousands of accurate masses, which in principle can be transformed into real elemental composition (Aharoni *et al.*, 2002; Marshall, 2004; Brown *et al.*, 2005; Junot *et al.*, 2010). Therefore, it permits high-throughput screening of intracellular and extracellular metabolites providing overall information on bacterial metabolism.

This technique was successfully employed to analyze the variation in the endo-metabolome during bacterial growth, in studies of metabolic diversity among different ecotypes and in analyzing bacterial response to stress conditions (Rosselló-Móra *et al.*, 2008; Takahashi *et al.*, 2008; Brito-Echeverria *et al.*, 2011; Antón *et al.*, 2013). However, studies that analyze the bacterial exo-metabolome

during growth and in response to nutrient limitation are missing. In the present manuscript, we report a detailed analysis of the exo-metabolome of strain *Pseudovibrio* sp. FO-BEG1, a heterotrophic alphaproteobacterium potential symbiont of marine sponges (Bondarev *et al.*, 2013; **Contributed works**). Bacteria belonging to this genus have often been shown to produce bioactive secondary metabolites, and they are considered a potential source of new molecules of medical interest (Kennedy *et al.*, 2009; O'Halloran *et al.*, 2011; Flemer *et al.*, 2012; Bondarev *et al.*, 2013; **Contributed works**).

We investigated the composition of the secreted metabolites during bacterial growth and we analyzed the effect of phosphate (P_i) limitation. Phosphate limitation was chosen because it is a common environmental condition encountered in many marine systems (Cotner *et al.*, 1997; Wu *et al.*, 2000; Thingstad *et al.*, 2005), and it has been described to have a significant effect not only on the overall cell physiology, but also on secondary metabolite production (Martín, 2004 and references therein). We report here the astonishing diversity of the exo-metabolome of strain FO-BEG1 and the drastic effect that phosphate limitation has on its composition. These data shed new light onto the complexity of the metabolites secreted by heterotrophic marine bacteria and onto the effect that their metabolic state can have on the composition of DOM in the ocean.

Materials and Methods

Growth conditions

Strain FO-BEG1 was cultivated in the carbohydrate/mineral medium (CM) as described by Shieh *et al.* (2004) and modified by Bondarev *et al.* (2013;

Contributed works). For the phosphate-surplus conditions (+P_i) phosphate was added to a final concentration of 1.4 mmol L^{-1}, whereas no phosphate was added to the phosphate-limited (−P_i) medium. Under –P_i conditions the sole phosphate source was the buffer used for the vitamin solutions, resulting in a final concentration of 0.1 mmol L^{-1} phosphate. Erlenmeyer flasks of 250 mL were filled with 100 mL of medium and inoculated with 100 µL of a pre-culture grown under +P_i conditions. Cultures were incubated at 28 °C in the dark and shaken at 120 rpm. We monitored bacterial growth by means of Optical Density (OD) measured at 600 nm using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). The OD_{600} was then correlated with the cell number, determined by counting cells in a Thoma chamber (Brand GmbH, Wertheim, Germany; data not shown).

Solid phase extraction of dissolved organic matter (SPE-DOM), dissolved organic carbon (DOC) measurements, and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) of DOM

For both −P_i and +P_i cultures, samples were collected immediately after the inoculation (T0) and in the exponential growth phase (T1). Two more samples during the late exponential phase (T2) and the middle stationary phase (T3) were collected for the –P_i cultures, and one more was collected in stationary phase (T2) of +P_i cultures. Samples were centrifuged at 10,000 × *g* for 10 min at 5 °C. The supernatant was filtered into 150 mL combusted glass serum-bottles using Acrodisc 25 mm syringe filters with a 0.2 µm pore size GHP membrane

(Pall LifeSciences, Ann Arbor, MI, USA), acidified to pH 2.5 with 2 mol L^{-1} HCl, and stored at 4 °C until further analysis. We collected the samples from all biological triplicates in both $+P_i$ and $-P_i$ conditions, with the exception of T0.

DOM of the cell-free supernatants was extracted according to the solid phase extraction of dissolved organic matter (SPE-DOM) method described by Dittmar *et al.* (2008). The extraction was performed using Bond Elute PPL (Agilent Technologies, Wildbronn, Germany) cartridges with a styrene-divinylbenzene (SDVB) polymer modified with a proprietary surface able to retain also the most polar classes of analytes. DOC content of each extract was analyzed using a Shimadzu TOC-VCPH total organic carbon analyzer (Shimadzu, Kyoto, Japan). The extracted DOM samples were then diluted with a mixture of methanol (MS grade) and ultrapure water (50:50 v/v) to yield a DOC concentration of 20 mg L^{-1} carbon, filtered using a 0.2 µm pore size PTFE filter (Rotilabo, Carl Roth GmbH, Karlsruhe, Germany), and analyzed in the negative ion mode with a solariX FT-ICR-MS (Bruker Daltonik GmbH, Bremen, Germany) with a 15.0 Tesla magnet and equipped with an electrospray ionization (ESI) source. All data were acquired with a time domain size of 4 megawords with a detection range of *m/z* 150 to 2,000. For each run, 500 broadband scans were accumulated. The acquired mass spectra were analyzed with the Data Analysis software Version 4.0 SP4 (Bruker Daltonik GmbH).

Calibration of the mass spectra was performed as follows: one replicate of $-P_i$ T3 was spiked with 0.05 ppm L-arginine (Sigma-Aldrich, Steinheim, Germany). The resulting mass spectra were calibrated internally with a reference mass list for L-arginine, and molecular formulae were assigned for

the remaining peaks in the spectrum using the Data Analysis software. The molecular formulae were assigned to an elemental composition in the following ranges: C 1-∞, H 1-∞, O 1-∞, N 0-4, S 0-2, P 0-1, allowing an error of 0.15 ppm. A mass list with more than 300 masses in the range 150-800 *m/z* was obtained, and used to calibrate all other acquired mass spectra. Due to the diversity of the samples, the calibration list was adjusted manually to always cover the full detected mass range with at least 40 calibration points. All linear calibrations resulted in an average mass error of below 0.05 ppm. Additionally, the instrument was externally calibrated with an in-house marine deep sea DOM reference sample (mass accuracy of less than 0.1 ppm). Before each sample set, blank checks with methanol/ultrapure water 1:1 were measured.

Samples comparison, molecular formulae assignment, filtration of the dataset, and statistical analysis

Comparison of the mass spectra, formulae assignment and isotope (^{13}C) identification were performed using an in-house Matlab routine developed by the Max Planck Research Group for Marine Geochemistry. The molecular formulae were assigned in the elemental composition in the following ranges: C 1-40, H 1-∞, O 1-∞, N 0-4, S 0-2, P 0-1, no Na, Fe, Cl, and allowing a mass error of 500 ppb. Only peaks with signal to noise ratio higher than 4 were considered and only formulae with a minimum H/C ratio of 0.3 and a maximum O/C ratio of 1 were accepted. All detected ions were singly charged, as indicated by the mass difference between isotopologues (of ^{12}C versus ^{13}C). Therefore, all detected *m/z* values were equivalent to molecular masses.

Ion intensities were normalized by dividing the intensity of each mass by the sum of the 500 highest intensities measured in the respective sample. This normalization process was performed independently for each sample. After normalization, singlets were removed, i.e. masses detected only in one sample out of the seventeen analyzed. In order to have an overview of the similarity among the samples, we performed a non-metric multidimensional scaling (NMDS), using the Bray-Curtis similarity index for the calculation of the distance matrix. Minimum-spanning tree between all samples was constructed to visualize pairwise sample similarities. Nearest neighbors, the most similar samples, were connected according to their degree of similarity. In order to reduce contingent noise and to consider only the molecules produced by the bacteria, we further filtered the dataset using the following criteria. We removed all masses detected in the samples $-P_i$ and $+P_i$ T0 that did not at least double their normalized intensity during the experiment; we removed all masses that were not present in at least all triplicates of one condition in a specific time point; we removed all masses which could contain an isotope ^{13}C. The filtered dataset was newly analyzed by means of NMDS, but the samples collected at T0 for both growth conditions were not considered, due to the significant alteration in their *m/z* composition derived by the filtration of the dataset described above. A minimum-spanning tree between all samples was newly constructed. NMDSs, the relative stress values, which is a measure that reflects the degree of deviation of NMDS distances from the original matrix distances, and the minimum-spanning trees were carried out by means of the PAST program (Hammer *et al.*, 2001). Subsequently, in order to identify the unique masses

present per time point under both conditions and the masses shared among the growth stages, we created a Venn diagram always considering masses present in all triplicates at the respective time point.

The elemental composition and the modified aromaticity index (AI_{mod}; Koch and Dittmar, 2006) of each molecular formula were used to divide them into molecular categories according to the criteria modified after Šantl-Temkiv *et al.* (2013). For this analysis we excluded all masses for which multiple molecular formulae were obtained. We divided the molecular formulae in the following categories: peptides (if the molecular formula has a H/C ratio between 1.5 and 2, an O/C ratio lower than 0.9 and includes N), sugars (if the molecular formula has an O/C ratio higher or equal to 0.9 and an AI_{mod} lower than 0.5), saturated fatty acids (if the molecular formula has H/C ratio higher or equal to 2 and an O/C ratio lower or equal to 0.9), unsaturated aliphatic compounds (if the molecular formula has a H/C ratio between 1.5 and 2, an O/C ratio lower than 0.9, and does not contain N), highly unsaturated compounds (if the molecular formula has an AI_{mod} lower than 0.5, an H/C ratio lower than 1.5, and an O/C ratio lower than 0.9), phenols (if the molecular formula has an AI_{mod} higher or equal than 0.5 and less than 12 C atoms), and polyphenols (if the molecular formula has an AI_{mod} higher or equal than 0.5 and 12 or more C atoms).

Metabolite and pathway annotation

Masses detected in all three biological replicates at each time point were putatively annotated (i.e. level 2 of metabolite identification as defined by the Metabolomics Standards Initiative; Summer *et al.*, 2007) using the

"transformation mapping" approach (Weber and Viant, 2010), after correcting the mass values for the H^+ loss. This method is based on mapping an experimentally-derived empirical formula difference for a pair of peaks to a known empirical formula difference between substrate-product pairs derived from the KEGG database (Kyoto Encyclopedia of Genes and Genomes; Kanehisa and Goto, 2000). To reduce the number of false positive assignments even more only metabolites that occurred in one of the *Pseudovibrio* sp. FO-BEG1 pathways (KEGG identifier: psf) were selected for annotation (as listed in KEGG on July 2013).

Results

Measurement of the DOC released during bacterial growth and FT-ICR-MS analysis

Phosphate limitation repressed the growth of *Pseudovibrio* sp. FO-BEG1, leading to a final cell density 2.5-3.5 times lower than under phosphate-surplus conditions (**Fig. 3.1A**). Under $-P_i$ conditions a slightly higher amount of solid phase extractable dissolved organic carbon (SPE-DOC) was produced during the first half of the exponential phase (T1; **Fig. 3.1B**). As observed in T0, the SPE extraction did not retain the provided glucose, which would correspond alone to 60 mmol L^{-1} DOC. Therefore the measured DOC represented the organic compounds produced and secreted by *Pseudovibrio* sp. FO-BEG1. At T1 under both conditions only around 2 mmol L^{-1} of glucose was taken up by the cells (**Chapter 2**), resulting in a conversion of the initial carbon

source in SPE-DOC of 0.4% for $-P_i$ cultures and 0.3% for $+P_i$ cultures. Surprisingly, in the samples collected in the late exponential (T2) and in the middle stationary phase (T3) under $-P_i$ conditions the SPE-DOC production increased to 266.9 ± 57.9 μmol L^{-1} and 510.6 ± 191.9 μmol L^{-1}, respectively. At T2 the glucose consumed was around 5 mmol L^{-1}, which was then taken up by the cells during the rest of the growth (**Chapter 2**). Consequently, the SPE-DOC produced represented in both growth stages 0.9% of the used glucose. Compared to this, the SPE-DOC content under $+P_i$ conditions during the stationary phase was more than 3 times lower (144.3 ± 17.6 μmol L^{-1}) (**Fig. 3.1B**), representing 0.2% of the consumed glucose.

The raw data obtained from the FT-ICR-MS analysis consisted of 23,892 masses ranging from 154 *m/z* to 1,930 *m/z*. After normalization of the ion intensity, we performed a non-metrical multidimensional scaling (NMDS) in order to evaluate the similarities among the samples (**Fig. 3.2**). As the stress value of the NMDS plot was 0.06, it can be considered a good representation of the calculated distance matrix and thus, of the similarity among the samples. The samples collected at T1 for each biological triplicate under both $-P_i$ and $+P_i$ conditions clustered together and were clearly separated from the samples collected during the rest of the growth period (**Fig. 3.2**). All biological triplicates of the $-P_i$ conditions collected in the late exponential and in the stationary phase (T2 and T3) were completely divergent from the triplicates of the $+P_i$ stationary phase (T2). Moreover, the samples T2 and T3 for the $-P_i$ conditions also clustered separately in the plot (**Fig. 3.2**).

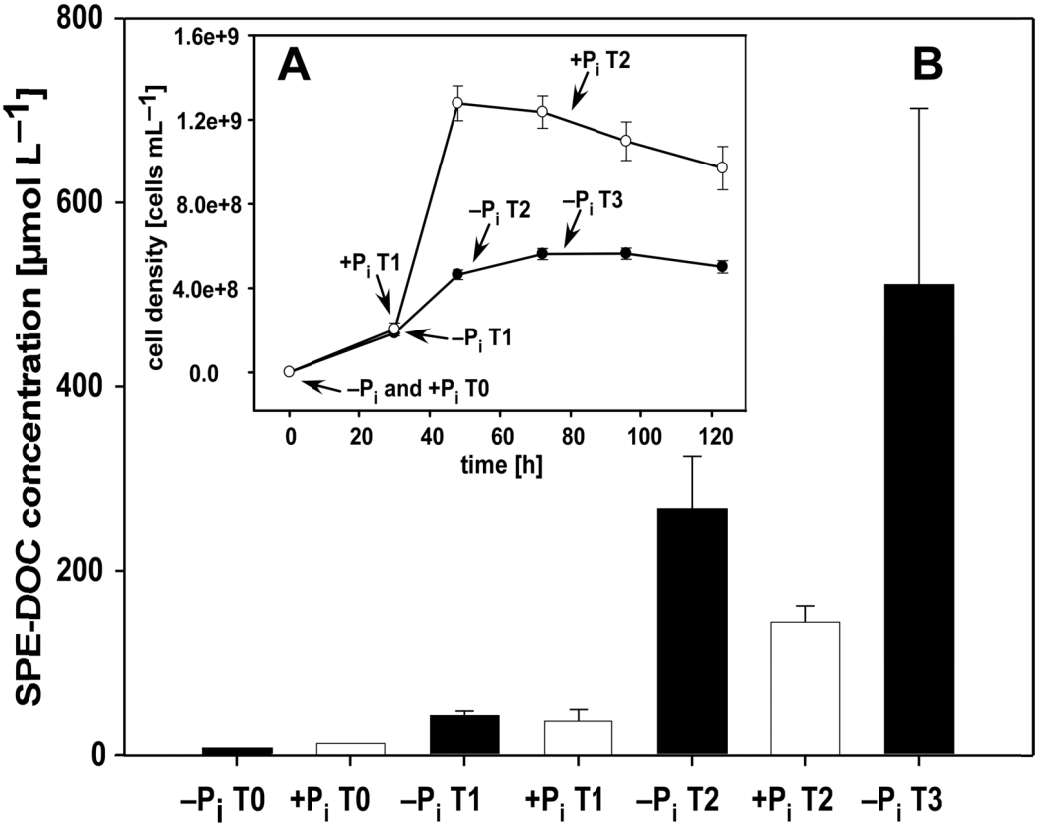


Figure 3.1 | Cell growth and solid phase extractable dissolved organic carbon (SPE-DOC). The inner panel (**A**) shows cell growth, measured as cell density over time, for the two tested conditions. Empty circles represent the cultures grown under $+P_i$ conditions and filled circles represent the cultures grown under $-P_i$ conditions. The bars (**B**) represent the average amount of DOC measured in the solid phase extract of the biological triplicates collected during growth under both $+P_i$ (white) and $-P_i$ conditions (black). Error bars represent the standard deviation of biological triplicates.

In order to consider only those metabolites that were produced by the strain under the tested conditions, we removed all compounds from the dataset that were already present at T0 and did not at least double their ion intensities during the investigated growth period. Moreover, only compounds present in all biological triplicates at a certain time point and growth condition were further considered. This filtration reduced the dataset to 8,381 masses ranging from an

m/z value of 154 to 998. The NMDS plot (**Fig. S3.1**) performed for this new dataset showed the same clustering pattern as the unreduced dataset (**Fig. 3.2**).

In the Venn diagram (**Fig. 3.3**), it was evident that the samples collected during the exponential growth phase under $+P_i$ and $-P_i$ conditions presented 23 and 100 unique masses, respectively, and 202 shared masses never detected in the other samples collected during the later growth phases. Independent of the condition and the growth phase, we detected 573 masses shared among all samples. The samples collected in the late exponential and in the stationary phase under $-P_i$ conditions (T2 and T3), together showed 1,088 unique masses never detected in the other samples, whereas in the samples collected in the stationary phase under $+P_i$ conditions we detected 832 unique masses not present in the other samples (**Fig. 3.3**).

Conversion of masses in molecular formulae and annotation of metabolites

Of the 8,381 detected masses, we were able to assign molecular formulae to 4,914. Of these, 4,122 were unique molecular formulae, corresponding to 49% of the *m/z* of the filtered dataset. A greater number of molecular formulae could be assigned to the masses obtained from samples collected at T1 under both $+P_i$ and $-P_i$ conditions (**Table 3.1**). Under $+P_i$ conditions an increase in the relative number of formulae containing nitrogen was observed from exponential to stationary phase, whereas the percentage of these compounds decreased under $-P_i$ conditions. Interestingly, during bacterial growth under $-P_i$ conditions the relative amount of formulae containing sulfur

increased strongly from 44.5% to 64.6% of the assigned molecular formulae (**Table 3.1**).

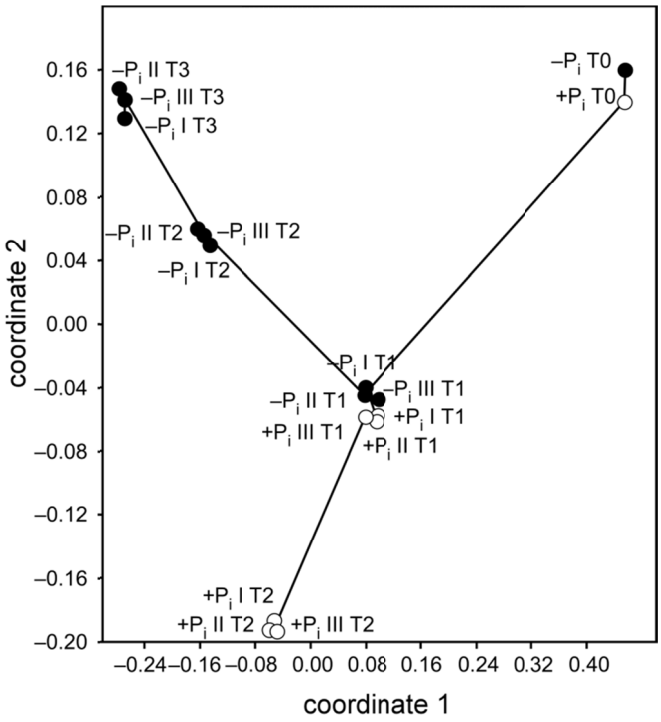


Figure 3.2 | Non metrical multidimensional scaling (NMDS) plot performed employing the Bray-Curtis similarity index and using the data obtained from all biological triplicates of +P_i (empty circles) and –P_i (filled circles) conditions. Nearest neighbor samples (i.e. most similar) are connected in order to visualize pairwise sample similarities. Stress value: 0.06.

After calculating the modified aromaticity index (AI_{mod}) we assigned the obtained molecular formulae to specific molecular categories, and calculated their relative abundance at different time points (**Fig. 3.4**). At T1, the composition of the secreted metabolites was similar in both treatments. The major components of the exo-metabolome were compounds with molecular formulae assigned to peptides and highly unsaturated molecules. Interestingly, when we

compared the composition of the secreted molecules during stationary phase, we observed a pronounced increase of highly unsaturated, phenolic and polyphenolic compounds under $-P_i$ conditions and a decrease in peptides and unsaturated aliphatic compounds.

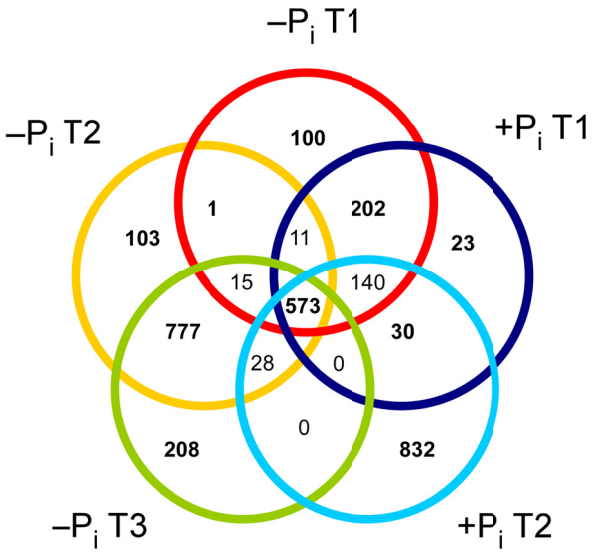


Figure 3.3 | Venn diagram showing unique and shared masses detected under -P_i and +P_i conditions. Only masses detected in all biological triplicates for each time point were considered.

The ultra-high resolution of the FT-ICR-MS results in precise masses that can be compared and assigned to known compounds present in pathways described for the considered organism and collected in target databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes). Using the metabolite names reported in the pathways of strain *Pseudovibrio* sp. FO-BEG1 in this database we annotated the masses obtained from the FT-ICR-MS analysis. The annotation strategy was based on mapping an experimentally-derived empirical formula difference for a pair of peaks to a known empirical formula difference between

substrate-product pairs retried from KEGG. It was previously shown that this approach reduced the false positive rate of identification by more than four-fold versus a traditional peak by peak search using accurate mass measurements only, while maintaining a minimal false negative rate (Weber and Viant, 2010). A molecular name could be assigned only to a minor proportion of compounds (less than 3%; **Dataset S3.1**). For the sample $-P_i$ T1, we could annotate 85 masses with 55 assigned to unique metabolites (1.8% of the detected masses in all triplicates). The number of masses assigned to unique metabolites decreased to 46 (65 total annotated masses) for the samples $-P_i$ T2 and to 30 for $-P_i$ T3 (37 total annotated masses), representing 1.3% and 1.2% of the detected masses in all triplicates, respectively. 49 and 64 masses could be assigned to unique metabolites (73 and 97 total annotated masses) in the samples $+P_i$ T1 and $+P_i$ T2 (1.8% and 1.5% of the detected masses in all triplicates), respectively. Most of the annotated compounds were intermediates in the metabolism of the amino acids lysine, tyrosine, tryptophan, and phenylalanine (**Dataset S3.2** and **Fig. S3.2**). In all samples, except $-P_i$ T3, several metabolites were also annotated in the pathway of the purine metabolism (**Dataset S3.2** and **Fig. S3.2**).

Discussion

In order to quantify and characterize the metabolites secreted by strain *Pseudovibrio* sp. FO-BEG1, and to evaluate the effect of phosphate limitation on them, we performed an ultra-high resolution mass spectrometry analysis of the bacterial exo-metabolome. Mass spectrometry is the most widely used approach in metabolomic studies (Want *et al.*, 2007). In particular high resolution accurate

mass (HRAM) mass spectrometry instruments are receiving progressively more attention, owing to their ability to resolve highly complex samples, yielding accurate mass measurements, which allow precise calculation of their elemental composition (Aharoni *et al.*, 2002; Marshall, 2004; Junot *et al.*, 2010).

Table 3.1 | Overview of the data obtained from the FT-ICR-MS analysis
In the table the number of masses detected in all biological triplicates of each specific time point are reported. The values in brackets represent the percentage of masses to which a unique molecular formula could be assigned and the percentage of unique molecular formulae containing heteroatoms.

	$+P_i$ T1	$+P_i$ T2	$-P_i$ T1	$-P_i$ T2	$-P_i$ T3
detected masses	2596	4206	3112	3566	2479
unique molecular formulae	1578 (60.8%)	2426 (57.7%)	1931 (62.1%)	1876 (52.6%)	1241 (50.1%)
formulae containing nitrogen	1193 (75.6%)	2085 (85.9%)	1362 (70.5%)	1387 (73.9%)	813 (65.5%)
formulae containing sulfur	648 (41.0%)	1087 (44.8%)	859 (44.5%)	1138 (60.7%)	802 (64.6%)
formulae containing phosphorus	221 (14.0%)	374 (15.4%)	247 (12.8%)	301 (16.0%)	176 (14.2%)

When cells growing under $-P_i$ conditions entered stationary phase, they released three times more solid phase extractable dissolved organic carbon (SPE-DOC) than cells grown under $+P_i$ conditions (**Fig. 3.1**). We are confident that the SPE-DOC concentrations and the number of metabolites obtained are not biased by the composition of the cultivation medium because, as shown by the amount of SPE-DOC at T0, the SPE process did not retain significant amounts of organic compounds present in the medium (**Fig. 3.1**). Moreover, during the

filtration of our dataset we removed all *m/z* which were detected in T0 and did not at least double their ion intensity during the experiment. Therefore, all compounds originally present in the medium were excluded from the analyses.

It has been known for several years that low phosphate concentrations can induce the production of secondary metabolites (Martín, 2004 and references therein), which would suggest that under $-P_i$ conditions a higher fraction of the carbon source provided was used by *Pseudovibrio* sp. FO-BEG1 for the production of such compounds. In addition, it is known that phosphate limitation can trigger membrane lipid rearrangement, with the substitution of phospho-containing with phospho-free lipids (Minnikin *et al.*, 1972; Benning *et al.*, 1995), a phenomenon that we also observed for *Pseudovibrio* sp. FO-BEG1 (**Chapter 2**). Therefore, it is reasonable to hypothesize that due to the membrane rearrangement more cytosolic metabolites could leak out from the cells, justifying the higher production of SPE-DOC under $-P_i$ conditions. Consistently, nutrient leakage was also described in a marine yeast strain growing under phosphate-limited conditions (Robertson and Button, 1979). Other studies showed that bacteria can convert 5 to 15% of the provided carbon to DOC (Ogawa *et al.*, 2001; Gruber *et al.*, 2006; Kawasaki and Benner, 2006), which is one order of magnitude higher than observed in our experiments. However, a precise comparison is difficult because in all mentioned experiments different medium composition, growth parameters, and analytic procedures were used.

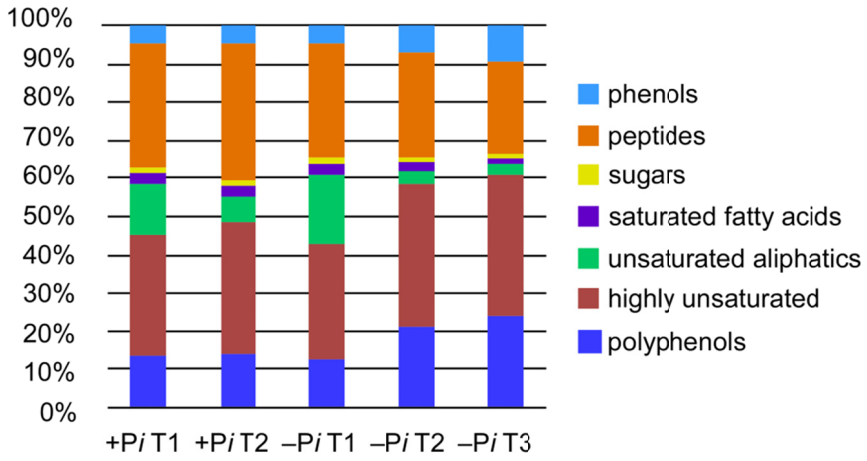


Figure 3.4 | Percentage of molecular formulae, detected during the bacterial growth under $+P_i$ and $-P_i$ conditions at different time points, attributed to molecular categories.

Rosselló-Móra *et al.* (2008) and Antón *et al.* (2013) analyzed the endo- and the exo-metabolome of different *Salinibacter ruber* isolates during the classification of different ecotypes, and reported that the isolates can be distinguished by their metabolic profiles. Moreover, Brito-Echeverria *et al.* (2011) analyzed the endo- and exo-metabolome of different *Salinibacter* strains in response to different stress conditions, and reported that the exo-metabolome was affected to a greater extent than the endo-metabolome. In all studies the analysis was performed via FT-ICR-MS and they are the first reports that give information about the complexity of the bacterial exo-metabolome. In line with these observations, our analysis revealed that *Pseudovibrio* sp. FO-BEG1 produced and released at least many hundreds of compounds into the medium, and that the composition of this DOC was greatly affected by phosphate

limitation. In this respect, FT-ICR-MS represents an ideal and powerful technique to unravel this complexity. We could clearly show that the exo-metabolome composition differs during different growth phases and between the two tested conditions (**Fig. 3.2, S3.1**). These data are consistent with previous studies, which using low resolution techniques reported variation in the secreted metabolites due to the growth phase or to environmental stress (Shnayderman *et al.*, 2005; Coucheney *et al.*, 2008; Takahashi *et al.*, 2008; Barreto *et al.*, 2011). Moreover, our data indicate that phosphate limitation also affects the sulfur metabolism of *Pseudovibrio* sp. FO-BEG1, leading to an increased release of compounds containing this element (**Table 3.1**).

The presence of unique masses detected only at specific time points under both conditions shows a dynamic cycling of organic compounds. Molecules produced during the beginning of the exponential growth phase were then taken up again when cells entered stationary phase. A similar phenomenon was observed in a study that investigated the grazing effect on the DOC production in a pure culture of *Pseudomonas chlororaphis* (Gruber *et al.*, 2006). Interestingly, even though for each sampling point and each condition we identified hundreds of unique masses, we also detected 573 masses that were always present in our samples independent from the growth stage or the growth condition. It will be interesting to verify if this "core" exo-metabolome is affected by other environmental changes or if it represents a distinctive "metabolic signature" of the strain.

It has been suggested that the trophic status of the environment affects DOM composition via shaping the ecological processes that are responsible for

its production (Kujawinski, 2011). Productive, nutrient rich regions have significant DOM production directly from photosynthesis, whereas oligotrophic, nutrient poor regions have significant DOM production from grazing processes (Nagata, 2000; Maranón *et al.*, 2004). This difference was attributed to the complexity of the microbial food web in different environments, with the oligotrophic region having a more effective microbial loop compared to the classical food web described in the productive regions (Teira *et al.*, 2001). Our data suggest that to understand DOM composition the effect of the environmental nutrient regimes on bacterial physiology should not be underestimated. As we show, it can greatly affect both the amount and the composition of the produced organic compounds.

Comparing the variation of the metabolome of *Escherichia coli* and *Saccharomyces cerevisiae* in response to carbon and nitrogen limitation, Brauer *et al.* (2006) showed global metabolic trends remarkably conserved among these two distantly related microorganisms. Therefore, in order to verify the presence of shared metabolic responses, which could indicate the presence of highly conserved regulatory schemes, it would be of great interest to compare the variations of the exo-metabolome in response to nutrient limitation among different bacteria. This information could also help to better understand the influences of microbes on DOM composition in natural environments characterized by particular nutrient regimes.

The molecular formula assignment allowed us to classify the detected masses in molecular categories, giving a broad overview of the compounds released during the growth under both $-P_i$ and $+P_i$ conditions. Under phosphate

limitation, we observed a higher production of phenolic and polyphenolic compounds when cells entered stationary phase (**Fig. 3.4**). Production of phenol was described for the strains *Pseudovibrio* sp. D323 and L4-8 (Penesyan *et al.*, 2011; Roué *et al.*, 2012). The crude extract of the spent medium of the latter strain showed a strong antioxidant activity, which is consistent with our finding that strain FO-BEG1 produces different types of phenols and polyphenols, known for their antioxidant properties (Scalbert *et al.*, 2005). Higher production of these compounds under $-P_i$ conditions could be related to the increased oxidative stress that cells growing under phosphate limitation can experience (Gérard *et al.*, 1999; Moreau, 2004; Yuan *et al.*, 2005), and which we also inferred for strain FO-BEG1 from the comparison of the protein expression between $+P_i$ and $-P_i$ conditions (**Chapter 2**).

Some of the detected phenolic and polyphenolic compounds can be, for instance, tropone derivates. These molecules are commonly produced by bacteria members of the *Roseobacter* clade and can have algaecide and antibacterial activity, such as, for example, the potent antibiotic tropodithietic acid (TDA; Thiel *et al.*, 2010; Seyedsayamdost *et al.*, 2011). Previous high-performance liquid chromatography experiments suggested that a compound with the same retention time and UV-visible spectra as the TDA standard was produced by *Pseudovibrio* sp. FO-BEG1 under $-P_i$ conditions when cells entered stationary phase (**Chapter 2**). Also during the FT-ICR-MS analysis, we identified a *m/z* 210.952904 with the molecular formula assigned $C_8H_4O_3S_2$, which is, also considering its peculiar isotopic patterns due to the presence of two sulfur atoms per molecule, consistent with being TDA. As observed

previously (**Chapter 2**), this compound was detected only in stationary phase under phosphate limitation, and its ion intensity increased from T2 to T3. Members of the genus *Pseudovibrio* possess several traits in common with members of the abundant and ubiquitous *Roseobacter* clade, such as the ability to utilize dimethylsulfoniopropionate, the symbiotic lifestyle, and the ability to produce bioactive secondary metabolites such as TDA (Buchan *et al.*, 2005; Bondarev *et al.*, 2013; **Contributed works**). Members of the *Roseobacter* clade produce TDA together with an uncharacterized yellow pigment (Buchan *et al.*, 2005), and consistently also the *Pseudovibrio* cultures growing under $-P_i$ conditions developed an intense yellow coloration when entered stationary phase (**Chapter 2**). Considering these similarities, it is reasonable to speculate that among the phenolic and polyphenolic fractions of the detected compounds there were some molecules with similar characteristics to the one described for the *Roseobacter* clade, which might be of biotechnological interest.

The ultra-high resolution of the FT-ICR-MS helps to provide the elemental composition of the measured masses and, therefore, also to perform metabolic annotations using existing databases. We used the KEGG database to interpret the information concerning the metabolic pathways of strain FO-BEG1. Only a small fraction of the detected masses were registered in KEGG, and most of the annotated metabolites, in all samples, were compounds involved in the synthesis of mainly aromatic amino acids (e.g. tyrosine, tryptophan, phenylalanine) and nucleotides. Release of these compounds was also observed in the analysis of the exo-metabolome of other bacterial and yeast strains (Behrends *et al.*, 2009; Paczia *et al.*, 2012). In conditions of "overflow metabolism",

meaning in conditions with an excess of carbon or energy source or in presence of nutrient limitation, intermediates of different metabolic pathways can be released (Kramer, 1994). Recent evidence suggests that this is a common phenomenon in different microorganisms when cultivated under conditions of non-inhibited carbon uptake (Paczia *et al.*, 2012). Aromatic amino acids are key intermediates in the production of aromatic secondary metabolites (Herrmann, 1995) suggesting that strain FO-BEG1 is potentially producing such compounds, which, however, are of unknown structure.

Production and release of amino acids by bacterial communities was also reported by Kawasaki and Benner (2006), and these compounds are important constituents of DOC in some coastal environments (Coble, 1996; Yamashita and Tanoue, 2003). It is worth pointing out that comparing the list of molecular formulae retrieved from our exo-metabolome study with a list of formulae detected in DOM of the deep North Pacific Ocean (Rossel *et al.*, 2013), we found only 83 shared compounds. However, comparing our data with a list of molecular formulae detected in DOM during and after a phytoplankton bloom in the North Sea (Dittmar *et al.*, unpublished data), we detected 729 matches and 90.8% of them were always present in the natural samples, independently from the bloom of the phytoplankton (**Dataset S3.3**). This indicates that, at least on a molecular formula level, the compounds we were able to detect are indeed part of natural DOM, and their presence does not seem to be directly related to the activity of the primary producers of the community. Consistently, also Kujawinski *et al.* (2009), showed that molecules detected in a pure culture of "*Candidatus* Pelagibacter ubique" were also detected in open ocean DOM.

We were able to detect several hundreds of masses for each sample, but we only managed to annotate less than 3% of them using the KEGG database as reference. Among the identified compounds, a smaller number of metabolites could be annotated for the samples collected at T3 under $-P_i$ conditions. However, the majority of the annotated compounds belonged to the same pathways also identified in the other samples. Under phosphate limitation the number of formulae annotated in the pathway "Tyrosine metabolism" and "Tryptophan metabolism" decreased strongly from T1 to T3, indicating a reuptake of such molecules by the cells when they enter stationary phase.

Although our approach represents a high-throughput way of performing metabolomic studies, it is limited by the incompleteness of the targeted database. For instance, even though we have strong indications that TDA production occurred under $-P_i$ conditions (**Chapter 2**) and we also detected the respective ion in the FT-ICR-MS analysis, we could not identify this compound during the annotation process using the KEGG database. The reason is the absence of the pathway for TDA production among the annotated one in the KEGG pathways of *Pseudovibrio* sp. FO-BEG1. This represents a limit of this approach and, at the same time, underlines the lack of knowledge we have about the biosynthetic ability of marine bacteria. Since databases such as KEGG are mostly restricted to genome-reconstruction pathways, wrongly annotated genes and absence of compounds for which the biosynthetic pathways have not yet been elucidated for the target organism can decrease the number of identified molecules in metabolic studies and limit the capabilities of techniques such as FT-ICR-MS.

