Microbial iron metabolism as revealed by gene expression profiles in contrasted Southern Ocean regimes

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Summary
Iron (Fe) is a limiting nutrient in large regions of the ocean, but the strategies of prokaryotes to cope with this micronutrient are poorly known. Using a gene-specific approach from metatranscriptomics data, we investigated seven Fe-related metabolic pathways in microbial communities from high nutrient low chlorophyll and naturally Fe-fertilized waters in the Southern Ocean. We observed major differences in the contribution of prokaryotic groups at different taxonomic levels to transcripts encoding Fe-uptake mechanisms, intracellular Fe storage and replacement and Fe-related pathways in the tricarboxylic acid (TCA) cycle. The composition of the prokaryotic communities contributing to the transcripts of a given Fe-related pathway was overall independent of the in situ Fe supply, indicating that microbial taxa utilize distinct Fe-related metabolic processes. Only a few prokaryotic groups contributed to the transcripts of more than one Fe-uptake mechanism, suggesting limited metabolic versatility. Taxa-specific expression of individual genes varied among prokaryotic groups and was substantially higher for all inspected genes in Fe-limited as compared to naturally fertilized waters, indicating the link between transcriptional state and Fe regime. Different metabolic strategies regarding low Fe concentrations in the Southern Ocean are discussed for two abundant prokaryotic groups, Pelagibacteraceae and Flavobacteriaceae.

Introduction
Since John Martin’s ‘iron hypothesis’ was introduced in the late 1980s to solve the high nutrient low chlorophyll (HNLC) paradox in the ocean, the micronutrient iron (Fe) has been recognized as a major factor in the regulation of ocean primary productivity (Martin, 1990; Tagliabue et al., 2017). The Southern Ocean as the largest HNLC area has been subject to multiple mesoscale artificial Fe fertilization studies focusing on enhanced phytoplankton blooms through Fe input (reviewed in the study by Boyd et al., 2007). Together with investigations in naturally Fe-fertilized regions (Blain et al., 2007; Pollard et al., 2009), the control by Fe of primary productivity and subsequent carbon dioxide (CO₂) drawdown in this ocean has been confirmed.

Heterotrophic microorganisms rapidly respond to phytoplankton blooms induced by Fe-fertilization (Cochlan, 2001; Hall and Safi, 2001; Oliver et al., 2004; Obernosterer et al., 2008) and they remineralize a substantial fraction of phytoplankton-derived dissolved organic matter (Christaki et al., 2014). Fe is essential for microbial heterotrophic metabolism, the access to this micronutrient by various taxa will therefore affect the processing of organic carbon. The limited number of measurements indicates that heterotrophic prokaryotes have cellular Fe quotas that are similar or higher than those of phytoplankton (Tortell et al., 1999; Sarthou et al., 2008; Fourquez et al., 2012). The majority of Fe (> 90%) in heterotrophic prokaryotic cells is located in the respiratory chain (Andrews et al., 2003) and as a consequence Fe limitation results in a prokaryotic reduction in prokaryotic respiration and growth rates (Tortell et al., 1999; Smith et al., 2010; Fourquez et al., 2014; Koedooder et al., 2018). Experimental studies testing the effect of Fe on natural prokaryotic communities have revealed both positive and negative bulk metabolic responses.
(summarized in the study by Obernosterer et al., 2015), likely reflecting temporal and spatial variability of the bioavailability and the cellular requirements of this micronutrient. In the region off Kerguelen Island, heterotrophic prokaryotic growth and production were limited by Fe and organic carbon in early spring (Obernosterer et al., 2015), leading to competition between heterotrophic and photohetero trophic microorganisms for this micronutrient (Fourquez et al., 2015).

These observations raise the question of the mechanisms used by microbial taxa to acquire and to metabolize this micronutrient in cellular processes. An increasing number of prokaryotic genomes and of metagenomes originating from global ocean surveys has provided insights to the inventories of Fe-related pathways (Desai et al., 2012; Hopkinson and Barbeau, 2012; Toulza et al., 2012; Hogle et al., 2016). These studies have shown that the genomic potential for Fe-uptake mechanisms varies among prokaryotic taxa (Hopkinson and Barbeau, 2012; Hogle et al., 2016) and that the prevalence of Fe-related pathways in prokaryotes reflects Fe concentrations across ocean regions (Toulza et al., 2012). The single-cell approach MICRO-CARD-FISH using $^{55}$Fe revealed that the community taking up Fe in the Southern Ocean was dominated by Gammaproteobacteria and FCB, while SAR11 and Roseobacter had overall lower contributions (Fourquez et al., 2016). Fe limitation has been shown to induce the glyoxylate shunt in heterotrophic bacterial model organisms (Smith et al., 2010; Fourquez et al., 2014; Koedooder et al., 2018), a pattern that was also observed for SAR11 in the HNLC Southern Ocean (Beier et al., 2015). The glyoxylate shunt bypasses two decarboxylation steps and the coupled release of CO$_2$ and reducing equivalents (NADH$_2$) of the TCA cycle, with important consequences on ATP production and processing of organic carbon (Koedooder et al., 2018).

