Phosphate Limitation Triggers the Dissolution of Precipitated Iron by the Marine Bacterium *Pseudovibrio* sp. FO-BEG1

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Phosphorus is an essential nutrient for all living organisms. In bacteria, the preferential phosphorus source is phosphate, which is often a limiting macronutrient in many areas of the ocean. The geochemical cycle of phosphorus is strongly interconnected with the cycles of other elements and especially iron, because phosphate tends to adsorb onto iron minerals, such as iron oxide formed in oxic marine environments. Although the response to either iron or phosphate limitation has been investigated in several bacterial species, the metabolic interplay between these two nutrients has rarely been considered. In this study we evaluated the impact of phosphate limitation on the iron metabolism of the marine bacterium *Pseudovibrio* sp. FO-BEG1. We observed that phosphate limitation led to an initial decrease of soluble iron in the culture up to three times higher than under phosphate surplus conditions. Similarly, a decrease in soluble cobalt was more pronounced under phosphate limitation. These data point toward physiological changes induced by phosphate limitation that affect either the cellular surface and therefore the metal adsorption onto it or the cellular metal uptake. We discovered that under phosphate limitation strain FO-BEG1, as well as selected strains of the *Roseobacter* clade, secreted iron-chelating molecules. This leads to the hypothesis that these bacteria might release such molecules to dissolve iron minerals, such as iron-oxyhydroxide, in order to access the adsorbed phosphate. As the adsorption of phosphate onto iron minerals can significantly decrease phosphate concentrations in the environment, the observed release of iron-chelators might represent an as yet unrecognized link between the biogeochemical cycle of phosphorus and iron, and it suggests another biological function of iron-chelating molecules in addition to metal-scavenging.

**Keywords:** *Pseudovibrio*, phosphate limitation, siderophores, *Roseobacter*, chelators, metabolomics, bacterial physiology, mass spectrometry
INTRODUCTION

Phosphorus (P) is an important component of biomolecules and a fundamental element in cellular regulatory processes. Bacteria acquire phosphorus via phosphate (P$_i$), which in natural environments is often scarce. In marine waters, the concentration of P$_i$ may be in the nanomolar range, thus limiting bacterial growth and productivity in several areas of the ocean, at least during part of the year (Cotner et al., 1997; Wu et al., 2000; Thingstad et al., 2005). Bacteria evolved a sophisticated system to overcome suboptimal P availability, and it has been shown that P$_i$ limitation affects not only cellular P metabolism, but also central metabolism, production of secondary metabolites, and the expression of virulence-related genes (Martin, 2004; Hsieh and Wanner, 2010). But while this response has been well-studied in model organisms and organisms of biotechnological interest, such as Escherichia coli, Sinorhizobium meliloti, and Streptomyces coelicolor, less is known about the response of heterotrophic marine bacteria (Ishige et al., 2003; Krol and Becker, 2004; Rodriguez-Garcia et al., 2007; Romano et al., 2015).

In the ocean, the geochemical P cycle is tightly linked to the geochemical cycles of other elements, especially iron (Fe), given that P$_i$ strongly adsorbs onto Fe minerals. Indeed, in some parts of the ocean this process removes up to 40% of the total soluble P$_i$ input (Berner, 1973; Froelich et al., 1977; Feely et al., 1998; Châtellier et al., 2013). This phenomenon has been also described in freshwater systems (Cosmidis et al., 2014). Like P, Fe is often a limiting micronutrient in marine surface waters. Under oxic conditions Fe(III) is the only thermodynamically stable form, but its solubility at circumneutral pH is several orders of magnitude lower than the average amount of Fe required for bacterial growth (Millero, 1998; Andrews et al., 2003). Due to its low solubility and concentration in oxic environments, microorganisms produce and secrete organic molecules, siderophores, that bind Fe(III) with high affinity and are able to dissolve Fe particles, thus facilitating Fe acquisition. These molecules are produced by microorganisms in response to Fe limitation, and allow them to scavenge Fe from the environment (Wandersman and Delepelaire, 2004).

Biogeochemical studies have linked the concentration of P$_i$ with the concentrations of other micronutrients in different areas of the ocean (Saito and Moffett, 2002; Paytan and McLaughlin, 2007; Jakuba et al., 2008). Moreover, molecular studies have underlined how different enzymes involved in P$_i$ and organo-P$_i$ metabolism have metal ions as cofactor (Kulakova et al., 2003; Sebastian and Ammerman, 2009; Kathuria and Martiny, 2011; Yong et al., 2014). All together these data suggest that the metabolism of P$_i$ is closely coupled to that of other micronutrients in microorganisms. However, little is known about the effect of P$_i$ limitation on the metabolism of Fe and other micronutrients in bacteria, and, to the best of our knowledge there have been no such studies in marine heterotrophic bacteria. In previous proteomic and metabolomic studies we showed that the activation of the P$_i$ starvation response, as a consequence of P$_i$-limited growth, has a drastic impact on both the central and the secondary metabolism of strain FO-BEG1 (Romano et al., 2014, 2015), a marine heterotrophic bacterium belonging to the broadly distributed and metabolically versatile genus Pseudovibrio (Bondarev et al., 2013; Schwedt et al., 2015; Romano et al., 2016). In this study we aimed to investigate the impact of P$_i$ limitation on the micronutrient metabolism, especially that of Fe, of strain FO-BEG1. Surprisingly, we observed that P$_i$ limitation triggered the secretion of Fe-chelating molecules into the medium, a phenomenon which was also observed to a certain extent in cultures of strains belonging to the abundant and ubiquitous Roseobacter clade.