Conclusions

In this work for the first time the exo-metabolome of a marine heterotrophic bacterium was investigated in detail using ultrahigh-resolution mass spectrometry. We show that the exo-metabolome is unexpectedly large and diverse, it is characterized by a dynamic recycling of compounds, and it is drastically affected by the physiological state of the strain. Our data clearly illustrate that phosphate limitation triggered a pronounced increase in the secretion of DOC and at the same time greatly affected its composition. We reported that a part of the molecular formulae discovered in the exo-metabolome were detected in natural DOM. Future studies on the exo-metabolomes of different strains and DOM from different locations may help to understand to what extent the compounds secreted by heterotrophic bacteria influence DOM composition in the ocean. By assigning unique molecular formulae, we were able to chemically categorize the detected masses and we showed that phosphate limitation leads to an increased production of phenols and polyphenols, which could represent new metabolites of biotechnological interest. Our work shows that HRAM instruments represent a promising technique to unravel the complexity of the metabolites secreted from microorganisms. However, the great discrepancy between the number of measured masses and the number of annotated molecules using the KEGG database underlines the large gap in our knowledge concerning the biosynthetic ability of marine bacteria, indicating the necessity of further work directed to the chemical characterization of secreted metabolites. At the same time the large exo-metabolome would represent a big "chemical reservoir", of bacterial origin, for the discovery of new molecules of

biotechnological interest. Our data underline the great biosynthetic ability of heterotrophic bacteria and suggest that, using the words of Traxler and Kolter (2012), ''*the chemical landscape inhabited and manipulated by bacteria is vastly more complex and sophisticated than previously thought*''.

Acknowledgments

We are very grateful to K. Klaproth for technical support in the FT-ICR-MS analysis and I. Ulber and M. Friebe for the help in the DOM extraction and DOC measurement. We are indebted to Dr. J. Niggemann for sharing DOM data of the North Sea. This study was funded by the European Research Council, Grant No. 203364, and the Max Planck Society.

References

Aharoni, A., Ric de Vos, C.H., Verhoeven, H.A., Maliepaard, C.A., Kruppa, G., Bino, R., and Goodenowe, D.B. (2002) Nontargeted metabolome analysis by use of Fourier transform ion cyclotron mass spectrometry. *Omics* **6**: 217-234.

Allen, J., Davey, H.M., Broadhurst, D., Heald, J.K., Rowland, J.J., Oliver, S.G., and Kell, D.B. (2003) High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. *Nature Biotechnol* **21**: 692-696.

Antón, J., Lucio, M., Peña, A., Cifuentes, A., Brito-Echeverria, J., Moritz, F., Tziotis, D., López, C., Urdiain, M., Schmitt-Kopplin, P., and Rosselló-Móra, R. (2013) High metabolomic microdiversity within co-occurring Isolates of the extremely halophilic bacterium *Salinibacter ruber*. *Plos One* **8**: e64701. doi:64710.61371/journal.pone.0064701.

Barreto, M.C., Frisvad, J.C., Larsen, T.O., Mogensen, J., and San-Romão, M.V. (2011) Exo-metabolome of some fungal isolates growing on cork-based medium. *European Food Research and Technology* **232**: 575-582.

Behrends, V., Ebbels, T.M.D., Williams, H.D., and Bundy, J.G. (2009) Time-resolved metabolic footprinting for nonlinear modeling of bacterial substrate utilization. *Applied and Environmental Microbiology* **75**: 2453-2463.

Benning, C., Huang, Z.H., and Gage, D.A. (1995) Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. *Archives of Biochemistry and Biophysics* **317**: 103-111.

Boersma, M.G., Solyanikova, I.P., Van Berkel, W.J.H., Vervoort, J., Golovleva, L.A., and Rietjens, I.M.C.M. (2001) F-19 NMR metabolomics for the elucidation of microbial degradation pathways of fluorophenols. *Journal of Industrial Microbiology & Biotechnology* **26**: 22-34.

Bondarev, V., Richter, M., Romano, S., Piel, J., Schwedt, A., and Schulz-Vogt, H.N. (2013) The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. *Environmental Microbiology* **15**: 2095-2113.

Brauer, M.J., Yuan, J., Bennett, B.D., Lu, W.Y., Kimball, E., Botstein, D., and Rabinowitz, J.D. (2006) Conservation of the metabolomic response to starvation across two divergent microbes. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 19302-19307.

Brito-Echeverria, J., Lucio, M., López-López, A., Antón, J., Schmitt-Kopplin, P., and Rosselló-Móra, R. (2011) Response to adverse conditions in two strains of the extremely halophilic species *Salinibacter ruber*. *Extremophiles* **15**: 379-389.

Brown, S.C., Kruppa, G., and Dasseux, J.L. (2005) Metabolomics applications of FT-ICR mass spectrometry. *Mass Spectrometry Reviews* **24**: 223-231.

Buchan, A., González, J.M., and Moran, M.A. (2005) Overview of the marine *Roseobacter* lineage. *Applied and Environmental Microbiology* **71**: 5665-5677.

Carlier, J.P. (1990) Identification by quantitative gas-chromatography of common *Fusobacterium* species isolated from clinical specimens. *Letters in Applied Microbiology* **11**: 133-136.

Carlson, C.A. (2002) Production and removal processes. In *Biogeochemistry of marinine Dissolved Organic Matter*. Hansell, D.A., and Carlson, C.A. (eds). San Diego: CA: Elsevier, pp. 91-152.

Coble, P.G. (1996) Characterization of marine and terrestrial DOM in seawater using excitation emission matrix spectroscopy. *Marine Chemistry* **51**: 325-346.

Cotner, J.B., Ammerman, J.W., Peele, E.R., and Bentzen, E. (1997) Phosphorus-limited bacterioplankton growth in the Sargasso Sea. *Aquatic Microbial Ecology* **13**: 141-149.

Coucheney, E., Daniell, T.J., Chenu, C., and Nunan, N. (2008) Gas chromatographic metabolic profiling: a sensitive tool for functional microbial ecology. *Journal of Microbiological Methods* **75**: 491-500.

Cundy, K.V., Willard, K.E., Valeri, L.J., Shanholtzer, C.J., Singh, J., and Peterson, L.R. (1991) Comparison of traditional Gas-Chromatography (Gc), headspace Gc, and the microbial identification library Gc system for the identification of *Clostridium difficile*. *Journal of Clinical Microbiology* **29**: 260-263.

Dittmar, T., Koch, B., Hertkorn, N., and Kattner, G. (2008) A simple and efficient method for the solid-phase extraction of dissolved organic matter (SPE-DOM) from seawater. *Limnology and Oceanography-Methods* **6**: 230-235.

Fiehn, O. (2001) Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comparative and Functional Genomics* **2**: 155-168.

Flemer, B., Kennedy, J., Margassery, L.M., Morrissey, J.P., O'Gara, F., and Dobson, A.D.W. (2012) Diversity and antimicrobial activities of microbes from two Irish marine sponges, Suberites carnosus and Leucosolenia sp. *Journal of Applied Microbiology* **112**: 289-301.

Gérard, F., Dri, A.M., and Moreau, P.L. (1999) Role of *Escherichia coli* RpoS, LexA and H-NS global regulators in metabolism and survival under aerobic, phosphate-starvation conditions. *Microbiology* **145**: 1547-1562.

Gruber, D.F., Simjouw, J.P., Seitzinger, S.P., and Taghon, G.L. (2006) Dynamics and characterization of refractory dissolved organic matter produced by a pure bacterial culture in an experimental predator-prey system. *Applied and Environmental Microbiology* **72**: 4184-4191.

Hammer, O., Harper, D.A.T., and Ryan, P.D. (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4(1)**: 4:9.

Herrmann, K.M. (1995) The shikimate pathway as an entry to aromatic secondary metabolism. *Plant Physiology* **107**: 7-12.

Junot, C., Madalinski, G., Tabet, J.C., and Ezan, E. (2010) Fourier transform mass spectrometry for metabolome analysis. *Analyst* **135**: 2203-2219.

Kanehisa, M., and Goto, S. (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**: 27-30.

Kawasaki, N., and Benner, R. (2006) Bacterial release of dissolved organic matter during cell growth and decline: molecular origin and composition. *Limnology and Oceanography* **51**: 2170-2180.

Kennedy, J., Baker, P., Piper, C., Cotter, P.D., Walsh, M., Mooij, M.J., Bourke, M.B., Rea, M.C., O'Connor, P.M., Ross, R.P., Hill, C., O'Gara, F., Marchesi, J.R., and Dobson, A.D. (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. *Marine Biotechnology (NY)* **11**: 384-396.

Koch, B.P., and Dittmar, T. (2006) From mass to structure: an aromaticity index for high-resolution mass data of natural organic matter. *Rapid Communications in Mass Spectrometry* **20**: 926-932.

Kramer, R. (1994) Secretion of amino-acids by bacteria - Physiology and mechanism. *FEMS Microbiology Reviews* **13**: 75-93.

Kujawinski, E.B. (2011) The impact of microbial metabolism on marine Dissolved Organic Matter. *Annual Review of Marine Science* **3**: 567-599.

Kujawinski, E.B., Longnecker, K., Blough, N.V., Del Vecchio, R., Finlay, L., Kitner, J.B., and Giovannoni, S.J. (2009) Identification of possible source markers in marine dissolved organic matter using ultrahigh resolution mass spectrometry. *Geochimica Et Cosmochimica Acta* **73**: 4384-4399.

Mapelli, V., Olsson, L., and Nielsen, J. (2008) Metabolic footprinting in microbiology: methods and applications in functional genomics and biotechnology. *Trends in Biotechnology* **26**: 490-497.

Maranón, E., Cermeno, P., Fernández, E., Rodríguez, J., and Zabala, L. (2004) Significance and mechanisms of photosynthetic production of dissolved organic carbon in a coastal eutrophic ecosystem. *Limnology and Oceanography* **49**: 1652-1666.

Marshall, A.G. (2004) Accurate mass measurement: taking full advantage of nature's isotopic complexity. *Physica B-Condensed Matter* **346**: 503-508.

Martín, J.F. (2004) Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *Journal of Bacteriology* **186**: 5197-5201.

Minnikin, D.E., Baddiley, J., and Abdolrah.H (1972) Variation of polar lipid composition of *Bacillus subtilis* (Marburg) with different growth conditions. *FEBS Letters* **27**: 16-18.

Moreau, P.L. (2004) Diversion of the metabolic flux from pyruvate dehydrogenase to pyruvate oxidase decreases oxidative stress during glucose metabolism in nongrowing *Escherichia coli* cells incubated under aerobic, phosphate starvation conditions. *Journal of Bacteriology* **186**: 7364-7368.

Nagata, T. (2000) *Production mechanisms of dissolved organic matter*. In *Microbial ecology of the ocean*. Kirchman, D.L. (eds). Hoboken: NJ: Wiley & Sons, Inc, pp. 207-242

O'Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O'Gara, F., and Dobson, A.D.W. (2011) Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *Journal of Applied Microbiology* **110**: 1495-1508.

Ogawa, H., Amagai, Y., Koike, I., Kaiser, K., and Benner, R. (2001) Production of refractory dissolved organic matter by bacteria. *Science* **292**: 917-920.

Oliver, S.G., Winson, M.K., Kell, D.B., and Baganz, F. (1998) Systematic functional analysis of the yeast genome. *Trends in Biotechnology* **16**: 373-378.

Paczia, N., Nilgen, A., Lehmann, T., Gätgens, J., Wiechert, W., and Noack, S. (2012) Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. *Microbial Cell Factories* **11**: 122.

Penesyan, A., Tebben, J., Lee, M., Thomas, T., Kjelleberg, S., Harder, T., and Egan, S. (2011) Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio* sp. D323 and related sponge-associated bacteria. *Marine Drugs* **9**: 1391-1402.

Rinas, U., Hellmuth, K., Kang, R.J., Seeger, A., and Schlieker, H. (1995) Entry of *Escherichia coli* into stationary phase is indicated by endogenous and exogenous accumulation of nucleobases. *Applied and Environmental Microbiology* **61**: 4147-4151.

Robertson, B.R., and Button, D.K. (1979) Phosphate-limited continuous culture of *Rhodotorula rubra* - Kinetics of transport, leakage, and growth. *Journal of Bacteriology* **138**: 884-895.

Rossel, P.E., Vähätalo, A.V., Witt, M., and Dittmar, T. (2013) Molecular composition of dissolved organic matter from a wetland plant (*Juncus effusus*) after photochemical and microbial decomposition (1.25 yr): Common features with deep sea dissolved organic matter. *Organic Geochemistry* **60**: 62-71.

Rosselló-Móra, R., Lucio, M., Peña, A., Brito-Echeverria, J., López-López, A., Valens-Vadell, M., Frommberger, M., Antón, J., and Schmitt-Kopplin, P. (2008) Metabolic evidence for biogeographic isolation of the extremophilic bacterium *Salinibacter ruber*. *The ISME Journal* **2**: 242-253.

Roué, M., Quévrain, E., Domart-Coulon, I., and Bourguet-Kondracki, M.L. (2012) Assessing calcareous sponges and their associated bacteria for the discovery of new bioactive natural products. *Natural Product Reports* **29**: 739-751.

Šantl-Temkiv, T., Finster, K., Dittmar, T., Hansen, B.M., Thyrhaug, R., Nielsen, N.W., and Karlson, U.G. (2013) Hailstones: a window into the microbial and chemical inventory of a storm cloud. *Plos One* **8**: e53550. doi:53510.51371/journal.pone.0053550.

Scalbert, A., Johnson, I.T., and Saltmarsh, M. (2005) Polyphenols: antioxidants and beyond. *The American Journal of Clinical Nutrition* **81**: 215s-217s.

Seyedsayamdost, M.R., Carr, G., Kolter, R., and Clardy, J. (2011) Roseobacticides: small molecule modulators of an algal-bacterial symbiosis. *Journal of the American Chemical Society* **133**: 18343-18349.

Shieh, W.Y., Lin, Y.T., and Jean, W.D. (2004) *Pseudovibrio denitrificans* gen. nov., sp. nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2307-2312.

Shnayderman, M., Mansfield, B., Yip, P., Clark, H.A., Krebs, M.D., Cohen, S.J., Zeskind, J.E., Ryan, E.T., Dorkin, H.L., Callahan, M.V., Stair, T.O., Gelfand, J.A., Gill, C.J., Hitt, B., and Davis, C.E. (2005) Species-specific bacteria identification using differential mobility spectrometry and bioinformatics pattern recognition. *Analytical Chemistry* **77**: 5930-5937.

Sumner, L.W., Amberg, A., Barrett, D., Beale, M.H., Beger, R., Daykin, C.A., Fan, T.W., Fiehn, O., Goodacre, R., Griffin, J.L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A.N., Lindon, J.C., Marriott, P., Nicholls, A.W., Reily, M.D., Thaden, J.J., and Viant, M.R. (2007) Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics: Official journal of the Metabolomic Society* **3**: 211-221.

Takahashi, H., Kai, K., Shinbo, Y., Tanaka, K., Ohta, D., Oshima, T., Altaf-Ul-Amin, M., Kurokawa, K., Ogasawara, N., and Kanaya, S. (2008) Metabolomics approach for determining growth-specific metabolites based on Fourier transform ion cyclotron resonance mass spectrometry. *Analytical and Bioanalytical Chemistry* **391**: 2769-2782.

Teira, E., Pazó, M.J., Serret, P., and Fernández, E. (2001) Dissolved organic carbon production by microbial populations in the Atlantic Ocean. *Limnology and Oceanography* **46**: 1370-1377.

Thiel, V., Brinkhoff, T., Dickschat, J.S., Wickel, S., Grunenberg, J., Wagner-Döbler, I., Simon, M., and Schulz, S. (2010) Identification and biosynthesis of tropone derivatives

and sulfur volatiles produced by bacteria of the marine *Roseobacter* clade. *Organic & Biomolecular Chemistry* **8**: 234-246.

Thingstad, T.F., Krom, M.D., Mantoura, R.F.C., Flaten, G.A.F., Groom, S., Herut, B., Kress, N., Law, C.S., Pasternak, A., Pitta, P., Psarra, S., Rassoulzadegan, F., Tanaka, T., Tselepides, A., Wassmann, P., Woodward, E.M.S., Riser, C.W., Zodiatis, G., and Zohary, T. (2005) Nature of phosphorus limitation in the ultraoligotrophic eastern Mediterranean. *Science* **309**: 1068-1071.

Traxler, M.F., and Kolter, R. (2012) A massively spectacular view of the chemical lives of microbes. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 10128-10129.

Want, E.J., Nordstrom, A., Morita, H., and Siuzdak, G. (2007) From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. *Journal of Proteome Research* **6**: 459-468.

Weber, R.J.M., and Viant, M.R. (2010) MI-Pack: increased confidence of metabolite identification in mass spectra by integrating accurate masses and metabolic pathways. *Chemometrics and Intelligent Laboratory Systems* **104**: 75-82.

Wu, J.F., Sunda, W., Boyle, E.A., and Karl, D.M. (2000) Phosphate depletion in the western North Atlantic Ocean. *Science* **289**: 759-762.

Yamashita, Y., and Tanoue, E. (2003) Distribution and alteration of amino acids in bulk DOM along a transect from bay to oceanic waters. *Marine Chemistry* **82**: 145-160.

Yuan, Z.C., Zaheer, R., and Finan, T.M. (2005) Phosphate limitation induces catalase expression in *Sinorhizobium meliloti*, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. *Molecular Microbiology* **58**: 877-894.

Supplementary figures

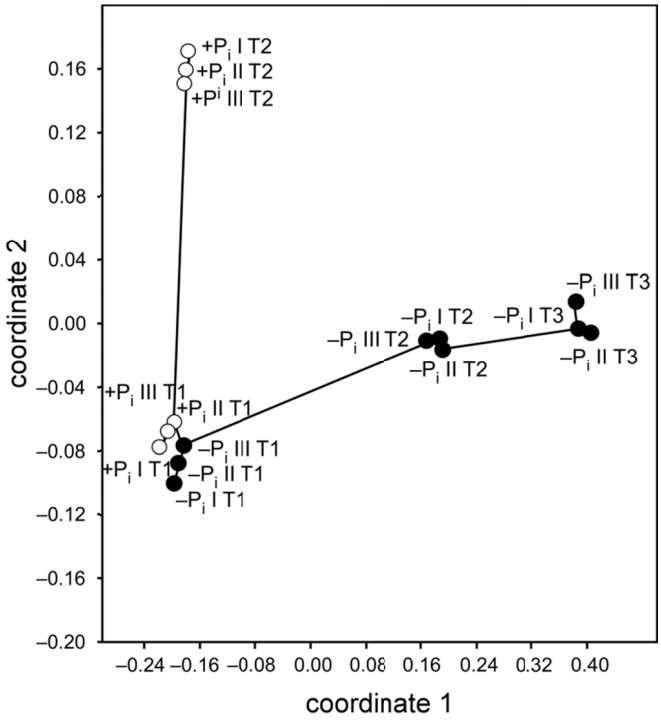


Figure S3.1 | Non metrical multidimensional scaling (NMDS) plot performed after the filtration of the dataset. The Bray-Curtis similarity index was used considering the data obtained from all biological triplicates of $+P_i$ (empty circles) and $-P_i$ (filled circles) conditions. Nearest neighbor samples (i.e. most similar) are connected in order to visualize pairwise sample similarities. Stress value: 0.06.

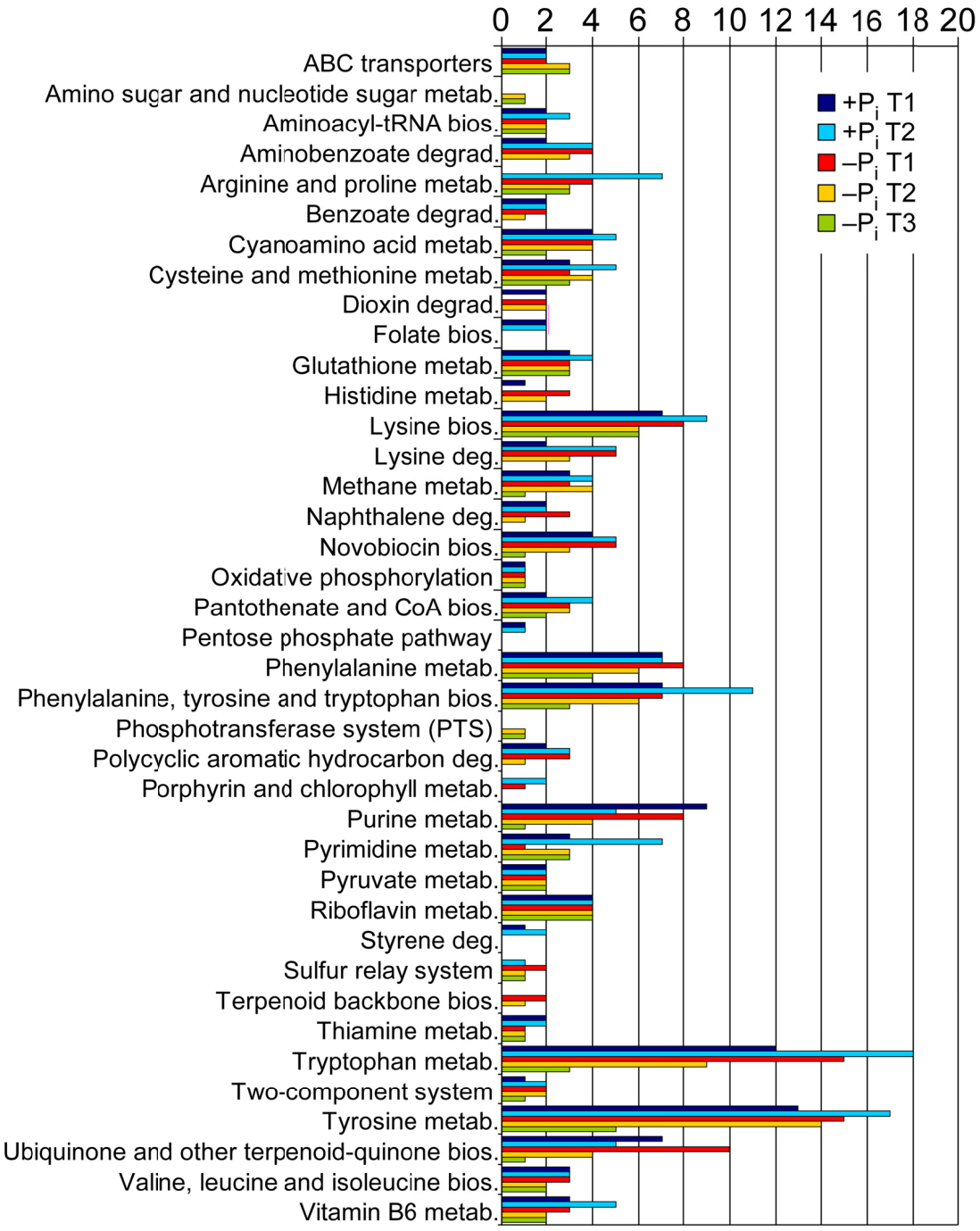


Figure S3.2 | Number of metabolites annotated in the metabolic pathways of *Pseudovibrio* sp. FO-BEG1 collected in the KEGG database. The masses obtained from the FT-ICR-MS analysis were annotated using the MI-Pack package. The bars of each color, representing the different time points, indicate the absolute number of metabolites annotated in the respective pathway reported in the KEGG database.

Supplementary tables are provided as digital material

Dataset S3.1 | Metabolites annotated using as target the metabolic pathways of *Pseudovibrio* sp. FO-BEG1 reported in the KEGG database.

Dataset S3.2 | Metabolic pathways present in the KEGG database, which contain the annotated metabolites.

Dataset S3.3 | Molecular formulae shared between the experiments conducted in the present work and the DOM retrieved from the North Sea.

Chapter 4

Solubilization of precipitated iron by strain *Pseudovibrio* sp. FO-BEG1 growing under phosphate limitation

Stefano Romano[1*], Vladimir Bondarev[1], Thorsten Dittmar[2], Heide N. Schulz-Vogt[3]

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany.

[2] Research Group for Marine Geochemistry, Institute for Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Oldenburg, Germany.

[3] Leibniz-Institute for Baltic Sea Research Warnemuende (IOW), Section Biological Oceanography, Rostock, Germany.

*corresponding authors

Contributions:
I developed the concept of the study with the support of Vladimir Bondarev. All experiments were conducted by me with the help of Vladimir Bondarev. All bioinformatic and phylogenetic analyses were performed by me and I wrote the manuscript including comments from Heide N. Schulz-Vogt.

Manuscript in preparation

Abstract

Iron and phosphate are fundamental nutrients for all living organisms and in nature their biogeochemical cycles are intimately connected. In aerobic systems the only stable iron form is Fe(III), however, its solubility is extremely low at pH values found in seawater. Therefore, Fe(III) tends to aggregate forming iron oxyhydroxides, which rapidly precipitate. These represent an "iron trap" for phosphate since it can adsorb on the oxyhydroxide surface and can therefore be removed from the surrounding environment. Environmental concentrations of both iron and phosphate have a drastic effect on the bacterial cell physiology, controlling, for example, the production of secondary metabolites and the expression of virulence related genes. In the course of our studies on the physiological response of *Pseudovibrio* sp. FO-BEG1 to phosphate limitation, we detected an increase in the total soluble iron in the cell-free medium when phosphate starved cells entered stationary phase. We proved that this undescribed phenomenon was due to the secretion of molecules with chelating properties. First attempts to characterize the chelators via chemical tests, size exclusion chromatography, reverse phase liquid chromatography, and mass spectrometry revealed the presence of compounds with molecular masses of around 1 kDa and containing catechol subunits. To a certain extent a similar response was also observed in strains belonging to the *Roseobacter* clade, suggesting that this phenomenon could be more widespread in nature.

Introduction

Cells rely on iron (Fe) for a wide range of key metabolic and signaling functions. Under oxic conditions Fe(III) is the only thermodynamically stable form. However, the solubility of Fe(III) at circumneutral pH is below 10^{-18} mol L^{-1} (Neilands, 1981), which is several orders of magnitude lower than the average amount of iron required for bacterial growth (Andrews *et al.*, 2003). Iron concentration in the open ocean is generally below 1 nmol L^{-1} and it exhibits a nutrient-like vertical profile, with lower values detected in surface waters (Tortell *et al.*, 1999). Unlike most of the other trace metals, iron is not present freely soluble, but it is found in form of particulate material (silicates, aluminosilicates, and oxyhydroxides) or dissolved as complexes with organic matter (Price and Morel, 1998; Macrellis *et al.*, 2001).

Generally, bacteria living in aerobic aquatic systems take up Fe(III) complexed with organic molecules. Due to its low solubility and concentration in oxic environments, microorganisms produce and secrete organic molecules that complex Fe(III) with high affinity and are able to solubilize iron particles (Byers and Arceneaux, 1998). These molecules are called siderophores, and they are low molecular weight compounds with masses generally lower than 1,000 Da. They are produced in response to iron limitation in order to facilitate iron acquisition (reviewed in Wandersman and Delepelaire, 2004). Siderophores can be classified according to their chemical characteristics or to their bio-synthetic mechanisms. Considering their structures, they are divided into hydroxamate or catechol containing molecules and polyhydroxy carboxylic acid. The structural variety is big and often molecules with mixed sub-unit types are produced (Neilands,

1993). Siderophores can either be synthesized by non-ribosomal peptide synthetases (NRPS) or by NRPS-independent pathways (Barry and Challis, 2009). So far the most common siderophores are produced via NRPS systems, which are modular multienzymes involved in the production of a large variety of secondary metabolites (Moss *et al.*, 2004).

Due to the chemical characteristics of iron, its biogeochemical cycle is tightly connected with the cycles of other elements essential for bacterial growth. Among those, one of the most important is phosphorus (P), which can also be a limiting nutrient for bacterial growth in marine environments (Wu *et al.*, 2000; Thingstad *et al.*, 2005). Iron and phosphorus cycles are intimately linked because phosphate (P_i), the preferred phosphorus source of bacteria, strongly adsorbs to the surface of iron oxyhydroxides and is released when the oxides are degraded or reduced. The interaction of phosphate with hydrous iron oxides could remove from 5 to 40% of the total phosphate-input to the oceans, particularly in environments with high iron oxide concentrations (Berner, 1973; Froelich *et al.*, 1977; Feely *et al.*, 1990; Rudnicki and Elderfield, 1993; Kadko *et al.*, 1994; Blake *et al.*, 2001).

Changes in environmental concentrations of either iron or phosphate have a great effect on similar physiological traits of bacteria. For instance, phosphate limitation or iron limitation triggers the production of different kinds of secondary metabolites ranging from molecules involved in quorum sensing to siderophores and antibiotics (Vining, 1990; Martín, 2004). There are many areas in the ocean where bacterial growth was described to be limited by one of these two nutrients (Behrenfeld *et al.*, 1996; Wu *et al.*, 2000). Moreover, iron limitation is

a common condition that pathogenic bacteria have to overcome inside their hosts. A successful colonization is often associated with the production of iron-scavenging molecules, which, therefore, are considered virulence factors. Phosphate can also drastically influence bacterial virulence, and it was shown that virulent phenotypes can be induced by both iron and phosphate limiting conditions (Litwin and Calderwood, 1993; Lamarche *et al.*, 2008; Zaborin *et al.*, 2009; Chakraborty *et al.*, 2011).

We have been investigating the phosphate limitation response of strain FO-BEG1, an alphaproteobacterium belonging to the genus *Pseudovibrio* (**Chapter 2**). Members of this genus are typically thriving in close association with, and may even be potential symbionts of, marine invertebrates, and are thought to be a promising source for new bioactive compounds (Taylor *et al.*, 2007; O'Halloran *et al.*, 2011). We observed that phosphate limitation also has a significant influence on bacterial iron metabolism. Under these conditions we observed an increase in the concentration of the total soluble iron in the cell-free supernatant, starting when cells entered stationary phase. In order to understand the origin and mechanisms of this phenomenon, and its distribution among marine heterotrophic bacteria, we performed physiological and chemical experiments on *Pseudovibrio* sp. FO-BEG1 and on selected strains belonging to the *Roseobacter* clade. Moreover, bioinformatic analyses were conducted, aiming to identify genes potentially involved in siderophore production.

Materials and methods

Growth conditions

Pseudovibrio sp. FO-BEG1 was cultivated under two phosphate regimes, phosphate-limited ($-P_i$) and phosphate-surplus ($+P_i$), using the carbohydrate/mineral medium prepared as previously described (**Chapter 2** and **3**). In this medium iron was supplied together with other micronutrients from an acidified trace element solution without the addition of chelating agents (Widdel and Pfenning, 1984). This medium will be referred to as "normal CM". *Phaeobacter gallaeciensis* (DSM-17395) and *Phaeobacter inhibens* (DSM-16374) were cultivated under the same conditions, whereas *Ruegeria pomeroyi* (DSM-15171) was cultivated at 30 ^{o}C. Bacterial growth was monitored by means of Optical Density (OD) measured at 600 nm using an Eppendorf BioPhotometer (Eppendorf AG, Germany). The OD_{600} was then correlated with the cell number, determined by counting cells in a Thoma chamber (Brand GmbH, Germany; data not shown). During the whole growth period the UV-visible spectra of the cell-free supernatants were recorded using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

The effect of different iron concentrations on the development of the color and the UV-visible spectra of the $-P_i$ cell-free supernatant was investigated cultivating FO-BEG1 in $-P_i$ CM medium supplied with the same acidified trace element solution of the "normal CM", but prepared without iron addition. Three stock solutions of $FeSO_4$ and ethylenediaminetetraacetic acid (EDTA) were prepared in the following concentrations: 25 mmol L^{-1} $FeSO_4$ and

50 mmol L^{-1} EDTA, 45 mmol L^{-1} $FeSO_4$ and 90 mmol L^{-1} EDTA, 75 mmol $L^{-1}$$FeSO_4$ and 150 mmol L^{-1} EDTA. Iron was then added into the medium to obtain final concentrations of 2.5, 4.5, and 7.5 μmol L^{-1}. Growth was monitored via measuring $OD_{600,}$ and UV-visible spectra of the cell-free supernatant were recorded during the entire growth period.

In order to understand whether the observed iron increase was due to iron released from the cells or from a solubilization of the precipitated fraction, we performed two experiments to which we will refer to as "Refresh experiment" and "EDTA experiment". In the first experiment, we incubated strain FO-BEG1 in the "normal CM" medium under $+P_i$ and $-P_i$ conditions, up to the point where we previously detected the maximum iron uptake. Biomass of all $+P_i$ and $-P_i$ replicates were then harvested via centrifugation at 7,000 x *g* and 15 °C, for 10 min, using a J-26XP Beckmann centrifuge (Beckman Instruments, Inc., Palo Alto, CA). Cell pellets were washed with Fe-free sterile artificial seawater (ASW), and equally divided to re-inoculate fresh Fe-free phosphate-limited CM medium in biological triplicates. Cultures were incubated again at 28 °C and shaken at 120 rpm. This resulted in two series of cultures: cultures in $-P_i$ Fe-free medium pre-grown under "normal CM" $+P_i$, and cultures in $-P_i$ Fe-free medium pre-grown under "normal CM" $-P_i$ medium. The working hypothesis was that the elimination of external iron to the second cultures avoids the formation of iron precipitates. Therefore, an eventual increase in the total soluble iron in the medium could only be attributed to a cellular release. In the second culturing step, we monitored bacterial growth via OD_{600} measurement and the UV-visible spectra of the cell-free supernatants for 95 h. Iron concentrations in the cell-free

supernatants were determined via ICP-OES. For this second cultivation step all glassware was washed two times with 1 mol L^{-1} HCl and rinsed with MembraPure water (Optilab-Standard Water System, MembraPure, Bodenheim, Germany), in order to remove residual iron adsorbed to the glass walls.

In the "EDTA experiment" we cultivated strain FO-BEG1 in a $+P_i$ and $-P_i$ CM medium where we supplied iron complexed to EDTA to a final concentration of 7.5 µmol L^{-1} iron and 100 µmol L^{-1} EDTA. Cultures were incubated as described before. All glassware was acid washed prior to use. We hypothesized that supplying EDTA-Fe would avoid iron precipitation. Consequently, in case of increase in the concentration of total soluble iron in the cell-free supernatant, this could only come from cellular release.

Extraction of the chelating molecules for the isolation process was performed on the cell-free supernatant of a 10 L culture obtained using a Sartorius bioreactor with a Biostat B Plus control unit (Sartorious GmbH, Göttingen, Germany). The temperature was maintained at 28 °C and the culture was mixed at 300 rpm. Filtered air was injected into the chemostat at a constant flow rate of 1 L min^{-1}. Growth was monitored via OD_{600} measurement.

.

Iron measurement, dialyzation, and size exclusion chromatography (SEC) of the cell-free supernatants

Iron concentration in the supernatants was measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Samples were collected during bacterial growth, centrifuged at 11,000 x *g* and 5 °C for 10 min, filtered with 0.22 µm pore size Millipore filter (Millipore, Bedford, MA,

USA), diluted 1:10 in 1% supra-pure HNO_3 (Merck, Darmstadt, Germany), and stored at 4 ^oC until further processing. Analyses were performed with an Agilent 720 ICP-OES (Agilent Technologies, Palo Alto, CA, USA).

Cell-free supernatants of both $+P_i$ and $-P_i$ cultures were dialyzed over-night at 4 ^oC against MQ under stirring conditions using a dialysis tube Spectra/Por™ (molecular cut-off of 1000 Da; Serva, Heidelberg, Germany). Iron content and chelating activity were measured before and after dialysis.

SEC of the $-P_i$ cell-free supernatant was performed by injecting 1 mL of sample into an Äkta purifier system (GE Healthcare, Freiburg, Germany) equipped with a Superdex™ Peptide 10/300 GL column (GE Healthcare, Freiburg, Germany), detecting absorption at 280 nm. Samples were run using tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) at pH 7.8 as buffer with a flow rate of 0.25 mL min^{-1}. During the chromatographic run, 1 mL fractions were collected using a fraction collector Frac-950 (GE Healthcare, Piscataway, NJ, USA). Each fraction was then diluted 1:10 in 1% supra-pure HNO_3 and iron content was analyzed via ICP-OES. The column was calibrated using ribonuclease A, aprotinin, [D-Ala^2, D-Leu^5]-Enkephalin, and vitamin B12 (Sigma-Aldrich Chemie Gmbh, Munich, Germany), which were run under the same conditions. The total volume of the column was estimated considering the elution time of the ions in solution detected as increase in conductivity. Cell-free supernatants of the "Refresh experiment" were also tested via SEC using the same condition described above.

Characterization, extraction and isolation of the chelating compounds, and detection of tropodithietic acid (TDA)

Cell-free supernatants were tested with the Chromazurol-S (CAS) assay, modified from Schwyn and Neilands (1987). To 0.6 mL of 10 mmol L^{-1} hexadecyltrimethylammonium bromide (HDTMA) 0.15 mL of 1 mmol L^{-1} FeCl3 prepared in 0.1 mol L^{-1} HCl was slowly added under stirring. Subsequently, 0.75 mL of 2 mmol L^{-1} CAS were added drop-wise. After complete mixing of the solution 6.5 mL of 0.5 mmol L^{-1} piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), previously adjusted to pH 6.8, were added and the volume of the solution was filled up to 10 mL using MQ. To perform the CAS assay an equal volume of CAS solution and samples were mixed. Presence of catechol containing molecules was assessed using the chemical assay described in Rioux *et al.* (1983) and presence of hydroxamate derivate was verified with the method described by Csaky (1948) and modified by Gillam *et al.* (1981).