The aim of the present study was to extend these observations, by providing a detailed picture on the expression of genes responsible for Fe-uptake and Fe-related downstream processes in Southern Ocean natural prokaryotic communities. We screened the total mRNA from metatranscriptomes against a database that contained 10,411 protein sequences corresponding to seven bacterial phyla as well as archaea. Highest overall path counts were observed for the classes of Alpha-, Beta- and Gammaproteobacteria as well as the Fibrobacteres, Chlorobi, Bacteroidetes (FCB) (Figs. 2 and 3, study 2 (KEOPS2)-cruise that took place in November 2011. Station (R-2) was located in HNLC waters west of Kerguelen Island (Fig. 1), and two stations were situated east of the island in naturally Fe-fertilized waters south (Station A3-2) and north (Station F-L) of the polar front. Concentrations of dissolved Fe in the surface mixed layer varied between 0.13 ± 0.05 nM and 0.22 ± 0.06 nM (Quéroué et al., 2015) (Table 1). Chlorophyll a concentrations were low at station R-2 (Chl a, 0.25 ± 0.08 μg L$^{-1}$) and up to 16-fold higher in naturally fertilized waters (Lasbleiz et al., 2014) (Table 1). The abundance, production and respiration of heterotrophic prokaryotes were several fold enhanced at the fertilized sites as compared with HNLC waters. Concentrations of DOC, DON and DOP were not enhanced at the Fe-fertilized sites, probably due to the rapid consumption of organic matter by heterotrophic prokaryotes. Despite these pronounced differences in biomass and production, heterotrophic prokaryotes were shown to be limited by Fe at the HNLC-site and in Fe-fertilized waters (Obernosterer et al. 2015).

**Results**

*Environmental context*

The three stations considered in the present study were part of the Kerguelen Ocean and Plateau compared...
Chlorophyll a (able for siderophore- and Fe2+-uptake, and Fe3+-uptake distributed to Fe2+-and Fe3+-uptake, but this group had low Alphaproteobacteria, Rhodobacteracea and Shewanellaceae and Piscirickettsiaceae were major contributors to siderophore-uptake transcripts but neither Fe2+- nor Fe3+. Pelagibacteraceae transcripts were not detectable for siderophore- and Fe3+-uptake, and Fe3+-uptake transcripts belonging to this group accounted for 0.8% of alphaproteobacterial transcripts. Actinobacteriaceae and Archea were almost absent from siderophore-uptake but contributed to Fe2+- and Fe3+-uptake.

The flavodoxin switch and bacterioferritin transcripts revealed both high contributions of gammaproteobacterial groups, and smaller proportions of alphaproteobacterial and FCB transcripts, in particular for flavodoxin transcripts. Pronounced differences in the contribution of different gammaproteobacterial phylotypes to the two Fe-related processes were detectable. Shewanellaceae, Alcanivoracaceae and Aeromonadaceae were the most important contributors to flavodoxin switch transcripts, while Cellulibrionaceae, Halieaceae and Alteromonadaceae dominated the bacterioferritin transcripts. Flavobacteriaceae and Rhodobacteraceae contributed each substantially to bacterioferritin transcripts, while flavodoxin switch transcripts belonging to these groups were almost absent. Pelagibacteriales did not contribute to flavodoxin switch and bacterioferritin transcripts. Cyanobacteria accounted for a large portion of flavodoxin switch transcripts, in particular at the HNLC-site. The relative contribution of cyanobacteria to 16S rRNA sequences varied between 0.37% at the HNLC and 0.41% at the Fe-fertilized stations respectively (Landa et al. 2016). Taken together, these results construe specific Fe-uptake and Fe-processing mechanisms for several prokaryotic groups.

We identified two categories of prokaryotic groups contributing to transcripts of aconitase and isocitrate lyase, genes associated with the TCA cycle. Alphaproteobacterial phylotypes, in particular Pelagibacteriales and Actinobacteriaceae, each had similar contribution to aconitase and isocitrate lyase. In contrast, the proportions of

Supporting Information Figs. S2 and S3, Supporting Information Table S2). These bacterial phyla were shown to be abundant also in data sets derived from 16S rRNA amplicon sequencing (Fig. 4). We observed major differences in the contribution of prokaryotic groups to the different Fe-uptake mechanisms and this pattern was largely independent of site. Siderophore-uptake was dominated by Gammaproteobacteria (43% of total transcripts) and FCB (25%), while Fe2+- and Fe3+-uptake revealed an increased contribution of alphaproteobacterial groups, most pronounced for Fe3+-uptake (29%). Within Gammaproteobacteria noticeable differences in the contribution of different phylotypes to the three uptake mechanisms could be observed. While Alteromonadaceae, Cellulibrionaceae and Shewanellaceae were abundant contributors to siderophore-uptake, Shewanellaceae and Enterobacteriaceae, accounted for most gammaproteobacterial Fe3+-uptake transcripts and Pseudomonadaceae, Chromatiaceae and Piscirickettsiaceae were major contributors to Fe3+-uptake transcripts (Fig. 3, Supporting Information Table S2). FCB were mostly represented by Flavobacteriaceae for siderophore- and Fe2+-uptake, and Bacillaceae contributed additionally to Fe3+-uptake. Within Alphaproteobacteria, Rhodobacteraceae substantially contributed to Fe3+- and Fe3+-uptake, but this group had low siderophore uptake transcripts (<1.6% of alphaproteobacterial transcripts). A contrasting pattern was observed for Sphingomonadaceae and Erythrobacteraceae that contributed to siderophore uptake transcripts but neither Fe2+- nor Fe3+. Pelagibacteraceae transcripts were not detectable for siderophore- and Fe3+-uptake, and Fe3+-uptake transcripts belonging to this group accounted for 0.8% of