MATERIALS AND METHODS

Growth Conditions

Strain FO-BEG1 was cultivated under P$_i$-limited (−P$_i$; C:N:P ratio: 600:100:1) and P$_i$-surplus conditions (+P$_i$; C:N:P ratio: 43:7:1) in carbohydrate mineral medium (CMM), containing per liter: 15 g NaCl; 1.5 g MgCl$_2$ hexahydrate; 1 g K$_2$SO$_4$; 0.27 g (10 mmol L$^{-1}$) NH$_4$Cl; 0.005 g CaCl$_2$ (Bondarev et al., 2013; Romano et al., 2015). The medium was buffered using Tris-HCl (3.9 g L$^{-1}$), brought to pH 8, and after autoclaving Fe(II), and Co were supplied to a final concentration of 7.5 and 0.75 μmol L$^{-1}$, respectively, together with other micronutrients from an acidified trace-element solution (Widdel and Pfennig, 1984). Additionally, the medium was amended with glucose (10 mmol L$^{-1}$), 1 ml L$^{-1}$ tungsten/selenium solution (Brysch et al., 1987), 1 ml L$^{-1}$ of four vitamin solutions (prepared according to Aeckersberg et al., 1991), and for the P$_i$ surplus conditions, P$_i$ was added from a K$_2$HPO$_4$ solution, to a final concentration of 1.4 mmol L$^{-1}$. In the P$_i$-limited medium no P$_i$ was added, and the only P$_i$ source (100 μmol L$^{-1}$) derived from the buffer used to prepare the vitamin solutions. The cultures were incubated at 28°C in the dark under shaking conditions (120 rpm). All Roseobacter strains, were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), and were similarly cultivated and incubated at 28°C (Phaeobacter inhibens DSM-17395, DSM-16374) or 30°C (Rugeria pomeroyi DSM-15171). All experiments were performed using a 0.001% v/v inoculum, consisting of cells grown under +P$_i$ conditions. Bacterial growth was monitored by measuring the optical density (OD) at 600 nm using an Eppendorf BioPhotometer (Eppendorf AG, Germany). The OD$_{600}$ was then correlated with the cell number, determined using a counting chamber (Brand GmbH, Germany; data not shown). All cultivation experiments were performed in biological triplicates and included controls, consisting of sterile un-inoculated media, which were incubated under the same conditions described for the cultures. For all cultures and sterile controls, the Fe and Co concentrations in the cell-free supernatants were determined over the course of the incubations using inductively coupled plasma optical emission spectroscopy (ICP-OES), and P$_i$ was determined colorimetrically using the ammonium molybdate method (Hansen and Karoleff, 1999).

To assess whether the increase in the Fe concentration measured during P$_i$-limited growth of strain FO-BEG1 was due...
to dissolution of the precipitated Fe fraction, two experiments, hereafter referred to as the “refresh experiment” and the “EDTA experiment,” were performed. A schematic overview of the cultivation approaches used for strain FO-BEG1 is reported in Supplementary Figure S1. In the refresh experiment (Supplementary Figure S1B), strain FO-BEG1 was incubated in CMM under $-P_i$ conditions until Fe uptake reached the previously detected maximum. The biomass of all replicates was then harvested by centrifugation at 7,000 × g and 15°C for 10 min using a J-26XP Beckmann centrifuge and a JA-10 rotor (Beckman Instruments, Inc., Palo Alto, CA, USA). The resulting cell pellets were washed with Fe-free sterile artificial seawater and then equally divided to re-inoculate fresh Fe-free $P_i$-limited CMM in biological triplicates. These cultures were incubated again in the dark at 28°C with shaking at 120 rpm. Bacterial growth was then monitored for 95 h by measuring the OD$_{600}$.