As reported in Neilands (1981) liquid-liquid extraction using ethyl acetate as solvent is the most common way to extract catechol-like siderophores. Therefore, we employed this method to extract the metabolites from the cell-free supernatant of a 10 L culture. After obtaining a crude extract, we performed in collaboration with BioViotica GmbH (Göttingen, Germany) high performance liquid chromatography (HPLC) experiments aimed to isolate the chelating molecules. Fractions were collected and their chelating activity was tested using the CAS assay. In addition, we verified by means of reverse phase (RP)-HPLC the presence of the antibiotic tropodithietic acid (TDA) in the cell-free supernatants of the *Roseobacter* strains, which developed a yellow coloration. A

detailed description of the procedures is provided in the Supplementary materials and methods section.

Electrospray ionization mass spectrometry (ESI-MS) of the RP-HPLC fractions

The HPLC fraction that resulted positive to the CAS assay and the fraction containing TDA were further analyzed via mass spectrometry. The fractions were diluted 1 to 10 in 70% acetonitrile and filtered using a 0.2 μm pore size PTFE filter (Rotilabo, Carl Roth GmbH, Karlsruhe, Germany). Mass spectrometry analysis was performed in ESI positive mode with a solariX FT-ICR-MS (Bruker Daltonik GmbH, Bremen, Germany) with a 15.0 Tesla magnet. All data were acquired with a time domain size of 4 megawords with a detection range of *m/z* 150 to 2,500. For each run, 200 broadband scans were accumulated. The acquired mass spectra were analyzed with the Data Analysis software Version 4.0 SP4 (Bruker Daltonik GmbH). To facilitate the identification of chelating molecules both HPLC fractions were analyzed before and after adding analytic grade $GaCl_3$ (Sigma-Aldrich Chemie Gmbh, Munich, Germany). Ga(III) has chemical properties similar to Fe(III), but its two isotopes ^{69}Ga and ^{71}Ga have an abundance ratio of 3:2, generating a characteristic isotopic pattern in the MS scan. Spectra were manually analyzed and the mass shift and the isotopic pattern, resulted from the Ga addition, were considered for the identification of compounds with chelating properties. MS/MS of the molecules presenting the characteristic Ga isotopic pattern was performed using a collision induced dissociation (CID) ranging from 12 to 17.

In-silico **analysis for the detection of genes potentially involved in siderophore production**

Both genome and plasmid sequences of *Pseudovibrio* sp. FO-BEG1 (GeneBank ID: CP003147.1 and CP003148.1, respectively) were analyzed with the program antiSMASH 2.0 (Blin *et al.*, 2013). The same analysis was performed for the genomes and plasmids of *P. gallaeciensis* (GeneBank ID: CP002976.1, CP002977.1, CP002979.1, CP002978.1) and *R. pomeroyi* (CP000031.1, CP000032.1). Since the genome of *P. inhibens* (GeneBank ID: GCA_000473105.1) was still not completely assembled, the two longest scaffolds were downloaded, automatically annotated using the RAST server (Aziz *et al.*, 2008), and subsequently submitted to antiSMASH 2.0.

After identification of a hybrid non-ribosomal peptide polyketide synthetase (NRPS-PKS) gene cluster, potentially involved in the production of a chelating compound in *Pseudovibrio* sp. FO-BEG1, the respective genomic region was selected to perform a multigene BLAST using the MultiGeneBlast platform (Medema *et al.*, 2013). This analysis was conducted in order to identify homologous clusters in the genome of other bacteria. More details can be found in the Supplementary materials and methods section.

In order to obtain a first broad overview of the affiliation of the proteins identified by antiSMASH to be potentially involved in the synthesis and transport of a non-ribosomal peptide in *Pseudovibrio* sp. FO-BEG1, a phylogenetic analysis was conducted considering the full length of each protein. After retrieving homologous sequences, phylogenetic trees were calculated using the Maximum likelihood method. In order to have more accurate information concerning the affiliation of the proteins, we conducted an additional

phylogenetic analysis using the more important domains of the NRPS (A and C) and PKS (AT and KS) systems. Phylogenetic trees were calculated using the Maximum likelihood method. A detailed description of the phylogenetic analyses can be found in the Supplementary materials and methods section.

To clarify whether the gene cluster of interest was horizontally acquired by *Pseudovibrio* sp. FO-BEG1, the whole genome was analyzed using the integrated interface of IslandViewer (Langille and Brinkman, 2009). Tetranucleotide usage of the gene cluster was calculated using the program TETRA (Teeling *et al.*, 2004b), correlating the usage patterns of the genomic region containing the NRPS-PKS cluster of FO-BEG1 with the colibactin cluster identified in the genomes of *Escherichia coli* (CP001969.1) and *Citrobacter koseri* (CP000822.1). Moreover, the pattern was also correlated with the FO-BEG1 genome and with the genomes of four enterobacteria (CP001969.1; CP000822.1; CP000247.1; CP000308.1).

Finally, the genome of FO-BEG1 was screened for the presence of binding domains for the ferric-uptake-regulator (Fur) family proteins using a weighted matrix constructed for the Fur-box of *E. coli* and retrieved from the platform RegulonDB (Salgad*o et al.,* 2012). The weighted matrix was used to scan the intergenic regions of *Pseudovibrio* sp. FO-BEG1 genome using the program genome-patser (Hertz and Stormo, 1999).

Results

Pseudovibrio sp. FO-BEG1 solubilizes precipitated iron when growing under phosphate-limited conditions

As reported previously in **Chapter 2** and **3**, phosphate limitation repressed bacterial growth. During the investigated growth period, we monitored the concentration of total soluble iron in both $+P_i$ and $-P_i$ mediums (**Fig. 4.1**). In the sterile controls, iron concentrations decreased drastically during the first 24 h of incubation. The decrease was more evident under $+P_i$ conditions, where the concentration dropped from 6.7 ± 0.15 μmol L^{-1} to 1.8 ± 1.2 μmol L^{-1}. In contrast, under $-P_i$ conditions the iron concentration decreased from 7.0 ± 0.04 μmol L^{-1} to 4.6 ± 1.9 μmol L^{-1}. Comparing the residual iron in the sterile controls with the minimum iron concentrations detected in the cultures we could infer the amount of iron taken up by the cells under both phosphate regimes. Cells growing under phosphate limitation took up 2.5 times more iron than cells growing under phosphate-surplus. Surprisingly, only under $-P_i$ conditions the concentration of the total soluble iron in the medium increased again when cells entered stationary phase, reaching 4.7 ± 0.12 μmol L^{-1} (**Fig. 4.1**).

The increase could have been derived either from iron released by the cells or by solubilization of the precipitated iron fraction. To address this question we performed the "Refresh experiment" and the "EDTA experiment". During the first experiment *Pseudovibrio* sp. FO-BEG1 was cultivated in a "normal CM" medium under both $+P_i$ and $-P_i$ conditions up to the point where we previously detected the higher iron uptake. The entire biomass of the cultures

was then recovered and re-inoculated in a new $-P_i$ and Fe-free CM medium. Bacterial growth and iron concentrations were then monitored for 95 h. In the second cultures iron precipitates will be avoided by the elimination of the external iron source. Therefore, an eventual increase in the total soluble iron in the medium could only be derived from cellular release. In both series of cultures (from $+P_i$ and $-P_i$ pre-cultures) we observed an increase in OD_{600}, but we did not detect any variation in total soluble iron (data not shown). Moreover, for both series of cultures we observed the formation of a slightly yellow color and we could record UV-visible spectra comparable to the spectra measured for the "normal CM" $-P_i$ cultures, but lower in absorption (**Fig. S4.1**).

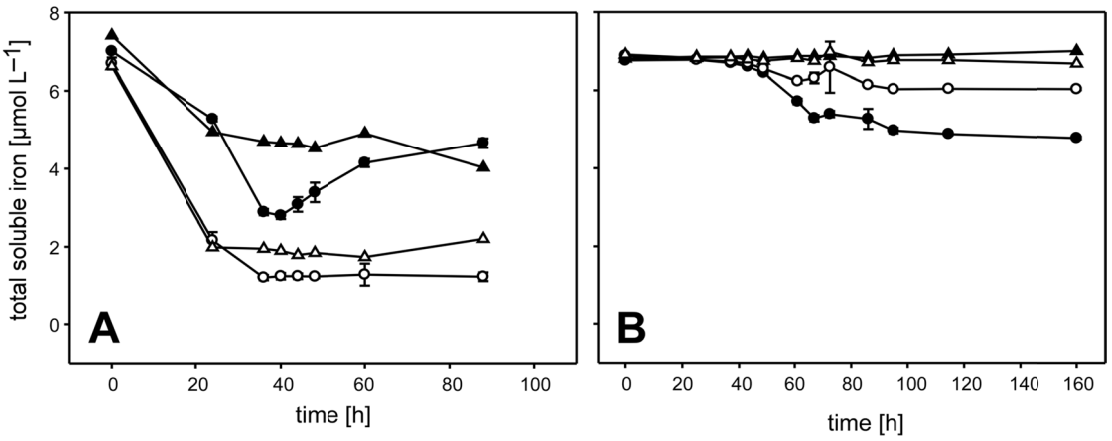


Figure 4.1 | Concentrations of total soluble iron over time in the sterile controls (triangles) and cultures (circles), of $+P_i$ (empty symbols) and $-P_i$ (filled symbols) conditions. A shows the concentrations measured during growth of *Pseudovibrio* sp. FO-BEG1 under "normal CM" medium. **B** shows the concentrations measured during the "EDTA experiment". The error bars represent the standard deviation of biological triplicates.

In the "EDTA experiment" we inoculated FO-BEG1 in a CM medium supplying iron in the form of EDTA complex. The working hypothesis was that supplying EDTA-Fe would avoid iron precipitation. Consequently, the eventual

increase in the concentration of total soluble iron in the cell-free supernatant could only be attributed to cellular release. As hypothesized, we did not observe iron precipitation in the sterile controls of both $+P_i$ and $-P_i$ conditions (**Fig. 4.1**). In agreement with the original cultivation approach ("normal CM" medium), cells took up 2.5 times more iron under phosphate limitation. In addition, we did not observe an increase in the concentration of total soluble iron under both $+P_i$ and $-P_i$ conditions, indicating that the cells did not release any iron.

Since the formation of the color seemed to be affected by the iron concentration in the medium, we monitored the color development and the UV-visible spectra of the cell-free supernatant in additional experiments, where *Pseudovibrio* sp. FO-BEG1 was cultivated under $-P_i$ conditions providing lower amounts of iron than the "normal CM" medium. Although bacterial growth was not affected, the intensity of the color and the absorption of the UV-visible spectra decreased under lower iron concentration (data not shown).

In order to verify whether the soluble iron was bound to organic molecules in solution, we performed a size exclusion chromatography (SEC) experiment using the $-P_i$ cell-free supernatant. We collected the eluted fractions and their iron content was measured via ICP-OES. The maximum iron amount was detected in the fractions that contained molecules in an apparent mass interval from 1,030 to 4,885 Da (data not shown). However, the chromatogram in this range was not well resolved. Therefore, it was not possible to derive a precise size of the organic complexes. Conversely, these compounds were not detected in the SEC experiments performed using the cell-free supernatants obtained from the "Refresh experiment" (data not shown).

Finally, we dialyzed both $-P_i$ and $+P_i$ cell-free supernatants using a molecular cut-off of 1 kDa. In contrast to the $+P_i$ supernatant, the iron content of

the $-P_i$ dialyzed cell-free supernatant increased from 3.9 µmol L^{-1} to 6.3 µmol L^{-1}. For both dialyzed supernatants we did not detect any significant activity with the CAS assay.

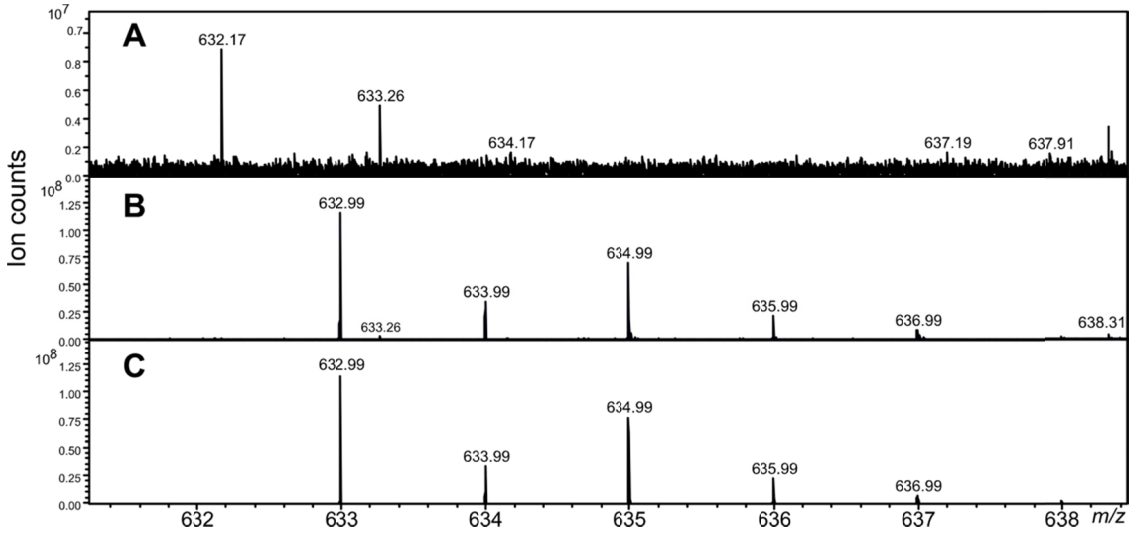


Figure 4.3 | Portion of the mass spectra of the RP-HPLC fraction (A) that resulted positive in the CAS assay. B shows the mass spectra of the same fraction after addition of $GaCl_3$ and it is compared with a theoretical isotopic pattern (**C**) of a molecule calculated allowing the presence of maximum one atom of Ga in the final molecular formula.

The cell-free supernatant of $-P_i$ cultures contains molecules with chelating properties

The universal CAS assay provides a quick method to verify the presence of Fe-chelating molecules in solution. The assay is based on the interaction of the chelators with the complex CAS-HDTMA-Fe. The tested chelating molecule removes iron from the CAS complex, which causes a variation in the UV-visible spectrum of the solution. We tested the cell-free supernatants of both $+P_i$ and $-P_i$ cultures, but only the last one resulted positive. In order to further characterize the chemical structure of the chelating molecule

we performed chemical assays for the two main categories of siderophores, hydroxamate and catechol derivates. Both $+P_i$ and $-P_i$ cell-free supernatants resulted positive for the catechol assay, but the $-P_i$ supernatant gave a signal with double the intensity than the $+P_i$ one. The assay for hydroxamate gave negative results for both supernatants, even though the test was not always reproducible.

We extracted the hypothetical catechol siderophore using ethyl acetate according to Neilands (1981). The extracted metabolites of the $-P_i$ supernatant of strain FO-BEG1 had an intense yellow-brown coloration and reacted immediately during the CAS assay. The crude extract was then analyzed via RP-HPLC and five fractions were isolated. Four of the five fractions showed a yellow or yellow-brown coloration (**Fig. S4.2**). The retention time and the UV-visible spectra of the compound contained in fraction 4 were consistent with the ones of tropodithietic acid (TDA), which was previously detected and quantified via HPLC during the growth of FO-BEG1 under $-P_i$ conditions (**Chapter 2**). The activity of all fractions was tested again using the CAS assay and only fraction 1 reacted immediately, whereas the fraction containing TDA started to react only after 2 h of incubation. Finally, we verified the purity of fraction 1 via analytical HPCL and we could detect eight different peaks. This condition did not allow us to proceed further in the isolation of the chelating compounds.

In order to better characterize the compounds with chelating activity, fraction 1 was analyzed via mass spectrometry before and after addition of $GaCl_3$. Moreover, since the fraction containing TDA mildly reacted with the CAS

solution, it was also analyzed using the same approach. Ga(III) has chemical properties similar to Fe(III) and can also interact with chelating molecules. Peculiarly, its two isotopes have an abundance ratio of 3:2, generating a characteristic isotopic pattern in the MS scan, which facilitate the identification of molecules bound to it. In fraction 1 we identified several masses that according to the analysis of the isotopic patterns contained unequivocally Ga (**Fig. 4.3B**). These masses were not detected in the same sample analyzed before Ga addition (**Fig. 4.3A**). Four of the *m/z* containing Ga (*m/z* 633; 874; 845; 887) were further analyzed via MS/MS. Unexpectedly, in all fragmentation patterns three *m/z* (311.00; 445.02; 455.02), still showing the Ga isotopic signature, were always detected (data not shown). Moreover, several other shared ions were detected in all patterns (e.g. *m/z* 413.28; 442.03; 478.19; 552.29; 574.27). In the TDA containing fraction we did not identify any mass showing the Ga isotopic pattern. In this fraction we detected a mass which was consistent, considering also the isotopic pattern, with the mass of TDA (*m/z* 234.9; $[TDA+Na]^+$)

Strains from the *Roseobacter* clade show different iron solubilizing activity under phosphate limitation

In order to verify whether the production of a chelating agent is a common physiological response to phosphate limitation among marine heterotrophic bacteria and thus could be of environmental relevance, we investigated this phenomenon in different strains belonging to the abundant and ubiquitous *Roseobacter* clade. We selected three strains: *Phaeobacter gallaeciensis* DSM-17395, *Phaeobacter inhibens* DSM-16374, and *Ruegeria pomeroyi* DSM-15171

and monitored their growth and the soluble iron concentration in the medium under the two phosphate regimes. *P. gallaeciensis* showed a response to phosphate limitation similar to *Pseudovibrio* sp. FO-BEG1. We observed a repressed cell growth and formation of a yellow-brown coloration when cells entered stationary phase (**Fig. S4.3**; **Fig. S4.4**). As observed for *Pseudovibrio* sp. FO-BEG1, also in the $-P_i$ cultures of *P. gallaeciensis* the total soluble iron increased when cells entered stationary phase (**Fig. 4.4**). Both $-P_i$ and $+P_i$ supernatants were tested using the CAS assay, and only the $-P_i$ was positive. Similarly, also its crude extract reacted immediately in the CAS assay.

P. inhibens and *R. pomeroyi* showed a different response to phosphate limitation. For *P. inhibens* a precise quantification of the bacterial growth was not possible due to the formation of compact cell aggregates (**Fig. S4.3**). Both $+P_i$ and $-P_i$ cultures developed a yellow-brown coloration (**Fig. S4.4**), and under both conditions the soluble iron concentration increased during bacterial growth (**Fig. 4.4**). In addition, both supernatants and their respective crude extracts resulted positive to the CAS assay. *R. pomeroyi* was characterized by a slower growth and we did not observe significant differences in the final cell density between the two treatments (**Fig. S4.3**). Moreover, no yellow coloration developed under the two phosphate regimes (data not shown). Also for this strain we observed an increase in the total soluble iron in the cell-free supernatant of $-P_i$ cultures, even though significantly less pronounced than in the other strains (**Fig. 4.4**). The UV-visible absorption spectra of the cell-free supernatants of all cultures that developed a yellow coloration resembled the spectra of the FO-BEG1 $-P_i$ supernatant (**Fig. S4.4**).

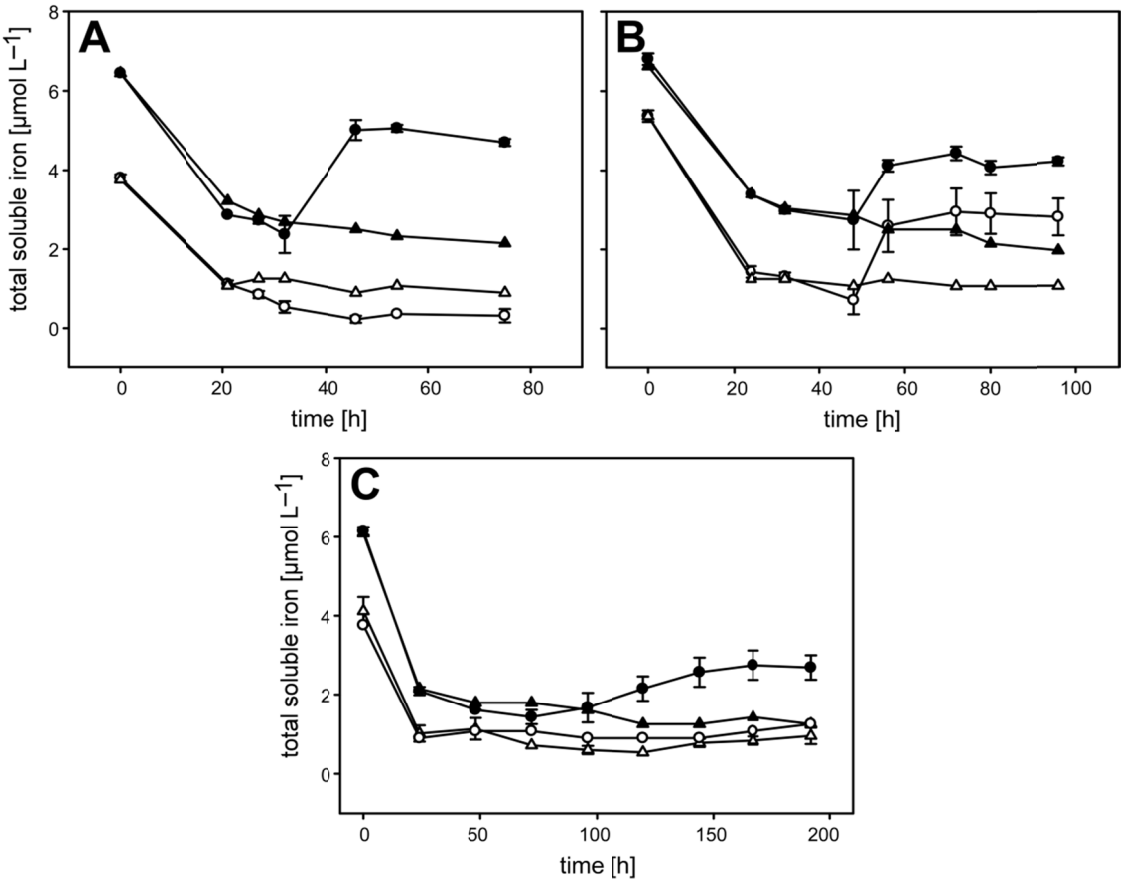


Figure 4.4 | Concentrations of total soluble iron over time in the sterile controls (triangles) and cultures (circles) of *P. gallaeciensis* (A), *P. inhibens* (B), *R. pomeroyi* (C). Filled symbols represent $-P_i$ and empty symbols represent $+P_i$ conditions. The error bars indicate the standard deviation of biological triplicates.

Members of the *Roseobacter* clade usually produce a yellow pigment together with the antibiotic TDA (Bruhn *et al.*, 2005). Since we observed a mildly positive reaction of TDA in the CAS assay, and since the development of the yellow color was always associated with increase in iron concentration, the crude extracts of the cell-free supernatant of all cultures that showed the formation of yellow color were analyzed via RP-HPLC to verify the presence of TDA. In all of

them we could detect the presence of a peak with retention time and UV-visible spectra consistent with the pure TDA standard (data not shown).

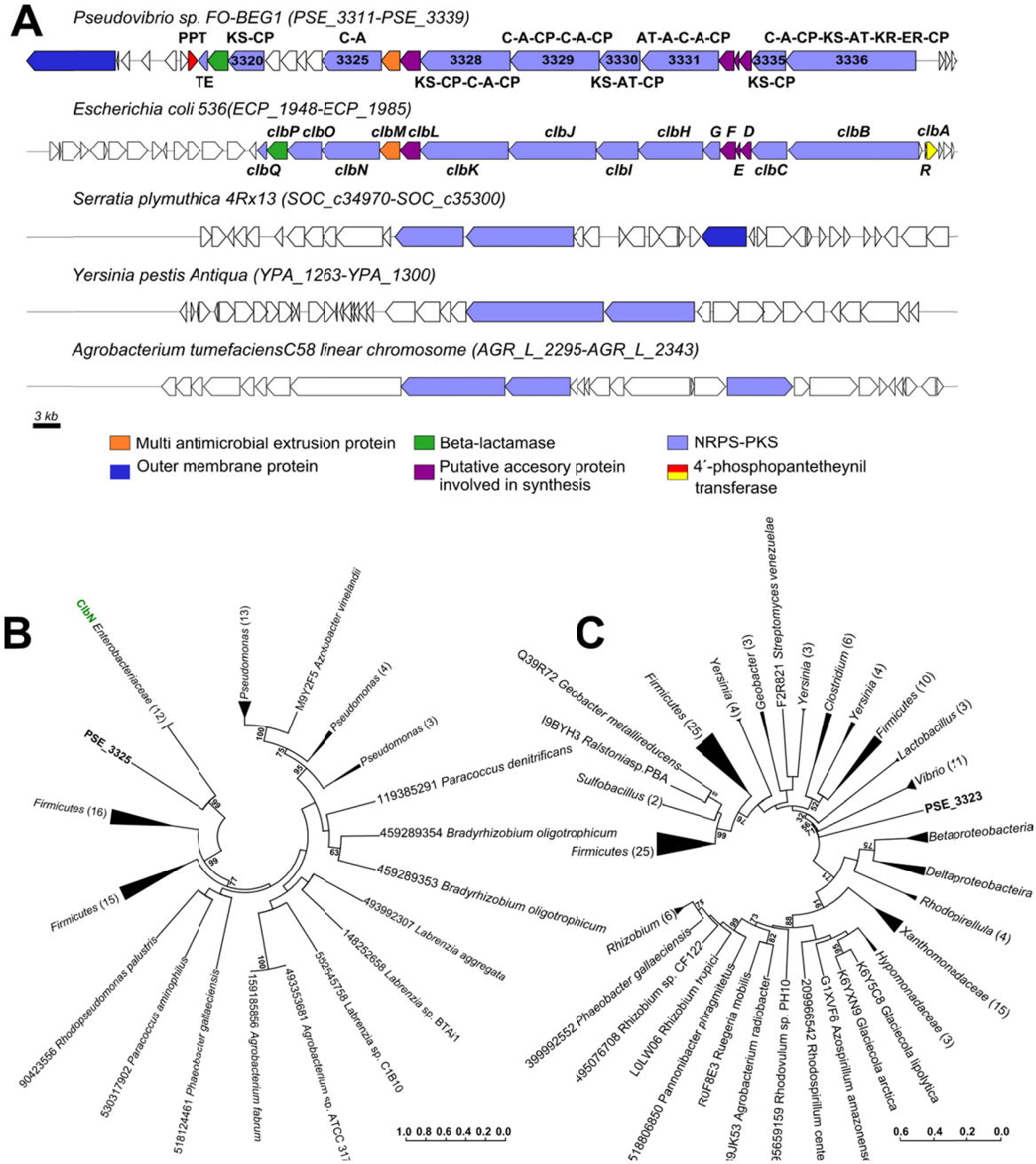


Figure 4.5 (displayed on the previous page) | Genomic region containing the gene cluster encoding the hybrid NRPS-PKS system in *Pseudovibrio* sp. FO-BEG1 (A). For comparison, homologous regions belonging to different bacteria are shown. The higher similarity is shared with the colibactin gene cluster of *Escherichia coli* strain 536. Genes encoding proteins with similar function are indicated by the same color. The gene *clbA* of *E. coli* strain 536 encodes a protein containing a PPT domain similar to the gene PSE_3317 of *Pseudovibrio* sp. FO-BEG1. However, the phylogenetic analysis revealed that the encoded protein in FO-BEG1 is affiliated to a homologous protein of *Sinorhizobium* sp. To underline the difference, these two genes are shown in different colors. For each gene of the *Pseudovibrio* NRPS-PKS system the respective domain content is reported. PPT phosphopantetheinyl transferase domain, C condensation domain, KS ketosynthase domain, A adenylation domain, AT acyltransferase domain, CP carrier peptide domain. **Figure B** and **C** show the phylogenetic trees for the proteins PSE_3323 and PSE_3325. Trees were constructed using the Maximum Likelihood method based on the JTT matrix-based model. Trees with the highest log likelihood are shown. The percentage of trees in which the associated taxa clustered together (bootstrap values) is shown next to the branches. Only values equal or higher than 50% are reported. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Due to space constraints only two trees are shown, but they are representative for the trees constructed for all other analyzed proteins.

Genes potentially involved in siderophore production

After verifying the presence in the cell-free supernatants of molecules with chelating properties, we analyzed the genome of FO-BEG1 and of the *Roseobacter* strains in the attempt to identify genes involved in siderophore production. The *in-silico* analysis gave positive results only for *P. gallaeciensis* (PGA_78p00410-PGA_78p00440). In all other strains, FO-BEG1 included, no known genes were detected. Interestingly, in the genome of *Pseudovibrio* sp. FO-BEG1 we identified a hybrid non-ribosomal peptide polyketide synthases (NRPS-PKS) gene cluster (PSE_3317-18, PSE_3320, PSE_3325, PSE_3328-31, PSE_3335-36). Since most of the known siderophores are produced via NRPS systems, this genomic region was further analyzed.

The NRPS and PKS systems are modularly organized multi-domain proteins, which represent at the same time template and biosynthetic machinery. Each domain catalyzes a specific reaction, representing the individual steps in

the synthesis of the final molecule (Schwarzer *et al.*, 2003). Some domains, e.g. adenylation domain (A) in NRPS and acyl-transferase domain (AT) in PKS, have high specificity for the single monomers which are used for the synthesis of the compounds, e.g. amino acids or carboxylic acids (Schwarzer *et al.*, 2003). Therefore, if the order of the domains in the synthetic process is known it is possible to predict, to a certain extent, the backbone composition of the product (Schwarzer *et al.*, 2003). Consequently, two systems that share similar proteins and domains are likely producing highly similar compounds. In terms of organization and gene content the NRPS-PKS cluster detected in the FO-BEG1 genome clearly showed a high degree of similarity with a genomic region responsible for the synthesis of colibactin in bacteria belonging to the family *Enterobacteriaceae* (**Fig. 4.5A**).

In order to gain a first glimpse into the phylogenetic affiliation of the proteins predicted by antiSMASH to be involved in the synthesis and transport of the non-ribosomal peptide (NRP), we performed a phylogenetic analysis considering their full sequences. All megasynthases belonging to the NRPS-PKS system clustered together with proteins involved in the synthesis of colibactin and belonging to the family *Enterobacteriacea*. However, they formed always an independent branch in the tree (**Fig. 4.5B**). The only exception was PSE_3317, which clustered together with a homologous protein of *Sinorizobium melliloti* (data not shown). The majority of the proteins annotated as members of transport systems (PSE_3323; PSE_3324; PSE_3350; **Fig. 4.5A**) were closely related to proteins belonging to *Alphaproteobacteria* and formed isolated branches in the phylogenetic trees (**Fig. 4.5B**). The only exception was PSE_3326, which clustered

together with homologous proteins of enterobacteria. In the NRPS-PKS genomic region also other enzymes not belonging to the NRPS-PKS system, and potentially involved in the synthesis of the NRP were identified (PSE_3314; PSE_3319; PSE_3327; PSE_3332-3334). The phylogenetic analysis revealed that the majority of these enzymes were related to homologous proteins belonging to *Enterobacteriaceae*, with the exception of PSE_3346 and PSE_3347, which clustered together with *Alphaproteobacteria* sequences (data not shown).

Due to the modularity of the NRPS-PKS systems, a high level of genetic recombination can occur. This can cause a reorganization of single domains in the megasynthases, leading to a difference in the synthetic process, and consequently to a difference in the final structure of the product (Moss *et al.*, 2004; Jenke-Kodama and Dittmann, 2009). Therefore, gene clusters with identical sequential organization, like the one detected in FO-BEG1 and the colibactin cluster, can differ at the domain level. For this reason, a phylogenetic analysis considering the single domains can give more accurate and indicative results. Therefore, we performed an additional phylogenetic analysis using the PKS domains KS, AT and the NRPS domains C, A. Consistently with the analysis conducted for the full sequences of the megasynthases, all domains clustered together with domains involved in the production of colibactin (**Fig. S4.5, Fig. S4.6**).

Due to the high similarity between the colibactin NRPS-PKS gene cluster and the one identified in *Pseudovibrio* sp. FO-BEG1 genome, we performed additional analyses to verify if this genomic region was acquired by strain FO-BEG1 via horizontal gene transfer (HGT). In the first place, we

calculated the GC content of the colibactin and the FO-BEG1 NRPS-PKS gene clusters. The difference in GC content between a specific genomic region and the overall genome of the bacterium represents a first common indicator for HGT. The GC content of the colibactin gene cluster (53%) was highly similar to the one of the FO-BEG1 NRPS-PKS cluster (51.1%) and of the overall genome of strain FO-BEG1 (52.5%). Additionally, we considered the tetranucleotide usage patterns, which were shown to contain a stronger phylogenetic signal than the GC content (Teeling *et al.*, 2004a). They have often been used to identify the relatedness among genomes and genome fragments considering the degree of correlation between the usage patterns of the respective sequences (Teeling *et al.*, 2004a). The usage patterns of the genomic region containing the NRPS-PKS was weakly correlated with the patterns of the FO-BEG1 genome (0.47). However, the values of correlation with the enterobacterial colibactin gene clusters and with the genomes of the respective strains were lower than 0.31. Finally, the genome of *Pseudovibrio* sp. FO-BEG1 was screened for the presence of known genomic islands (GIs) considering homology with GIs known in other species, presence of viral genes, and anomalies in the GC skew (**Fig. S4.7**). Apart from mobile elements in one flanking region (PSE_3337-3345), the NRPS-PKS cluster was not identified as a GI (**Fig. S4.7**).

Siderophores are regulated by the transcription regulator Fur, which controls the expression of genes involved in iron metabolism. It binds to specific sequences called Fur-boxes, which are located in the upstream region of the genes. Therefore, in order to clarify whether the expressions of the NRPS-PKS cluster was regulated according to this scheme, we screened the genome of

Pseudovibrio sp. FO-BEG1 for the presence of potential Fur-Boxes. 388 genes that presented a potential Fur-box in their upstream region were identified (data not shown). Among these there were several gene encoding proteins involved in iron metabolism (e.g. ferric uptake regulator, PSE_0496), confirming that the approach we applied represented a valid preliminary identification. However, none of the genes belonging to the hybrid NRPS-PKS cluster presented a potential Fur-box.

Discussion

Under phosphate limitation *Pseudovibrio* sp. FO-BEG1 secretes molecules with chelating properties, which solubilize precipitated iron

Our investigation on the effect of phosphate limitation on the physiology of *Pseudovibrio* sp. FO-BEG1 showed that this nutrient condition strongly influences the iron metabolism of the strain. The concentration of total soluble iron under $-P_i$ conditions decreased drastically during the first 40 h of incubation and increased again during the rest of the growth (**Fig. 4.1**). The main aim of the presented study was to understand the origin and the mechanisms behind this increase.

In the used medium iron was provided in the form of Fe(II) from an acidified trace element solution (Widdel and Pfenning, 1984). During the incubation time under aerobic conditions Fe(II) oxidized to Fe(III) and, since the solubility of the latter at pH 7.0 is 10^{-17} to 10^{-18} mol L^{-1} (Neilands, 1981), it is

reasonable to state that it was removed from the solution due to precipitation. This was clearly observed in the sterile controls of both $-P_i$ and $+P_i$ conditions (**Fig. 4.1**). Under $+P_i$ conditions a higher iron decrease was observed and, since the only difference between the two treatments was the phosphate concentration in the medium, we can conclude that higher phosphate concentration enhanced iron precipitation. In both controls during the entire incubation time a portion of iron remained in solution. This could be due to the presence of TRIS-HCl, used as a buffer system, which was described to have slight chelating properties (Taylor *et al.*, 1993).

Unexpectedly, cells growing under phosphate-limited conditions took up 2.5 times more iron than cells proliferating under phosphate-surplus conditions. Although iron is an essential nutrient for cells it can also be toxic due to its participation in the Haber-Weiss/Fenton reaction, which generates highly reactive hydroxyl radicals (Haber and Weiss, 1934; Cabiscol *et al.*, 2000). Therefore, the concentration of free iron within the cell is tightly controlled. It was proposed that cells growing under phosphate limitation experience increased oxidative stress (Gerard *et al.*, 1999; Moreau *et al.*, 2001; Moreau, 2004; Yuan *et al.*, 2005). We also have indications that this phenomenon occurred in strain FO-BEG1 since proteins involved in protection against reactive oxygen species (ROS) were up-regulated under $-P_i$ conditions (**Chapter 2**). Consequently a higher iron uptake might represent a risk for the cells. However, under $-P_i$ conditions two proteins homologous to bacterioferritin were up-regulated (**Chapter 2**). These proteins are involved in iron storage and protection against oxidative stress via iron detoxification (Carrondo, 2003), and likely allocate the

iron taken up by FO-BEG1 under –P_i conditions. This accumulation could represent a reservoir to support growth in future conditions of iron restriction. A similar luxury uptake was described in diatoms (Sunda, 2001) and it might be an important strategy especially in environments where iron availability is variable in time and space such as coastal waters, where *Pseudovibrio* related bacteria were often isolated (Shieh *et al.*, 2004; Hosoya and Yokota, 2007).

In general iron accumulates in bacterioferritin in granules containing phosphate in a Fe/P_i ratio varying between 1.4:1 to 1.7:1(Le Brun *et al.*, 2010). Sometimes the amount of phosphate can even be higher, so that these proteins have also been considered as phosphate storage systems (reviewed in Harrison and Arosio, 1996). Therefore, cells facing phosphate shortage could theoretically disassemble the iron core in the bacterioferritin to recover phosphate. Consequently, due to the toxicity and insolubility of iron in the cytoplasm, cells should secrete it into the external environment. However, the "Refresh experiment" and the "EDTA experiment" clearly showed that the increase in iron observed during the growth of strain FO-BEG1 under –P_i conditions was due to solubilization of precipitated iron. The "Refresh experiment" together with the growth test we performed with increasing iron concentrations also showed that the color observed under –Pi conditions (**Chapter 2**) depends on the availability of this element. Consistently, the SEC separation of the cell-free supernatants unequivocally showed the presence of organic-Fe complexes, not present in the "Refresh experiment" where it could not be formed due to the absence of iron precipitate. Finally, the

CAS assay together with the mass spectrometry analysis proved the presence of one or more molecules with chelating properties in the $-P_i$ cell-free supernatant.