Table 1. Location, date, biogeochemical properties and bulk prokaryotic parameters at the three study sites. All parameters are mean ± SD for the surface mixed layer.

|                      | R-2         | F-L         | A3-2        |
|----------------------|-------------|-------------|-------------|
| Latitude S           | 50.3590     | 48.5222     | 51.0333     |
| Longitude E          | 66.7170     | 74.6500     | 72.0833     |
| Date of sampling     | 26 October 2011 | 7 November 2011 | 16 November 2011 |
| Sampling depth (m)   | 60          | 20          | 20          |
| Surface mixed layer (m) | 105 ± 15    | 38 ± 7      | 153 ± 15    |
| Dissolved and particulate nutrients |
| DOC (μM)             | 47.8 ± 0.4  | 49.6 ± 1.3  | 51.3 ± 1.5  |
| DON (μM)             | 6.1 ± 0.04  | 5.47 ± 1.33 | 6.44 ± 2.2  |
| DOP (μM)             | 0.3 ± 0.02  | 0.26 ± 0.12 | 0.36 ± 0.04 |
| POC (μM)             | 6.5 ± 1.8   | 11.5 ± 1.2  | 13.5 ± 1.8  |
| DFe (nmol L⁻¹)       | 0.13 ± 0.05 | 0.22 ± 0.06 | 0.16 ± 0.03 |
| Chlorophyll a (μg L⁻¹) | 0.25 ± 0.08 | 4.00 ± 1.58 | 2.03 ± 0.34 |
| Prokaryotic abundance (×10¹⁵ cells mL⁻¹) | 2.72 ± 0.3  | 6.06¹⁵⁷  | 3.16 ± 0.5  |
| Prokaryotic production (ng C L⁻¹ h⁻¹) | 2.59 ± 0.53 | 65.7 ± 1.62 | 19.9 ± 3.4  |
| Prokaryotic respiration (μmol O₂ L⁻¹ d⁻¹) | 0.25 ± 0.12 | 1.37 ± 0.64 | 0.63 ± 0.45 |

a. From Tremblay et al. (2015).
b. From Blain et al. (2015).
c. From Queroüe et al. (2016).
d. From Lasbleiz et al. (2014).
e. From Christaki et al. (2014).
f. Only one measurement for the surface mixed layer available.
transcripts accounted for by Flavobacteriaceae and gammaproteobacterial phylotypes varied considerably between the two genes. Flavobacteriaceae had higher contributions to aconitase transcripts than to those of isocitrate lyase, and gammaproteobacterial phylotypes, such as Pseudoalteromonadaceae, Moraxellaceae, Oceanospirillaceae, Alteromonadaceae and Colwelliaceae showed the opposite pattern. Burkholderiaceae, a group that had low contributions to all other transcripts, accounted for up to 30% of the aconitase and isocitrate lyase transcripts.

Transcriptional activity in HNLC and Fe-fertilized waters

Among site comparison of pathway-specific transcripts revealed that $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, the flavodoxin switch and bacterioferritin had higher proportions at R-2 as compared to the Fe-fertilized sites (Fig. 5). In contrast, no such pattern was observed for siderophore-uptake, aconitase and isocitrate lyase. Comparison among genes encoding for the different Fe-uptake mechanisms was possible due to the similar gene length (Supporting Information Table S4). The relative transcript abundance of siderophore-uptake was roughly 6- and 2-fold higher than those of $\text{Fe}^{2+}$- and $\text{Fe}^{3+}$-uptake respectively.

In order to assess a potential per cell transcriptional activity, results from qPCR expression of isocitrate lyase in SAR11 at two sites, R-2 and F-L, were used as correction factors (see Experimental procedures). This normalization step was chosen in order to answer the following question: Does gene expression vary between HNLC and Fe-fertilized waters? And further, how variable is cell specific expression of a given gene among the different prokaryotic
groups? This step was done for representatives from the prokaryotic phyla (presented in Fig. 2) for which the gene transcripts and 16S relative abundances were available for a given phylogenetic level (Fig. 4 and Supporting Information Fig. S5). To verify the approach, ribosomal proteins, essential in cellular processes of translation, were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG) and screened against our data sets (Fig. 6 and Supporting Information Fig. S4).