In the EDTA experiment (Supplementary Figure S1C), strain FO-BEG1 was cultivated in $+P_i$ and $-P_i$ CMM, both prepared using an acidified trace element solution not containing Fe, which was instead supplied separately as EDTA-complex to a final concentration of 7.5 µmol L$^{-1}$ and 100 µmol EDTA L$^{-1}$. The cultures were incubated as described above and all glassware was acid-washed prior to use. The Fe concentrations in the cell-free supernatants were determined by ICP-OES. Fe-chelating molecules were extracted from the cell-free supernatant of a 10 L culture grown in the $-P_i$ CMM, supplemented with the original acidified trace element solution, in 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 bioreactor at a constant flow rate of 1 L min$^{-1}$. Throughout the incubation time, both pH and bacterial growth (OD$_{600}$ nm) were monitored.

Trace Metal Measurement, Dialysis, and Size-Exclusion Chromatography (SEC) of the Cell-Free Supernatants

The Fe and Co concentrations in the supernatants were measured using ICP-OES. The samples were collected during bacterial growth, centrifuged at 11,000 × g and 5°C for 10 min, filtered through Millipore filters (0.22-µm pore size; Millipore, Bedford, MA, USA), diluted 1:10 in 1% supra-pure HNO$_3$ (Merck, Darmstadt, Germany), and stored at 4°C until further processing. They were analyzed using an Agilent 720 ICP-OES (Agilent Technologies, Palo Alto, CA, USA). Cell-free supernatants of the $-P_i$ cultures were dialyzed overnight at 4°C against MQ under stirring conditions using a Spectra/Por TM dialysis tube (molecular cut-off: 1000 Da; Serva, Heidelberg, Germany). The Fe content was measured before and after dialysis via ICP-OES.

Size-exclusion chromatography (SEC) of the $-P_i$ cell-free supernatant was performed by injecting 1 mL of sample into a Äkta purifier system (GE Healthcare, Freiburg, Germany) equipped with a Superdex$^TM$ peptide 10/300 GL column (GE Healthcare, Freiburg, Germany), detecting absorption at 280 nm. The samples were run using the buffer Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), at pH 7.8 and a flow rate of 0.25 mL min$^{-1}$. During the chromatographic run, 1 mL fractions were collected using a Frac-950 fraction collector (GE Healthcare, Piscataway, NJ, USA). The column was calibrated using ribonuclease A, aprotinin, [d-Ala$^2$, d-Leu$^3$]-enkephalin, and vitamin B12 (Sigma-Aldrich Chemie GmbH, Munich, Germany), all of which were run under the above-described conditions. The total volume of the column was estimated based on the elution time of the ions in solution, detected as an increase in conductivity. Each fraction was then diluted 1:10 in 1% supra-pure HNO$_3$ and its Fe content was analyzed using ICP-OES.

Assay of the Chelating Activity and Partial Purification of the Chelating Compounds

The presence of Fe-chelating molecules in the cell-free supernatants of cultures grown in CMM, supplemented with the original acidified trace element solution, was tested using 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$^{-1}$ FeCl$_3$, prepared in 0.1 mol L$^{-1}$ HCl, was slowly added under stirring, followed by the dropwise addition of 0.75 mL of 2 mmol L$^{-1}$ CAS. 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, was added. MembraPure water was then added to achieve a final volume of 10 mL. The CAS solution was always prepared fresh before use, and the assay was performed using a mixture of equal volumes of CAS solution and sample.

A crude extract was prepared from a 10 L cell-free supernatant as follows. The cells from a 10 L culture were harvested by centrifugation at 11,000 × g and 5°C for 20 min using a J-26XP Beckmann centrifuge and a JA-10 rotor (Beckman Instruments) and filtered through a 0.22-µm membrane filter (Sartorius AG, Göttingen, Germany). The cell-free supernatant was then acidified to pH 2.6 using 5 mol HCl L$^{-1}$ and mixed in the dark for 1 h with an equal volume of ethyl acetate. The solvent phase was collected and the organic phase was evaporated in a Laborota 4,000 rotary evaporation system (Heidolph, Schwabach, Germany) at 45°C. Evaporation was completed in a speed-vacuum centrifuge (Eppendorf Concentrator 5301, Eppendorf, Hamburg, Germany). Prior to isolating the compounds using high performance liquid chromatography (HPLC), the activity of the crude extract was confirmed using the CAS assay.

The chelating molecules were isolated in collaboration with BioViotica GmbH (Göttingen, Germany), using the extract obtained from the above mentioned 10 L culture. The complexity
of the crude extract was verified in an analytical reverse-phase HPLC run using a C18 column (Nucleodur 100-5 C18ec, 250 mm × 3 mm). Eluents A and B were 100% MQ and 70% MQ and 30% acetonitrile (ACN), respectively, both containing 0.1% trifluoroacetic acid, which was handled under chemical safety conditions. 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, at a flow rate of 2.5 mL min⁻¹. The same conditions were then applied to isolate the metabolites using a preparative C18 column (Kromasil 100-7 C18, 250 mm × 20 mm) at a flow rate of 18 mL min⁻¹ and recording the absorption at 300 nm. The fractions were manually collected during the preparative HPLC runs every time an increase in the absorption was observed. The eluent was evaporated and the fractions were re-dissolved in 70% ACN to test their activity in the CAS assay. The composition of the active fraction 1 was verified by performing two more analytical runs, using two C18 analytical columns (Nucleodur 100-5 C18ec, 250 mm × 3 mm; Phenomenex aqua C18, 250 mm × 2.0 mm) and the above-described running conditions.

Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR-MS) of the Reverse-Phase HPLC Fractions

The HPLC fractions resulted positive in the CAS-assay, fractions 1 and 4, were further analyzed via mass spectrometry (MS). The fractions were diluted 1:10 in 70% ACN and filtered through a PTFE filter (0.2-µm pore size; Rotilabo, Carl Roth GmbH, Karlsruhe, Germany). MS analysis was performed in electrospray ionization (ESI) positive mode with a solariX FT-ICR-MS (Bruker Daltonik GmbH, Bremen, Germany) equipped 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 150–2,500. For each run, 200 broadband scans were accumulated. To facilitate the identification of chelating molecules both HPLC fractions were analyzed before and after the addition of analytical grade GaCl₃ (Sigma-Aldrich Chemie GmbH, Munich, Germany). The acquired mass spectra were analyzed using the Data Analysis software v4.0 SP4 (Bruker Daltonik GmbH, Bremen, Germany). An external mass calibration was performed for all spectra, which were then manually inspected to identify masses showing the characteristic Ga isotopic signature. Since, the two Ga isotopes, ⁶⁹Ga and ⁷¹Ga, have an abundance ratio of 3:2, Ga-containing compounds can be unambiguously identified considering the mass differences and the ratio of the signal intensities of the isotopologues.

Bioinformatics Analysis

The in silico prediction of gene clusters involved in the biosynthesis of siderophores was performed by analyzing both genomic and plasmid sequences of *Pseudovibrio* sp. FO-BEG1 (GenBank ID: CP003147.1 and CP003148.1, respectively) with antiSMASH v3.0 (Weber et al., 2015). The same analysis was performed for the genomes and plasmids of *P. inhibens* strain DSM-17395 (GenBank ID: CP002976.1, CP002977.1, CP002979.1, CP002978.1) and of *R. pomeroyi* (CP000031.1, CP000032.1). The assembly of *P. inhibens* strain DSM-16374 (GenBank ID: GCA_000154765.2) was annotated using PROKKA v1.10 (Seemann, 2014) and then submitted to antiSMASH as well.

RESULTS

Micronutrient Measurements

Pᵢ limitation repressed *Pseudovibrio* growth, leading to a final cell density 2.5- to 3.5-fold lower observed under +Pᵢ conditions (Figure 1). Cells growing under Pᵢ surplus did not metabolize all of the Pᵢ provided, whereas Pᵢ was taken up completely under −Pᵢ conditions during the first ∼30 h of growth, reaching our detection limit of ∼3 µmol L⁻¹ (Figure 1). In the sterile controls abiotic Fe precipitation occurred in both the +Pᵢ and −Pᵢ media, but it was more pronounced under +Pᵢ conditions (Figure 2A). Fe was added to the medium as Fe(II), which oxidized to Fe(III) during the oxic incubation. The solubility of Fe(III) in natural seawater at circumneutral pH is below 1 nmol L⁻¹ (Millero, 1998), which explains the strong precipitation in the CMM. The presence of residual Fe in solution might be explained by the presence of the Tris buffer, which can interact with metal ions (Morrow et al., 1992). Considering that the only difference in medium composition between the two treatments was the Pᵢ concentration, it is most likely that the higher Pᵢ concentrations, or higher concentrations of its counter cations K, were responsible for the increased Fe precipitation. In the cultures both abiotic and biotic processes influenced the Fe concentrations. The biotic processes include cellular uptake and adsorption onto cellular surface, and can be estimated by calculating the difference between the minimum Fe concentrations measured in the cultures and the sterile controls. The biotic Fe removal was 1.5- to 3-fold higher under Pᵢ limitation than under Pᵢ surplus conditions (Figure 2A). Surprisingly, also the biotic Co removal from the media showed significant differences between the two Pᵢ regimes. Under Pᵢ-limited conditions biotic Co removal was 3- to 5-fold higher than that observed under Pᵢ surplus (Figure 2B). In contrast to this, in none of the cultures of the *Roseobacter* strains biotic Fe and Co removal differed between the two Pᵢ regimes (Figure 3, data not shown for Co).