The SEC experiment indicates that the chelator-Fe complexes have an apparent molecular size ranging from 1 to 4.8 KDa. This is consistent with the dialysis experiment, where the iron concentration did not decrease, indicating that the Fe-complexes are greater than 1 kDa. During this experiment a slight increase in iron concentration in the $-P_i$ cell-free supernatant was observed, confirming the presence of chelating molecules able to bind the residual iron in solution (as reported by the manufacturing company, dialysis membranes contain traces of heavy metals). Interestingly, the dialyzed $-P_i$ cell-free supernatant did not react in the CAS assay. This indicates that either the Fe-free chelating molecules have a molecular size lower than 1 kDa, and, therefore, diffused out of the membrane, or that the chelators completely reacted with available iron, and the residual concentration was not sufficient to observe positive reaction in the CAS assay.

Comparing our results with the molecular size of known siderophores (e.g. enterobactin 669 Da) it could be hypothesized that the Fe-free chelator has a molecular size lower than 1 kDa, but the configuration during the chelating process involves the coordination of more than one molecule leading to a greater complex. However, the apparent molecular size inferred from the SEC experiment exceeds by far the size of known siderophores. Possibly, the chelator-Fe complexes form molecular-clusters generating aggregates of bigger dimensions, a phenomenon also observed in siderophores such as yersiniabactin and marinobactin E (Drechsel *et al.*, 1995; Butler and Theisen, 2010).

During the MS analysis, we detected *m/z* as being perfectly consistent with the size of known siderophores. However, these data were partially contrasting with the SEC and the dialysis experiments, since the MS analysis showed that the chelator-Ga complexes have a molecular mass lower than 1 kDa. On the one hand, this could result from partial degradation of the molecules during the extraction process, but on the other hand it supports the idea that the chelator-Fe complexes form greater molecular-clusters in solution. Intriguingly, we identified more than one *m/z* showing the Ga isotopic pattern. If we assume that this does not represent an artifact due to degradation, it is consistent with recent findings concerning the siderophores produced by members of the genus *Marinobacter.* These molecules have hydrophobic chains of different lengths resulting in compounds with the same chelating scaffold but with a different final size (Butler and Theisen, 2010). This is exactly the information that the MS/MS scan showed, where irrespective of the initial parent ion we always obtained several shared sister ions containing Ga, which might represent the chelating scaffolds.

The genome of *Pseudovibrio* sp. FO-BEG1 does not contain known genes for the synthesis of siderophores

Siderophores can be distinguished on the basis of the enzymatic machinery involved in their bio-synthesis. One group derives from non-ribosomal peptide synthetase (NRPS) or hybrid NRPS-polyketide synthetase (PKS) modular multienzymes (e.g. enterobactin, yersiniabactin), and the other can be produced through NRPS-independent pathways (e.g. aerobactin, alcaligin;

Barry and Challis, 2009). After confirming the production of one or more chelating molecules under phosphate limitation, we verified the presence of genes known to be involved in the synthesis of siderophores. Surprisingly, no genes were identified in the genome of FO-BEG1. However, a large hybrid NRPS-PKS containing region caught our attention, and we performed bioinformatic analyses in order to elucidate whether its proteins could share homologies with other proteins involved in siderophore production. Confirming a previous analysis (Bondarev *et al.*, 2013; **Contributed works**) we showed that it is highly similar to the gene cluster encoding the cyto-toxin colibactin (**Fig. 4.5**). This compound is produced by pathogenic and non pathogenic members of the family *Enterobacteriaceae* and was shown to induce breaks in the eukaryotic dsDNA, arresting the cells in G2 phase (Nougayrède *et al.*, 2006; Putze *et al.*, 2009).

Intriguingly, there are few reports that refer to colibactin as a new siderophore produced by enterobacteria (Seltmann, 1990; Rabsch *et al.*, 1991; Kresse *et al.*, 2007). So far, the structures of both toxin-colibactin and siderophore-colibactin have not yet been elucidated, and there is no evidence that the siderophore-colibactin corresponds to the molecule encoded by the hybrid NRPS-PKS gene cluster, which, instead, was shown to be the toxin-colibactin (Nougayrède *et al.*, 2006). Apparently, the toxin-colibactin alone is not able to chelate iron in *in-vitro* experiments (Martin *et al.*, 2013), and it was reported to be produced only when *Escherichia coli* is in contact with eukaryotic cells (Nougayrède *et al.*, 2006).

Interestingly, in the FO-BEG1 NRPS-PKS gene cluster gene PSE_3336 and the flanking genes PSE_3314 and PSE_3310 have a potential Pho-box in their upstream regions (Table S2.4; **Chapter 2**), suggesting that their transcription is directly regulated by the environmental phosphate concentration. In the proteomic analyses we performed previously (**Chapter 2**), we detected the proteins PSE_3328 and PSE_3336, but their relative expression could not be quantified. The same is true for other genes located in close proximity to the NRPS cluster (PSE_3326; PSE_3344; PSE_3316; PSE_3327).

The NRPS and PKS systems are formed by synthetic proteins containing multiple domains, such as carrier domains (A, AT) and condensation domains (C, KS). Carrier domains have a high specificity for defined substrates, allowing a prediction of the composition of the molecule backbone if the precise domain order is known (Schwarzer *et al.*, 2003). All synthetic domains, which define the backbone of the molecule, and all known genes involved in the maturation of colibactin were identified in *Pseudovibrio* sp. FO-BEG1 (**Fig. 4.5**). In the phylogenetic analysis all NRPS-PKS synthetic proteins and domains clustered together with proteins belonging to enterobacteria, even though they formed independent branches in the trees and never occurred within the family *Enterobacteriaceae* (**Fig. 4.5, Fig. S4.5-S4.6**). In addition, the composition of the molecular backbone, predicted with antiSMASH, was similar for both the colibactin and the FO-BEG1 NRPS-PKS gene cluster (data not shown). Altogether these data suggest that the FO-BEG1 NRPS-PKS cluster is involved in the synthesis of a molecule highly similar to the toxin-colibactin and undermine the

hypothesis of a possible involvement in the synthesis of a siderophore-like molecule.

The whole colibactin genomic region is described to be laterally transferred among species belonging to the family *Enterobacteriaceae* (Putze *et al.*, 2009), and it is likely that this process also occurred in *Pseudovibrio* sp. FO-BEG1. To verify the lateral acquisition we further analyzed this genomic region, using different approaches. The GC content, GC skew, tetranucleotide usage and the screening for the presence of known genomic islands did not indicate that this region was laterally acquired by FO-BEG1 (**Fig. S4.7**). So far the colibactin NRPS-PKS gene cluster was described only in enterobacteria (Putze *et al.*, 2009) and in the draft genome of strain JE062, closely related to FO-BEG1, homologous genes were not identified (Bondarev *et al.*, 2013; **Contributed works**). Phylogenetic studies performed on a vast number of PKS domains showed that these genes are mainly acquired by proteobacteria via HGT, explaining their random distribution among strains belonging to the same species (Jenke-Kodama *et al.*, 2005). Therefore, it is reasonable to assume that this region was horizontally acquired by *Pseudovibrio* sp. FO-BEG1 and subsequently it underwent amelioration processes, characteristically occurring for horizontally acquired genes (Lawrence and Ochman, 1997).

The high similarity between the enterobacteria and the FO-BEG1 NRPS-PKS cluster suggests a similar end-product. However, in *Rhodococcus* and *Serratia* it was shown that siderophore production resulted from the coordination of more than one gene cluster (Bosello *et al.*, 2011; Seyedsayamdost *et al.*, 2012). In addition, non-ribosomal peptides undergo several processes of post synthesis

modification and maturation, which cannot be predicted at the genomic level. Variation in the modular synthesis and iterative processes can also occur, and these increase the number of products derived from one cluster (Moss *et al.*, 2004). Therefore, it could be hypothesized that all these mechanisms, involved in the formation of a functional compound, could be responsible for the formation in FO-BEG1 of a final product which is substantially different from the colibactin of enterobacteria. Indeed, in the FO-BEG1 genome another PKS cluster (PSEp_0217-0220; not identified in the *Enterobacteriacea*) and a protein containing a polyketide cyclase domain (PSE_3283) are present. Additionally, different beta-lactamases and peptidases, potentially involved in maturation of the NRP, were identified in proximity to the NRPS-PKS region.

It is important to mention that the cell-free supernatant of the $-P_i$ cultures gave negative results when tested for the presence of colibactin, using eukaryotic cells as a bio-indicator (Bondarev *et al.*, 2012). This would suggest that either the whole NRPS-PKS island is not expressed under phosphate limitation, and, therefore, is not involved in the synthesis of the chelating molecule, or that it can produce a molecule structurally different from colibactin. Recently, it was shown that a mutation in the *clbA* gene (homologous of PSE_3317) inhibited colibactin synthesis in the probiotic *E. coli* str. Nissle 1917. However, this also reduced the probiotic effect of the strain. Both were restored when the *clbA* mutant was complemented (Olier *et al.*, 2012). These data showed that the colibactin NRPS-PKS island is required for toxicity, but also for probiotic activity. For this reason the authors suggested that colibactin may consist of more than

one molecule, supporting our hypothesis of structural variability in colibactin-related compounds.

Genes for siderophore production are regulated by the ferric-uptake regulator family protein (Fur), which binds to a specific sequence in the upstream region of the genes, regulating their transcription (Hantke, 2001). Therefore, genes having a Fur-box are likely to be controlled by extracellular iron concentrations. If the NRPS-PKS cluster produces a chelator that is used by *Pseudovibrio* sp. FO-BEG1 as siderophore it is likely to be part of this regulon, therefore, Fur-boxes should be present in the upstream regions of its genes. With the approach applied we did not identify any Fur-boxes in the upstream regions of the NRPS-PKS genes, indicating that they are not members of the FO-BEG1 Fur-regulon. Even though these results have to be cautiously interpreted, due to the variability of the Fur-proteins in the *Alphaproteobacteria* (Cornelis *et al.*, 2011), they suggest that either the cluster is not involved in the synthesis of a chelating molecule, or that the produced chelating molecule has not the primary function of scavenging iron in response to iron limitation.

Chelating molecules are produced by bacteria of the *Roseobacter* clade not only as a response to phosphate limitation

In order to clarify whether the production of chelating molecules in response to phosphate limitation can also be observed in other heterotrophic marine bacteria, and could be, therefore, of broader environmental relevance, we investigated this phenomenon in strains of the *Roseobacter* clade. These and bacteria belonging to the *Pseudovibrio* genus are phylogenetically related

(**Figure 1.1**; **Chapter 1**) and have several physiological traits in common. Members of this clade make up 15 to 20% of the coastal and oceanic bacterioplankton, having, therefore, a significant influence on marine element cycles (Buchan *et al.*, 2005; Moran *et al.*, 2007). The different responses observed in the *Roseobacter* strains tested in this study (**Fig. 4.4**) suggest that although the production of chelating molecules can occur under phosphate-limited condition, this phenomenon is species-specific and can be triggered by different mechanisms as apart from the response to phosphate limitation.

As reported previously (Thole *et al.*, 2012), a gene cluster involved in siderophore production was identified in the plasmid of *P. gallaeciensis,* which could potentially be responsible for the production of the putative chelating molecule detected in this study. Besides this, there is no further available evidence concerning the ability of the other tested *Roseobacter* strains to produce siderophores. Additionally, the hybrid NRPS-PKS gene cluster detected in FO-BEG1 could not be identified in the *Roseobacter* genomes. This weakens the hypothesis that this cluster is responsible for the synthesis of the chelating molecule. However, it cannot be excluded that different type of chelators are produced.

Tropodithietic acid (TDA) is not responsible for the solubilization of precipitated iron

Even more puzzling was the correlation between the increase in soluble iron and the production of TDA, which was detected in all crude extracts of the yellow cultures characterized by iron increase. TDA can have mild

chelating properties especially in its tautomeric form thiotropocin (Kintaka *et al.*, 1984; Greer *et al.*, 2008). However, when we tested the chelating properties of pure TDA with the CAS assay it reacted only when incubated for at least 2 h and only in concentrations higher than normally produced (data not shown). In addition, the fraction containing TDA, obtained from the preparative HPLC, was CAS negative and a mild reaction occurred only after 2 h of incubation. A positive reaction of TDA in the CAS assay was also observed by D´Alvise (2013). However, this was detected only after 16-24 h of incubation and only with a surplus of TDA (≈ 0.2 g L^{-1}). Both observations are not consistent with the rapid reaction of fraction 1 in the CAS assay. TDA has a molecular mass of 211.96 Da. Assuming that one molecule of TDA can form a bidentate ligand with Fe(III) using the oxygen in the carboxylic and carbonyl group, three molecules would be required to form a stable exadentate complex. This generates a Fe-complex of 691.73 Da, which is not consistent with our dialysis and SEC experiments.

Eventually, the MS analysis of the active fraction revealed the presence of molecules able to chelate Ga(III) in the mass range 600 to 900 *m/z*. These are in the same range of the hypothesized TDA-Fe complex. However, the MS/MS analysis did not show a fragmentation pattern ascribable to a TDA derivate. D´Alvise proposed that a "pre-TDA" molecule could be responsible for the interaction with iron, which would then catalyze the formation of a functional TDA. However, in our experiments the only fraction which immediately reacted in the CAS assay (fraction 1) did not show the TDA mass in the MS analysis, even after Fe(III) or Ga(III) addition. Instead, this mass was identified as sodium adduct ($[M+Na]^+$; 234.9 m/z) in the fraction that, according

to the chromatographic properties, was thought to contain TDA. Therefore, at this stage our data do not support the hypothesis that TDA is responsible for the iron chelation in FO-BEG1 phosphate-limited cultures, strengthening the idea that novel and undescribed molecules with chelating properties were produced.

Many hypotheses could be proposed to explain this phenomenon. For example, chelating molecules could help to dissolve iron oxyhydroxide minerals, allowing the desorption of phosphate anchored to their surface. In this way, an extra source of bio-available phosphate could be generated and used to support growth under phosphate-limited conditions. Although at this stage all hypotheses remain highly speculative, our results could open new perspectives on the understanding of the regulation of iron and phosphorus metabolisms and on the biogeochemical cycle of these two nutrients.

Acknowledgement

We are grateful to M. Meyer and S. Menger for technical support. Silvana Pape and Martin Kölling are greatly acknowledged for the help with the ICP-OES measurements. We thank M. Medema for being promptly available to fix minor issues in the MultiGeneBlast program. Katrin Klaproth is also acknowledged for the technical assistance during the MS analysis. This study was funded by the European Research Council, Grant No. 203364, and the Max Planck Society. The authors have no conflict of interest to declare.

References

Andrews, S.C., Robinson, A.K., and Rodríguez-Quiñones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiology Reviews* **27**: 215-237.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R.,

Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. (2008) The RAST server: Rapid annotations using subsystems technology. *BMC Genomics* **9**: 75.

Barry, S.M., and Challis, G.L. (2009) Recent advances in siderophore biosynthesis. *Current Opinion in Chemical Biology* **13**: 205-215.

Behrenfeld, M.J., Bale, A.J., Kolber, Z.S., Aiken, J., and Falkowski, P.G. (1996) Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. *Nature* **383**: 508-511.

Berner, R.A. (1973) Phosphate removal from sea-water by adsorption on volcanogenic ferric oxides. *Earth and Planetary Science Letters* **18**: 77-86.

Blake, R.E., Alt, J.C., and Martini, A.M. (2001) Oxygen isotope ratios of PO4: an inorganic indicator of enzymatic activity and P metabolism and a new biomarker in the search for life. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 2148-2153.

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., and Weber, T. (2013) antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Research* **41**: W204-W212.

Bondarev, V. (2012) Physiology of *Pseudovibrio* sp. FO-BEG1 - a facultatively oligotrophic and metabolically versatile bacterium. Bremen: Universität Bremen.

Bondarev, V., Richter, M., Romano, S., Piel, J., Schwedt, A., and Schulz-Vogt, H.N. (2013) The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. *Environmental Microbiol*ogy **15**: 2095-2113.

Bosello, M., Robbel, L., Linne, U., Xie, X.L., and Marahiel, M.A. (2011) Biosynthesis of the siderophore rhodochelin requires the coordinated expression of three Independent gene clusters in *Rhodococcus jostii* RHA1. *Journal of the American Chemical Society* **133**: 4587-4595.

Bruhn, J.B., Nielsen, K.F., Hjelm, M., Hansen, M., Bresciani, J., Schulz, S., and Gram, L. (2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. *Applied and Environmental Microbiology* **71**: 7263-7270.

Buchan, A., González, J.M., and Moran, M.A. (2005) Overview of the marine *Roseobacter* lineage. *Applied and Environmental Microbiology* **71**: 5665-5677.

Butler, A., and Theisen, R.M. (2010) Iron(III)-siderophore coordination chemistry: Reactivity of marine siderophores. *Coordination Chemistry Reviews* **254**: 288-296.

Byers, B.R., and Arceneaux, J.E.L. (1998) Microbial iron transport: Iron acquisition by pathogenic microorganisms. *Metal Ions in Biological Systems* **35**: 37-66.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E., and Ros, J. (2000) Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **275**: 27393-27398.

Carrondo, M.A. (2003) Ferritins, iron uptake and storage from the bacterioferritin viewpoint. *The EMBO Journal* **22**: 1959-1968.

Chakraborty, S., Sivaraman, J., Leung, K.Y., and Mok, Y.K. (2011) Two-component PhoB-PhoR Regulatory System and Ferric Uptake Regulator Sense Phosphate and Iron to Control Virulence Genes in Type III and VI Secretion Systems of Edwardsiella tarda. *Journal of Biological Chemistry* **286**: 39417-39430.

Cornelis, P., Wei, Q., Andrews, S.C., and Vinckx, T. (2011) Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics* **3**: 540-549.

Csaky, T.Z. (1948) On the estimation of bound hydroxylamine in biological materials. *Acta Chemica Scandinavica* **2**: 450-454.

D´Alvise, P. (2013) Aquaculture application and ecophysiology of marine bacteria from the *Roseobacter* clade. Søborg: Technical University of Denmark.

Drechsel, H., Stephan, H., Lotz, R., Haag, H., Zahner, H., Hantke, K., and Jung, G. (1995) Structure elucidation of yersiniabactin, a siderophore from highly virulent *Yersinia* strains. *Liebigs Annalen*: 1727-1733.

Feely, R.A., Massoth, G.J., Baker, E.T., Cowen, J.P., Lamb, M.F., and Krogslund, K.A. (1990) The Effect of Hydrothermal Processes on Midwater Phosphorus Distributions in the Northeast Pacific. *Earth and Planetary Science Letters* **96**: 305-318.

Froelich, P.N., Bender, M.L., and Heath, G.R. (1977) Phosphorus accumulation rates in metalliferous sediments on east Pacific Rise. *Earth and Planetary Science Letters* **34**: 351-359.

Gerard, F., Dri, A.M., and Moreau, P.L. (1999) Role of Escherichia coli RpoS, LexA and H-NS global regulators in metabolism and survival under aerobic, phosphate-starvation conditions. *Microbiology* **145**: 1547-1562.

Gillam, A.H., Lewis, A.G., and Andersen, R.J. (1981) Quantitative-Determination of Hydroxamic Acids. *Analytical Chemistry* **53**: 841-844.

Greer, E.M., Aebisher, D., Greer, A., and Bentley, R. (2008) Computational studies of the tropone natural products, thiotropocin, tropodithietic acid, and troposulfenin. Significance of thiocarbonyl-enol tautomerism. *Journal of Organic Chemistry* **73**: 280-283.

Haber, F., and Weiss, J. (1934) The catalytic decomposition of hydrogen peroxide by iron salts. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences* **Se. A**: 331-352.

Hantke, K. (2001) Iron and metal regulation in bacteria. *Current Opinion in Microbiology* **4**: 172-177.

Harrison, P.M., and Arosio, P. (1996) Ferritins: molecular properties, iron storage function and cellular regulation. *Biochimica Et Biophysica Acta-Bioenergetics* **1275**: 161-203.

Hertz, G.Z., and Stormo, G.D. (1999) Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. *Bioinformatics* **15**: 563-577.

Hosoya, S., and Yokota, A. (2007) *Pseudovibrio japonicus* sp nov., isolated from coastal seawater in Japan. *International Journal of Systematic and Evolutionary Microbiology* **57**: 1952-1955.

Jenke-Kodama, H., and Dittmann, E. (2009) Bioinformatic perspectives on NRPS/PKS megasynthases: Advances and challenges. *Natural Product Reports* **26**: 874-883.

Jenke-Kodama, H., Sandmann, A., Müller, R., and Dittmann, E. (2005) Evolutionary implications of bacterial polyketide synthases. *Molecular Biology and Evolution* **22**: 2027-2039.

Kadko, D., Feely, R., and Massoth, G. (1994) Scavenging of Th-234 and Phosphorus Removal from the Hydrothermal Effluent Plume over the North Cleft Segment of the Juan-De-Fuca Ridge. *Journal of Geophysical Research-Solid Earth* **99**: 5017-5024.

Kintaka, K., Ono, H., Tsubotani, S., Harada, S., and Okazaki, H. (1984) Thiotropocin, a new sulfur-containing 7-membered-ring antibiotic produced by a *Pseudomonas* sp. *Journal of Antibiotics* **37**: 1294-1300.

Kresse, A.U., Rienäcker, I., Valle, A.M., Steinrück, H., Claus, H., Payne, S.M., Tschäpe, H., Williams, P.H., and Reissbrodt, R. (2007) Enterohaemorrhagic *Escherichia coli* O157 and non-O157 serovars differ in their mechanisms for iron supply. *International Journal of Medical Microbiology* **297**: 9-15.

Lamarche, M.G., Wanner, B.L., Crepin, S., and Harel, J. (2008) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiology Reviews* **32**: 461-473.

Langille, M.G.I., and Brinkman, F.S.L. (2009) IslandViewer: an integrated interface for computational identification and visualization of genomic islands. *Bioinformatics* **25**: 664-665.

Lawrence, J.G., and Ochman, H. (1997) Amelioration of bacterial genomes: rates of change and exchange. *Journal of Molecular Evolution* **44**: 383-397.

Le Brun, N.E., Crow, A., Murphy, M.E.P., Mauk, A.G., and Moore, G.R. (2010) Iron core mineralisation in prokaryotic ferritins. *Biochimica Et Biophysica Acta-General Subjects* **1800**: 732-744.

Litwin, C.M., and Calderwood, S.B. (1993) Role of iron in regulation of virulence genes. *Clinical Microbiology Reviews* **6**: 137-149.

Macrellis, H.M., Trick, C.G., Rue, E.L., Smith, G., and Bruland, K.W. (2001) Collection and detection of natural iron-binding ligands from seawater. *Marine Chemistry* **76**: 175-187.

Martín, J.F. (2004) Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *Journal of Bacteriology* **186**: 5197-5201.

Martin, P., Marcq, I., Magistro, G., Penary, M., Garcie, C., Payros, D., Boury, M., Olier, M., Nougayrède, J.P., Audebert, M., Chalut, C., Schubert, S., and Oswald, E. (2013) Interplay between siderophores and colibactin genotoxin biosynthetic pathways in *Escherichia coli*. *Plos Pathogens* **9**: doi:10.1371/journal.ppat.1003437.

Medema, M.H., Takano, E., and Breitling, R. (2013) Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Molecular Biology and Evolution* **30**: 1218-1223.

Moran, M.A., Belas, R., Schell, M.A., González, J.M., Sun, F., Sun, S., Binder, B.J., Edmonds, J., Ye, W., Orcutt, B., Howard, E.C., Meile, C., Palefsky, W., Goesmann, A., Ren, Q., Paulsen, I., Ulrich, L.E., Thompson, L.S., Saunders, E., and Buchan, A. (2007) Ecological genomics of marine roseobacters. *Applied and Environmental Microbiology* **73**: 4559-4569.

Moreau, P.L. (2004) Diversion of the metabolic flux from pyruvate dehydrogenase to pyruvate oxidase decreases oxidative stress during glucose metabolism in nongrowing *Escherichia coli* cells incubated under aerobic, phosphate starvation conditions. *Journal of Bacteriology* **186**: 7364-7368.

Moreau, P.L., Gerard, F., Lutz, N.W., and Cozzone, P. (2001) Non-growing *Escherichia coli* cells starved for glucose or phosphate use different mechanisms to survive oxidative stress. *Molecular Microbiology* **39**: 1048-1060.

Moss, S.J., Martin, C.J., and Wilkinson, B. (2004) Loss of co-linearity by modular polyketide synthases: a mechanism for the evolution of chemical diversity. *Natural Product Reports* **21**: 575-593.

Neilands, J.B. (1981) Microbial iron compounds. *Annual Review of Biochemistry* **50**: 715-731.

Neilands, J.B. (1993) Siderophores. *Archives of Biochemistry and Biophysics* **302**: 1-3.

Nougayrède, J.P., Homburg, S., Taieb, F., Boury, M., Brzuszkiewicz, E., Gottschalk, G., Buchrieser, C., Hacker, J., Dobrindt, U., and Oswald, E. (2006) *Escherichia coli* induces DNA double-strand breaks in eukaryotic cells. *Science* **313**: 848-851.

O'Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O'Gara, F., and Dobson, A.D.W. (2011) Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *Journal of Applied Microbiology* **110**: 1495-1508.

Olier, M., Marcq, I., Salvador-Cartier, C., Secher, T., Dobrindt, U., Boury, M., Bacquié, V., Penary, M., Gaultier, E., Nougayrède, J.P., Fioramonti, J., and Oswald, E. (2012) Genotoxicity of *Escherichia coli* Nissle 1917 strain cannot be dissociated from its probiotic activity. *Gut Microbes* **3**: 501-509.

Price, N.M., and Morel, F.M.M. (1998) Biological cycling of iron in the ocean. *Metal Ions in Biological Systems* **35**: 1-36.

Putze, J., Hennequin, C., Nougayrède, J.P., Zhang, W.L., Homburg, S., Karch, H., Bringer, M.A., Fayolle, C., Carniel, E., Rabsch, W., Oelschlaeger, T.A., Oswald, E., Forestier, C., Hacker, J., and Dobrindt, U. (2009) Genetic structure and distribution of the colibactin genomic island among members of the family *Enterobacteriaceae*. *Infection and Immunity* **77**: 4696-4703.

Rabsch, W., Tkacik, J., Lindemann, W., Mikula, I., and Reissbrodt, R. (1991) Different systems for iron supply of *Salmonella typhimurium* and *Escherichia coli* wild strains as tool for typing. *Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases* **274**: 437-445.

Rioux, C., Jordan, D.C., and Rattray, J.B.M. (1983) Colorimetric determination of catechol siderophores in microbial cultures. *Analytical Biochemistry* **133**: 163-169.

Rudnicki, M.D., and Elderfield, H. (1993) A Chemical-Model of the Buoyant and Neutrally Buoyant Plume above the Tag Vent Field, 26 Degrees-N, Mid-Atlantic Ridge. *Geochimica Et Cosmochimica Acta* **57**: 2939-2957.

Salgado, H., Peralta-Gil, M., Gama-Castro, S., Santos-Zavaleta, A., Muñiz-Rascado, L., García-Sotelo, J.S., Weiss, V., Solano-Lira, H., Martínez-Flores, I., Medina-Rivera, A., Salgado-Osorio, G., Alquicira-Hernández, S., Alquicira-Hernández, K., López-Fuentes, A., Porrón-Sotelo, L., Huerta, A.M., Bonavides-Martínez, C., Balderas-Martínez, Y.I., Pannier, L., Olvera, M., Labastida, A., Jiménez-Jacinto, V., Vega-Alvarado, L., Del Moral-Chávez, V., Hernández-Alvarez, A., Morett, E., and Collado-Vides, J. (2012) RegulonDB v8.0: omics data sets, evolutionary conservation, regulatory phrases, cross-validated gold standards and more. *Nucleic Acids Research* **41**: D203-213.

Schwarzer, D., Finking, R., and Marahiel, M.A. (2003) Nonribosomal peptides: from genes to products. *Natural Product Reports* **20**: 275-287.

Seltmann, G. (1990) Significance of composition and structure of the enterobacterial outer-membrane to the transport of desired and undesired substances and its inhibition. *Acta Biotechnologica* **10**: 107-115.

Seyedsayamdost, M.R., Cleto, S., Carr, G., Vlamakis, H., Vieira, M.J., Kolter, R., and Clardy, J. (2012) Mixing and matching siderophore clusters: structure and biosynthesis of serratiochelins from *Serratia* sp. V4. *Journal of the American Chemical Society* **134**: 13550-13553.

Shieh, W.Y., Lin, Y.T., and Jean, W.D. (2004) *Pseudovibrio denitrificans* gen. nov., sp nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2307-2312.

Sunda, W.G. (2001) Bioavailability and bioaccumulation of iron in the sea. In *The biogeochemstry of iron in seawater*. Turner, D.R., and Hunter, K.A. (eds). Chichester: John Wiley and Sons, LTD, pp. 42-84.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews* **71**: 295-347.

Taylor, S.W., Hawkins, C.J., and Winzor, D.J. (1993) Spectrophotometric evidence for involvement of aromatic residues in the interaction of ferreascidin with ferric ion. *Inorganic Chemistry* **32**: 422-427.

Teeling, H., Meyerdierks, A., Bauer, M., Amann, R., and Glöckner, F.O. (2004a) Application of tetranucleotide frequencies for the assignment of genomic fragments. *Environmental Microbiology* **6**: 938-947.

Teeling, H., Waldmann, J., Lombardot, T., Bauer, M., and Glöckner, F.O. (2004b) TETRA: a web-service and a stand-alone program for the analysis and comparison of tetranucleotide usage patterns in DNA sequences. *BMC Bioinformatics* **5**: 163.

Thingstad, T.F., Krom, M.D., Mantoura, R.F.C., Flaten, G.A.F., Groom, S., Herut, B., Kress, N., Law, C.S., Pasternak, A., Pitta, P., Psarra, S., Rassoulzadegan, F., Tanaka, T., Tselepides, A., Wassmann, P., Woodward, E.M.S., Riser, C.W., Zodiatis, G., and Zohary, T. (2005) Nature of phosphorus limitation in the ultraoligotrophic eastern Mediterranean. *Science* **309**: 1068-1071.

Thole, S., Kalhoefer, D., Voget, S., Berger, M., Engelhardt, T., Liesegang, H., Wollherr, A., Kjelleberg, S., Daniel, R., Simon, M., Thomas, T., and Brinkhoff, T. (2012) *Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptation to surface life. *The ISME Journal* **6**: 2229-2244.

Tortell, P.D., Maldonado, M.T., Granger, J., and Price, N.M. (1999) Marine bacteria and biogeochemical cycling of iron in the oceans. *FEMS Microbiology Ecology* **29**: 1-11.

Vining, L.C. (1990) Functions of secondary metabolites. *Annual Review of Microbiology* **44**: 395-427.

Wandersman, C., and Delepelaire, P. (2004) Bacterial iron sources: from siderophores to hemophores. *Annual Review of Microbiology* **58**: 611-647.

Widdel, F., and Pfenning, N. (1984) Dissimilatory sulfate- or sulfur-reducing bacteria. In *Bergey´s Manual of Systematic Bacteriology*. Krieg, N.R., and Holt, J.G. (eds). Baltimora, MD, USA: Williams & Wilkins, pp. 663-679.

Wu, J.F., Sunda, W., Boyle, E.A., and Karl, D.M. (2000) Phosphate depletion in the western North Atlantic Ocean. *Science* **289**: 759-762.

Yuan, Z.C., Zaheer, R., and Finan, T.M. (2005) Phosphate limitation induces catalase expression in *Sinorhizobium meliloti*, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. *Molecular Microbiology* **58**: 877-894.

Zaborin, A., Romanowski, K., Gerdes, S., Holbrook, C., Lepine, F., Long, J., Poroyko, V., Diggle, S.P., Wilke, A., Righetti, K., Morozova, I., Babrowski, T., Liu, D.C., Zaborina, O., and Alverdy, J.C. (2009) Red death in Caenorhabditis elegans caused by Pseudomonas aeruginosa PAO1. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 6327-6332.

Supplementary materials and methods

Characterization, extraction, and isolation of the chelating compounds, and detection of TDA

Cells from 10 L culture were harvested in stationary phase, after about 120 h of incubation. Cells were removed by means of centrifugation at 11,000 x *g* and 5 °C for 20 min, the supernatant was filtered with a 0.22 µm filter (Sartorius AG, Göttingen, Germany), and the activity was tested using the CAS assay. The cell-free supernatant was then acidified to pH 2.6 using 5 mol L^{-1} HCl. This facilitates the release of the iron from the chelator-Fe complex, due to the protonation of the hydroxyl and carboxyl groups, which are generally interacting with iron. Subsequently, the acidified supernatant was mixed in the dark with an equal volume of ethyl acetate for 1 h. The solvent phase was collected and the solvent was evaporated in the rotary evaporation system Laborota 4,000 (Heidolph, Schwabach, Germany) at 45 °C. Evaporation was completed in a speed-vacuum centrifuge (Eppendorf Concentrator 5301, Eppendorf, Hamburg, Germany) at 45 °C. Before performing the isolation via high performance liquid chromatography (HPLC), the activity of the crude extract was tested using the CAS assay.

In order to verify the complexity of the crude extract we performed a first analytical reverse phase (RP)-HPLC run using a C18 column (Nucleodur 100-5 C18ec, 250 mm x 3mm). The eluents were A: 100% MQ and B: 70% MQ and 30% acetonitrile (ACN) both containing 0.1% trifluoroacetic aicd (TFA). The program for the separation was: 0-20 min from 20% B to 100% B,

20-30 min 100% B, and 30-35 min from 100% B to 20% B, using a flow rate of 2.5 mL min^{-1}. The same conditions were then applied for the isolation of the metabolites using a preparative column C18 (Kromasil 100-7 C18, 250mm x 20 mm), a flow rate of 18 mL min^{-1}, and recording the absorption at 300 nm. Fractions were manually collected every time an increase in absorption was observed. Eluent was evaporated and the fractions were re-dissolved in 70% acetonitrile to test their activity using the CAS assay. For the active fraction we performed two more analytical runs, in order to verify its composition. We used two C18 analytical columns (Nucleodur 100-5 C18ec, 250 mm x 3mm; Phenomenex aqua C18, 250 x 2.0 mm) using the running condition described above.

In order to verify the production of tropodithietic acid (TDA) by the strains of the *Roseobacter* clade, metabolites were extracted as described above from 300 mL of cell-free supernatant of the cultures that developed a yellow coloration and showed the higher iron increase under phosphate limitation. TDA was detected via RP-HPLC using a Nucleodur Isis C18 column (100-5 C18ec, 250 mm x 3mm). The eluents were: 100% acetonitrile (A) and 100% MQ (B), both containing 0.1% acetic acid. The program was: 0-5 min 1% B, 5-10 min from 1% B to 42% B, 10-35 min from 42% B to 57% B, 35-40 min from 57% B to 1% B, using a flow rate of 1 mL min^{-1}. Identification of TDA was based on the known retention time and absorption spectra obtained analyzing a TDA standard with purity ≥ 98% (BioViotica GmbH, Gottingen, Germany) under the same condition as applied for the crude extracts.

***In-silico* analysis for the detection of genes potentially involved in siderophore production**

The genomic region containing the non-ribosomal peptide polyketide synthase system (NRPS-PKS) identified by the antiSMASH analysis, and potentially involved in the production of a chelating compound in *Pseudovibrio* sp. FO-BEG1, was used to perform a multigene BLAST using the MultiGeneBlast platform (Medema *et al.*, 2013). At first the region of interest (PSE_3310-50) was blasted against all available entries in GenBank (October 2013). Subsequently, to avoid redundancy in the dataset, a specific database containing the genomes in which genes belonging to the query cluster were identified was created. In this second database, genomes representing the main phylogenetic groups present in the result of the first analysis were included.

The affiliation of the proteins, identified by antiSMASH to be potentially involved in synthesis and transport of the non-ribosomal peptide, was investigated performing a phylogenetic analysis considering in the first instance their full length sequences. They were singularly used to perform a WU-BLAST search against the Uniprot Knowledge database. To further identify proteins distantly related to the query, PSI-BLAST was used (Altschul *et al.*, 1997), reiterating the search until no further sequences affiliated to new taxa could be obtained. Sequences were retrieved and aligned using the program MUSCLE (Edgar, 2004). All alignments were then imported in the software MEGA 5.2 (Tamura *et al.*, 2011) and were manually checked. Phylogenetic trees were calculated using the Maximum likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). Bootstrapping was performed allowing 100 reiterations

and the tree with the highest log likelihood was chosen. Positions with less than 100% amino acid coverage were not considered.

Phylogenetic analysis of the A, C, AT, and KS domains were performed after arbitrarily selecting homologous domains involved in the synthesis of siderophores and antibiotic in other bacteria. Sequences were retrieved from the databases CluterMine360 (Conway and Boddy, 2013) and SBSPKS (Anand *et al.*, 2010), or after direct submission of bacterial full genomes to antiSMASH 2.0 (Blin *et al.*, 2013). Sequences were aligned using the program PROMALS3D (Pei *et al.*, 2008) and phylogenetic trees were calculated using the Maximum likelihood method based on the JTT matrix-based model with 500 bootstrap re-iterations. Sequences of the FadD and FadH domains of the fatty acid synthetase (FAS) were used to root the trees. All sequences used for the phylogenetic analyses are available upon request from the authors

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Anand, S., Prasad, M.V.R., Yadav, G., Kumar, N., Shehara, J., Ansari, M.Z., and Mohanty, D. (2010) SBSPKS: structure based sequence analysis of polyketide synthases. *Nucleic Acids Research* **38**: W487-W496.