Cell-specific expression of all genes considered in the present study was considerably higher (10- to 1000-fold) at Station R-2 in comparison to F-L for all prokaryotic groups (Fig. 6 and Supporting Information Table S3). Exceptions were Pseudomonadales that consistently revealed an inverse pattern, and Burkholderiales for which differences between sites were in most cases small. In comparison, the ribosomal proteins showed the lowest fold-change (1.5) between R-2 and F-L. For clarity, the following comparison of taxa-specific expression levels among prokaryotic groups is focused on the HNLC site R-2.

Alteromonadales had the highest taxa-specific expression levels for all genes, while taxa-specific expression levels of a given gene was more variable for the other prokaryotic groups. Flavobacteriales, Oceanospirillales and Rhodobacterales had similar taxa-specific siderophore-uptake expression while Rhodobacterales and Actinomycetales had substantially higher taxa-specific Fe\(^{3+}\)-uptake as compared with Flavobacteriales (8- to 6-fold respectively) and Oceanospirillales (15- to 12-fold). Rhodobacterales, Flavobacteriales and Oceanospirillales had similar bacterioferritin expression levels, and flavodoxin switch expression levels belonging to Flavobacteriales were considerably lower than those of the other groups. Taxa-specific gene expression of aconitase was similar for Rhodobacterales, Flavobacteriales and Oceanospirillales. In contrast, taxa-specific expression levels of isocitrate lyase were highly variable among groups, with Actinomycetales and Pelagibacterales at the higher and lower ranges respectively.

Discussion

We present here taxon-specific strategies of Fe-uptake and intracellular processes dependent on Fe, a crucial, yet growth-limiting element for microbial heterotrophs in large areas of the ocean. By investigating the in situ expression patterns of candidate genes from metatranscriptomics data, we provide novel insights into these metabolic traits of Southern Ocean microbial communities in contrasting Fe- and C-regimes. We observe major differences in the contribution of prokaryotic groups to the pathways investigated, indicating distinct metabolic capabilities for Fe-related processes and downstream carbon metabolism for microbial taxa. Our finding that taxa-specific expression levels are substantially higher under Fe-limited conditions suggests that the transcriptional states related to Fe-uptake and Fe cell-content reduction or control are associated with the in situ Fe-supply.

Our observation that gamma- and flavobacterial groups accounted for a large fraction of siderophore-
uptake transcripts, while alphaproteobacterial groups had an increased contribution to Fe^{3+}-uptake transcripts corroborates previous findings on the genomic potential of representatives of these classes (Hopkinson and Barbeau, 2012; Tang et al., 2012; Hogle et al., 2016). A survey of 206 bacterial genomes revealed that Ton-B-dependent transporters (TBDTs), many of which are known as siderophore type transporters, are common in Gammaproteobacteria and Bacteroidetes but less abundant in Alphaproteobacteria, and absent in Pelagibacter ubique (Hopkinson and Barbeau, 2012; Tang et al., 2012). Within Alphaproteobacteria, Erythrobacteraceae and Sphingomonadaceae were major contributors to siderophore-uptake transcripts in the present study, an observation supported by the genomic potential of representative strains (Tang et al., 2012). Fe^{3+}-uptake gene expression revealed a contrasting pattern, with high contributions of Rhodobacteraceae and Actinomycetales and a minor contribution of FCB. While Fe^{3+} transporters were abundant in many of the 206 bacterial genomes investigated, only 1 of 16 Bacteroidetes genomes contained this type of transporter (Hopkinson and Barbeau, 2012). Despite its dependency on inorganic Fe-uptake, Pelagibacteraceae had a minor contribution to the overall Fe^{3+}-uptake gene expression (0.8% of total Fe^{3+} transcripts) and the lowest cell-specific Fe^{3+}-uptake (Fig. 6). The lower copy numbers of Fe^{3+} transporters in SAR11 as compared with Roseobacter genomes (Hogle et al., 2016) and potentially lower Fe requirements could explain this observation.

The dominating microbial contributors to the transcripts of a given Fe-uptake mechanism were overall similar among sites and thus largely independent of the in situ Fe supply. This contrasts with the different relative contributions of the observed groups at the sites (Fig. 4, Landa et al., 2016) and suggests that our observations on the transcriptome level are mainly driven by the metabolic potential of the respective prokaryotic groups (see Supporting Information Fig. S5). In support of this conclusion, our results illustrate that only a few prokaryotic
groups contribute substantially to the transcripts of more than one Fe-uptake mechanism. This was the case for *Shewanellaceae* transcripts that were present for the three Fe-uptake mechanisms, and for *Rhodobacteraceae* and the FCB cluster that accounted both for substantial proportions of Fe$^{2+}$- and Fe$^{3+}$-uptake transcripts.