Total Soluble Iron Increases during Bacterial Growth under Pᵢ Limitation

Following the Fe concentration in Figure 2A, it can be seen that only under −Pᵢ conditions the concentration of the total soluble Fe increased again during the late bacterial growth. This phenomenon always started in the late exponential phase and continued during the stationary phase. Theoretically, the increase in Fe may have derived from either the cellular release or the dissolution of the precipitated fraction. We hypothesized that strain FO-BEG1 released molecules able to dissolve the Fe that abiotically precipitated in the medium. In order to test this hypothesis we performed two experiments, hereafter referred to as the “refresh experiment” and the “EDTA experiment”
FIGURE 1 | Growth and Pi uptake of Pseuvibrio sp. strain FO-BEG1 under the two Pi regimes. Pi-limited and Pi-surplus conditions are indicated with filled and empty symbols, respectively. Triangles indicate the cell densities and circles indicate the Pi concentrations over time. Error bars represent the standard deviations of the data as determined from biological triplicates. The data refer to a different experiment than the one described in Figure 2. (Supplementary Figure S1). In the refresh experiment, cells were pre-grown under −Pi conditions, until the previously detected maximum Fe uptake was reached (Figure 2A). Cells were then harvested and re-inoculated into fresh Fe-free −Pi CMM. In this experiment, we hypothesized that since the fresh medium did not contain Fe, any Fe increase would have indicated cellular release. In the EDTA-experiment, strain FO-BEG1 was cultivated in +Pi and −Pi CMM containing an excess of EDTA, in order to complex Fe. Consequently, Fe precipitation would not occur neither in the sterile controls nor in the cultures and a release of Fe-chelators by the cells would not result in an increase of total soluble Fe in the cultures. On the other hand, a release of stored Fe by the cells would increase the total soluble Fe in the cultures, as this Fe would be kept in solution by the excess EDTA.

The results of both experiments clarified that the Fe increase, observed in the original cultivation experiments (Supplementary Figures S1A, 2A), derived from the dissolution of the precipitated fraction, because we did not observe any Fe increase in both the refresh experiment (data not shown) and the EDTA experiment (Figure 2C). As hypothesized, in the EDTA experiment, the addition of EDTA prevented Fe precipitation (see the data for the sterile controls in Figure 2C) and, consistent with the original cultivation approach (Figure 2A), Pi limitation induced a ~2.5-fold higher biotic Fe removal. Also, the EDTA experiment showed that the biotic Fe removal did not continue throughout the late phase of growth, when Fe increased in the first cultivation experiments performed using a CMM amended with uncomplexed Fe(II) (Figures 2A,C and Supplementary Figures S1A,C). This suggests that the bacteria did not continuously took up Fe during the late growth phase. In order to understand whether the secretion of Fe-chelators under Pi limitation is a general phenomenon observed amongst heterotrophic marine bacteria, we performed similar analyses in three strains of the abundant and ubiquitous Roseobacter clade: P. inhibens DSM-17395, DSM-16374 and R. pomeroyi DSM-15171. As in strain FO-BEG1, in strains DSM-17395 and DSM-15171 the soluble Fe increased during the late growth phase only under −Pi conditions (Figures 3A,C and Supplementary Figure S2) whereas in strain DSM-16374 Fe increased in both the −Pi and +Pi cultures (Figure 3B). It is worth noting that strain DSM-16374 increasingly formed tight flocs throughout growth, creating a bias in the cell density estimation (Supplementary Figure S2B).

Detection of Chelating Molecules in the Cell-Free Supernatant of Pi-Limited Pseudovibrio Cultures

The secretion of Fe-chelating molecules by Pseudovibrio under Pi limitation was then confirmed via chromatographic and mass spectrometric techniques. Fractionation of the −Pi supernatant (Figure 2C) and, consistent with the original cultivation approach (Figure 2A), Pi limitation induced a ~2.5-fold higher biotic Fe removal. Also, the EDTA experiment showed that the biotic Fe removal did not continue throughout the late phase of growth, when Fe increased in the first cultivation experiments performed using a CMM amended with uncomplexed Fe(II) (Figures 2A,C and Supplementary Figures S1A,C). This suggests that the bacteria did not continuously took up Fe during the late growth phase. In order to understand whether the secretion of Fe-chelators under Pi limitation is a general phenomenon observed amongst heterotrophic marine bacteria, we performed similar analyses in three strains of the abundant and ubiquitous Roseobacter clade: P. inhibens DSM-17395, DSM-16374 and R. pomeroyi DSM-15171. As in strain FO-BEG1, in strains DSM-17395 and DSM-15171 the soluble Fe increased during the late growth phase only under −Pi conditions (Figures 3A,C and Supplementary Figure S2) whereas in strain DSM-16374 Fe increased in both the −Pi and +Pi cultures (Figure 3B). It is worth noting that strain DSM-16374 increasingly formed tight flocs throughout growth, creating a bias in the cell density estimation (Supplementary Figure S2B).
chelating molecules were detected only in the \(-P_i\) supernatant, a finding confirmed by ultra-high resolution mass spectrometry (FT-ICR-MS). The compounds in the crude extract of the supernatant obtained from a 10 L \(-P_i\) culture, were separated using HPLC into five fractions (Figure 4A). Amongst them, fraction 4 contained a compound with a retention time and UV-visible spectrum consistent with tropodithietic acid (TDA), previously reported to be produced by strain FO-BEG1 under...
−P\textsubscript{i} conditions (Romano et al., 2014, 2015). Chelating properties of all fractions were tested again via the CAS-assay. Fraction 1 reacted quickly in the assay, but successive HPLC analyses showed the presence of multiple compounds, which hindered a complete characterization of the chelating agents enriched in this sample. Additionally, the TDA-containing fraction started to react after ∼2 h of incubation.