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., and Weber, T. (2013) antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Research* **41**: W204-W212.

Conway, K.R., and Boddy, C.N. (2013) ClusterMine360: a database of microbial PKS/NRPS biosynthesis. *Nucleic Acids Research* **41**: D402-D407.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792-1797.

Jones, D.T., Taylor, W.R., and Thornton, J.M. (1992) The rapid generation of mutation data matrices from protein sequences. *Computer Applications in the Biosciences* **8**: 275-282.

Medema, M.H., Takano, E., and Breitling, R. (2013) Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Molecular Biology and Evolution* **30**: 1218-1223.

Pei, J.M., Kim, B.H., and Grishin, N.V. (2008) PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Research* **36**: 2295-2300.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* **28**: 2731-2739.

Supplementary figures

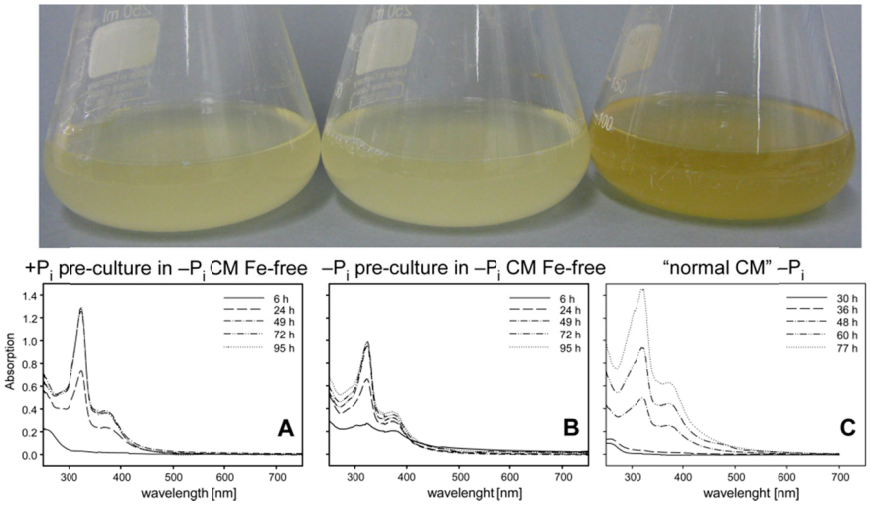


Figure S4.1 | UV-visible spectra of the cell-free supernatant recorded for cultures grown under –P_i conditions in "normal CM" medium (C) and for cultures of the "Refresh experiment" (A, B). The picture shows the visual appearance of the cultures at the end of the stationary phase.

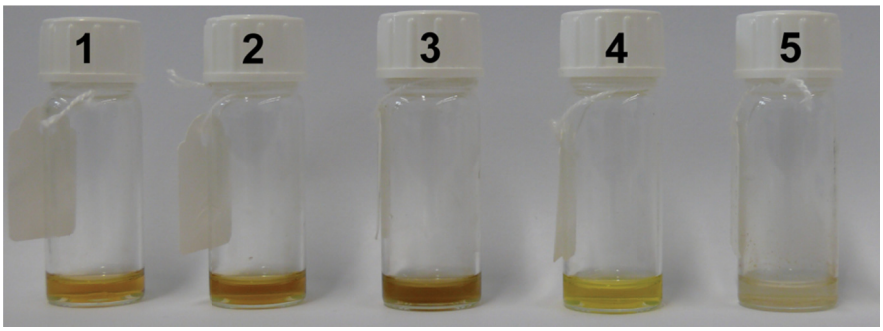


Figure S4.2 | Fractions collected during the preparative RP-HPLC run performed for the crude extract of the FO-BEG1 phosphate limited cell-free supernatant. Fraction one resulted positive to the CAS assay. Fraction four contained a compound with retention time and UV-visible spectra consistent with the ones of the tropodithietic acid (TDA).

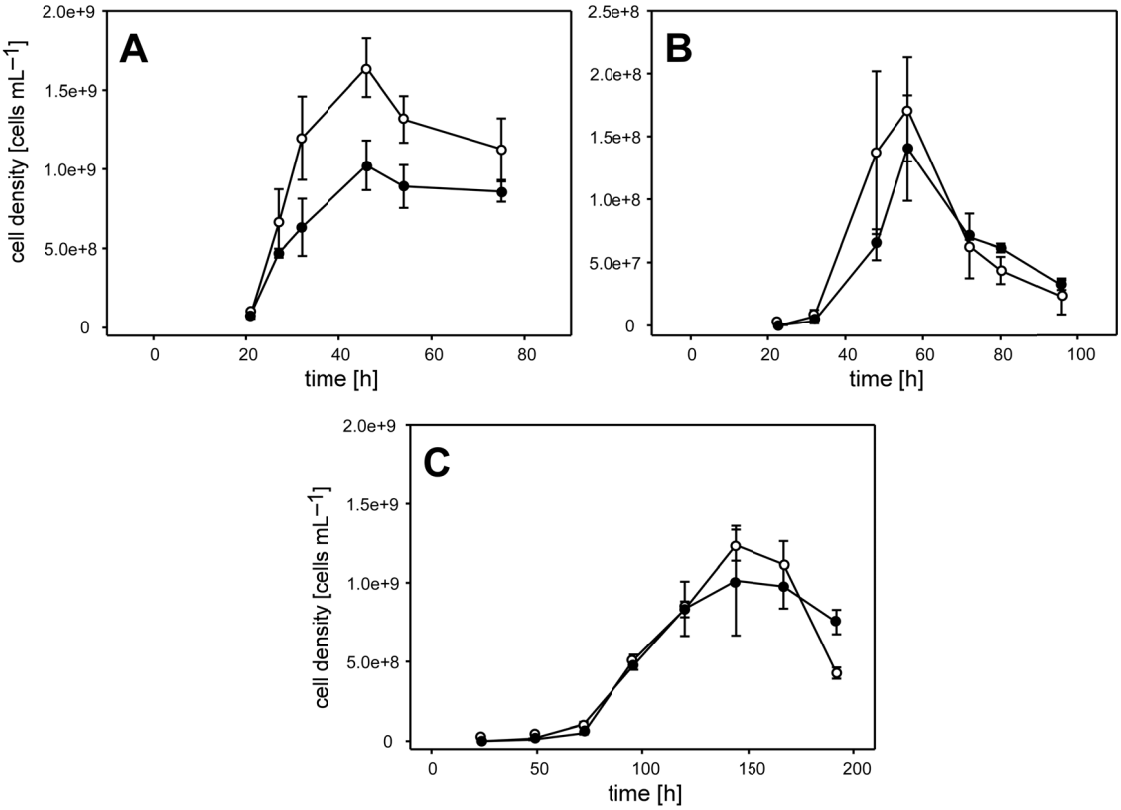


Figure S4.3 | Cell density over time of cultures grown under +P_i (empty symbols) and −P_i (filled symbols) conditions, of *P. gallaeciensis* (A), *P. inhibens* (B), *R. pomeroyi* (C). The error bars represent the standard deviation of biological triplicates.

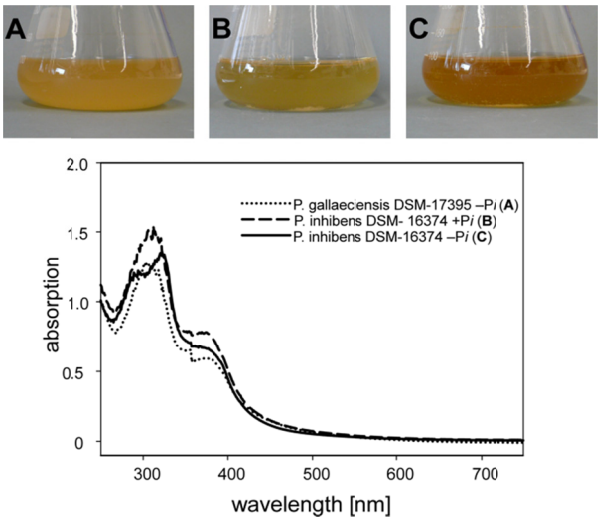


Figure S4.4 | UV-visible spectra of the cell-free supernatant recorded for the cultures of *P. gallaeciensis* (A) and *P. inhibens* (B) grown under –P_i conditions and *P. inhibens* (C) grown under +P_i conditions. The pictures show the visual appearance of the cultures at the end of the stationary phase.

Figure S4.5 (displayed on the next page) | Phylogentic trees obtained using Maximum Likelihood method for the A domain of the NRPS system and AT domain of the PKS system. The trees with the highest log likelihood are shown. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are shown at the root of each branch. Only values equal or higher than 50% are shown. For each sequence the relative species and the name of the respective protein are indicated. For the domains involved in siderophore production the name of the compound is reported in red. The names of the proteins of *Pseudovibrio* sp. FO-BEG1 in bold and the names of the colibactin proteins are shown in bold and bold green, respectively.

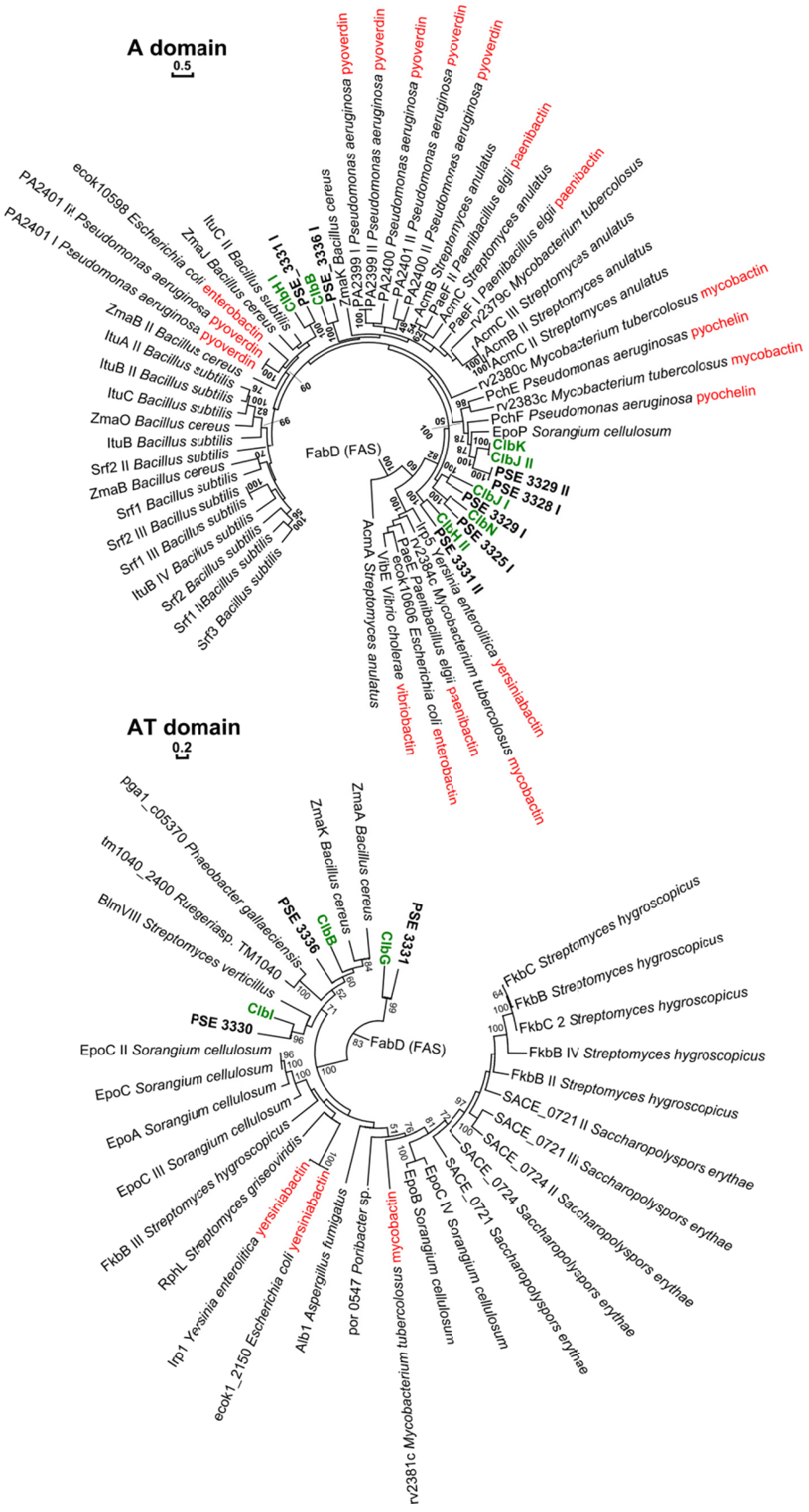
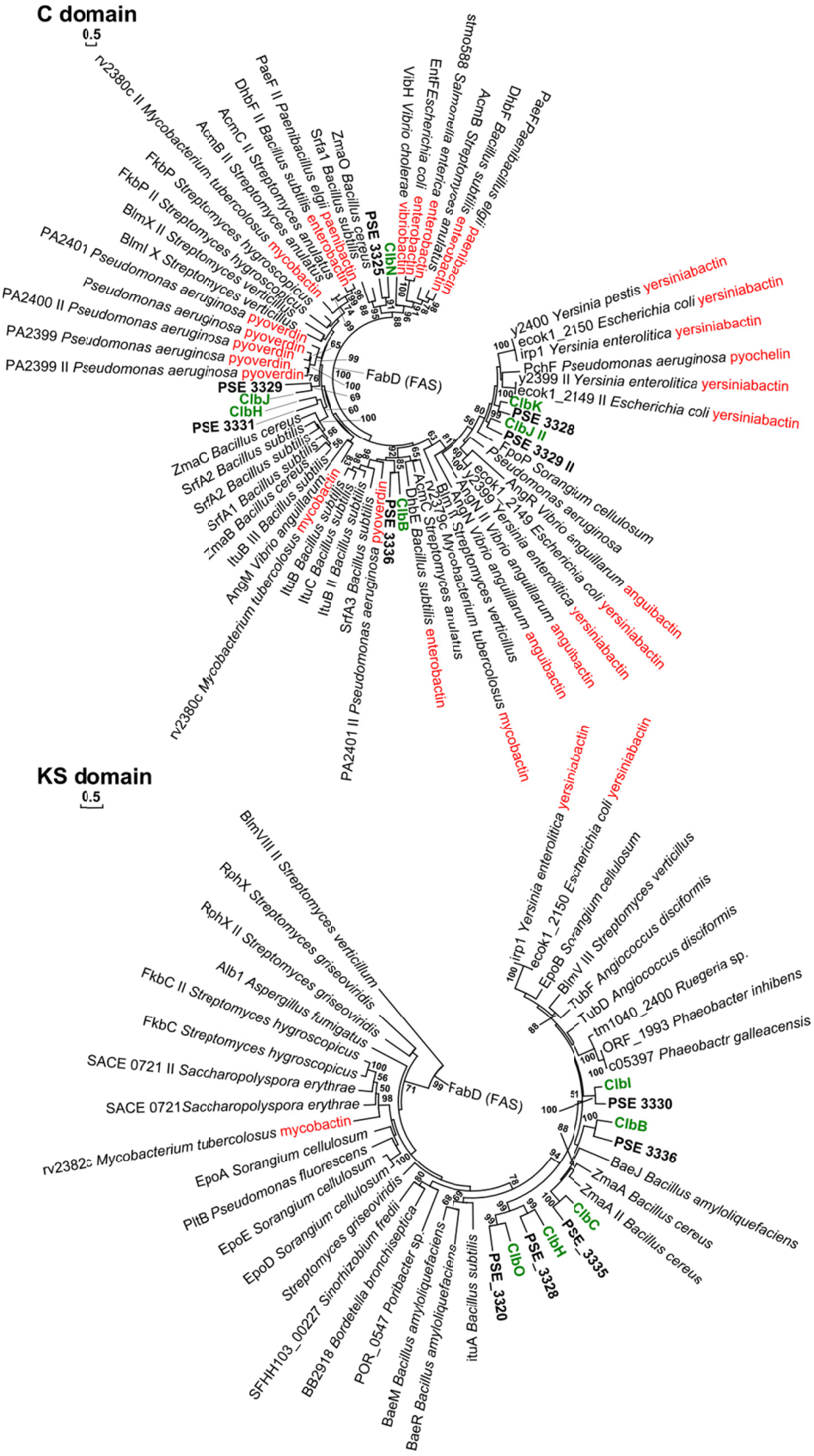

A domain
0.5
AT domain
0.2
FabD (FAS)
FabD (FAS)
PSE 3329 II
PSE 3328 I
PSE 3329 I
PSE 3325 I
PSE 3331 II
PSE 3331
PSE 3336
PSE 3330
ClbK
ClbJ II
ClbJ I
ClbN
ClbH II
ClbG
ClbB
ClbI
PSE 3331 I
PSE 3336 I
ClbH I
ClbB
ClbI
AcmA Streptomyces anulatus
VibE Vibrio cholerae vibriobactin
PaeE Paenibacillus elgii paenibactin
ecok10606 Escherichia coli enterobactin
Irp3 Yersinia enterolitica yersiniabactin
rv2384c Mycobacterium tubercolosus mycobactin
ZmaA Bacillus cereus
ZmaK Bacillus cereus
pga1_c05370 Phaeobacter gallaeciensis
tm1040_2400 Ruegeriasp. TM1040
BlmVIII Streptomyces verticillus
EpoC II Sorangium cellulosum
EpoC Sorangium cellulosum
EpoA Sorangium cellulosum
EpoC III Sorangium cellulosum
FkbB III Streptomyces hygroscopicus
RphL Streptomyces griseoviridis
Irp1 Yersinia enterolitica yersiniabactin
ecok1_2150 Escherichia coli yersiniabactin
Alb1 Aspergillus fumigatus
por 0547 Poribacter sp.
rv2381c Mycobacterium tubercolosus mycobactin
EpoB Sorangium cellulosum
EpoC IV Sorangium cellulosum
SACE_0721 Saccharopolyspors erythae
SACE_0724 II Saccharopolyspors erythae
SACE_0724 Saccharopolyspors erythae
SACE_0721 III Saccharopolyspors erythae
SACE_0721 II Saccharopolyspors erythae
FkbB II Streptomyces hygroscopicus
FkbB IV Streptomyces hygroscopicus
FkbC 2 Streptomyces hygroscopicus
FkbB Streptomyces hygroscopicus
FkbC Streptomyces hygroscopicus
AcmB Streptomyces anulatus
AcmC II Streptomyces anulatus
AcmC III Streptomyces anulatus
rv2379c Mycobacterium tubercolosus
PaeF I Paenibacillus elgii paenibactin
PaeF II Paenibacillus elgii paenibactin
AcmC Streptomyces anulatus
AcmB II Streptomyces anulatus
AcmB Streptomyces aeruginosa pyoverdin
PA2401 II Pseudomonas aeruginosa pyoverdin
PA2400 Pseudomonas aeruginosa pyoverdin
PA2399 II Pseudomonas aeruginosa pyoverdin
ZmaK Bacillus cereus
PA2399 I Pseudomonas aeruginosa pyoverdin
PA2400 Pseudomonas aeruginosa pyoverdin
PA2401 II Pseudomonas aeruginosa pyoverdin
rv2380c Mycobacterium tubercolosus mycobactin
PchE Pseudomonas aeruginosas pyochelin
rv2383c Mycobacterium tubercolosus mycobactin
PchF Pseudomonas aeruginosa pyochelin
EpoP Sorangium cellulosum
ZmaB II Bacillus cereus
ZmaJ Bacillus cereus
ecok10598 Escherichia coli enterobactin
PA2401 III Pseudomonas aeruginosa pyoverdin
PA2401 I Pseudomonas aeruginosa pyoverdin
ItuC II Bacillus subtilis
ItuA II Bacillus cereus
ItuB II Bacillus subtilis
ItuC Bacillus subtilis
ZmaO Bacillus cereus
ItuB Bacillus subtilis
Srf2 II Bacillus subtilis
ZmaB Bacillus cereus
Srf1 Bacillus subtilis
Srf2 III Bacillus subtilis
Srf1 III Bacillus subtilis
ItuB IV Bacillus subtilis
Srf2 Bacillus subtilis
Srf1 II Bacillus subtilis
Srf3 Bacillus subtilis

Figure S4.6 (displayed on the next page) | Phylogentic trees obtained using Maximum Likelihood method for the C domain of the NRPS system and KS domain of the PKS system. The trees with the highest log likelihood are shown. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are shown at the root of each branch. Only values equal or higher than 50% are shown. For each sequence the relative species and the name of the respective protein are indicated. For the domains involved in siderophore production the name of the compound is reported in red. The names of the proteins of *Pseudovibrio* sp. FO-BEG1 and the names of the colibactin proteins are shown in bold and bold green, respectively.


C domain
0.5
rv2380c II Mycobacterium tubercolosus mycobactin
PaeF II Paenibacillus elgii paenibactin
DhbF II Bacillus subtilis enterobactin
AcmC II Streptomyces anulatus
AcmB II Streptomyces anulatus
FkbP Streptomyces hygroscopicus
FkbP II Streptomyces hygroscopicus
BlmX II Streptomyces verticillus
BlmI X Streptomyces verticillus
PA2401 Pseudomonas aeruginosa pyoverdin
PA2400 II Pseudomonas aeruginosa pyoverdin
PA2399 Pseudomonas aeruginosa pyoverdin
PA2399 II Pseudomonas aeruginosa pyoverdin
PSE 3329
ClbJ
ClbH
PSE 3331
ZmaC Bacillus cereus
SrfA2 Bacillus subtilis
SrfA2 Bacillus subtilis
SrfA1 Bacillus cereus
ZmaB Bacillus subtilis
ItuB III Bacillus subtilis
AngM Vibrio anguillarum
rv2380c Mycobacterium tubercolosus mycobactin
ItuB Bacillus subtilis
ItuC Bacillus subtilis
ItuB II Bacillus subtilis
SrfA3 Bacillus subtilis
PA2401 II Pseudomonas aeruginosa pyoverdin
ClbB
PSE 3336
DhbE Bacillus subtilis enterobactin
AcmC Streptomyces anulatus
BlmVIII Streptomyces verticillus
AngN II Vibrio anguillarum anguibactin
ecok1_2149 Escherichia coli yersiniabactin
AngR Vibrio anguillarum anguibactin
pop Sorangium cellulosum
Pseudomonas aeruginosa
ClbJ II
PSE 3329 II
ClbK
PSE 3328
ecok1_2149 II Escherichia coli yersiniabactin
y2399 II Yersinia enterolitica yersiniabactin
PchF Pseudomonas aeruginosa pyochelin
irp1 Yersinia enterolitica yersiniabactin
ecok1_2150 Escherichia coli yersiniabactin
y2400 Yersinia pestis yersiniabactin
FabD (FAS)
ClbN
PSE 3325
ZmaO Bacillus cereus
Srfa1 Bacillus subtilis
VibH Vibrio cholerae vibriobactin
EntFEscherichia coli enterobactin
stm0588 Salmonella enterica enterobactin
AcmB Streptomyces anulatus
DhbF Bacillus subtilis
PaeFPaenibacillus elgii paenibactin
KS domain
0.5
BlmVIII II Streptomyces verticillum
RphX Streptomyces griseoviridis
RphX II Streptomyces griseoviridis
Alb1 Aspergillus fumigatus
FkbC II Streptomyces hygroscopicus
FkbC Streptomyces hygroscopicus
SACE 0721 II Saccharopolyspora erythrae
SACE 0721Saccharopolyspora erythrae
rv2382c Mycobacterium tubercolosus mycobactin
EpoA Sorangium cellulosum
PltB Pseudomonas fluorescens
EpoE Sorangium cellulosum
EpoD Sorangium cellulosum
Streptomyces griseoviridis
SFHH103_00227 Sinorhizobium fredii
BB2918 Bordetella bronchiseptica
POR_0547 Poribacter sp.
BaeM Bacillus amyloliquefaciens
BaeR Bacillus amyloliquefaciens
ituA Bacillus subtilis
PSE 3320
ClbO
PSE_3328
ClbH
PSE_3335
ClbC
ZmaA II Bacillus cereus
ZmaA Bacillus cereus
BaeJ Bacillus amyloliquefaciens
PSE 3336
ClbB
PSE 3330
ClbI
c05397 Phaeobactr galleacensis
ORF_1993 Phaeobacter inhibens
tm1040_2400 Ruegeria sp.
TubD Angiococcus disciformis
TubF Angiococcus disciformis
BlmV III Streptomyces verticillus
EpoB Sorangium cellulosum
ecok1_2150 Escherichia coli yersiniabactin
irp1 Yersinia enterolitica yersiniabactin
FabD (FAS)

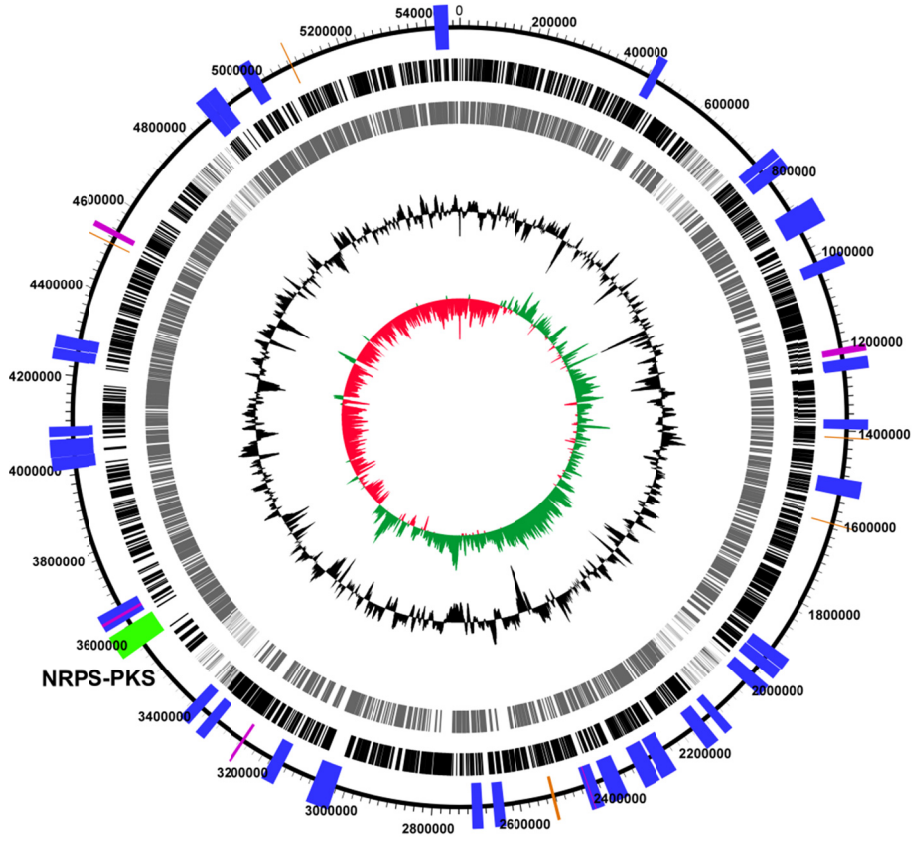


Figure S4.7 | Circular chromosome of *Pseudovibrio* sp. FO-BEG1. The outside outer ring shows the presence of genomic islands predicted by the integrated system of IslandViewer. IslandPick (blue) identifies potential genomic islands (GIs) by performing whole genome alignments and localizing possible unique unaligned regions in the query genome. These regions are then are further verified as possible GIs. SIGI-HMM (orange) identifies potential GIs analyzing the codon usage of each gene in the genome used as a query. IslandPath (purple) is an integrated tool which uses multiple genomic signatures, such as presence of mobile elements, to identify potential GIs. The genomic region containing the hybrid NRPS-PKS is indicated in green. The inside outer rings show the two DNA strands (black and grey) and their respective coding region. The center ring (black) shows a normalized plot of GC content and the inside ring (green and red) shows a normalized plot of GC skew.

5

General Discussion

"*nothing in biology makes sense if not in the light of evolution*"

(Dobzhansky, 1973)

Phosphate (P_i) has essential metabolic, structural and regulatory roles in living organisms. It represents the preferential phosphorus (P) source of bacteria, but its concentration is often in the nanomolar range in surface seawaters. Many studies have investigated the effects of different nutrient regimes on bacterial communities in marine systems and it was often shown that phosphate limitation occurs in several areas of the ocean and it affects

productivity, structure of phytoplankton communities, as well as density and composition of heterotrophic bacterioplankton (Rivkin and Anderson, 1997; Wu *et al.*, 2000; Sala *et al.*, 2002; Pinhassi *et al.*, 2006). Despite this growing evidence, which suggests that phosphate depletion is common in marine environments (reviewed in Dyhrman *et al.*, 2007) the physiological response of heterotrophic marine bacteria to this condition has been poorly investigated. The general assumption is that they exhibit similar adaptations to *Escherichia coli*.

In the present thesis, the response to phosphate limitation of the heterotrophic marine bacterium *Pseudovibrio* sp. FO-BEG1 was investigated considering multiple aspects of its physiology. In **Chapter 2** it was reported that this condition affects not only the phosphorus metabolism but also other important physiological traits such as cell morphology, carbon metabolism, and cellular lipid composition. In addition, a drastic effect of phosphate limitation on the secreted metabolites was reported in **Chapter 3**. This represents the first systematic study conducted on the exo-metabolome of a marine bacterium. Surprisingly, the iron (Fe) metabolism of *Pseudovibrio* sp. FO-BEG1 was also greatly affected by phosphate limitation, and in **Chapter 4** a description of the possible mechanisms responsible for the observed iron solubilization was reported. In the following, I will discuss the obtained data in a broader picture, framing the results in a more ecological context. Additionally, I will depict hypothetical scenarios that could explain the observed production of chelating molecules under phosphate limitation.

5.1 Ecological adaptation of marine bacteria to phosphate limitation

5.1.1 Effect of phosphate limitation on bacterial survival strategies

In **Chapter 2** it was shown that under phosphate limitation *Pseudovibrio* cells almost double their length during growth, reaching an average cell-length of around 6.5 μm by the end of the investigated period. Bacteria strictly control cell dimensions, and they have evolved complex genetic and biochemical programs to control cell shape (Young, 2006). Although morphology is mainly constant in all microorganisms, changes in environmental conditions can greatly affect cell shape, and there is growing evidence that filamentous phenotypes provide survival advantages (Young, 2006; Justice *et al.*, 2008). This phenomenon has been well studied in several pathogenic bacteria, and the strategy of cell elongation, which can vary from 2 to almost 50 times the normal length, was often associated with increased resistance to phagocytosis and to antimicrobial agents (reviewed in Justice *et al.*, 2008).

In natural environments the grazing activity of phagotrophic protists, which contributes significantly to the microbial mortality in the water column, is greatly affected by bacterial cell length and morphology. Heterotrophic nanoflagelates and bacteriovorous ciliates preferentially consume microbial cells that range in length from 1 to 3 μm, whereas smaller or larger cells are less affected (reviewed in Pernthaler, 2005) and filamentous bacteria longer than 7 μm are generally inedible for marine protists (Jürgens and Matz, 2002). At the end of the growth period under phosphate-limited conditions *Pseudovibrio* cells

with a length equal or higher than 7 µm made up 37% of the total cells. Such considerable lengths were never measured under phosphate-surplus conditions, under which the average cell length was 2.5 ± 0,45µm. Cell elongation has previously been observed in different bacteria growing under nutrient limitation (Pine and Boone, 1967; Gonin *et al.*, 2000; Steinberger *et al.*, 2002; Løvdal *et al.*, 2008; Rifat *et al.*, 2009; Goclaw-Binder *et al.*, 2012). It might represent a good adaptation to confer a selective advantage under conditions of reduced growth, and, therefore, potentially lower fitness, by reducing predator pressure. In addition, cell elongation increases the cellular uptake surface without changing the surface-to-volume ratio (Young, 2006). In both phosphate regimes the cellular surface-to-volume ratio of *Pseudovibrio* sp. FO-BEG1 was 0.17 ± 0,01 μm^{-1} throughout the entire growth period. Therefore, cellular elongation under phosphate limitation can increase the surface available for phosphate uptake. This process has been shown for *Caulobacter crescentus,* in which this morphological reaction to phosphate starvation was deeply investigated (Wagner *et al.*, 2006).

It was often reported that especially in conditions of inorganic nutrient limitation microorganisms accumulate storage compounds such as glycogen or polyhydroxyalkanoates (PHA) if a source of organic carbon is available (Lillie and Pringle, 1980; Malmcrona-Friberg *et al.*, 1986; Løvdal *et al.*, 2008). The physiological and proteomic data presented in **Chapter 2** showed that *Pseudovibrio* sp. FO-BEG1 accumulates PHA under phosphate limitation. According to Thingstad *et al.* (2005), this strategy can be seen as a way to use the non-limiting substrate (glucose in our experiments) to increase cell size without

increasing the cellular quota of the limiting factor (phosphate in our experiments). In environments where such nutrient combination occurs this adaptation would lead to a decreased predation pressure, to an accumulation of compounds advantageous to overcame future conditions of carbon limitation, and to a higher uptake capabilities for the limiting factor due to the increased surface. Under conditions of phosphate limitation cell elongation would require synthesis of new membrane phospholipids, which will increase the cellular phosphorus demand. However, we showed that under these conditions *Pseudovibrio* sp. FO-BEG1 changed its lipid composition in favor of phosphorus-free lipids. Therefore, accumulation of carbon storage compounds and cell elongation can be seen as strategies that can greatly increase the survival of *Pseudovibrio* when thriving as free-living organism in environments depleted in phosphate and characterized by fluctuating nutrient regimes.

5.1.2 Evolutionary adaptation of marine bacteria to different phosphate regimes

In **Chapter 2,** we showed that *Pseudovibrio* exhibited a complex reorganization of the phosphorus metabolism under phosphate limitation. Up-regulation of proteins involved in scavenging phosphate from the environment, degradation of stored poly-P_i, and phospholipid exchange suggest that FO-BEG1 has the potential to adapt to variable environmental phosphate concentrations, exploiting alternative nutrient sources and initiating a starvation response.

In the open ocean, a characteristic of bacteria adapted to thrive under phosphate-limiting conditions is the presence of high relative number of genes involved in phosphorus metabolism (Giovannoni *et al.*, 2005; Martiny *et al.*, 2009a). Comparison of the available genomes of different *Prochlorococcus* isolates revealed that the amount of genes present in the *phoB* gene cluster, which encodes several proteins involved in phosphate limitation response and phosphorus metabolism, was not consistent with the rRNA-based phylogenetic relationship of the isolates (Martiny *et al.*, 2006). A negative correlation was observed between the abundance and variety of genes and the phosphate concentration in the environments from which the strains were isolated. Therefore, it was proposed that phosphate regimes were one of the driving forces in the genomic divergence among the isolates (Martiny *et al.* 2006, Martiny *et al.*, 2009b). In addition, the heterogeneity in gene content within and between populations of *Prochlorococcus* and *Pelagibacter* from the Atlantic Ocean (phosphate-limited) and the Pacific Ocean (not phosphate-limited) was reflected mainly in the amount of genes related to phosphorus metabolism (Coleman and Chisholm, 2010). These data suggest that the environmental phosphate concentrations can represent a key selective pressure in the distribution and evolution of marine bacteria. In support of these observations, there are recent studies on bacterial metagenomes and metaproteomes that showed a significant enrichment in genes involved in phosphorus metabolism in environments that are characterized by low phosphate concentrations, such as the Sargasso Sea or the Mediterranean Sea (Sowell *et al.*, 2010; Temperton *et al.*, 2011; Kelly *et al.*, 2013; Thompson *et al.*, 2013).

In order to verify whether the genome of *Pseudovibrio* sp. FO-BEG1 presents a similar enrichment in genes involved in phosphorus metabolism as the bacteria highly adapted to thrive in marine environments depleted in phosphate, I compared it with 58 available bacterial genomes considering the total and the relative amount of genes involved in phosphorus metabolism (**Fig. 5.1)**. I retrieved the data from the "phosphorus metabolism" subsystem reported in the SEED database (Overbeek *et al.*, 2005), and I calculated the relative amount of genes belonging to this subsystem, dividing their absolute number by the total number of coding sequences in the respective bacterial genome. The genome of *Pseudovibrio* sp. FO-BEG1 is not significantly enriched in genes belonging to this category (1.02%), and shows a relative value just below the average percentage (1.06%, dashed blue line in **Fig. 5.1**). However, if the absolute number of genes involved in phosphorus metabolism is considered, *Pseudovibrio* sp. FO-BEG1 is the third strain among the 58 considered.

The included genomes of *Prochlorococcus* sp. and *Pelagibacter* sp. have 1.4 and 1.6% of genes devoted to phosphorus metabolism, respectively. However, these values are mainly due to the very small number of coding sequences present in their genomes. These bacteria are highly abundant in the open ocean, where nutrients are often limited. Therefore, among other adaptations, they show a drastic genome reduction that allows them to minimize replication costs, nitrogen, and phosphorus demand (Dufresne *et al.*, 2005; Giovannoni *et al.*, 2005). In contrast, the genome of *Pseudovibrio* sp. FO-BEG1 is one of the largest among the considered, and it shows several phosphorus related genes, e.g. genes for three different alkaline phosphatases, not annotated in the considered genome of

Pelagibacter sp. strain HTCC1062. In recent metagenome comparisons some of these genes (e.g. for phosphonate usage) were found in bacteria of the SAR11 clade thriving in waters with severe phosphorus depletion (Thompson *et al.*, 2013). This finding suggests that *Pseudovibrio* possesses good genetic potentials to retrieve phosphorus from the environment, similarly to bacteria ubiquitous and abundant in phosphorus-limited waters.