The 2- to 6-fold higher proportions of siderophore-uptake gene expression as compared with Fe$^{3+}$ and Fe$^{2+}$ could indicate that this mechanism was a more efficient pathway for prokaryotes to acquire Fe. Most dissolved Fe (99%) (Gledhill and van den Berg, 1994; Rue and Bruland, 1995) is complexed by organic ligands leaving extremely low steady-state concentrations of inorganic Fe$^{3+}$ noted as Fe$'$ . The steady-state concentrations of Fe$^{2+}$ are also typically extremely low in oxygenated surface waters although reduction mediated by different processes could locally produce enhanced concentrations. Fe$'$ has been considered a more bioavailable and thus more important form than siderophore-bound Fe for phytoplankton (Morel et al., 2008; Lis et al., 2015). Our observations suggest that heterotrophic prokaryotes favour the uptake of Fe bound to siderophores, which presents several advantages. First, once complexed by siderophores, Fe is hardly available for most phytoplankton (Lis et al., 2015), even though the idea that phytoplankton cannot directly use Fe-siderophore complexes has recently been challenged (Kazamia et al., 2018; McQuaid et al., 2018). Heterotrophic prokaryotes thereby avoid competition with phytoplankton for this scarce resource. Second, siderophores can contribute to access initially not available forms of Fe, such as particulate Fe or Fe-organic complexes (Kraemer, 2004). Third, Fe-siderophore-uptake could be stimulated by siderophore-production of the same microbial cell and thus provide an advantage to certain taxa (Martinez et al., 2003; Hopkinson and Barbeau, 2012; Sijercić and Price, 2015; Boiteau et al., 2016). Our taxa-specific transcripts point out that the expression of the genes encoding for siderophore-uptake was increased (20- to 135-fold) under Fe-limited conditions when the competition for the acquisition of this resource was highest. This latter observation agrees well with the previously reported global scale inverse relationship between siderophore-uptake gene occurrence and Fe concentrations (Toulza et al., 2012). Under strong Fe limitation, the Fe-uptake rate can only be increased by the number of Fe transporters as has been demonstrated for phytoplankton (Hudson and Morel, 1993). Our observations point to a similar conclusion for heterotrophic prokaryotes. Besides the capacity to optimize Fe acquisition in limited conditions, the second global strategy for prokaryotes is to decrease the Fe cellular content.

To explore differences in intracellular Fe-regulatory mechanisms among prokaryotic groups, we investigated the two genes encoding for flavodoxin and bacterioferritin. The non-Fe containing protein flavodoxin is an iso-functional protein, which can replace ferredoxin, an electron shuttle harbouring Fe-sulfur clusters. The expression of this protein by marine autotrophic plankton was proposed as a proxy for Fe scarcity in the oceans (Roche et al., 1996), and insight into the
mechanisms of how Fe availability regulates this protein was obtained from studies on various temporal and spatial scales (Erdner et al., 1999; Saito et al., 2011; Tara Oceans Coordinators et al., 2018). A metagenomic analysis revealed that prokaryotes lacking this flavoprotein are confined to coastal areas where Fe supply is high, while flavodoxin-containing marine prokaryotes are preferably located in open ocean sites (Toulza et al., 2012). Given the roughly 35-fold higher proportion of total flavodoxin transcripts and the higher cell-specific expression in HNLC waters compared with Fe-fertilized sites, our results extend the understanding of the regulation of this switch by Fe-availability for a large range of prokaryotic groups. In the present study, the dominant contributors were gammaproteobacterial taxa and Cyanobacteria, while Alphaproteobacteria and the FCB cluster had minor contributions to flavodoxin
transcripts. These latter groups revealed also lowest cell-specific expression patterns, suggesting that many FCB and alphaproteobacterial members might make use of other strategies to cope with Fe-limitation.

Ferritins are compounds that were shown to regulate the storage and the release of intracellular Fe in a number of eukaryotic microorganisms (Marchetti et al., 2009; Botebol et al., 2015). Bacterioferritins are known to be involved in the storage of Fe in Bacteria; however, the regulation and the exact physiological mechanisms of these compounds are not clear (Andrews et al., 2003; Carrondo, 2003). In the GOS data set, bacterioferritin gene abundance was higher at coastal sites with overall high Fe concentrations (Toulza et al., 2012). In the present study, all prokaryotic groups contributed to bacterioferritin transcripts. The differences in bacterioferritin transcripts between HNLC and Fe-fertilized waters were far less pronounced than the other Fe-related metabolisms. This could indicate that bacterioferritin-related processes are occurring at background levels, for instance, as a control of Fe homeostasis, rather than as storage of Fe in response to episodic Fe supply.

The higher cell-specific expression of siderophore-, Fe³⁺-, Fe³⁺-uptake, flavodoxin and bacterioferritin transcripts in HNLK as compared with Fe-fertilized waters highlights the increased investment in Fe-related metabolism when prokaryotic growth and production are limited by Fe and organic carbon (Obermosterer et al., 2015). But how can this affect cellular carbon metabolism? We addressed this question by investigating the two enzymes aconitase and isocitrate lyase belonging to a central metabolic pathway, the TCA cycle. Aconitase, an Fe-containing enzyme, transforms citrate to isocitrate, which can either serve as a substrate for the enzyme isocitrate dehydrogenase (IDH) in the TCA cycle or as a substrate for isocitrate lyase, a non-Fe-containing enzyme that induces the glyoxylate shunt, a bypass of the TCA cycle (Supporting Information Fig. S6). While the regulation of the glyoxylate shunt can be driven by a number of factors, its induction by Fe-limitation has been demonstrated in bacterial model organisms (Fourquez et al., 2014; Koedooder et al., 2018). Using genetic tools and bioinformatic analysis, we demonstrated that the isocitrate lyase knock-out strain of gammaproteobacterium Photobacterium angustum S14 had significantly lower growth and respiration rates as compared with the wild type under Fe-limited conditions (Koedooder et al., 2018). Using qPCR, SAR11 cell-specific isocitrate lyase gene expression was higher at Station R-2 as compared with F-L (Beier et al., 2015). The increased cell-specific isocitrate-lyase expression in Fe-limited as compared with Fe-fertilized waters observed in the present study extends this previous observation to several prokaryotic groups.