Fractions 1 and 4 were then analyzed by MS before and after GaCl\textsubscript{3} addition. Ga(III) has chemical properties similar to Fe(III), but its two isotopes, \textsuperscript{69}Ga and \textsuperscript{71}Ga, have an abundance ratio of 3:2 that facilitates the identification of chelating compounds (Gledhill et al., 2004). In fraction 1 we identified the molecular masses of several compounds (i.e., nominal masses 633; 845; 874), that according to the analysis of the isotopic patterns unequivocally contained Ga (Figures 4B–G). An additional peak with a strong intensity was identified at 311 m/z. However, a similar m/z with an intensity ∼20-fold lower was also detected in a control run performed by dissolving GaCl\textsubscript{3} in ACN. In the TDA fraction, masses showing the Ga signature were not identified. Instead a peak with a mass (m/z 234.9; [TDA+Na\textsuperscript{+}]\textasciitilde{)} and isotopic pattern consistent with TDA was detected.

DISCUSSION

**Pseudovibrio Secretes Unknown Fe-Chelating Molecules under Phosphate Limitation**

P\textsubscript{i} and Fe are both fundamental nutrients for all organisms. The interconnection between their geochemical cycles suggests that also their cellular metabolisms are tightly linked; however, there is a dearth of studies considering these metabolic connections in bacteria. By combining physiological and chemical experiments, we report in this work the impact of P\textsubscript{i} limitation on the Fe metabolism of the marine bacterium *Pseudovibrio* sp. FO-BEG1. The results of the growth experiments together with the chromatographic and mass spectrometry data clearly showed that under P\textsubscript{i} limitation *Pseudovibrio* sp. FO-BEG1 produces Fe-chelating molecules. This phenomenon was also observed in two out of three *Roseobacter* strains. A more extended screening including strains from different phylogenetic groups would be needed to understand how common is this process amongst marine bacteria. As yet, the nature of the compounds secreted by *Pseudovibrio* is unclear. Recently, D’Alvise et al. (2016) reported that TDA and an uncharacterised precursor molecule (pre-TDA) interact with Fe. *Pseudovibrio* produces TDA under P\textsubscript{i} limitation (fraction 4 and Romano et al., 2014, 2015). However, in our experiments fraction 1 (Figure 4), which reacted in the CAS-assay and with Ga in the MS analysis, did not contain TDA, whereas the purified fraction containing TDA (fraction 4; Figure 4A) reacted only mildly with the CAS reagent and did not bind to Ga during the MS experiments. D’Alvise et al. (2016) pointed out that acidification triggers the rapid conversion of pre-TDA into TDA. In our study, the partial purification was always performed under acidic conditions; thus, if pre-TDA was present in the supernatant it would have been converted to TDA. Therefore, the presence of a compound analogues to pre-TDA in the active fraction 1 is unlikely. Overall, our data suggest that *Pseudovibrio* sp. FO-BEG1 produces multiple compounds, other than TDA, with chelating properties during P\textsubscript{i}-limited growth.

Considering the geochemical interaction between P\textsubscript{i} and other Fe-minerals, such as Fe-oxyhydroxides, the increase of dissolved Fe at the end of the exponential growth phase and the secretion of Fe-chelating molecules (Figures 2A, 3) led us to the hypothesis that in the environment P\textsubscript{i}-limited bacteria might dissolve Fe-oxyhydroxides or other Fe-containing minerals in order to access the adsorbed P\textsubscript{i}. This process may serve as an as yet unrecognized link between the biogeochemical cycles of P and Fe, and may be an important microbially driven process, which would allow bacteria to access an additional P\textsubscript{i} pool trapped in Fe minerals. Such phenomenon might be of importance especially in environments where P\textsubscript{i} availability is reduced by adsorption onto different Fe-containing minerals, or in areas where allochthonous P\textsubscript{i}-coated Fe-minerals are found, such as in some oxic marine sediments, regions affected by hydrothermal vent plumes, and many coastal areas (Feely et al., 1998; Paytan and McLaughlin, 2007). Additional experiments, using different Fe:P\textsubscript{i} ratios, different nutrient regimes simulating various environmental conditions, and different bacterial strains and natural bacterial communities, together with the characterization of the Fe-chelating molecules produced under P\textsubscript{i} limitation, will allow to verify this hypothesis, providing novel insights into the biogeochemical cycles of both Fe and P.