These data together with the response described in **Chapter 2** underline that FO-BEG1 may have evolved a multifaceted ensemble of strategies to cope with variations in phosphorus concentration. This metabolic versatility confirms the overall physiological plasticity previously described in this bacterium (Bondarev *et al.*, 2013; **Contributed works**) and it is consistent with the description of "opportuni-trophs". This expression is used for bacteria able to exploit temporally and spatially variable resources owing to their flexible genome features (Polz *et al.*, 2006). Well known examples of this ecological group of bacteria can be found among members of the *Roseobacter* clade and the genus *Marinobacter*, which are ubiquitous in oceanic environments and play important roles in almost all biogeochemical cycles (Moran *et al.*, 2004; Moran *et al.*, 2007; Kaye *et al.*, 2011; Singer *et al.*, 2011). The metabolic versatility of *Pseudovibrio* FO-BEG1 is also underlined by the impressive number (363 encoded in the chromosome and 38 encoded in the plasmid) of transcription regulators annotated in its genome (data obtained from the P2tf database; Ortet *et al.*, 2012), which makes FO-BEG1 the leading marine strain regarding the number of transcription regulators among all *Alphaproteobacteria*.

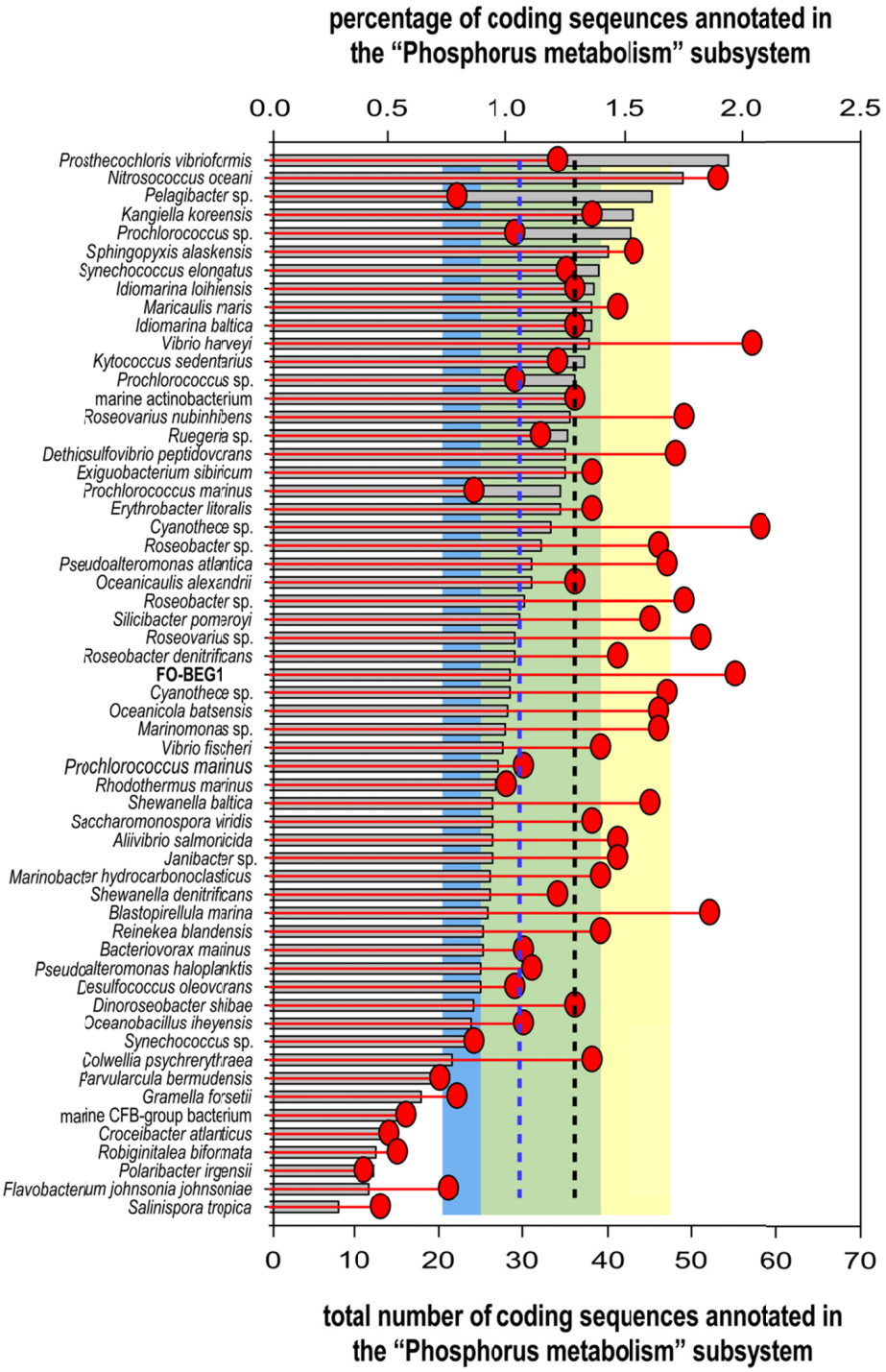


Figure 5.1 | Total (circles) and relative (bars) amounts of genes annotated in the category "phosphorus metabolism" of the SEED database. Black and blue dashed lines represent the average values of the absolute and relative number of genes, respectively. Yellow and blue areas indicate the standard deviation from the average of the absolute and relative number of genes, respectively. Accession numbers can be provided upon request.

The ability to thrive in environments characterized by low phosphate concentrations also requires physiological adaptations not directly related to phosphorus metabolism. In regions where phosphate is scarce it was shown that phytoplankton can reduce its cellular phosphorus requirement by substituting phospholipids with non-P containing lipids (Van Mooy *et al.*, 2009). In this process, the sulphoquinovosyl diacylglycerols (SQDG) and other glycolipids have been described to be of crucial importance. In marine environments exchange in lipid composition has been considered a strategy adopted by phytoplankton to compete with phospholipid rich heterotrophic bacteria in phosphorus-limited environments (Van Mooy *et al.*, 2006; Van Mooy *et al.*, 2009). In addition, several studies suggested that SQDG lipids detected in the North Atlantic, South Pacific, Sargasso Sea and Mediterranean Sea mainly originate from photoautotrophic organisms (Van Mooy *et al.*, 2006; Van Mooy and Fredricks, 2010; Popendorf *et al.*, 2011a; Popendorf *et al.*, 2011b). Even though recent data pointed out that heterotrophic marine bacteria have the genetic potential for SQDG synthesis, the conditions in which these lipids are synthesized have so far never been investigated (Villanueva *et al.*, 2013). In this respect, the data reported in **Chapter 2** represent a clear novelty, since we could show that *Pseudovibrio* sp. FO-BEG1 responded to phosphate limitation by exchanging its phospholipids with glycolipids, amino lipids, and SQDG. In addition, the proteomic experiments and the identification of a Pho-box in the upstream region of genes involved in SQDG synthesis strongly suggest that this class of lipids is synthesized by FO-BEG1 as a direct response to phosphate limitation.

The ability to modify the cellular lipid composition under phosphate limitation seems to be a prerogative of specific groups of *Proteobacteria*. For example, unlike *Pseudovibrio* members of the SAR11 clade were shown to be unable to synthesize SQDG and to exchange their membrane lipids composition in response to phosphate limitation (Van Mooy *et al*., 2009; Villanueva *et al*., 2013). Altogether, these data underline the different adaptation strategies observed in those bacteria highly specialized to thrive in the oligotrophic open ocean, such as the SAR11 related strains, and other metabolically versatile bacteria like *Pseudovibrio*, which are able to explore numerous resources and adopt different strategies to cope with variable nutrient regimes.

5.2 From dissolved organic matter (DOM) to system biology

5.2.1 Is there a possible effect of environmental phosphate concentrations on DOM composition in the ocean?

In **Chapter 3**, the drastic effect of phosphate limitation on the amount and composition of dissolved organic carbon (DOC) secreted by *Pseudovibrio* sp. FO-BEG1 was demonstrated. This underlines the importance of nutrient regimes in bacterial DOM production. There are many studies showing how the amount and composition of DOM can affect bacterial communities structure. Moreover, the ability of bacteria to release variable amounts of DOM characterized by different grades of bio-reactivity has also been investigated (Carlson, 2002; Jiao *et al*., 2010; Gómez-Consarnau *et al*., 2012). However, as pointed out by Kujawinski (2011), the chemical composition of DOM produced by heterotrophic marine

bacteria is mostly unknown. In this respect the data reported in **Chapter 3** represent the first detailed analysis concerning the metabolites secreted by a heterotrophic marine bacterium, considering in addition the effect of one environmentally important limiting nutrient.

As reported in **Chapter 3,** a surprising discovery was made by Brauer *et al.* (2006) who showed similar nutrient-specific starvation responses in the metabolome of two distantly related microorganisms. Consequently, it is reasonable to ask whether the drastic effect of phosphate limitation on the exo-metabolome of *Pseudovibrio* sp. FO-BEG1 could also be observed in other bacteria. If this were the case, it could be hypothesized that environments with similar nutrient regimes (e.g. phosphate limitation) can induce comparable metabolic responses in different members of the bacterial communities, affecting the secreted metabolites and, therefore, the composition of marine DOM. For example, *Pseudovibrio* released a higher number of phenolic and polyphenolic compounds into the medium during growth under phosphate limitation. If such response can also be observed in other heterotrophic marine bacteria, it would be interesting to compare the DOM composition in the surface water of oceanic regions characterized by low and high phosphate concentrations, and to clarify whether these classes of compounds are indeed more abundant in systems depleted in phosphate. This approach, which could be considered as a "geographical-metabolomic" analysis, could reveal the presence of geographic regions with shared DOM features, which resulted from specific metabolic adaptations of bacteria induced by similar environmental nutrient regimes.

One interesting aspect of the data obtained in **Chapter 3** was the identification of a high number of molecules with an AI_{mod} higher or equal to 0.66, which were included in the category of phenols and polyphenols. These compounds were more abundant under phosphate-limited conditions and increased during bacterial growth from 7.4% to 16.8% of the total assigned molecular formulae. Molecules with high AI_{mod} are described to be part of the refractory fraction of DOM, and are mainly regarded as thermally altered biomass composed of condensed polyaromatic moieties (Masiello, 2004; Koch and Dittmar, 2006; Dittmar, 2008). For the sake of clarity, to date the DOM and DOC are mainly classified according to their lifetime, which vary from hours to millennia in the labile and refractory (or recalcitrant) fractions, respectively (for more details refer to Jiao *et al.*, 2010 and Hansell, 2013). Remarkably, several studies showed that bacteria in marine and freshwater systems can release refractory DOM, which can resist microbial degradation for years (Brophy and Carlson, 1989; Tranvik, 1993; Ogawa *et al.*, 2001; Gruber *et al.*, 2006). Interestingly, there is growing evidence suggesting that bacteria, and their food web interactions, can play an important role in the production of recalcitrant DOC in the ocean (Jiao *et al.* 2011b; Hansell 2013). Recent estimations suggest that they could generate around 25% of the global DOC inventory and around 50% of the refractory DOC, building up the huge carbon reservoir in the ocean (reviewed in Hansell 2013). On the basis of these data, the theoretical framework of the "microbial carbon pump" (MCP) was recently proposed. In the MCP the heterotrophic activity of bacteria has an important role in converting labile and semi-labile DOM into refractory DOM, building up the big oceanic carbon pool

and influencing in this way the global carbon cycle (Jiao *et al.*, 2011b; Hansell, 2013).

If we assume that molecules characterized by high aromaticity (high AI_{mod}) are indeed less bio-reactive, then the data reported in **Chapter 3** indicate that their production could be enhanced under nutrient (in our case phosphate) limitation. These data stimulate the debate on the nature of the refractory DOM produced by marine bacteria and underline, as previously suggested (Jiao *et al.*, 2011b), the importance of classifying the different fractions of DOM according to chemical criteria and not only on the base of their lifetime. This would allow reliable comparisons between environmental DOM and bacterial exo-metabolome, and at the same time, it could permit a better estimation of the contribution of heterotrophic bacteria to the refractory DOM in the ocean.

Recently, the DOC compositions of the Sargasso Sea and the Ross Sea were compared (Hansell, 2013). A higher concentration of semi-refractory DOC could be found in the former one, whereas the Ross Sea was enriched in the semi-labile fraction. The main reasons for these differences can be found in the presence of a permanent pycnocline in the Sargasso Sea that prevents a constant mixing of the water masses, and in the differences in productivity (Hansell, 2013). Intriguingly, the Sargasso Sea is an oligotrophic environment, where phosphate limitation was often shown (e.g. Rivkin and Anderson 1997; Wu *et al.* 2000). Therefore, considering the data reported in **Chapter 3** and the assumption reported above, it is tempting to speculate that also the distinct phosphate regimes of the two areas could influence the metabolites released into the environment by the local bacterial community. Altogether these data point to the

existence of an additional variable to consider when explaining the differences in DOC composition between different regions, and suggest that phosphate limitation (or other nutrient limitations, as also proposed by Jiao *et al.*, 2011a) can have a direct effect on the MCP and, therefore, on the global carbon cycle.

5.2.2 Integration of metabolomic and proteomic data can provide a closer look into cell physiology

The ultra-high resolution of the FT-ICR-MS allows to identify several hundreds of metabolite masses with ppm or sub-ppm error. This information, or information derived from more focused MS/MS analyses, can be integrated with other "omics" data by sophisticated bio-informatic pipelines. This could help to obtain a more detailed picture of bacterial metabolism and understand specific metabolic pathways and regulatory circuits. Nowadays, the exponential increase of "omics" data opens the possibility to apply holistic approaches for the study of bacterial physiology via combining new with existing knowledge (Borodina and Nielsen, 2005; De Keersmaecker *et al.*, 2006; Joyce and Palsson, 2006). For example, transcriptomic, proteomic, and metabolomic data can be mapped onto the metabolic network of the studied strain inferred from its genome (McCloskey *et al.*, 2013). Those metabolic networks can be reconstructed from genome interpretation using tools like the ones provided by the KEGG and the MetaCyc databases (Kanehisa and Goto, 2000; Thiele and Palsson, 2010; Caspi *et al.*, 2012). The results of the mapping are, for example, protein and metabolite networks,

where the nodes represent the single molecule or protein and the edges represent metabolic reactions that occur between the nodes (**A** and **B** in **Fig. 5.2**).

Contrastively from the conservative approach applied in **Chapter 3**, where all molecules identified in the KEGG database were further filtered according to the MI-Pack strategy (Weber and Viant, 2010), in a network approach metabolites or proteins not directly detected in the experiments but belonging to, or potentially belonging to, the same detected pathway can be included. For example, proteins with a predicted general function comparable to what is required in a specific reaction in the pathway can be included in the networks. In the same way, molecules that have chemical characteristics consistent with a specific product or substrate in a certain reaction in the pathway could be included. Today protein networks, e.g. protein-protein interaction networks, and regulatory networks are becoming more and more common in system biology studies. They can rely on several tools that allow to identify known and/or predicted physical and functional interactions between proteins and DNA or among proteins (e.g. STRING, Franceschini *et al.*, 2013). These approaches would allow us to have a comprehensive view of the cellular processes active under the experimental conditions. In fact, "omics" data are often noisy and not complete, and do not always allow the identification of all molecules or proteins involved in a pathway. Therefore, analyzing high-throughput data in the framework of the metabolic network can facilitate the identification of the regulated pathways, allowing to understand the cellular behavior as a whole (Borodina and Nielsen, 2005; David *et al.*, 2006). For instance, visualizing the data in the context of pathways might show how the

up-regulation of one transcription regulator could explain the up-regulation of many proteins identified during the experiment, leading to a testable experimental hypothesis.

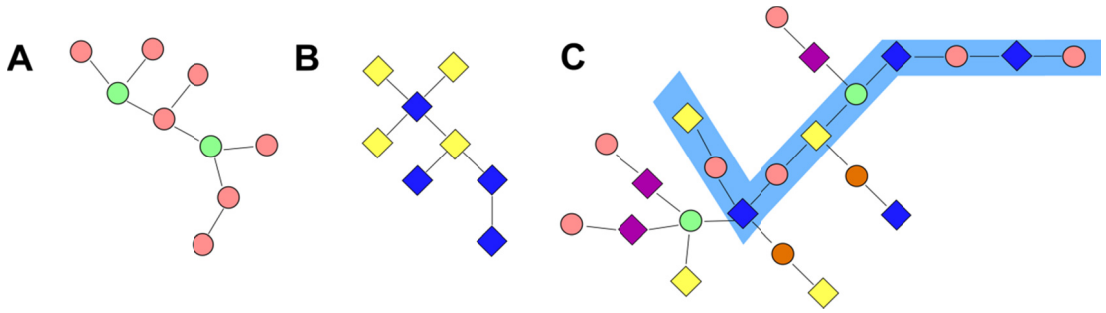


Figure 5.2 | Hypothetical network of metabolites (A) and proteins (B). Pink and blue represent the reported measured entities, whereas green and yellow denote the entities inferred from the mapping process. **C** shows the network resulting from the integration of the metabolite and the protein networks. New proteins (purple) and metabolites (orange) can be added to explain the formation and the functional interaction of metabolites and proteins, respectively. Light blue underlines a potential "metabolic route", which could explain the formation of a specific metabolite of interest.

Protein and metabolite networks are independent entities belonging to the same cellular metabolic model. Therefore, they can be combined and multiple types of biological data can be added. For example, cytosolic, membrane, and secreted proteins can also be integrated together with endo- and exo-metabolomes. The resulting networks would therefore contain multiple sources of information, e.g. proteins and metabolites, connected by biological reactions. This would represent a metabolic model, which can help to have a complete view of processes regulated under the specific tested conditions. In the cell, regulatory and metabolic processes are the result of the interaction between many types of molecules, which studied alone will not allow to predict the

function of the pathway in which they are involved (Hartwell *et al.*, 1999). Therefore, the model resulting from the integration could allow to visualize and identify potential regulative and metabolic circuits active under the tested conditions. This identification can occur because the integration of multiple biological data creates information not visible when considering the single datasets separately. Indeed, no single "omics" analysis can unravel the complexity of bacterial physiology and only the integration of multiple layers of information (multi-"omics" data) can allow to acquire a more detailed picture of microorganisms (Patil and Nielsen, 2005; Zhang *et al.*, 2010).

In the cell many levels of regulation occur (Gygi *et al.*, 1999), therefore, system biology approaches, such as the one proposed above, represent a powerful tool to gain an integrated view into cellular physiology (Joyce and Palsson, 2006). These have been successfully used for many eukaryotes (Ihmels *et al.*, 2004; Zhu *et al.*, 2012) and could help to better understand bacterial physiology and metabolic regulation in function of different environmental factors. For example, we can encounter a situation where, during a specific growth condition, the studied strain produces a specific known compound. However, the complete synthetic and secretory pathways, and the regulation network behind its production are not completely known. Previously, proteomic and metabolomic studies (as the one performed in **Chapter 2** and **Chapter 3**) have been conducted and now both these datasets could be independently mapped and then integrated into a network containing metabolites and proteins as proposed above (**Fig. 5.2**). From this integration we can now infer possible metabolic and regulatory "routes", which can explain the production (light blue

area in panel **C** of **Fig. 5.2**). Using these approaches new hypotheses can be proposed, and focused experiments can be performed to elucidate the process of interest.

5.3 Why does phosphate limitation induce the secretion of molecules with chelating properties?

5.3.1 Cross-regulation of phosphorus and iron metabolism

One of the most striking observations of the present thesis was the ability of *Pseudovibrio* sp. FO-BEG1 to release molecules able to solubilize precipitated iron when growing under phosphate limitation. This, together with the higher iron uptake, points to a combined regulation of these two metabolisms in *Pseudovibrio* sp. FO-BEG1. Interestingly, a potential PhoB binding site was identified in the upstream region of a *fur* gene (which encodes for the ferric uptake transcription regulator, Fur; PSE_0323; **Table S2.1**; **Chapter 2**). In addition, preliminary data recently obtained suggest that some proteins involved in iron uptake were regulated under $-P_i$ conditions (Romano *et al.*, unpublished data). This cross-regulation hypothesis is corroborated by studies reporting the presence of Fur-boxes in the upstream region of *phoB* genes in isolates of the genus *Acidithiobacillus* (Quatrini *et al.*, 2007; Osorio *et al.*, 2008). Moreover, there is evidence suggesting that the alteration of phosphorus metabolism in S*accharomyces cerevisiae* induces an iron starvation response (Rosenfeld *et al.*, 2010).

Another fascinating, although highly speculative, scenario that involves quorum-sensing (QS) could explain the observed interconnection between phosphorus and iron metabolism. QS is a term used to describe cell-to-cell communication mechanisms, which are cell-density dependent. These mechanisms rely on the synthesis by bacteria of small diffusible molecules. With the increase of the cell density the synthesis increases and consequently the concentration of the signaling molecules rises. Once a critical threshold concentration has been reached, target regulatory proteins are activated and gene regulation can be achieved (Williams *et al.*, 2007). Phosphate limitation was shown to trigger QS in *Pseudomonas* and *Serratia* strains (Slater *et al.*, 2003; Jensen *et al.*, 2006; Zaborin *et al.*, 2009) and astonishingly it was also shown that QS signaling molecules or their natural formed degradation products can chelate iron (Kaufmann *et al.*, 2005; Bredenbruch *et al.*, 2006; Diggle *et al.*, 2007). So far the biological meaning of this phenomenon has not yet been clarified, but some authors suggest that the Fe-chelating ability of QS signaling molecules can be a widespread feature so far overlooked (Schertzer *et al.*, 2009). In the genome of *Pseudovibrio* sp. FO-BEG1 no genes involved in the production of known QS signaling molecules were found (Bondarev *et al.*, 2013; **Contributed works**) and, differently from bacteria of the *Roseobacter* clade (Wagner-Döbler *et al.*, 2005), QS processes were never described for *Pseudovibrio* strains. However, in the recent years the great diversity of QS signaling molecules is emerging, therefore, as Decho *et al.* (2011) states, "*there are likely to be many as yet uncharacterized cues and perhaps novel cue classes*". These considerations bring to speculate that in the investigated strains phosphate limitation can trigger QS-like processes, which

can regulate, for example, secondary metabolite production (e.g. TDA) and virulence related genes (e.g. toxin). In addition, the signaling molecules could chelate iron and be responsible for the iron increase we observed during the experiments.

Chapter 4 focused on the different approaches used for the identification of the molecules secreted by *Pseudovibrio* sp. FO-BEG1, and involved in solubilization of precipitated iron. It is important to mention that the experiments presented there do not show that the chelating molecules are produced as a consequence of the activation of the iron starvation response, as implied in the definition of siderophores (Andrews *et al.*, 2003). Therefore, when possible, I prefer to refer to the produced compounds just as chelating molecules. In the following section, I will report some hypotheses that could explain such a physiological response, discussing possible scenarios that can support the interconnection between phosphate limitation response and iron metabolism.

5.3.2 Hypothesis 1: scavenging phosphate bound to iron oxyhydroxide

Iron is introduced into the open ocean mainly by rivers, hydrothermal systems, and aeolian dusts. However, the majority of iron from the first two sources precipitates and is trapped in sediments (Raiswell and Canfield, 2012). Therefore, aeolian dusts together with iron brought to the surface by upwelling phenomena have been considered as the major iron sources in the off-shore regions (Poulton *et al.*, 2002; Raiswell and Canfield, 2012). As described before

(**Chapter 1**, **4**), phosphate can adsorb to the surface of iron oxyhydroxides and other Fe-oxide minerals, and these processes can remove up to 40% of the bio-available phosphate (Berner, 1973; Lijklema, 1980; Mclaughlin *et al.*, 1981; Parfitt, 1989; Feely *et al.*, 1998). As described in **Chapter 4,** siderophores have an extremely high affinity for Fe(III), and there are several reports describing their ability to dissolve different Fe-oxide minerals (Yoshida *et al.*, 2002; Kraemer, 2004; Kraemer *et al.*, 2005). Obviously, in environments where phosphate is primarily trapped by Fe-oxide minerals, it is reasonable to assume that more phosphate will be released and become bio-available when the Fe-oxides will be dissolved. Therefore, it is tempting to speculate that, in conditions of phosphate limitation, bacteria thriving in environments rich in particulate iron could produce siderophores in order to dissolve the iron minerals and consequently trigger phosphate desorption.

Bacterial phosphate mobilization is a process well described for soil microorganisms. In all cases the solubilization was mainly due to the production of acids, which enhanced the dissolution of phosphate minerals via decreasing the pH of the surrounding environment (e.g. Rodríguez and Fraga, 1999). This process was also described in bacteria isolated from marine environments and from corals (Kannapiran and Ravindran, 2012). Interestingly, there is evidence suggesting that the fungus *Trichoderma harzianum* is able to solubilize iron, phosphate, and manganese minerals via secreting unknown molecules with chelating properties (Altomare *et al.*, 1999).

To date the interconnection between environmental phosphate concentration and siderophore production seems to be complex and largely

unexplained. For example, the siderophore production of some *Streptomyces* species was significantly reduced when the bacteria were grown under very high or very low phosphate concentrations (Bendale *et al.*, 2009). In addition, it was reported that in *Sinorhizobium meliloti* and in *Corynebacterium glutamicum* phosphate limitation leads to a down-regulation of proteins involved in siderophore production and iron uptake (Ishige *et al.*, 2003; Krol and Becker, 2004). In the experiments reported in **Chapter 4**, only *P. gallaeciensis*, similarly to *Pseudovibrio*, released chelating molecules exclusively under phosphate limitation. In the rest of the *Roseobacter* strains this correspondence was not observed. Altogether these data indicate that, although the dissolution of iron minerals mediated by siderophores is a process well described, the direct connection between phosphate limitation and siderophore production needs to be further investigated.

If we assume that the production of chelating molecules is a strategy adopted to access an extra phosphate-pool in conditions of phosphate starvation, it is reasonable to suggest that genes involved in the synthesis of these molecules will be part of the Pho-regulon, or at least their transcription will be up-regulated under phosphate limitation. However, the data available for other bacteria suggest a different scenario, where most of these genes, together with other genes involved in iron uptake, are down-regulated under phosphate limitation (Ishige *et al.*, 2003; Krol and Becker, 2004). It is important to mention that there are data suggesting that in *Edwardsiella tarda*, PhoB negatively regulates the *fur* gene (Chakraborty *et al.*, 2011). This means that, in theory, under conditions of phosphate limitation the Fur transcription regulator is down-regulated by PhoB,

and consequently it does not repress anymore genes involved in the synthesis of siderophores. This regulation cascade could explain the hypothesis proposed above.

5.3.3 Hypothesis 2: role of chelating molecules in the protection against oxidative stress

In **Chapter 2** we observed the up-regulation of several proteins involved in protecting the *Pseudovibrio* cells against reactive oxygen species (ROS). Several studies reported that cells thriving under phosphate limitation experience an increased oxidative stress (Gérard *et al.*, 1999; Moreau *et al.*, 2001; Moreau, 2004; Yuan *et al.*, 2005). Interestingly, there is evidence suggesting that siderophores can decrease the toxic effect of ROS. In a recent article Adler *et al.* (2012) proposed that catechol containing siderophores can protect bacteria against ROS. It was shown that the catechol siderophore enterobactin decreased the toxicity of pyochelin in *Escherichia coli* by reducing the formation of ROS that this molecule generates. Mutation in the enterobactin bio-synthetic pathway rendered *E. coli* sensitive to pyochelin (Adler *et al.* 2012). The authors suggested that catechol siderophores could function as hydrogen donors, terminating, in this way, the radical chain reactions. The protective function of catechol siderophores against oxidative stress was confirmed in a recent study, which showed that the production of enterobactin and salmochelin defends *Salmonella* sp. against ROS (Achard *et al.*, 2013). In this case, however, the catechols had to be situated intracellularly to exploit their anti-oxidant function.

In addition, in *Azotobacter vinelandii* catechol biosynthesis was under control of the transcription activator of the superoxide dismutase (SoxS) and was significantly increased when iron starved cultures experienced increased oxidative stress (Cornish and Page, 1998; Tindale *et al.*, 2000). Consistently, catechol siderophores were described to be highly susceptible to oxidation (Hider, 1984).

Besides scavenging radicals, siderophores could also be involved in oxidative stress protection via regulating the free iron concentration in the cells. Fe(II) can react with hydrogen peroxide generating hydroxyl radicals in the Fenton reaction (Winterbourn, 1995). Hydroxyl radicals are extremely reactive and are able to oxidize any organic molecule, generating lethal damage to the cell. Usually, Fe(II) is not freely present in the cell; however, superoxide can interact with Fe-S clusters or with Fe(III), generating the reduced iron form during the so called Haber-Weiss reaction (Haber and Weiss, 1934). In *A. vinelandii*, it was shown that Fe(III) chelated by azotochelin or protochelin does not react with superoxide, and, therefore, Fe(II) ions are not generated (Cornish and Page 1998). There is additional evidence suggesting that oxidative stress could play an important role in regulating siderophore synthesis. For example, Lee *et al.* (2011) reported that the production of the siderophore petrobactin under iron starvation in *Bacillus anthracis* increased under elevated aeration condition and under mildly induced oxidative stress. A similar effect was observed for an intracellular siderophore in the fungus *Aspergillus nidulans* (Eisendle *et al.*, 2006). There is also evidence indicating that in *Anabaena* sp. genes involved in siderophore production were activated under oxidative stress (Jeanjean *et al.*, 2008). In

contrast, production of the siderophore pyoverdin in *Pseudomonas aeruginosa* decreased when iron-limited cells were exposed to mild artificial oxidative stress (Dao *et al.*, 1999).

Altogether, these data clearly show the strong interconnection between iron metabolism, siderophore production, and oxidative stress. It seems likely, that siderophores can be used to tightly regulate the amount of free iron available for the Haber-Weiss/Fenton reaction, reducing the risk of hydroxyl radical formation. On the other hand, the presence of catechol sub-units makes this class of siderophores able to act directly as radical scavengers, representing an additional protection against oxidative damage. These data generate another line of argument to explain the production of chelating molecules under phosphate limitation. This strategy would protect *Pseudovibrio*, according to the mechanisms described above, from the increased oxidative stress that the cells likely experience during phosphate limitation. However, the picture becomes more complicated when the data concerning the *Roseobacter* strains are considered (**Chapter 4**), indicating that this response, although widespread, is regulated in a species-specific way.

Intuitively, the protective mechanisms will be beneficial when situated inside the cell, as described for *Salmonella* sp. (Achard *et al.* 2013), since the amount of oxidative stress that a cell experiences depends upon the rate at which radicals are produced intracellularly (Imlay, 2003). In all our experiments we detected chelating activity in the cell-free supernatant of the cultures grown under phosphate limitation, suggesting an extracellular mechanism of action, which is more difficult to explain. However, it is important to point out, that

recently it was shown that ecologically and taxonomically diverse bacteria produce extracellular superoxide, representing a vast source of this compound, and likely other ROS, in the environment (Diaz *et al.*, 2013). Consequently, the production of extracellular scavenging molecules would represent an advantage for the producing strains and for the entire bacterial population associated with it.

5.3.4 Hypothesis 3: the "Trojan-horse" strategy

Bacteria can produce a large variety of antibacterial compounds and among them a special group is represented by bacteriocins. They are loosely defined as ribosomal synthesized peptides with a narrow toxicity on strains of the same species, or specific isolates of a closely related species (Riley and Wertz, 2002; Gillor *et al.*, 2008). According to Klaenhammer (1988), 99% of bacteria may produce at least one bacteriocin. However, to date only around 200 bacteriocins have been characterized, and most of them were described in enterobacetria, lactic acid bacteria, and Gram-positive bacteria, even though they were detected in all lineages of prokaryotes (Riley and Wertz, 2002; Gillor *et al.*, 2008; Desriac *et al.*, 2010). Enterobacteria produce two types of bacteriocins, which can be mainly differentiated according to their molecular size in microcins and colicins. Microcins are the smallest class and are characterized by peptides generally smaller than 10 KDa. They are extremely stable to heat, pH, and proteases and are generally produced under conditions of stress, such as nutrient limitation (Duquesne *et al.*, 2007). These peptides can undergo significant post-translational modification and some microcins can carry a siderophore-like molecule as, for

example, in the microcin MccE49 produced by *Klebsiella pneumoniae*, which is tailored with a linear and monoglycosylated derivate of enterobactin (Duquesne *et al.*, 2007; Nolan *et al.*, 2007).

Siderophores are in general always synthesized via NRPS systems and are subsequently modified and added to the antibacterial peptide. Therefore, these molecules are considered as a "bridge" between the ribosomal and non-ribosomal synthetic machinery (McIntosh *et al.*, 2009). In addition to siderophore-conjugate microcins, other smaller siderophore-conjugate antibiotics were described. However, only few have been isolated so far (Braun *et al.*, 2009; Ji *et al.*, 2012). Both these groups of compounds are referred to as sideromycin. They act as a "Trojan-horse", entering the competing cells via the corresponding siderophore uptake systems. This is an active and highly specialized process, and it allows to reduce the minimal inhibitory concentration of these antibiotics by several orders of magnitude (Braun *et al.* 2009). The ecological role of bacteriocins is not yet completely clear. It was suggested that they serve as anti-competitors, enabling the producing strains to invade an established microbial community. On the other hand, they could also play a defensive role and avoid the invasion of other strains into an already occupied niche (Riley and Wertz, 2002). Overall, bacteriocins would preferentially be efficient when bacteria live in close contact.

Interestingly, as reported in **Chapter 2**, phosphate limitation can enhance bacterial virulence and also biofilm formation. In the proteomic analysis, we identified several proteins that might indeed help *Pseudovibrio* to establish and maintain a symbiotic relationship with marine invertebrates. In this respect, the production of the wide-spectrum antibiotic TDA can represent a great

advantage, allowing *Pseudovibrio* sp. FO-BEG1 to outcompete a major part of the established bacterial community, as well as the concurrent bacteria for the host colonization. However, this antibiotic will most likely not be effective against other bacteria able to produce TDA as other *Pseudovibrio* and *Roseobacter* strains, which have often been isolated from sponges as well (Buchan *et al.*, 2005). Therefore, a narrower spectrum of strain/species-specific antibacterial compounds would give *Pseudovibrio* sp. FO-BEG1 an additional advantage allowing it to outcompete other strains in the race for the colonization of the host.

Microcins are generally smaller than 10 kDa and can be as small as 1 kDa (Duquesne *et al.* 2007). These sizes correlate well with the SEC experiments performed in **Chapter 4**. However, the MS analysis of the active fraction isolated during the HPLC experiment did not show the presence of molecules in the high mass range 500- 3500 *m/z* (data not shown). Even though, the analytical data do not provide direct proof that the active compounds were in the mass range of the known microcins, an interesting observation was obtained in **Chapter 2**. Under phosphate-limited conditions we detected a 2.6 fold up-regulated protein homologous to PmbA, which is a protein that was characterized in *E. coli*, and which was proposed to play a role in the export of microcins (Rodríguez-Sáinz *et al.*, 1990). This suggests that a microcin-like compound could have been produced by *Pseudovibrio* sp. FO-BEG1 under phosphate limitation.

Usually, the fundamental genes necessary for microcin production are organized in gene clusters. I considered the more common gene clusters found in enterobacteria reported in the review of Duquesne *et al.* (2007), and I screened the genome and the plasmid of *Pseudovibrio* sp. FO-BEG1 for the presence of

homologous regions (analysis performed using MultiGeneBlast). However, none of the synthetic genes were identified apart from genes encoding for components of ABC transport systems. Surprisingly, the antiSMASH analysis, performed using the genomes of all strains used in the experiments reported in **Chapter 4**, revealed the presence of genomic regions that have homologies with bacteriocin synthetic clusters mainly related to *Burkholderia* sp. and *Bradyrhizobium* sp. Consistently, genes potentially involved in bacteriocin synthesis were also recently found in the genome of *Phaeobacter articus* (Freese *et al.*, 2013). Overall, these data give way to a new interpretation of the data reported in **Chapter 4**. Phosphate limitation, or the general stress response induced by phosphate limitation, could trigger the production of siderophore-tailed microcins-like molecules, or other sideromycins, in *Pseudovibrio* sp. FO-BEG1 and to a certain extent in strains of the *Roseobacter* clade. These compounds would enhance the strain's competitiveness, especially against closely related bacteria, and would explain the increase in soluble iron we observed during the bacterial growth.

5.3.5 Hypothesis 4: phosphate limitation enhances bacterial virulence, and iron is the key to success

The process of withholding iron from infecting bacteria has long been known to be of fundamental importance in the host defense mechanisms. Ferritins and transferrins are proteins used by vertebrates and many invertebrates to store iron and control its concentration within the cells and the extracellular space (Harrison and Arosio, 1996; Krasko *et al.*, 2002; Gaffney and

Valentine, 2012). However, bacteria can proliferate in the host, implying that they are able to recover iron from the available sources. One of the strategies they adopted is the secretion of siderophores, which help to recover iron. Almost all pathogenic bacteria are able to produce siderophores, and according to Ratledge and Dover (2000) "*the absence of siderophore formation by a pathogen may mean only that no one has investigated this phenomenon thoroughly enough*". These molecules are able to subtract iron from ferritins and transferrins, allowing bacteria to proliferate under iron-limited conditions. Moreover, bacteria also evolved ingenious strategies to overcome the presence of other defense proteins in the hosts, called siderocalin, which are able to catch the Fe-siderophore complex making it unavailable for bacteria. In enterobacteria, salmochelin is a siderophore immune to this defense process and derives mainly from glycosylation of enterobactin, the most common siderophore in this group of bacteria, which could otherwise be easily scavenged by siderocalin (Ratledge and Dover, 2000; Fischbach *et al.*, 2006).