In the context of these recent findings, we focus in the following discussion on two prokaryotic groups with distinct patterns in the expression of aconitase and isocitrate lyase. Pelagibacteraceae contributed similarly to the expression of both genes; in contrast, Flavobacteriaceae revealed substantially higher contributions to aconitase (7%–13% of prokaryotic transcripts) as compared with isocitrate lyase transcripts (0.7%–2.2% of prokaryotic transcripts). In addition, Flavobacteriaceae had 18.5-fold higher cell-specific aconitase expression than Pelagibacteraceae, but both groups had similar cell-specific isocitrate lyase expression. These observations could indicate that the entire TCA cycle is more preferentially used in Flavobacteriaceae than in the members of Pelagibacteraceae with consequences on the production of NADH and ATP equivalents.

The combined information obtained by the present results and previous knowledge on characteristics of these bacterial groups lead us to propose two distinct ecological strategies with respect to Fe-related processes for Pelagibacteraceae and Flavobacteriaceae. Members of Pelagibacteraceae appear to be the thriftiest group, characterized by the unique use of Fe³⁺-uptake, performed by ABC-type transporters that do not require the costly outer membrane receptors (Andrews et al., 2003) but do require ATP. Additionally, members of this group lack Fe storage and the flavodoxin switch for which no transcripts were detectable in the present study. Despite its dependency on Fe³⁺-uptake, Pelagibacteraceae had a minor contribution to the total transcripts, suggesting low Fe requirements of this group. These characteristics extend those described previously of the most prominent representatives such as the streamlined SAR11 (reviewed in the study by Giovannoni, 2017). The SAR11 clade has been shown to possess high-affinity uptake systems for a range of small molecules present at low concentrations, including two-carbon compounds, known to induce the glyoxylate shunt. This strategy allows to maintain cellular metabolism with low Fe requirements and to efficiently metabolize small molecules.

Members of Flavobacteriaceae appear to be characterized by different features. Our results point out that members of this group display the most competitive Fe-uptake systems. Also, they can potentially regulate homeostasis with bacterioferritin in particular under Fe limited conditions. This group has a moderate use of the glyoxylate shunt, because the biosynthesis of TBDTs, and their transport to the cytoplasm renders the acquisition of siderophore-bound Fe a process that is costlier in terms of energy and carbon requirements than that of Fe³⁺-uptake. Siderophore biosynthesis, coupled in many bacterial genomes to TBDTs (Hopkinson and Barbeau, 2012), adds further energy requirements (Siđerić and Price, 2015). Besides Fe-siderophore-uptake, TBDTs were associated with the uptake of a range of substrates, such as carbohydrates, amino acids, amino sugars or vitamin B₁₂ (Schauer et al., 2008; Noinaj et al., 2010).
The highest number and most diverse types of TBDTs were associated with *Gammaproteobacteria* and FCB in the GOS data set (Tang et al., 2012). This genomic information, in combination with whole genome sequencing, culture-based studies and single-cell approaches have led to the characterization of members of FCB to be efficient degraders of polymeric organic matter (Kirchman et al., 2003; Bauer and Blodau, 2006; Kabisch et al., 2014). Even though members of both groups are well equipped to thrive in Fe-limited environments, *Pelagibacteraeae* are likely to have an advantage over other metabolic traits to clear prevalence for *Flavobacteriaceae* and *Flavobacteriaceae* when organic carbon is limiting. These contrasting characteristics for members of *Pelagibacteraeae* and *Flavobacteriaceae* extend those known for other metabolic traits to clear prevalence for Fe3+- and siderophore-uptake, respectively, and could be considered as ecological strategies in an ocean region where microbial activity is limited by Fe and organic carbon.

**Experimental procedures**

**Sample collection**

Seawater samples were collected during the KEOPS2 cruise (Kerguelen Ocean and Plateau Compared Study 2, 8 October to 30 November 2011) in the Indian sector of the Southern Ocean.

Seawater samples were collected with 12 l Niskin bottles mounted on a rosette equipped with a CTDO Seabird SBE911-plus. For nucleic acid extractions, seawater was sampled at one depth in the surface mixed layer, and the chemical and biological parameters were collected throughout the water column (Christaki et al., 2014; Lasbleiz et al., 2014; Blain et al., 2015; Quéroü et al., 2015; Tremblay et al., 2015) (Table 1).