The ability of microorganisms to mobilize elements via dissolving minerals is a known phenomenon in soil science (Uroz et al., 2009). The general accepted idea is that the dissolution of P\textsubscript{i}-containing minerals is most likely due to the decrease in pH resulting from the secretion of organic acids (i.e., oxalic acid, citric acid). Thus far, there is only indirect evidence and data from abiotic experiments suggesting that the chelating properties of some organic acids, or other chelating molecules, facilitate this process by dissolving the cations (i.e., Ca, Al) of P\textsubscript{i}-containing minerals (Whitelaw et al., 1999; Kim et al., 2005; Osorio and Habte, 2012; Koele et al., 2014). In our experiments, the pH of the medium during bacterial growth was always ≥7 (data not shown). Moreover, the SEC, as well as the data from both the dialysis experiment, and the MS suggested that Fe was bound to organic molecules with molecular masses mostly higher than those of the organic acids secreted by P\textsubscript{i}, mobilizing soil bacteria, even if the stoichiometric ratio of 2:2 common for the citric acid-Fe complex is considered (551.8 Da; Shweky et al., 1994). Thus, overall, our data are consistent with the presence of multiple compounds able to chelate Fe having molecular masses comparable to those of known siderophores (i.e., enterobactin, 669.6 Da).

Except for *P. inhibens*, gene clusters responsible for the production of siderophores could not be identified in the genomes of the investigated strains, suggesting that Fe chelation is mediated by molecules different from known siderophores. Generally, bacteria produce siderophore-like molecules in
response to Fe starvation, as a strategy to increase Fe uptake. In strain FO-BEG1, the EDTA-experiment along with previous proteomic data (Romano et al., 2015) showed that −P<sub>i</sub>-growing cells were not Fe starved and therefore that the secreted Fe-chelators had a biological function other than Fe-scavenging. While additional roles for siderophore-like compounds have been recently described (reviewed in Johnstone and Nolan, 2015), the production of these molecules in response to P<sub>i</sub> limitation has been reported only in the pathogenic bacterium Pseudomonas aeruginosa, in a study on the effect of P<sub>i</sub> limitation on virulence (Zaborin et al., 2009). To the best of our knowledge, the data we present provide the first evidence that P<sub>i</sub> limitation triggers the production of Fe-chelating molecules in marine bacteria.

**Evidence for an Interlink between the Metabolism of Phosphate, Iron, and Cobalt**

The increased biotic removal of Fe and Co observed under −P<sub>i</sub> conditions might have derived from a more pronounced cellular uptake or from a more intense adsorption onto cellular surface due to cellular surface modifications occurring under P<sub>i</sub> limitation. The absence of such an increased biotic removal in the P<sub>i</sub>-limited Roseobacter cultures suggests that either the growth conditions we used did not trigger a P<sub>i</sub>-limited response as pronounced as in Pseudovibrio, or that the process responsible for the metal decrease is strain specific and it might reflect...
the adaptation of *Pseudovibrio* to specific environmental niches. As yet, we cannot definitely estimate to what extent cellular adsorption and uptake contributed to the decrease of metals in the cultures. However, experiments on metal adsorption conducted on other bacteria, as well as previously proteomic and physiological data in *Pseudovibrio* suggest that cellular uptake might have played a major role in the decrease of Co and Fe in the −P<sub>i</sub> cultures. For example, for many bacteria it has been shown that the intensity of the metal adsorption onto cell surface is affected by the age of the cultures, rather than the nutrient regimes used for cultivation (Borrok et al., 2004; Haas, 2004; Guiné et al., 2007; Pokrovsky et al., 2008). This suggests that the metal adsorption onto cellular surface is probably responsible for a part of the Fe and Co decrease observed in the *Pseudovibrio* cultures, but unlikely explains alone the total decrease in metal concentrations observed under P<sub>i</sub>-limited conditions.

In previous proteomic analyses performed comparing the expression profiles of *Pseudovibrio* cells under the two P<sub>i</sub> regimes, two proteins homologous to bacterioferritin were strongly up-regulated under P<sub>i</sub> limitation (PSE_1030 and PSE_3844; Romano et al., 2015). These proteins have been described to be involved in Fe storage in other bacteria, strengthening the hypothesis that *Pseudovibrio* cells growing under P<sub>i</sub> limitation take up and store a larger amount of Fe than cells growing under P<sub>i</sub> surplus. Luxury Fe uptake was described in phytoplankton (Kustka et al., 2003; Shire and Kustka, 2015) and it might be an important strategy to overcome period of future Fe limitation, especially in environments in which the availability of this nutrient is variable both temporally and spatially, such as in coastal waters, where *Pseudovibrio* related bacteria often occur (Shieh et al., 2004; Hosoya and Yokota, 2007).