To further combat undesired bacteria, eukaryotes have evolved numerous articulate immune responses, which can be differentiated into the innate and adaptive systems. Until now, only the former has been identified in invertebrates. The first common line of defense observed in the innate immune response is phagocytosis (Mydlarz *et al.*, 2006). The phagocytic cells first surround and engulf the bacteria, and then carry out a microbicidal action via different mechanisms that can also require ROS formation. This is realized via the expression of specific enzymes responsible for the radical formation, and happens concomitant with the activation of protective systems for the host cells

(Nappi and Ottaviani, 2000). ROS represent one of the more conserved immune effectors among multicellular eukaryotes being produced inside the phagocytic cells or released into the extracellular space (Nyholm and Graf, 2012). Due to its interaction with ROS, Fe also plays an important role in these processes. For example, it was shown that in mammalian macrophages the cytoplasmatic Fe concentration greatly increases in the case of bacterial infection. It was suggested that this increase is the trigger for the formation of ROS (Schaible and Kaufmann, 2004).

In **Chapter 2** proteins potentially involved in the interaction with the host cells were detected (e.g. RTX-like protein). As discussed, phosphate limitation can have a drastic effect on bacterial virulence, inducing in some cases the formation of virulent phenotypes (Lamarche *et al.*, 2008; Zaborin *et al.*, 2009; Zaborin *et al.*, 2012). These data together suggest that the phosphate limitation response, or the general stress response induced by phosphate limitation can drive *Pseudovibrio* sp. FO-BEG1 to activate mechanisms for establishing a successful colonization of the host. This action will provide a constant supply of nutrients to the bacterium while the host will be supplied with vitamins, protection against other pathogens, and possibly additional cofactors.

In this symbiotic perspective, the production of chelating compounds can be seen as a strategy adopted by *Pseudovibrio* to successfully colonize its host when under certain stress such as nutrient-limiting conditions. These molecules could act both as weapons to scavenge iron and as control systems for iron concentrations, avoiding its participation in the Haber-Weiss/Fenton reaction during the immune response of the hosts. In support of the important role that

siderophores play during the colonization processes, there are several studies that repeatedly showed how strains not able to produce siderophores have a highly reduced virulent phenotype (Litwin *et al.*, 1996; Ratledge and Dover, 2000; Cendrowski *et al.*, 2004; Schrettl *et al.*, 2004). This "symbiotic hypothesis" can also be easily extended to the *Roseobacter* strains studied. These bacteria have often been described to be associated with marine phytoplankton, marine vertebrates, and invertebrates. In addition, they have also been reported as pathogenic, being responsible, for example, of juvenile oyster disease or black band disease in scleractinian corals (Buchan *et al.*, 2005). Considering these life styles, the strategies reported above will confer them additional colonization tools to successfully override the defense systems of their hosts.

5.4 Conclusions and perspectives

The physiological study conducted on the response of *Pseudovibrio* sp. FO-BEG1 to phosphate limitation reported in this thesis represents one of the few comprehensive studies conducted on the response to this nutrient condition by a heterotrophic marine bacterium. We confirmed that many regulatory processes that occur in *Pseudovibrio* are similar to the well studied cases described in *E. coli*. However, we also showed that other specific adaptations occur as the phospholipids exchange. As discussed above, these responses could represent important and widespread strategies adopted by marine heterotrophic bacteria to thrive in environments depleted in phosphate. These findings underline the importance of further investigations into the physiological adaptation of marine

heterotrophic bacteria to fluctuating phosphate nutrient regimes. This would help to gain a clearer picture of the adaptations evolved by different bacteria under these conditions and of the potential effects of this nutrient regime on the trophic relationships in the ecosystem. In this thesis it was shown how phosphate limitation had a significant influence also on the carbon, sulfur, and iron metabolism of *Pseudovibrio* sp. FO-BEG1. Therefore, systematic studies on a larger number of bacteria together with direct measurements in nature could help to understand to what extent these interconnections affect the natural biogeochemical cycles in the environment.

The novel approach applied in **Chapter 3** for the study of the exo-metabolome of FO-BEG1 revealed an astonishing diversity of metabolites secreted by this bacterium. Phosphate limitation proved to be a condition that greatly affected these processes. Therefore, as a start, more focused studies on exo-metabolomes of single strains and comparative studies between exo-metabolomes and natural DOM are inevitably required to identify which effects different nutrient regimes have on the secreted metabolites, and to understand to what extent heterotrophic marine bacteria influence the oceanic DOM. In addition, it was shown how high resolution mass spectrometry techniques are ideal tools to unravel the metabolomic diversity of bacteria. A promising challenge would be the bioinformatic integration of these data, or of more focused MS/MS analyses, with other "omic" approaches, which could greatly improve our understanding of metabolic processes. The mapping procedure discussed above is a simple example of a useful method for an integrated data analysis. Other approaches can be used as well, for example, the

construction of *ab-initio* networks based on protein co-expression or chemical interconnection among metabolites. These studies could produce new information and models uncolored by previous knowledge.

One intriguing aspect of the data presented in this work is the effect of phosphate limitation on secondary metabolite production and expression of virulence genes in *Pseudovibrio* sp. FO-BEG1 (**Chapter 2**,**3**,**4**). As discussed previously (**Chapter 1**), it has long been known that this condition has a great effect on these two physiological traits in other bacteria. The results of this thesis (e.g. production of TDA and chelating molecules; **Chapter 2**,**3**,**4)** strongly suggest that phosphate limitation represents a promising condition for the discovery of new compounds of biotechnological interest produced by *Pseudovibrio*. The drastic effect that this condition had on the secreted metabolites underlines also the necessity of more focused molecular analyses to understand the regulatory processes controlling the production of these compounds.

The detection of virulence related proteins under the tested condition showed that *Pseudovibrio* is indeed able to express these virulence factors. This also opens up several new perspectives for the study of symbiotic relationships. *Pseudovibrio* sp. FO-BEG1 could become a model organism for these kinds of studies as it is a versatile and easily culturable bacterium. It could help to better understand the molecular processes responsible for the interaction between bacteria and marine invertebrates. In addition, new hypotheses could be tested regarding the relatedness of nutrient limitation and symbiosis. As observed in other pathogenic bacteria, we can ask if these are common conditions that trigger the development of more virulent phenotypes also among marine bacteria. It

remains to be clarified whether the symbiotic relationship is driven, in the first place, by nutrient limitation and by the necessity of the bacteria to colonize a more hospitable environment.

Finally, the effect of phosphate limitation, or the general stress response induced by this condition, on the iron metabolism opens a new and broad field of research. The complete isolation and description of the chelating molecules together with mutagenesis experiments will be required to understand the function and the synthetic and regulatory processes behind this puzzling phenomenon. The production of chelating molecules as a response to phosphate limitation and the putative interconnection between iron and phosphate metabolisms need to be better investigated in other marine bacteria in order to draw comprehensive conclusions on the ecological importance that the phenomena described in this work might have in natural environments.

References of Chapter 1 and 5

Achard, M.E., Chen, K.W., Sweet, M.J., Watts, R.E., Schroder, K., Schembri, M.A., and McEwan, A.G. (2013) An antioxidant role for catecholate siderophores in *Salmonella*. *Biochemical Journal* **454**: 543-549.

Adler, C., Corbalán, N.S., Seyedsayamdost, M.R., Pomares, M.F., de Cristóbal, R.E., Clardy, J., Kolter, R., and Vincent, P.A. (2012) Catecholate siderophores protect bacteria from pyochelin toxicity. *Plos One* **7**: e46754. doi:46710.41371/journal.pone.0046754.

Agogué, H., Casamayor, E.O., Bourrain, M., Obernosterer, I., Joux, F., Herndl, G.J., and Lebaron, P. (2005) A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. *FEMS Microbiol Ecology* **54**: 269-280.

Altomare, C., Norvell, W.A., Björkman, T., and Harman, G.E. (1999) Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied and Environmental Microbiology* **65**: 2926-2933.

Andrews, S.C., Robinson, A.K., and Rodríguez-Quiñones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiology Reviews* **27**: 215-237.

Aoyama, T., Takanami, M., Makino, K., and Oka, A. (1991) Cross-talk between the virulence and phosphate regulons of *Agrobacterium tumefaciens* caused by an unusual interaction of the transcriptional activator with a regulatory DNA element. *Molecular and General Genetics MGG* **227**: 385-390.

Baek, J.H., and Lee, S.Y. (2006) Novel gene members in the Pho regulon of *Escherichia coli*. *FEMS Microbiology Lett*ers **264**: 104-109.

Bendale, M.S., Chaudhari, B.L., and Chincholkar, S.B. (2009) Influence of environmentla factors on siderophore production by *Streptomyces fulvissimus* ATCC 27431. *Curremt Trends in Biotechnology and Pharmacy* **3**: 362-371.

Benitez-Nelson, C.R. (2000) The biogeochemical cycling of phosphorus in marine systems. *Earth-Science Reviews* **51**: 109-135.

Berner, R.A. (1973) Phosphate removal from sea-water by adsorption on volcanogenic ferric Oxides. *Earth and Planetary Science Letters* **18**: 77-86.

Björkman, K., and Karl, D.M. (1994) Bioavailability of inorganic and organic phosphorus-compounds to natural assemblages of microorganisms in Hawaiian coastal waters. *Marine Ecology Progress Series* **111**: 265-273.

Björkman, K.M., and Karl, D.M. (2003) Bioavailability of dissolved organic phosphorus in the euphotic zone at station ALOHA, North Pacific Subtropical Gyre. *Limnology and Oceanography* **48**: 1049-1057.

Bondarev, V., Richter, M., Romano, S., Piel, J., Schwedt, A., and Schulz-Vogt, H.N. (2013) The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis. *Environmental Microbiology* **15**: 2095-2113.

Borodina, I., and Nielsen, J. (2005) From genomes to in silico cells via metabolic networks. *Current Opinion in Biotechnol*ogy **16**: 350-355.

Brauer, M.J., Yuan, J., Bennett, B.D., Lu, W.Y., Kimball, E., Botstein, D., and Rabinowitz, J.D. (2006) Conservation of the metabolomic response to starvation across two divergent microbes. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 19302-19307.

Braun, V., Pramanik, A., Gwinner, T., Köberle, M., and Bohn, E. (2009) Sideromycins: tools and antibiotics. *Biometals* **22**: 3-13.

Bredenbruch, F., Geffers, R., Nimtz, M., Buer, J., and Häussler, S. (2006) The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environmental Microbiology* **8**: 1318-1329.

Brinkman, A.G. (1993) A double-layer model for ion adsorption onto metal oxides, applied to experimental data and to natural sediments of Lake Veluwe, The Netherlands. *Hydrobiologia* **253**: 31–45.

Brock, J., and Schulz-Vogt, H.N. (2011) Sulfide induces phosphate release from polyphosphate in cultures of a marine *Beggiatoa* strain. *The ISME Journal* **5**: 497-506.

Brophy, J.E., and Carlson, D.J. (1989) Production of biologically refractory dissolved organic-carbon by natural seawater microbial-populations. *Deep-Sea Research Part a-Oceanographic Research Papers* **36**: 497-507.

Buchan, A., González, J.M., and Moran, M.A. (2005) Overview of the marine *Roseobacter* lineage. *Applied and Environmental Microbiology* **71**: 5665-5677.

Carlson, C.A. (2002) Production and removal processes. In *Biogeochemistry of marinine Dissolved Organic Matter*. Hansell, D.A., and Carlson, C.A. (eds). San Diego: CA: Elsevier, pp. 91-152.

Caspi, R., Altman, T., Dreher, K., Fulcher, C.A., Subhraveti, P., Keseler, I.M., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L.A., Ong, Q., Paley, S., Pujar, A., Shearer, A.G., Travers, M., Weerasinghe, D., Zhang, P.F., and Karp, P.D. (2012) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Research* **40**: D742-D753.

Cendrowski, S., MacArthur, W., and Hanna, P. (2004) *Bacillus anthracis* requires siderophore biosynthesis for growth in macrophages and mouse virulence. *Molecular Microbiology* **51**: 407-417.

Chakraborty, S., Sivaraman, J., Leung, K.Y., and Mok, Y.K. (2011) Two-component PhoB-PhoR regulatory system and ferric uptake regulator sense phosphate and iron to control virulence genes in type III and VI secretion systems of *Edwardsiella tarda*. *Journal of Biological Chemistry* **286**: 39417-39430.

Coleman, M.L., and Chisholm, S.W. (2010) Ecosystem-specific selection pressures revealed through comparative population genomics. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 18634-18639.

Cornish, A.S., and Page, W.J. (1998) The catecholate siderophores of *Azotobacter vinelandii*: their affinity for iron and role in oxygen stress management. *Microbiology* **144**: 1747-1754.

Cotner, J.B., Ammerman, J.W., Peele, E.R., and Bentzen, E. (1997) Phosphorus-limited bacterioplankton growth in the Sargasso Sea. *Aquatic Microbial Ecology* **13**: 141-149.

Crépin, S., Houle, S., Charbonneau, M.E., Mourez, M., Harel, J., and Dozois, C.M. (2012) Decreased expression of type 1 fimbriae by a pst mutant of uropathogenic *Escherichia coli* reduces urinary tract infection. *Infection and Immunity* **80**: 2802-2815.

Dao, K.H.T., Hamer, K.E., Clark, C.L., and Harshman, L.G. (1999) Pyoverdine production by *Pseudomonas aeruginosa* exposed to metals or an oxidative stress agent. *Ecological Applications* **9**: 441-448.

David, H., Hofmann, G., Oliveira, A.P., Jarmer, H., and Nielsen, J. (2006) Metabolic network driven analysis of genome-wide transcription data from *Aspergillus nidulans*. *Genome Biology* **7**: R108.

De Keersmaecker, S.C.J., Thijs, I.M.V., Vanderleyden, J., and Marchal, K. (2006) Integration of omics data: how well does it work for bacteria? *Molecular Microbiology* **62**: 1239-1250.

Decho, A.W., Frey, R.L., and Ferry, J.L. (2011) Chemical challenges to bacterial AHL signaling in the environment. *Chemical Reviews* **111**: 86-99.

Delaney, M.L. (1998) Phosphorus accumulation in marine sediments and the oceanic phosphorus cycle. *Global Biogeochemical Cycles* **12**: 563-572.

Desriac, F., Defer, D., Bourgougnon, N., Brillet, B., Le Chevalier, P., and Fleury, Y. (2010) Bacteriocin as weapons in the Marine Animal-Associated Bacteria Warfare: inventory and potential applications as an aquaculture probiotic. *Marine Drugs* **8**: 1153-1177.

Diaz, J.M., Hansel, C.M., Voelker, B.M., Mendes, C.M., Andeer, P.F., and Zhang, T. (2013) Widespread production of extracellular superoxide by heterotrophic bacteria. *Science* **340**: 1223-1226.

Diggle, S.P., Matthijs, S., Wright, V.J., Fletcher, M.P., Chhabra, S.R., Lamont, I.L., Kong, X., Hider, R.C., Cornelis, P., Cámara, M., and Williams, P. (2007) The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chemical Biology* **14**: 87-96.

Dittmar, T. (2008) The molecular level determination of black carbon in marine dissolved organic matter. *Organic Geochemistry* **39**: 396-407.

Dobzhansky, T. (1973) Nothing in Biology Makes Sense except in Light of Evolution. *American Biology Teacher* **35**: 125-129.

Dufresne, A., Garczarek, L., and Partensky, F. (2005) Accelerated evolution associated with genome reduction in a free-living prokaryote. *Genome Biology* **6**: R14.

Dupont, S., Carré-Mlouka, A., Descarrega, F., Ereskovsky, A., Longeon, A., Mouray, E., Florent, I., and Bourguet-Kondracki, M.L. (2013) Diversity and biological activities of the bacterial community associated with the marine sponge *Phorbas tenacior* (Porifera, Demospongiae). *Letters in Applied Microbiology* **58**: 45-52

Duquesne, S., Destoumieux-Garzón, D., Peduzzi, J., and Rebuffat, S. (2007) Microcins, gene-encoded antibacterial peptides from enterobacteria. *Natural Product Reports* **24**: 708-734.

Dyhrman, S.T., Ammerman, J.W., and Van Mooy, B.A.S. (2007) Microbes and the marine phosphorus cycle. *Oceanography* **20**: 110-116.

Dyhrman, S.T., Benitez-Nelson, C.R., Orchard, E.D., Haley, S.T., and Pellechia, P.J. (2009) A microbial source of phosphonates in oligotrophic marine systems. *Nature Geoscience* **2**: 696-699.

Dyhrman, S.T., Chappell, P.D., Haley, S.T., Moffett, J.W., Orchard, E.D., Waterbury, J.B., and Webb, E.A. (2006) Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. *Nature* **439**: 68-71.

Eisendle, M., Schrettl, M., Kragl, C., Müller, D., Illmer, P., and Haas, H. (2006) The intracellular siderophore ferricrocin is involved in iron storage, oxidative-stress resistance, germination, and sexual development in *Aspergillus nidulans*. *Eukaryotic Cell* **5**: 1596-1603.

Elser, J.J., Watts, J., Schampel, J.H., and Farmer, J. (2006) Early Cambrian food webs on a trophic knife-edge? A hypothesis and preliminary data from a modern stromatolite-based ecosystem. *Ecology Letters* **9**: 295-303.

Enticknap, J.J., Kelly, M., Peraud, O., and Hill, R.T. (2006) Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied and Environmental Microbiology* **72**: 3724-3732.

Fanning, K.A. (1992) Nutrient provinces in the sea - Concentration ratios, reaction-rate ratios, and ideal covariation. *Journal of Geophysical Research-Oceans* **97**: 5693-5712.

Feely, R.A., Trefry, J.H., Lebon, G.T., and German, C.R. (1998) The relationship between P/Fe and V/Fe ratios in hydrothermal precipitates and dissolved phosphate in seawater. *Geophysical Research Letters* **25**: 2253-2256.

Filippelli, G.M. (2008) The global phosphorus cycle: Past, present, and future. *Elements* **4**: 89-95.

Fischbach, M.A., Lin, H.N., Liu, D.R., and Walsh, C.T. (2006) How pathogenic bacteria evade mammalian sabotage in the battle for iron. *Nature Chemical Biology* **2**: 132-138.

Flemer, B., Kennedy, J., Margassery, L.M., Morrissey, J.P., O'Gara, F., and Dobson, A.D.W. (2012) Diversity and antimicrobial activities of microbes from two Irish marine sponges, Suberites carnosus and Leucosolenia sp. *Journal of Applied Microbiology* **112**: 289-301.

Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., Lin, J., Minguez, P., Bork, P., von Mering, C., and Jensen, L.J. (2013) STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Research* **41**: D808-815.

Freese, H.M., Dalingault, H., Petersen, J., Pradella, S., Davenport, K., Teshima, H., Chen, A., Pati, A., Ivanova, N., Goodwin, L.A., Chain, P., Detter, J.C., Rohde, M., Gronow, S., Kyrpides, N.C., Woyke, T., Brinkhoff, T., Göker, M., Overmann, J., and Klenk, H.-P. (2013) Genome sequence of the phage-gene rich marine *Phaeobacter arcticus* type strain DSM 23566 T. *Standards in Genomic Science* **8**.

Froelich, P.N., Bender, M.L., and Heath, G.R. (1977) Phosphorus accumulation rates in metalliferous sediments on east Pacific Rise. *Earth and Planetary Science Letters* **34**: 351-359.

Froelich, P.N., Bender, M.L., Luedtke, N.A., Heath, G.R., and Devries, T. (1982) The marine phosphorus cycle. *American Journal of Science* **282**: 474-511.

Fukunaga, Y., Kurahashi, M., Tanaka, K., Yanagi, K., Yokota, A., and Harayama, S. (2006) *Pseudovibrio ascidiaceicola* sp. nov., isolated from ascidians (sea squirts). *International Journal of Systematic and Evolutionary Microbiology* **56**: 343-347.

Gaffney, J.P., and Valentine, A.M. (2012) Beyond bilobal: transferrin homologs having unusual domain architectures. *Biochim Biophys Acta* **1820**: 212-217.

Gérard, F., Dri, A.M., and Moreau, P.L. (1999) Role of *Escherichia coli* RpoS, LexA and H-NS global regulators in metabolism and survival under aerobic, phosphate-starvation conditions. *Microbiology* **145**: 1547-1562.

Gilbert, J.A., Thomas, S., Cooley, N.A., Kulakova, A., Field, D., Booth, T., McGrath, J.W., Quinn, J.P., and Joint, I. (2009) Potential for phosphonoacetate utilization by marine bacteria in temperate coastal waters. *Environmental Microbiology* **11**: 111-125.

Gillor, O., Etzion, A., and Riley, M.A. (2008) The dual role of bacteriocins as anti- and probiotics. *Applied Microbiology and Biotechnol*ogy **81**: 591-606.

Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D., Bibbs, L., Eads, J., Richardson, T.H., Noordewier, M., Rappé, M.S., Short, J.M., Carrington, J.C., and Mathur, E.J. (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**: 1242-1245.

Goclaw-Binder, H., Sendersky, E., Shimoni, E., Kiss, V., Reich, Z., Perelman, A., and Schwarz, R. (2012) Nutrient-associated elongation and asymmetric division of the cyanobacterium *Synechococcus* PCC 7942. *Environmental Microbiology* **14**: 680-690.

Gómez-Consarnau, L., Lindh, M.V., Gasol, J.M., and Pinhassi, J. (2012) Structuring of bacterioplankton communities by specific dissolved organic carbon compounds. *Environmental Microbiology* **14**: 2361-2378.

Gonin, M., Quardokus, E.M., O'Donnol, D., Maddock, J., and Brun, Y.V. (2000) Regulation of stalk elongation by phosphate in *Caulobacter crescentus*. *Journal of Bacteriology* **182**: 337-347.

Gristwood, T., Fineran, P.C., Everson, L., Williamson, N.R., and Salmond, G.P. (2009) The PhoBR two-component system regulates antibiotic biosynthesis in *Serratia* in response to phosphate. *BMC Microbiology* **9**: doi:10.1186/1471-2180-1189-1112.

Gruber, D.F., Simjouw, J.P., Seitzinger, S.P., and Taghon, G.L. (2006) Dynamics and characterization of refractory dissolved organic matter produced by a pure bacterial culture in an experimental predator-prey system. *Applied and Environmental Microbiology* **72**: 4184-4191.

Gunnarsson, N., Bruheim, P., and Nielsen, J. (2003) Production of the glycopeptide antibiotic A40926 by *Nonomuraea* sp ATCC 39727: influence of medium composition in batch fermentation. *Journal of Industrial Microbiology & Biotechnology* **30**: 150-156.

Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology* **19**: 1720-1730.

Haber, F., and Weiss, J. (1934) The catalytic decomposition of hydrogen peroxide by iron salts. *Proceeding of the Royal Society of London* **Se. A**: 331-352.

Hansell, D.A. (2013) Recalcitrant Dissolved Organic Carbon fractions. *Annual Review of Marine Science, Vol 5* **5**: 421-445.

Harrison, P.M., and Arosio, P. (1996) Ferritins: Molecular properties, iron storage function and cellular regulation. *Biochimica Et Biophysica Acta-Bioenergetics* **1275**: 161-203.

Hartwell, L.H., Hopfield, J.J., Leibler, S., and Murray, A.W. (1999) From molecular to modular cell biology. *Nature* **402**: C47-52.

Heindl, H., Wiese, J., Thiel, V., and Imhoff, J.F. (2010) Phylogenetic diversity and antimicrobial activities of bryozoan-associated bacteria isolated from Mediterranean and Baltic Sea habitats. *Systematic and Applied Microbiology* **33**: 94-104.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., and Hacker, J. (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges Aplysina aerophoba and *Aplysina cavernicola*. *FEMS Microbiol Ecol* **35**: 305-312.

Hider, R.C. (1984) Siderophore mediated absorption of iron. *Structure and Bonding* **58**: 25-87.

Hochmuth, T., Niederkrüger, H., Gernert, C., Siegl, A., Taudien, S., Platzer, M., Crews, P., Hentschel, U., and Piel, J. (2010) Linking chemical and microbial diversity in marine sponges: possible role for *Poribacteria* as producers of methyl-branched fatty acids. *Chembiochem* **11**: 2572-2578.

Hosoya, S., and Yokota, A. (2007) *Pseudovibrio japonicus* sp nov., isolated from coastal seawater in Japan. *International Journal of Systematic and Evolutionary Microbiology* **57**: 1952-1955.

Hsieh, Y.J., and Wanner, B.L. (2010) Global regulation by the seven-component P_i signaling system. *Current Opinion in Microbiology* **13**: 198-203.

Hubbard, M.J., and Cohen, P. (1993) On target with a new mechanism for the regulation of protein-phosphorylation. *Trends in Biochemical Sciences* **18**: 172-177.

Ihmels, J., Levy, R., and Barkai, N. (2004) Principles of transcriptional control in the metabolic network of *Saccharomyces cerevisiae*. *Nature Biotechnol*ogy **22**: 86-92.

Imlay, J.A. (2003) Pathways of oxidative damage. *Annual Review of Microbiology* **57**: 395-418.

Ishige, T., Krause, M., Bott, M., Wendisch, V.F., and Sahm, H. (2003) The phosphate starvation stimulon of *Corynebacterium glutamicum* determined by DNA microarray analyses. *Journal of Bacteriology* **185**: 4519-4529.

Jeanjean, R., Talla, E., Latifi, A., Havaux, M., Janicki, A., and Zhang, C.C. (2008) A large gene cluster encoding peptide synthetases and polyketide synthases is involved in production of siderophores and oxidative stress response in the cyanobacterium *Anabaena* sp strain PCC 7120. *Environmental Microbiology* **10**: 2574-2585.

Jensen, H.S., and Thamdrup, B. (1993) Iron-bound phosphorus in marine sediments as measured by bicarbonate-dithionite extraction. *Hydrobiologia* **253**: 47-59.

Jensen, V., Lóns, D., Zaoui, C., Bredenbruch, F., Meissner, A., Dieterich, G., Munch, R., and Häussler, S. (2006) RhlR expression in *Pseudomonas aeruginosa* is modulated by the *Pseudomonas* quinolone signal via PhoB-dependent and -independent pathways. *Journal of Bacteriology* **188**: 8601-8606.

Ji, C., Juárez-Hernández, R.E., and Miller, M.J. (2012) Exploiting bacterial iron acquisition: siderophore conjugates. *Future Medicinal Chemistry* **4**: 297-313.

Jiao, N., Herndl, G.J., Hansell, D.A., Benner, R., Kattner, G., Wilhelm, S.W., Kirchman, D.L., Weinbauer, M.G., Luo, T.W., Chen, F., and Azam, F. (2010) Microbial production of recalcitrant dissolved organic matter: long-term carbon storage in the global ocean. *Nature Reviews Microbiology* **8**: 593-599.

Jiao, N.Z., Tang, K., Cai, H.Y., and Mao, Y.J. (2011a) Increasing the microbial carbon sink in the sea by reducing chemical fertilization on the land. *Nature Reviews Microbiology* **9**: 75-75.

Jiao, N.Z., Herndl, G.J., Hansell, D.A., Benner, R., Kattner, G., Wilhelm, S.W., Kirchman, D.L., Weinbauer, M.G., Luo, T.W., Chen, F., and Azam, F. (2011b) The microbial carbon pump and the oceanic recalcitrant dissolved organic matter pool. *Nature Reviews Microbiology* **9**: 555-555.

Joyce, A.R., and Palsson, B.Ø. (2006) The model organism as a system: integrating 'omics' data sets. *Nature Reviews Molecular Cell Biology* **7**: 198-210.

Jürgens, K., and Matz, C. (2002) Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 413-434.

Justice, S.S., Hunstad, D.A., Cegelski, L., and Hultgren, S.J. (2008) Morphological plasticity as a bacterial survival strategy. *Nature Reviews Microbiology* **6**: 162-168.

Kanehisa, M., and Goto, S. (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**: 27-30.

Kannapiran, E., and Ravindran, J. (2012) Dynamics and diversity of phosphate mineralizing bacteria in the coral reefs of Gulf of Mannar. *Journal of Basic Microbiology* **52**: 91-98.

Karl, D.M. (2000) Aquatic ecology - Phosphorus, the staff of life. *Nature* **406**: 31-33.

Karl, D.M., and Björkman, K.M. (2002) Dynamics of DOP. In *Biogeochemistry of Marine Dissolved Matter*. Hansell, D.A., and C.A., C. (eds). Boston: Elsevier Science, pp. 249-366.

Karl, D.M., Björkman, K.M., Dore, J.E., Fujieki, L., Hebel, D.V., Houlihan, T., Letelier, R.M., and Tupas, L.M. (2001) Ecological nitrogen-to-phosphorus stoichiometry at station ALOHA. *Deep-Sea Research Part Ii-Topical Studies in Oceanography* **48**: 1529-1566.

Kaufmann, G.F., Sartorio, R., Lee, S.H., Rogers, C.J., Meijler, M.M., Moss, J.A., Clapham, B., Brogan, A.P., Dickerson, T.J., and Janda, K.D. (2005) Revisiting quorum sensing: discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 309-314.

Kaye, J.Z., Sylvan, J.B., Edwards, K.J., and Baross, J.A. (2011) *Halomonas* and *Marinobacter* ecotypes from hydrothermal vent, subseafloor and deep-sea environments. *FEMS Microbiol Ecol* **75**: 123-133.

Kelly, L., Ding, H.M., Huang, K.H., Osburne, M.S., and Chisholm, S.W. (2013) Genetic diversity in cultured and wild marine cyanomyoviruses reveals phosphorus stress as a strong selective agent. *The ISME Journal* **7**: 1827-1841.

Kennedy, J., Baker, P., Piper, C., Cotter, P.D., Walsh, M., Mooij, M.J., Bourke, M.B., Rea, M.C., O'Connor, P.M., Ross, R.P., Hill, C., O'Gara, F., Marchesi, J.R., and Dobson, A.D. (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. *Marine Biotechnology (NY)* **11**: 384-396.

Kittredg, J.S., and Roberts, E. (1969) A carbon-phosphorus bond in nature. *Science* **164**: 37-42.

Klaenhammer, T.R. (1988) Bacteriocins of lactic-acid bacteria. *Biochimie* **70**: 337-349.

Koch, B.P., and Dittmar, T. (2006) From mass to structure: an aromaticity index for high-resolution mass data of natural organic matter. *Rapid Communications in Mass Spectrometry* **20**: 926-932.

Kolowith, L.C., Ingall, E.D., and Benner, R. (2001) Composition and cycling of marine organic phosphorus. *Limnology and Oceanography* **46**: 309-320.

Koren, O., and Rosenberg, E. (2006) Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. *Applied and Environmental Microbiology* **72**: 5254-5259.

Kraemer, S.M. (2004) Iron oxide dissolution and solubility in the presence of siderophores. *Aquatic Sciences* **66**: 3-18.

Kraemer, S.M., Butler, A., Borer, P., and Cervini-Silva, J. (2005) Siderophores and the dissolution of iron-bearing minerals in marine systems. *Molecular Geomicrobiology* **59**: 53-84.

Krasko, A., Schroder, H.C., Batel, R., Grebenjuk, V.A., Steffen, R., Muller, I.M., and Muller, W.E.G. (2002) Iron induces proliferation and morphogenesis in primmorphs from the marine sponge *Suberites domuncula*. *DNA and Cell Biology* **21**: 67-80.

Krol, E., and Becker, A. (2004) Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Molecular Genetics and Genomics* **272**: 1-17.

Krom, M.D., Kress, N., Brenner, S., and Gordon, L.I. (1991) Phosphorus limitation of primary productivity in the eastern Mediterranean-Sea. *Limnology and Oceanography* **36**: 424-432.

Kujawinski, E.B. (2011) The impact of microbial metabolism on marine Dissolved Organic Matter. *Annual Review of Marine Science* **3**: 567-599.

Kujawinski, E.B., Longnecker, K., Blough, N.V., Del Vecchio, R., Finlay, L., Kitner, J.B., and Giovannoni, S.J. (2009) Identification of possible source markers in marine dissolved organic matter using ultrahigh resolution mass spectrometry. *Geochimica Et Cosmochimica Acta* **73**: 4384-4399.

Lafi, F.F., Garson, M.J., and Fuerst, J.A. (2005) Culturable bacterial symbionts isolated from two distinct sponge species (*Pseudoceratina clavata* and *Rhabdastrella globostellata*) from the Great Barrier Reef display similar phylogenetic diversity. *Microbial Ecology* **50**: 213-220.

Lamarche, M.G., Wanner, B.L., Crepin, S., and Harel, J. (2008) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiology Reviews* **32**: 461-473.

Lee, J.Y., Passalacqua, K.D., Hanna, P.C., and Sherman, D.H. (2011) Regulation of petrobactin and bacillibactin biosynthesis in *Bacillus anthracis* under iron and oxygen variation. *Plos One* **6**: e20777. doi:20710.21371/journal.pone.0020777.

Levering, J., Musters, M.W., Bekker, M., Bellomo, D., Fiedler, T., de Vos, W.M., Hugenholtz, J., Kreikemeyer, B., Kummer, U., and Teusink, B. (2012) Role of phosphate in the central metabolism of two lactic acid bacteria-a comparative systems biology approach. *FEBS Journal* **279**: 1274-1290.

Lijklema, L. (1980) Interaction of ortho-phosphate with iron(III) and aluminum hydroxides. *Environmental Science & Technology* **14**: 537-541.

Lillie, S.H., and Pringle, J.R. (1980) Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *Journal of Bacteriology* **143**: 1384-1394.

Litwin, C.M., Rayback, T.W., and Skinner, J. (1996) Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. *Infection and Immunity* **64**: 2834-2838.

Long, J., Zaborina, O., Holbrook, C., Zaborin, A., and Alverdy, J. (2008) Depletion of intestinal phosphate after operative injury activates the virulence of *P. aeruginosa* causing lethal gut-derived sepsis. *Surgery* **144**: 189-197.

Løvdal, T., Skjoldal, E.F., Heldal, M., Norland, S., and Thingstad, T.F. (2008) Changes in morphology and elemental composition of *Vibrio splendidus* along a gradient from carbon-limited to phosphate-limited growth. *Microbial Ecology* **55**: 152-161.

Lucotte, M.D., and d'Anglejan, B. (1988) Seasonal changes in the phosphorus-iron geochemistry of the St. Lawrence Estuary. *Journal of Coastal Research* **4**: 339–349.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.H. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32**: 1363-1371.

Luo, H., Benner, R., Long, R.A., and Hu, J. (2009) Subcellular localization of marine bacterial alkaline phosphatases. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 21219-21223.

Luo, H.W., Zhang, H.M., Long, R.A., and Benner, R. (2011) Depth distributions of alkaline phosphatase and phosphonate utilization genes in the North Pacific Subtropical Gyre. *Aquatic Microbial Ecology* **62**: 61-69.

Malmcrona-Friberg, K., Tunlid, A., Mårdén, P., Kjelleberg, S., and Odham, G. (1986) Chemical changes in cell envelope and poly-β-hydroxybutyrate during short-term starvation of a marine bacterial Isolate. *Archives of Microbiology* **144**: 340-345.

Mapelli, V., Olsson, L., and Nielsen, J. (2008) Metabolic footprinting in microbiology: methods and applications in functional genomics and biotechnology. *Trends in Biotechnology* **26**: 490-497.

Martín, J.F. (2004) Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *Journal of Bacteriology* **186**: 5197-5201.

Martinez, A., Tyson, G.W., and DeLong, E.F. (2010) Widespread known and novel phosphonate utilization pathways in marine bacteria revealed by functional screening and metagenomic analyses. *Environmental Microbiology* **12**: 222-238.

Martiny, A.C., Coleman, M.L., and Chisholm, S.W. (2006) Phosphate acquisition genes in *Prochlorococcus* ecotypes: evidence for genome-wide adaptation. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 12552-12557.

Martiny, A.C., Huang, Y., and Li, W. (2009a) Occurrence of phosphate acquisition genes in *Prochlorococcus* cells from different ocean regions. *Environmental Microbiology* **11**: 1340-1347.

Martiny, A.C., Tai, A.P., Veneziano, D., Primeau, F., and Chisholm, S.W. (2009b) Taxonomic resolution, ecotypes and the biogeography of *Prochlorococcus*. *Environmental Microbiology* **11**: 823-832.

Masiello, C.A. (2004) New directions in black carbon organic geochemistry. *Marine Chemistry* **92**: 201-213.

Mason, P.W., Carbone, D.P., Cushman, R.A., and Waggoner, A.S. (1981) The importance of inorganic phosphate in regulation of energy metabolism of *Streptococcus lactis*. *Journal of Biological Chemistry* **256**: 1861-1866.

McCloskey, D., Palsson, B.Ø., and Feist, A.M. (2013) Basic and applied uses of genome-scale metabolic network reconstructions of *Escherichia coli*. *Molecular System Biology* **9**: 661.

McCutcheon, J.P., and Moran, N.A. (2012) Extreme genome reduction in symbiotic bacteria. *Nature Reviews Microbiology* **10**: 13-26.

McIntosh, J.A., Donia, M.S., and Schmidt, E.W. (2009) Ribosomal peptide natural products: bridging the ribosomal and nonribosomal worlds. *Natural Product Reports* **26**: 537-559.

Mclaughlin, J.R., Ryden, J.C., and Syers, J.K. (1981) Sorption of inorganic-phosphate by iron-containing and aluminum-containing components. *Journal of Soil Science* **32**: 365-377.