**RNA extraction**

For RNA extractions, volumes varying between 15 l and 30 l of pre-filtered water (200 μm nylon screen and 5 μm polycarbonate isopore filters) were collected onto 0.2 μm SuperPlus Membranes using a 142 mm filtration system (geotech equipment) and a peristaltic pump. The filtration procedure did not exceed 10 min and 10 ml of RNA-later was added before storage at −80 °C. All nucleic acid extractions were performed in triplicates by cutting the filter in three parts.

Total prokaryotic and eukaryotic RNA was extracted using the NucleoSpin® RNA Midi kit (Macherey-Nagel, Düren, Germany). Filters stored in RNA later were defrosted, removed from the RNA later solution, refrozen in liquid nitrogen and shattered using a mortar. The obtained ‘powder-like’ filter-pieces were added together with low binding zirconium beads (OPS Diagnostics, Lebanon, NJ, USA) to the denaturing lysis buffer supplied by the NucleoSpin® RNA Midi kit and cells were disrupted by vortexing for 2 min. Beads were discarded by centrifugation. The extraction with the NucleoSpin® RNA Midi kit include an on-column DNA digestion step. However, in order to ensure the absence of DNA in the sample, a control PCR reaction was performed without the retrotranscription (RT) step. Samples with DNA contamination, as indicated by amplification products were treated with a second DNA digestion step using the Turbo DNA-free kit (Ambion Life Technologies, Carlsbad, CA, USA). This additional DNAse treatment was followed by purification with the RNasefree MinElute Clean Up kit (Qiagen, Hilden, Germany). The extracted RNA was quantified with the Agilent 2100 Bioanalyzer/Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA) and duplicates were chosen for sequencing.

Prior to sequencing, ribosomal RNA was treated enzymatically with the Ribozero rRNA stranded RNA protocol to ensure sequencing of primarily messenger RNA followed by cDNA library construction using Illumina TruSeq Stranded mRNA Library Prep kit (Fasteri SA). Libraries were sequenced using paired-end 2 × 125 read length on one Illumina HiSeq 2500 lane.

**Bioinformatic analysis**

The raw Illumina reads were checked with FastQC (Andrews 2010; http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and adapters were eliminated using Cutadapt (Martin, 2011). Remaining ribosomal RNA sequences were removed by the riboPicker (Schmieder et al., 2012) tool and sequences were checked by interlacing and de-interlacing paired-end reads ensuring that the same sequences were removed from each R1 and R2 files and finally retaining only R1 (performed in Galaxy, Afgan et al., 2016). Randomized subsets of 1% of the data were affiliated using BLASTX (Altschul et al., 1990) against the non-redundant (nr) protein database followed by the visualization in MEGAN6 (Huson et al., 2007) and proportions of unassigned to not assigned sequences as well as prokaryotic to eukaryotic sequences were retrieved (Supporting Information Fig. S1). These sequence data have been submitted to the EMBL databases under accession number PRJEB30315.

**Database construction**

A database containing sequences of genes involved into Fe-related metabolic pathway was retrieved from the study by Toulza et al. (2012). This database was constructed by screening for bacterial sequences from NCBI.
with the gene name as query, as well as the protein sequences from the Moore Microbial Genome database (http://www.moore.org/microgenome/) for genes involved in Fe metabolism. For the purpose of this study, the specific sequences for the following pathways were retrieved from the database and updated by searching for these in NCBI protein clusters: Flavodoxin switch (FL), Fe\(^{2+}\)-uptake (F2), Fe\(^{3+}\)-uptake (F3), siderophore-uptake (SU), and storage (ST) (Supporting Information Table S2). These five together are further named as ‘Fe’ database.

In addition, protein sequences for two supplementary enzymes were chosen for pathway specific analysis. Aconitase that catalyses the isomerization of citrate to isocitrate via cis-aconitlate in the tricarboxylic acid cycle and isocitrate lyase an enzyme in the glyoxylate shunt which catalyses the cleavage of isocitrate to succinate and glyoxylate. For maximal phylogenetic coverage, all available bacterial protein sequences were retrieved from protein clusters (proteins grouped on taxonomic groups which are non-redundant) using the NCBI search tool with the protein name as query.

In order to include more sequences from environmental marine bacteria the two databases were aligned using BLASTX tool (Altschul et al., 1990) against the Global Ocean Sampling (GOS) protein database (downloaded January 2018) and each custom database sequences with an e-value of <1e-5 were chosen for additional analysis and annotated from their KEGG-lid (Supporting Information Table S3). Finally, each database contained FASTA sequences with the taxonomic affiliation in the header as well as the KEGG-lid. For SU only Fe-related siderophore KEGG-lids were retained.

Additionally, all ribosomal protein sequences (n = 261 980) stored in the KEGG database were broadly (order level) annotated by retrieving taxonomic information for sequences from KEGG. These sequences were used to recruit ribosomal protein transcripts from our metatranscriptome data that served as verification of the normalization approach.