Under conditions of P<sub>i</sub> limitation, bacteria produce an array of enzymes to recover P<sub>i</sub> from organic molecules. The majority of these enzymes have metal cations as cofactors, and this might explain why P<sub>i</sub> limitation affects the uptake of trace elements in *Pseudovibrio* cultures. For example, most of the known forms of alkaline phosphatases (APs), an enzyme that breaks C-O-P bonds in phosphoesters, need zinc (Zn) or calcium (Ca) for activity, and it was recently discovered that PhoX, the most common AP amongst marine bacteria, requires Fe (Sebastian and Ammerman, 2009; Kathuria and Martiny, 2011; Yong et al., 2014). Co as well was found to be required in some APs and in metalloenzymes involved in phosphonate degradation (molecules containing C-P bonds; Simpson and Vallee, 1969; Hulett et al., 1991; Wojciechowski et al., 2002; Kulakova et al., 2003; Gong et al., 2005; Jakuba et al., 2008). In the genome of *Pseudovibrio* sp. FO-BEG1 different APs, including PhoA (PSE_2813) and PhoX (PSE_1012), and phosphonate degrading enzymes are encoded. Consistent with the P<sub>i</sub> starvation response described in other bacteria, under P<sub>i</sub> limitation we detect an increase in the APs activity and in the expression of enzymes involved in phosphonate metabolism in the *Pseudovibrio* cultures (PSE_3629, PSE_3630, PSE_4852, PSE_4857; Romano et al., 2015). Overall, these data suggest that under P<sub>i</sub> limitation *Pseudovibrio* cells would require a higher amount of Co and Fe for the metalloenzymes needed to scavenge P<sub>i</sub> from organic molecules, accounting, at least partially, for the more pronounced decrease of Co and Fe in the −P<sub>i</sub> cultures. This conclusion is consistent with what has been previously observed for Co in freshwater phytoplankton (Ji and Sherrell, 2008), and strengthens the hypothesis that the low level of this element measured in some P<sub>i</sub>-limited regions, such as the Sargasso Sea, reflects the physiological response of microorganisms to P<sub>i</sub> limitation.

**CONCLUSION**

Our previous investigations on *Pseudovibrio* sp. strain FO-BEG1 demonstrated the drastic effect of P<sub>i</sub> limitation on central metabolism, antibiotic production, expression of virulence-related genes, and the overall pattern of secreted metabolites (Romano et al., 2014, 2015). The changes in the metabolism of micronutrients we herein report underlines the pleiotropic effect that P<sub>i</sub> limitation has on the overall cell physiology, and strongly points toward a tight interlink between the P<sub>i</sub> metabolism and the metabolism of other micronutrients in marine bacteria. Our findings led us to purpose a new mechanism that bacteria might use to access P<sub>i</sub> via the dissolution of Fe-minerals, such as Fe-oxhydroxides, which trap P<sub>i</sub> in many terrestrial and aquatic environments. The extend and the environmental relevance of this phenomenon will need to be experimentally verified, and if confirmed, this process will represent an additional aspect to take into account when considering the mechanistic interconnection between the biogeochemical cycles of P<sub>i</sub> and Fe. Moreover, the secretion of Fe-chelators under P<sub>i</sub> limitation will open the need to a re-interpretation of the role that siderophore-like molecules have in the environment, especially considering the regulatory processes behind their production under not Fe-limiting conditions.

**AUTHOR CONTRIBUTIONS**

The work was designed by SR, HS-V, and VB. SR and VB performed the experiments. All data were analyzed by SR in collaboration with VB, HS-V, MK, and TD. SR wrote the manuscript, including comments of all co-authors. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00364/full#supplementary-material

FIGURE S1 | Schematic overview of the cultivation experiments performed with Pseudovibrio sp. strain FO-BEG1. (A) Description of the original cultivation experiments performed using CM medium amended with an acidified trace element solution. (B) In the refreshment experiment, cells were pre-grown under −P conditions, until the previously detected maximum Fe uptake was reached. Cells were then harvested and re-inoculated into fresh Fe-free −P CM medium. (C) In the EDTA-experiment, strain FO-BEG1 was cultivated in +P and −P CM medium containing an excess of EDTA, in order to complex Fe, and avoid Fe precipitation.

FIGURE S2 | Growth curve and phosphate uptake measured in the Roseobacter cultures. Cell density (triangles) and phosphate concentrations (circles) over time in cultures grown under +P (empty symbols) and −P (filled symbols) conditions. (A) Pseudobacter inhibens strain DSM-17395. (B) Pseudobacter inhibens strain DSM-16374. (C) Ruegeria pomeroyi strain DSM-15171. The error bars represent the standard deviation of at least biological triplicates. Strain DSM-16374 formed tight flocs that increased the variability of the cell counting data.

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