Mendes, M.V., Tunca, S., Antón, N., Recio, E., Sola-Landa, A., Aparicio, J.F., and Martín, J.F. (2007) The two-component phoR-phoP system of *Streptomyces natalensis*: Inactivation or deletion of phoP reduces the negative phosphate regulation of pimaricin biosynthesis. *Metabolic Engineering* **9**: 217-227.

Mills, M.M., Ridame, C., Davey, M., La Roche, J., and Geider, R.J. (2004) Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* **429**: 292-294.

Moran, M.A., Belas, R., Schell, M.A., González, J.M., Sun, F., Sun, S., Binder, B.J., Edmonds, J., Ye, W., Orcutt, B., Howard, E.C., Meile, C., Palefsky, W., Goesmann, A., Ren, Q., Paulsen, I., Ulrich, L.E., Thompson, L.S., Saunders, E., and Buchan, A. (2007) Ecological genomics of marine roseobacters. *Applied and Environmental Microbiology* **73**: 4559-4569.

Moran, M.A., Buchan, A., González, J.M., Heidelberg, J.F., Whitman, W.B., Kiene, R.P., Henriksen, J.R., King, G.M., Belas, R., Fuqua, C., Brinkac, L., Lewis, M., Johri, S., Weaver, B., Pai, G., Eisen, J.A., Rahe, E., Sheldon, W.M., Ye, W., Miller, T.R., Carlton, J., Rasko, D.A., Paulsen, I.T., Ren, Q., Daugherty, S.C., Deboy, R.T., Dodson, R.J., Durkin, A.S., Madupu, R., Nelson, W.C., Sullivan, S.A., Rosovitz, M.J., Haft, D.H., Selengut, J., and Ward, N. (2004) Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. *Nature* **432**: 910-913.

Moreau, P.L. (2004) Diversion of the metabolic flux from pyruvate dehydrogenase to pyruvate oxidase decreases oxidative stress during glucose metabolism in nongrowing *Escherichia coli* cells incubated under aerobic, phosphate starvation conditions. *Journal of Bacteriology* **186**: 7364-7368.

Moreau, P.L., Gérard, F., Lutz, N.W., and Cozzone, P. (2001) Non-growing *Escherichia coli* cells starved for glucose or phosphate use different mechanisms to survive oxidative stress. *Molecular Microbiology* **39**: 1048-1060.

Moreira, A.P., Chimetto Tonon, L.A., do Valle, P.P.C., Alves, N., Jr., Amado-Filho, G.M., Francini-Filho, R.B., Paranhos, R., and Thompson, F.L. (2013) Culturable heterotrophic bacteria associated with healthy and bleached scleractinian *Madracis decactis* and the fireworm *Hermodice carunculata* from the remote St. Peter and St. Paul archipelago, Brazil. *Current Microbiology* **68**: 38-46.

Muscholl-Silberhorn, A., Thiel, V., and Imhoff, J.F. (2008) Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean sea. *Microbial Ecology* **55**: 94-106.

Mydlarz, L.D., Jones, L.E., and Harvell, C.D. (2006) Innate immunity environmental drivers and disease ecology of marine and freshwater invertebrates. *Annual Review of Ecology Evolution and Systematics* **37**: 251-288.

Nappi, A.J., and Ottaviani, E. (2000) Cytotoxicity and cytotoxic molecules in invertebrates. *Bioessays* **22**: 469-480.

Nolan, E.M., Fischbach, M.A., Koglin, A., and Walsh, C.T. (2007) Biosynthetic tailoring of microcin e492m: post-translational modification affords an antibacterial siderophore-peptide conjugate. *Journal of the American Chemical Society* **129**: 14336-14347.

Nyholm, S.V., and Graf, J. (2012) Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. *Nature Reviews Microbiology* **10**: 815-827.

O'Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O'Gara, F., and Dobson, A.D.W. (2011) Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *Journal of Applied Microbiology* **110**: 1495-1508.

O'Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., Dobson, A.D., and O'Gara, F. (2013) *Pseudovibrio axinellae* sp. nov., isolated from an Irish marine sponge. *International Journal of Systematic and Evolutionary Microbiology* **63**: 141-145.

Ogawa, H., Amagai, Y., Koike, I., Kaiser, K., and Benner, R. (2001) Production of refractory dissolved organic matter by bacteria. *Science* **292**: 917-920.

Olson, J.B., Harmody, D.K., and McCarthy, P.J. (2002) α-proteobacteria cultivated from marine sponges display branching rod morphology. *FEMS Microbiol Lett* **211**: 169-173.

Ortet, P., De Luca, G., Whitworth, D.E., and Barakat, M. (2012) P2TF: a comprehensive resource for analysis of prokaryotic transcription factors. *BMC Genomics* **13**: 628.

Osorio, H., Martínez, V., Nieto, P.A., Holmes, D.S., and Quatrini, R. (2008) Microbial iron management mechanisms in extremely acidic environments: comparative genomics evidence for diversity and versatility. *BMC Microbiology* **8**: 203.

Overbeek, R., Begley, T., Butler, R.M., Choudhuri, J.V., Chuang, H.Y., Cohoon, M., de Crécy-Lagard, V., Diaz, N., Disz, T., Edwards, R., Fonstein, M., Frank, E.D., Gerdes, S., Glass, E.M., Goesmann, A., Hanson, A., Iwata-Reuyl, D., Jensen, R., Jamshidi, N., Krause, L., Kubal, M., Larsen, N., Linke, B., McHardy, A.C., Meyer, F., Neuweger, H., Olsen, G., Olson, R., Osterman, A., Portnoy, V., Pusch, G.D., Rodionov, D.A., Rückert, C., Steiner, J., Stevens, R., Thiele, I., Vassieva, O., Ye, Y., Zagnitko, O., and Vonstein, V. (2005) The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Research* **33**: 5691-5702.

Parfitt, R.L. (1989) Phosphate reactions with natural allophane, ferrihydrite and goethite. *Journal of Soil Science* **40**: 359-369.

Patil, K.R., and Nielsen, J. (2005) Uncovering transcriptional regulation of metabolism by using metabolic network topology. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 2685-2689.

Paytan, A., and McLaughlin, K. (2007) The oceanic phosphorus cycle. *Chemical Reviews* **107**: 563-576.

Penesyan, A., Tebben, J., Lee, M., Thomas, T., Kjelleberg, S., Harder, T., and Egan, S. (2011) Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio* sp. D323 and related sponge-associated bacteria. *Marine Drugs* **9**: 1391-1402.

Pernthaler, J. (2005) Predation on prokaryotes in the water column and its ecological implications. *Nature Reviews Microbiology* **3**: 537-546.

Piel, J. (2004) Metabolites from symbiotic bacteria. *Natural Product Reports* **21**: 519-538.

Pine, L., and Boone, C.J. (1967) Comparative cell wall analyses of morphological forms within the genus *Actinomyces*. *Journal of Bacteriology* **94**: 875-883.

Pinhassi, J., Gómez-Consarnau, L., Alonso-Sáez, L., Sala, M.M., Vidal, M., Pedrós-Alió, C., and Gasol, J.M. (2006) Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea. *Aquatic Microbial Ecology* **44**: 241-252.

Polz, M.F., Hunt, D.E., Preheim, S.P., and Weinreich, D.M. (2006) Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. *Philosophical Transactions of the Royal Society B-Biological Sciences* **361**: 2009-2021.

Popendorf, K.J., Lomas, M.W., and Van Mooy, B.A.S. (2011a) Microbial sources of intact polar diacylglycerolipids in the Western North Atlantic Ocean. *Organic Geochemistry* **42**: 803-811.

Popendorf, K.J., Tanaka, T., Pujo-Pay, M., Lagaria, A., Courties, C., Conan, P., Oriol, L., Sofen, L.E., Moutin, T., and Van Mooy, B.A.S. (2011b) Gradients in intact polar diacylglycerolipids across the Mediterranean Sea are related to phosphate availability. *Biogeosciences* **8**: 3733-3745.

Poulton, S.W., Krom, M.D., Van Rijn, J., and Raiswell, R. (2002) The use of hydrous iron (III) oxides for the removal of hydrogen sulphide in aqueous systems. *Water Research* **36**: 825-834.

Pratheepa, V.K., Silva, M., and Vasconcelos, V. (2013) Viscera-associated bacterial diversity among intertidal gastropods from Northern-Atlantic coast of Portugal. *Current Microbiology* **68**: 140-148.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glöckner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* **35**: 7188-7196.

Quatrini, R., Lefimil, C., Veloso, F.A., Pedroso, I., Holmes, D.S., and Jedlicki, E. (2007) Bioinformatic prediction and experimental verification of Fur-regulated genes in the extreme acidophile *Acidithiobacillus ferrooxidans*. *Nucleic Acids Research* **35**: 2153-2166.

Quin, L.D. (1965) Persence of compounds with a carbon-phosphorus bond in some marine invertebrates. *Biochemistry* **141**: 568-591.

Quinn, J.P., Kulakova, A.N., Cooley, N.A., and McGrath, J.W. (2007) New ways to break an old bond: the bacterial carbon-phosphorus hydrolases and their role in biogeochemical phosphorus cycling. *Environmental Microbiology* **9**: 2392-2400.

Raiswell, R., and Canfield, D. (2012) The iron biogeochemical cycle past and present. *Geochemical Perspective* **1**: 1-220.

Ratledge, C., and Dover, L.G. (2000) Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology* **54**: 881-941.

Ribes, M., Jiménez, E., Yahel, G., López-Sendino, P., Díez, B., Massana, R., Sharp, J.H., and Coma, R. (2012) Functional convergence of microbes associated with temperate marine sponges. *Environmental Microbiology* **14**: 1224-1239.

Riesenfeld, C.S., Murray, A.E., and Baker, B.J. (2008) Characterization of the microbial community and polyketide biosynthetic potential in the palmerolide-producing tunicate *Synoicum adareanum*. *Journal of Natural Products* **71**: 1812-1818.

Rifat, D., Bishai, W.R., and Karakousis, P.C. (2009) Phosphate depletion: a novel trigger for *Mycobacterium tuberculosis* persistence. *Journal of Infectious Diseases* **200**: 1126-1135.

Riley, M.A., and Wertz, J.E. (2002) Bacteriocins: evolution, ecology, and application. *Annual Review of Microbiology* **56**: 117-137.

Rivkin, R.B., and Anderson, M.R. (1997) Inorganic nutrient limitation of oceanic bacterioplankton. *Limnology and Oceanography* **42**: 730-740.

Rizzo, C., Michaud, L., Hormann, B., Gerce, B., Syldatk, C., Hausmann, R., De Domenico, E., and Lo Giudice, A. (2013) Bacteria associated with sabellids (Polychaeta: Annelida) as a novel source of surface active compounds. *Marine Pollution Bulletin* **70**: 125-133.

Rodríguez-García, A., Sola-Landa, A., Apel, K., Santos-Beneit, F., and Martín, J.F. (2009) Phosphate control over nitrogen metabolism in *Streptomyces coelicolor*: direct and indirect negative control of glnR, glnA, glnII and amtB expression by the response regulator PhoP. *Nucleic Acids Research* **37**: 3230-3242.

Rodríguez-Sáinz, M.C., Hernándezchico, C., and Moreno, F. (1990) Molecular characterization of Pmba, an *Escherichia coli* chromosomal gene required for the production of the antibiotic peptide Mccb17. *Molecular Microbiology* **4**: 1921-1932.

Rodríguez, H., and Fraga, R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* **17**: 319-339.

Romanowski, K., Zaborin, A., Valuckaite, V., Rolfes, R.J., Babrowski, T., Bethel, C., Olivas, A., Zaborina, O., and Alverdy, J.C. (2012) *Candida albicans* isolates from the gut of critically ill patients respond to phosphate limitation by expressing filaments and a lethal phenotype. *Plos One* **7**: e30119. doi:30110.31371/journal.pone.0030119.

Rosenfeld, L., Reddi, A.R., Leung, E., Aranda, K., Jensen, L.T., and Culotta, V.C. (2010) The effect of phosphate accumulation on metal ion homeostasis in *Saccharomyces cerevisiae*. *Journal of Biological Inorganic Chemistry* **15**: 1051-1062.

Roué, M., Quévrain, E., Domart-Coulon, I., and Bourguet-Kondracki, M.L. (2012) Assessing calcareous sponges and their associated bacteria for the discovery of new bioactive natural products. *Natural Product Reports* **29**: 739-751.

Rypien, K.L., Ward, J.R., and Azam, F. (2010) Antagonistic interactions among coral-associated bacteria. *Environmental Microbiology* **12**: 28-39.

Sabarathnam, B., Manilal, A., Sujith, S., Seghal Kiran, G., Selvin, J., Thomas, A., and Ravji, R. (2010) Role of sponge associated actinomycetes in the marine phosphorrous biogeochemical cycles. *American-Eurasian Journal of Agriculture & Environmental Science* **8 (3)**: 253-256.

Sala, M.M., Peters, F., Gasol, J.M., Pedrós-Alió, C., Marrasé, C., and Vaqué, D. (2002) Seasonal and spatial variations in the nutrient limitation of bacterioplankton growth in the northwestern Mediterranean. *Aquatic Microbial Ecology* **27**: 47-56.

Sannigrahi, P., Ingall, E.D., and Benner, R. (2006) Nature and dynamics of phosphorus-containing components of marine dissolved and particulate organic matter. *Geochimica Et Cosmochimica Acta* **70**: 5868-5882.

Santos, O.C., Pontes, P.V., Santos, J.F., Muricy, G., Giambiagi-deMarval, M., and Laport, M.S. (2010) Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. *Research in Microbiology* **161**: 604-612.

Sañudo-Wilhelmy, S.A., Kustka, A.B., Gobler, C.J., Hutchins, D.A., Yang, M., Lwiza, K., Burns, J., Capone, D.G., Raven, J.A., and Carpenter, E.J. (2001) Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. *Nature* **411**: 66-69.

Schaible, M.E., and Kaufmann, S.H.E. (2004) Iron and microbial infection. *Nature Reviews Microbiology* **2**: 946-953.

Schembri, M.A., Bayly, R.C., and Davies, J.K. (1995) Phosphate concentration regulates transcription of the *Acinetobacter* polyhydroxyalkanoic acid biosynthetic genes. *Journal of Bacteriology* **177**: 4501-4507.

Schertzer, J.W., Boulette, M.L., and Whiteley, M. (2009) More than a signal: non-signaling properties of quorum sensing molecules. *Trends in Microbiology* **17**: 189-195.

Schrettl, M., Bignell, E., Kragl, C., Joechl, C., Rogers, T., Arst, H.N., Jr., Haynes, K., and Haas, H. (2004) Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. *Journal of Experimental Medicin* **200**: 1213-1219.

Schwedt, A. (2011) Physiology of a marine *Beggiatoa* strain and the accompanying organism *Pseudovibrio* sp. – a facultatively oligotrophic bacterium. Bremen: Universität Bremen.

Sertan-de Guzman, A.A., Predicala, R.Z., Bernardo, E.B., Neilan, B.A., Elardo, S.P., Mangalindan, G.C., Tasdemir, D., Ireland, C.M., Barraquio, W.L., and Concepcion, G.P. (2007) *Pseudovibrio denitrificans* strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate. *FEMS Microbiology Letters* **277**: 188-196.

Shieh, W.Y., Lin, Y.T., and Jean, W.D. (2004) *Pseudovibrio denitrificans* gen. nov., sp nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2307-2312.

Silva, T.R., Verde, L.C.L., Neto, E.V.S., and Oliveira, V.M. (2013) Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. *International Biodeterioration & Biodegradation* **81**: 57-70.

Singer, E., Webb, E.A., Nelson, W.C., Heidelberg, J.F., Ivanova, N., Pati, A., and Edwards, K.J. (2011) Genomic potential of *Marinobacter aquaeolei*, a biogeochemical "opportunitroph". *Applied and Environmental Microbiology* **77**: 2763-2771.

Slater, H., Crow, M., Everson, L., and Salmond, G.P. (2003) Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. *Molecular Microbiology* **47**: 303-320.

Smith, S.V. (1984) Phosphorus versus nitrogen limitation in the marine-environment. *Limnology and Oceanography* **29**: 1149-1160.

Souza, V., Eguiarte, L.E., Siefert, J., and Elser, J.J. (2008) Microbial endemism: does phosphorus limitation enhance speciation? *Nature Reviews Microbiology* **6**: 559-564.

Sowell, S.M., Abraham, P.E., Shah, M., Verberkmoes, N.C., Smith, D.P., Barofsky, D.F., and Giovannoni, S.J. (2010) Environmental proteomics of microbial plankton in a highly productive coastal upwelling system. *The ISME Journal* **5**: 856-865.

Steinberger, R.E., Allen, A.R., Hansma, H.G., and Holden, P.A. (2002) Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa*-unsaturates biofilms. *Microbial Ecology* **43**: 416-423.

Stock, J.B., Ninfa, A.J., and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiological Reviews* **53**: 450-490.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews* **71**: 295-347.

Temperton, B., Gilbert, J.A., Quinn, J.P., and McGrath, J.W. (2011) Novel analysis of oceanic surface water metagenomes suggests importance of polyphosphate metabolism in oligotrophic environments. *Plos One* **6**: e16499. doi:16410.11371/journal.pone.0016499.

Thakur, A.N., Thakur, N.L., Indap, M.M., Pandit, R.A., Datar, V.V., and Müller, W.E.G. (2005) Antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria. *Marine Biotechnology* **7**: 245-252.

Thakur, N.L., Hentschel, U., Krasko, A., Pabel, C.T., Anil, A.C., and Müller, W.E.G. (2003) Antibacterial activity of the sponge *Suberites domuncula* and its primmorphs: potential basis for epibacterial chemical defense. *Aquatic Microbialogy Ecology* **31**: 77-83.

Thiel, V., and Imhoff, J.F. (2003) Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges. *Biomolecular Engineering* **20**: 421-423.

Thiele, I., and Palsson, B.Ø. (2010) A protocol for generating a high-quality genome-scale metabolic reconstruction. *Nature Protoc*ols **5**: 93-121.

Thingstad, T.F., Zweifel, U.L., and Rassoulzadegan, F. (1998) P limitation of heterotrophic bacteria and phytoplankton in the northwest Mediterranean. *Limnology and Oceanography* **43**: 88-94.

Thingstad, T.F., Krom, M.D., Mantoura, R.F.C., Flaten, G.A.F., Groom, S., Herut, B., Kress, N., Law, C.S., Pasternak, A., Pitta, P., Psarra, S., Rassoulzadegan, F., Tanaka, T., Tselepides, A., Wassmann, P., Woodward, E.M.S., Riser, C.W., Zodiatis, G., and Zohary, T. (2005) Nature of phosphorus limitation in the ultraoligotrophic eastern Mediterranean. *Science* **309**: 1068-1071.

Thomas, S., Burdett, H., Temperton, B., Wick, R., Snelling, D., McGrath, J.W., Quinn, J.P., Munn, C., and Gilbert, J.A. (2010) Evidence for phosphonate usage in the coral holobiont. *The ISME Journal* **4**: 459-461.

Thompson, L.R., Field, C., Romanuk, T., Ngugi, D.K., Siam, R., El Dorry, H., and Stingl, U. (2013) Patterns of ecological specialization among microbial populations in the Red Sea and diverse oligotrophic marine environments. *Ecology and Evolution* **3**: 1780-1797.

Thoms, C., Horn, M., Wagner, M., Hentschel, U., and Proksch, P. (2003) Monitoring microbial diversity and natural product profiles of the sponge *Aplysina cavernicola* following transplantation. *Marine Biology* **142**: 685-692.

Thomson-Bulldis, A., and Karl, D. (1998) Application of a novel method for phosphorus determinations in the oligotrophic North Pacific Ocean. *Limnology and Oceanography* **43**: 1565-1577.

Tindale, A.E., Mehrotra, M., Ottem, D., and Page, W.J. (2000) Dual regulation of catecholate siderophore biosynthesis in *Azotobacter vinelandii* by iron and oxidative stress. *Microbiology* **146**: 1617-1626.

Tranvik, L.J. (1993) Microbial transformation of labile Dissolved Organic-Matter into Humic-Like Matter in seawater. *FEMS Microbiol Ecol* **12**: 177-183.

Tyrrell, T. (1999) The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature* **400**: 525-531.

Van Mooy, B.A.S., and Fredricks, H.F. (2010) Bacterial and eukaryotic intact polar lipids in the eastern subtropical South Pacific: water-column distribution, planktonic sources, and fatty acid composition. *Geochimica Et Cosmochimica Acta* **74**: 6499-6516.

Van Mooy, B.A.S., Rocap, G., Fredricks, H.F., Evans, C.T., and Devol, A.H. (2006) Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 8607-8612.

Van Mooy, B.A.S., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblížek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappé, M.S., and Webb, E.A. (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature* **458**: 69-72.

VanBogelen, R.A., Olson, E.R., Wanner, B.L., and Neidhardt, F.C. (1996) Global analysis of proteins synthesized during phosphorus restriction in *Escherichia coli*. *Journal of Bacteriology* **178**: 4344-4366.

VanCappellen, P., and Ingall, E.D. (1996) Redox stabilization of the atmosphere and oceans by phosphorus-limited marine productivity. *Science* **271**: 493-496.

Villanueva, L., Bale, N., Hopmans, E.C., Schouten, S., and Damsté, J.S. (2013) Diversity and distribution of a key sulpholipid biosynthetic gene in marine microbial assemblages. *Environmental Microbiology* **Article first published online**: DOI: 10.1111/1462-2920.12202.

von Kruger, W.M.A., Humphreys, S., and Ketley, J.M. (1999) A role for the PhoBR regulatory system homologue in the *Vibrio cholerae* phosphate limitation response and intestinal colonization. *Microbiology* **145**: 2463-2475.

Wagner-Döbler, I., Thiel, V., Eberl, L., Allgaier, M., Bodor, A., Meyer, S., Ebner, S., Hennig, A., Pukall, R., and Schulz, S. (2005) Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine alphaproteobacteria. *Chembiochem* **6**: 2195-2206.

Wagner, J.K., Setayeshgar, S., Sharon, L.A., Reilly, J.P., and Brun, Y.V. (2006) A nutrient uptake role for bacterial cell envelope extensions. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 11772-11777.

Wanner, B.L. (1996) Phosphorus assimilation and control of the phosphate regulon. In *Escherichia coli and Salmonella. Cellular and molecular biology*. Neidhardt, F.C., Curtis III, R., Ingraham, J.L., Lin, E.C.C., Brooks low, K., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M., and Umbarger, H.E. (eds). Washington, D.C.: ASM press, pp. 1357-1381.

Want, E.J., Nordström, A., Morita, H., and Siuzdak, G. (2007) From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. *Journal of Proteome Research* **6**: 459-468.

Weber, R.J.M., and Viant, M.R. (2010) MI-Pack: Increased confidence of metabolite identification in mass spectra by integrating accurate masses and metabolic pathways. *Chemometrics and Intelligent Laboratory Systems* **104**: 75-82.

Webster, N.S., and Hill, R.T. (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. *Marine Biology* **138**: 843-851.

Williams, P., Winzer, K., Chan, W.C., and Camara, M. (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philosophical Transaction of the Royal Society London B: Biological Science* **362**: 1119-1134.

Winterbourn, C.C. (1995) Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology Letters* **82-83**: 969-974.

Wu, J.F., Sunda, W., Boyle, E.A., and Karl, D.M. (2000) Phosphate depletion in the western North Atlantic Ocean. *Science* **289**: 759-762.

Xu, J., Kim, J., Danhorn, T., Merritt, P.M., and Fuqua, C. (2012) Phosphorus limitation increases attachment in *Agrobacterium tumefaciens* and reveals a conditional functional redundancy in adhesin biosynthesis. *Research in Microbiology* **163**: 674-684.

Yahel, G., Whitney, F., Reiswig, H.M., Eerkes-Medrano, D.I., and Leys, S.P. (2007) In situ feeding and metabolism of glass sponges (Hexactinellida, Porifera) studied in a deep temperate fjord with a remotely operated submersible. *Limnology and Oceanography* **52**: 428-440.

Yoshida, T., Hayashi, K., and Ohmoto, H. (2002) Dissolution of iron hydroxides by marine bacterial siderophore. *Chemical Geology* **184**: 1-9.

Young, C.L., and Ingall, E.D. (2010) Marine dissolved organic phosphorus composition: insights from samples recovered using combined electrodialysis/reverse osmosis. *Aquatic Geochemistry* **16**: 563-574.

Young, K.D. (2006) The selective value of bacterial shape. *Microbiology and Molecular Biology Reviews* **70**: 660-703.

Yuan, Z.C., Zaheer, R., and Finan, T.M. (2005) Phosphate limitation induces catalase expression in *Sinorhizobium meliloti*, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. *Molecular Microbiology* **58**: 877-894.

Yuan, Z.C., Zaheer, R., Morton, R., and Finan, T.M. (2006) Genome prediction of PhoB regulated promoters in *Sinorhizobium meliloti* and twelve proteobacteria. *Nucleic Acids Research* **34**: 2686-2697.

Zaborin, A., Gerdes, S., Holbrook, C., Liu, D.C., Zaborina, O.Y., and Alverdy, J.C. (2012) *Pseudomonas aeruginosa* overrides the virulence inducing effect of opioids when it senses an sbundance of phosphate. *Plos One* **7**: e34883. doi:34810.31371/journal.pone.0034883.

Zaborin, A., Romanowski, K., Gerdes, S., Holbrook, C., Lepine, F., Long, J., Poroyko, V., Diggle, S.P., Wilke, A., Righetti, K., Morozova, I., Babrowski, T., Liu, D.C., Zaborina, O., and Alverdy, J.C. (2009) Red death in *Caenorhabditis elegans* caused by *Pseudomonas aeruginosa* PAO1. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 6327-6332.

Zhang, W., Li, F., and Nie, L. (2010) Integrating multiple 'omics' analysis for microbial biology: application and methodologies. *Microbiology* **156**: 287-301.

Zhang, X., Nakahara, T., Murase, S., Nakata, H., Inoue, T., and Kudo, T. (2013) Physiological characterization of aerobic culturable bacteria in the intestine of the sea cucumber *Apostichopus japonicus*. *Journal of General and Applied Microbiology* **59**: 1-10.

Zhu, J., Sova, P., Xu, Q., Dombek, K.M., Xu, E.Y., Vu, H., Tu, Z., Brem, R.B., Bumgarner, R.E., and Schadt, E.E. (2012) Stitching together multiple data dimensions reveals interacting metabolomic and transcriptomic networks that modulate cell regulation. *PLoS Biology* **10**: e1001301. doi:1001310.1001371/journal.pbio.1001301.

Contributed works

The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis

Bondarev, V.[1], Richter, M.[2], **Romano, S.[1]**, Piel, J.[3 5], Schwedt, A.[1], Schulz-Vogt, H.N.[4],

Manuscript published in *Environmental microbiology*, 2013, 15(7): 2095-2133

[1] Max Planck Institute for Marine Microbiology, Ecophysiology Group and [2] Microbial Genomics and Bioinformatics Research Group, Bremen, Germany
[3] University of Bonn, Kekulé-Institute of Organic Chemistry and Biochemistry, Bonn, Germany
[4] Leibniz-Institute for Baltic Sea Research Warnemuende (IOW), Section Biological Oceanography, Rostock, Germany
[5] ETH Zurich, Institute of Microbiology, Zurich, Switzerland

Contributions:

I performed the phylogenetic analyses based on the 16S rRNA genes. I performed the denitrification experiments and the experiments for the production of tropodithietic acid for strain *Pseudovibrio* sp. FO-BEG1. I actively contributed to the writing of the paper.

Summary

Bacteria belonging to the *Pseudovibrio* genus have been isolated worldwide, especially from coastal waters and from marine invertebrates. Nevertheless, little is known about their genomic features and their overall physiology. In the present study, we sequenced and analyzed the genome of *Pseudovibrio* sp. FO-BEG1 and we compared it with the available draft genome of *Pseudovibrio* sp. JE062. The first strain was isolated from an enrichment culture of *Beggiatoa* sp. 35Flor and the second was isolated off the coast of Florida from the sponge *Mycale laxissima*. In addition to the genome comparison, most of the physiological features identified at the genomic level were experimentally verified. The presented data show that both strains are generalistic bacteria capable of importing and oxidizing a wide range of organic and inorganic compounds to meet their carbon, nitrogen, phosphorous, and energy requirements under both, oxic and anoxic conditions. The high metabolic versatility was also reflected in the high number of genes for TRAP and ABC transporters and in the ability of both strains to synthesize a big variety of vitamins. Besides the versatile metabolic abilities, our study reveals the presence in both *Pseudovibrio* genomes of a large number of open reading frames and gene clusters potentially involved in the synthesis of bioactive secondary metabolites and in the production of factors important for the interaction with the host. Both *Pseudovibrio* strains have the genomic potential to attach to host cells, interact with the eukaryotic cell machinery, produce secondary metabolites, and supply the host with cofactors.

Substrate use of *Pseudovibrio* sp. growing in ultraoligotrophic seawater

Schwedt, A.[1], Seidel, M.[2†], Dittmar, T.[2], Simon, M.[2], Bondarev, V.[1], **Romano, S.[1]**, Lavik, G.[1], Schulz-Vogt, H.N.[3]

Manuscript in preparation

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany
[2] Institute of Chemistry and Biology of the Marine Environment, Carl von Ossietzky University of Oldenburg, Germany
[3] Leibniz-Institute for Baltic Sea Research Warnemuende (IOW), Section Biological Oceanography, Rostock, Germany
[†]current address: University of Georgia, Athens, GA, USA

Contributions:
I performed the Biolog™ experiments and I provided comments on the manuscript.

Summary

Marine planktonic bacteria often thrive in habitats characterized by extremely low concentrations of dissolved organic matter (DOM). The composition of DOM in the ocean can vary greatly and bacteria are able to use only a portion of its molecules according to their metabolic features. In order to investigate the substrate usage under ultraoligotrophic conditions of the heterotrophic marine bacterium *Pseudovibrio* sp. FO-BEG1, we analyzed the DOM composition before and after bacterial growth in artificial and natural seawater. The compositional comparison of both setups was performed investigating (i) the dissolved organic carbon (DOC) and the total dissolved nitrogen (TDN), (ii) the composition of DOM by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, and (iii) the amino acid content by high performance liquid chromatography. Despite special precautions during the preparation of the artificial seawater medium, a DOC concentration of 0.06 mg C L^{-1} (5 µmol C L^{-1}) was measured, which was an order or magnitude below natural seawater, which has 0.9 mg C L^{-1} (75 µmol C L^{-1}). Under both conditions, DOC concentrations did not decrease measurably during growth, yet, cell numbers increased from about 20 cells mL^{-1} to 2×10^4 cells mL^{-1} in artificial and to 8×10^5 cells mL^{-1} in natural seawater. No nitrogen fixation was detectable and only 1% of cellular carbon derived from CO_2 fixation, even though no fixed nitrogen or carbon source was added. The ultra-high resolution analysis of the DOM composition revealed that *Pseudovibrio* sp. FO-BEG1 was able to proliferate under nutrient-poor conditions mainly using traces of sugars, amino acids, and detergents present in the cultivation media. This catabolic flexibility observed

under oligotrophic conditions was also confirmed during the incubation of oligotrophically pre-grown cells in Biolog™ plates. In these experiments cells showed a completely different catabolic pattern than cells pre-grown under nutrient rich conditions. This study suggests that heterotrophic bacteria are able to thrive under extremely low DOC concentrations, even lower than natural oligotrophic seawater, via the diversification of the spectra of metabolized molecules.

Isolation of facultatively oligotrophic bacteria

Schwedt, A.[1Δ], **Romano, S**.[1Δ], Bondarev, V.[1], Seidel, M.[2†], Dittmar, T.[2], Schulz-Vogt, H.N.[3]

[Δ] **these authors contributed equally to this work**

Manuscript in preparation

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany

[2] Institute of Chemistry and Biology of the Marine Environment, Carl von Ossietzky University of Oldenburg, Germany

[3] Leibniz-Institute for Baltic Sea Research Warnemuende (IOW), Section Biological Oceanography, Rostock, Germany

[†]current address: University of Georgia, Athens, GA, USA

Contributions:

I applied the isolation method developed by Anne Schwedt and Heide N. Schulz-Vogt for the isolation of marine heterotrophic bacteria from different environmental samples. I performed growth experiments under oligotrophic and eutrophic conditions and I carried out a phylogenetic analysis based on the 16S rRNA genes of the obtained isolates. I collaborated with Anne Schwedt in writing the manuscript

Summary

The dissolved organic carbon (DOC) in the open ocean is several orders of magnitude lower than in other environments and apparently bacteria are able to use only a fraction of its molecules. Therefore, in many marine systems bacteria thrive under oligotrophic conditions. Due to this particular low availability of nutrients the isolation and cultivation of heterotrophic marine bacteria using common cultivation media and techniques is challenging. In the present study, we developed the CANgrow-method (changing availability of nutrients - growth method) as a strategy to isolate facultatively oligotrophic bacteria from different marine habitats. In this method, the three initial transfer steps of cultures grown in pure artificial seawater preselected for bacteria multiplying under oligotrophic conditions. Pure cultures were obtained by three subsequent transfers of single colonies on organic rich solid medium. Finally, at least seven transfers in pure artificial seawater were performed to confirm the ability of the isolates to grow under these conditions. All obtained isolates were able to grow in pure artificial seawater with a residual organic carbon content ranging from 15 ± 5 to μmol C L^{-1} to 38 ± 5 to μmol C L^{-1}. These concentrations were more the two times lower than what observed in natural seawater. Most of the isolates, which were able to grow under these oligotrophic conditions, were affiliated to common and ubiquitous marine heterotrophic bacteria belonging to the *Actinobacteria*, *Alpha*- and *Gammaproteobacteria*. These data suggest that the ability to proliferate under extreme nutrient limitation, which is a condition commonly encountered in the open ocean, is a shared feature among

heterotrophic bacteria, and it is likely to be more widespread than previously thought.

Acknowledgments

To be honest I am a bit nervous to write this section, because this is the only part that everyone who will have this thesis in his/her hands will read. I will try to do my best, and I hope I will not forget anyone.

First of all I would like to thank Prof. Heide N. Schulz-Vogt for giving me the opportunity to work with this fascinating bacterium in this prestigious institute. Thank you, Prof. Dr. Fischer for your agreement to review my thesis, for your patience, and for your wise advice. I would like to thank especially Jens and Thorsten for all the scientific discussions we had and for the advices you always gave me.

Many thanks also to all the Geochemistry group at the ICBM in Oldenburg for the support during the MS experiments. Special thanks to Katrin Klaproth for the support and the patience during the uncountable analysis you helped with.

Dr. Martin Kölling, thank you for allowing us to measure our samples with the ICP-OES and thank you Silvana Pape, for the support during the measurements.

Mark Viant and Ralf Weber, thank you very much for the bioinformatic assistance during the exo-metabolome study.

Thank to the Bioviotica members for helping in the many HPLC runs we did in the attempt to isolate the "famous siderophore".

I would like to thank all members of the ex-Ecophysiology group, Anne, Anne-Christin, Sandra, Martina, Susanne, Christin, Jörg, Sascha, Artur, Vladimir and Verena for the fun, the inspiring discussion, and the help during these years.

I would especially like to thank Verena and Artur for being of great support during this last phase of my Ph.D inside and outside the MPI.

A special acknowledgment is for Vladimir. Thank you, "dude" for the help with the German burocracy and with my talkative landlord. Thank you for all the scientific and non-scientific discussions and most of all for being a good friend.

Aknowledgments

Jan, Johannes, Carsten, Sebastian, Rebecca thank you for being such nice office-mates. Jan and Johannes, I especially thank you for many discussions about science and life. Thank you for being the sensitive persons you are and for the epic evenings spent together with J.R.R. Tolkien.

Of course, I would like to thank also all the members of the Microbiology Department, for creating such a nice working environment. Prof. Widdel, I would like to thank you for the many times I came to you for asking all sorts of nifty questions.

There are many people that I met during these three and half years. Many people with whom I shared ideas and enthusiasm for science. Laura van Nifrtik and Sara Neumann, Alban, Hanno, Alexander, Antonio, Marnix, Michael, Robert, Florin, Sten, Tim, Clara, thank you all.

Special thanks to Bernd, to be always ready and eager to help in finding all sorts of unusual and rare articles.

I would also like to spent few words for Luigi Michaud. We spent working together 1 months, it was really fun. I got to know an honest and sensitive person. Now you are sleeping in the Antartic, which you loved so much. Ciao Luigi, we will miss you.

Thank you, Nico and Francesco for keeping alive the "December-metal" spirit!

I think that all the pages of this thesis will not be enough to thank you. Thank you for being there always, thank you for shining every morning close to me, thank you for the support and the strength you gave me, thank you for sharing your life with me. Thank you, Rebecca.

Aknowledgments

And last but not least, I would like to truly thank my family. Thank you, Laura and Gianluca, for being there to show me that after all you can find happiness in small things. Thank you, mamma and papà, for supporting me no matter what.

Infine vorrei ringraziare la mia famiglia. Grazie Laura e Gianluca per esserci, per avermi fatto ridere e per avermi mostrato una volta ancora che la felicità la puoi trovare anche nelle piccole cose. Grazie Alessio, perchè "tichitichitaca..". Grazie mamma e grazie papà. Grazie per il supporto, grazie per aver imparato ad usare internet, grazie per aver combattuto contro Skype per mantenervi in contatto con me in questi anni, e grazie per aver preso l'aereo per la prima volta per raggiungermi. Alla fine ci sono riuscito, forse perchè sono un Romano?

Erklärung der selbstständigen Erarbeitung

Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen vom 14.03.2007 für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche.

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel "Response to phosphate limitation of *Pseudovibrio* sp. FO-BEG1, a versatile bacterium with the potential for a symbiotic lifestyle"

1. ohne unerlaubte fremde Hilfe angefertigt habe,
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, den 3. Februar 2014

Stefano Romano