**Sequence alignment**

The final curated databases (n = 4, Fe, aconitase, isocitrate lyase and ribosomal proteins) containing information on the annotated taxonomic levels (phylum, class, order, family and genus) were aligned to the short-read translated DNA query sequences for each station and duplicate using diamond blastx (parameters used -k 1 -e 10 -p 12) (Buchfink et al., 2015). Total counts per phylum, class, order, family and genus for each database, and in case of Fe, for each pathway, were summed and relative proportions to all prokaryotic reads for bacterial groups were calculated (Supporting Information Fig. S7). Bacterial groups defined at the taxonomic order level with the highest abundances of pathway specific transcripts were taken for further analysis.

**Normalization approach**

In an attempt to estimate the absolute number of transcripts per sample, we followed the principals published elsewhere (Satinsky et al., 2013): the number of reads per sample is normalized by the number of reads obtained from an internal standard added with a known number of RNA molecules to the RNA extraction. However, in our case, we did not add internal standard RNA molecules, but instead based our calculations on the number of SAR11 isocitrate lyase transcripts for normalization, which were quantified earlier via qPCR (Beier et al., 2015).

While the number of SAR11 isocitrate lyase transcripts per L water derived from the qPCR approach might be biased, for instance, due to primer miss matches, such biases are strongly reduced for the ratio of SAR11-isocitrate lyase gene transcripts to SAR11-isocitrate lyase gene copies (Beier et al., 2015). For the normalization step, we therefore assumed that the above-mentioned ratio derived from qPCR data equals the ratio of SAR11-isocitrate lyase gene transcripts (metatranscriptome; RNA) to the number of SAR11 cell per L estimated by CARD-FISH (as described in the study by Fourquez et al. 2016):

\[
\frac{qT_{iso}}{qC_{iso}} = \frac{mT_{iso}}{nC_{SAR11}} \tag{1}
\]

where, for the purpose of this study, \(qT_{iso}/qC_{iso}\) is the ratio of SAR11-isocitrate lyase gene transcripts L\(^{-1}\) (\(qT_{iso}\)) to SAR11-isocitrate lyase gene copies L\(^{-1}\) (\(qC_{iso}\)) estimated by qPCR (Beier et al., 2015), \(mT_{iso}\) is the number of SAR11-isocitrate lyase gene transcripts L\(^{-1}\), and \(nC_{SAR11}\) is the SAR11 cells L\(^{-1}\) estimated by CARD-FISH (Fourquez et al. 2016).

The ratio \(qT_{iso}/qC_{iso}\) as well as \(nC\) are known variables and Eq. 1 can accordingly be resolved by \(mT_{iso}\). We subsequently related \(mT_{iso}\) to the number of metatranscriptome reads coding for SAR11 isocitrate lyase gene transcripts (\(mT_{iso}\)) and used this factor to estimate the absolute transcript numbers per L water or all remaining genes (\(mT\)).

qPCR data were only available for Station R-2 and F-L, thus samples from A3-2 were excluded from these calculations. Operational taxonomic units (OTUs) obtained by 16S rRNA gene sequencing were retrieved for Station R-2 and F-L from an already published data set from the same sampling date (Landa et al., 2016). The OTUs were
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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1.** Manuscript Codes

**Supplementary Figure 1.** Blast results of subsets for assigned to non-assigned reads.

**Supplementary Figure 2.** Relative contribution of prokaryotic groups to pathway specific transcripts. Prokaryotic group is defined until taxonomic family level. Number 1 and 2 refer to the sequencing results of duplicates per station.

**Supplementary Figure 3 A-C.** Detailed view of relative contribution of *Alpha* - and Gammaproteobacteria and FCB cluster to pathway specific transcripts. Note the different y-axis for *Gammaproteobacteria*. Number 1 and 2 refer to the sequencing results of duplicates per station.

**Supplementary Figure 4.** Percentage of ribosomal protein transcripts to all prokaryotic transcripts.

**Supplementary Figure 5 A-G.** Relative contribution of prokaryotic groups to pathway specific transcripts and...
relative abundance of 16S rRNA gene sequences. Prokaryotic group is defined until taxonomic order level.

**Supplementary Figure 6.** Simple illustration of the glyoxylate shunt (inside of the circle) in which isocitrate lyase cleaves isocitrate into glyoxylate and succinate.

**Supplementary Figure 7.** Detailed plots for duplicates on the phylum, class and order level for each pathway.

**Supplementary Table 1.** General information on sequencing results and reads.

**Supplementary Table 2.** Relative contribution of prokaryotic groups to pathway specific transcripts. For each group, mean value ± standard deviation of 2 replicates are shown. Error estimates are provided for all prokaryotic groups illustrated in Figs 2 & 3, and groups are listed by alphabetic order.

**Supplementary Table 3.** Taxa-specific transcript abundance (per $10^5$ cells) of a given pathway at Station F-L and R-2. For each group, mean value ± standard deviation of 2 replicates are shown. Error estimates are provided for all prokaryotic groups illustrated in Figs 6, and groups are listed by alphabetic order.

**Supplementary Table 4.** Information of databases constructed or modified from Toulza et al. (2012) and retrieved by NCBI.

**Supplementary Table 5.** Pathways and corresponding KEGG-Id numbers that were chosen for further analysis.

**Supplementary Table 6.** Cells per L for prokaryotic groups in Fig. 6 for station R-2 and F-L, calculated as described in experimental procedures.