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## Effects of iron limitation on growth and carbon metabolism in oceanic and coastal heterotrophic bacteria

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### Abstract

We investigated the metabolic response to iron (Fe) limitation of two bacterial strains of *Alteromonas macleodii*, isolated from a coastal and an oceanic marine environment. Bacteria were grown under Fe-limited and Fe-replete conditions, and comparative analyses of cellular properties and total proteomes were conducted. Respiration was reduced by a factor of two in both strains, but the growth rate of the oceanic strain was less affected by Fe limitation (reduced by 1.2-fold) than the coastal strain (reduced by 2-fold). Fe limitation led to significant changes in the expression of several key enzymes associated with carbon catabolism, specifically those involved in the citric acid cycle, glycolysis, and oxidative phosphorylation. The strain-specific overall responses to Fe limitation were in part reflected in different metabolic strategies of the carbon metabolism and energy acquisition. Our study provides novel insights on how Fe limitation can affect bacterial heterotrophic metabolism, and how this could influence the coupling of the Fe and carbon cycles in the ocean.

Of all the metals, iron (Fe) is required in the largest amounts by the most microorganisms on Earth. Because of its ability to gain or lose electrons, Fe has been integrated into a large set of proteins involved in major metabolisms, such as photosynthesis or nitrogen (N) fixation. In heterotrophic microorganisms, one particularly important role of Fe is related to carbon (C) catabolism and oxidative phosphorylation in the respiratory system. In aerobic cells, the complete oxidation of glucose and the associated production of adenosine triphosphate (ATP) are achieved by glycolysis, the citric acid cycle (CAC), and oxidative phosphorylation. Among these pathways, Fe is essential to the CAC and oxidative phosphorylation as they possess Fe-containing enzymes, including aconitase (Kennedy et al. 1983), succinate dehydrogenase (Singer and Johnson 1985), and fumarase (Flint et al. 1992). Moreover, components of the respiratory chain, the site of oxidative phosphorylation, represent the major sink of Fe in bacterial cells. Tortell et al. (1999) estimated that more than 90% of cellular Fe in *Escherichia coli* is located in the respiratory chain, where this metal is required in prosthetic groups of many electron carriers such as cytochromes and Fe–sulfur proteins.

However, Fe is found only in trace levels in ocean waters. Biological uptake of Fe, combined with the

subsequent sinking of part of this organic matter and the scavenging of Fe on biological or lithogenic material, contributes to the severe depletion of Fe in the surface layer of the ocean (Boyd and Ellwood 2010). The chemical properties of Fe render this micronutrient poorly available for microorganisms given that Fe(II) is rapidly oxidized to insoluble Fe(III) complexes in seawater (Millero et al. 1989). It is now well established that the exceedingly low concentrations of dissolved Fe (DFe) in many surface waters limit phytoplankton growth. This is typically the case in high-nutrient, low-chlorophyll (HNLC) regions. Consequences of the scarcity of Fe on phytoplankton metabolism and related biogeochemical processes have been extensively studied on model organisms, such as for example the diatom *Thalassiosira oceanica* (Strzepek and Harrison 2004; Bucciarelli et al. 2010) and the marine diazotroph *Crocospaera watsonii* (Saito et al. 2011).

By contrast, the effect of Fe limitation on heterotrophic bacterial metabolism is still poorly documented, but evidence exists that bacterial assemblages may be Fe limited in the ocean. In the Southern Ocean, bacterial growth was shown to be stimulated by the addition of Fe (Pakulski et al. 1996) and C plus Fe (Church et al. 2000). Tortell et al. (1996) reported that bacterial Fe quotas in the subarctic Pacific were similar to those of Fe-limited laboratory cultures, and more recent studies have shown that bacterial communities were Fe limited in the HNLC subantarctic (Mioni et al. 2005) and Antarctic (Bertrand et al. 2011) waters. However, field observations on the

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response of heterotrophic bacteria to Fe fertilization lead to ambiguous interpretations. A stimulation of heterotrophic bacteria can result from a direct effect of Fe on heterotrophic bacteria or from a cascading effect due to Fe-stimulated activity of phytoplankton providing more dissolved organic C (DOC). The coupling of Fe and C at the metabolic level also adds complexity.

Bacteria require large amount of Fe in their respiratory electron transport chain (ETS), which generates most of the ATP to fuel biosynthetic reactions. Previous reports have shown that Fe-limited marine heterotrophic bacteria have low ETS activities and reduced bacterial growth efficiencies (Tortell et al. 1996; Kirchman et al. 2003). This was explained by the respiration of a large proportion of C at the expense of biomass production. Thus, Fe availability for bacteria can affect the remineralization of organic C, influencing the fate of DOC and the flux of C in marine ecosystems. Given their high intracellular Fe demands, heterotrophic bacteria may also directly compete with phytoplankton for the acquisition of this scarce nutrient (Tortell et al. 1996; Maldonado and Price 1999). Heterotrophic bacteria are therefore key players of the coupling of the Fe and C cycles in the ocean, and further understanding of the responses of these microbes to Fe limitation is required.

The remodeling of the cellular metabolism in response to Fe availability has been extensively studied in organisms such as yeast (Kaplan et al. 2006), fungi (Parente et al. 2011; Philpott et al. 2012), and phytoplankton (Strzepek et al. 2011), but studies on marine heterotrophic bacteria have mainly been focused on Fe uptake systems (Armstrong et al. 2004; Hopkinson and Barbeau 2011). To date, the understanding of how Fe limitation in marine heterotrophic bacteria affects the pathways of C metabolism is a major challenge. Heterotrophic bacteria use C as a fuel for their metabolism, but a tight coupling between anabolism and catabolism does not exist, so that the relationship between ATP production and biomass is rather low (Russell and Cook 1995). This is explained by the existence of different metabolic pathways consuming ATP (del Giorgio and Cole 1998). Bacterial growth is closely related to ATP production in the cell, but ATP may also be consumed for maintenance expenses or active transport of substrates, and thus not used for biomass production. As a result, it is difficult to predict the effects of Fe limitation on the C metabolism in heterotrophic marine bacteria. A way to get answers is to study simultaneously the processes that generate (e.g., respiration) and consume (e.g., growth) ATP on a model organism, and to link them to the intrinsic cellular parameters (e.g., proteins).

In the present study, we investigated the effects of Fe limitation on heterotrophic bacterial metabolism using trace metal clean bioassays with two strains of the gammaproteobacterium *Alteromonas macleodii* (Baumann et al. 1972) isolated in contrasted marine environments (i.e., coastal and oceanic). The combined measurements of the overall cellular parameters, respiration and growth rates, and the total proteome analysis indicate a global common response in heterotrophic bacteria, with differences in the extent of Fe limitation at the strain level.

## Methods

*Study organism*—*A. macleodii* is a heterotrophic gram-negative gammaproteobacterium commonly identified in a variety of marine habitats (García-Martínez et al. 2002; Ivars-Martínez et al. 2008a), and it ranks among the 40 most abundant sequenced marine genomes present in the Global Ocean Sampling data set (Yooseph et al. 2010). *A. macleodii* harbors a large phylogenetic diversity (Pukall et al. 1999; García-Martínez et al. 2002). Many different strains belonging to this species have been isolated worldwide. This large diversity may in part be explained by adaptive mechanisms (Pukall et al. 1999). Despite strong similarity in the 16S ribosomal ribonucleic acid gene of two different strains of *A. macleodii*, Ivars-Martínez et al. (2008b) showed a large variability between their genomes, suggesting important genetic adaptability and flexibility. Given the current knowledge on *A. macleodii* and its potential in lab culture, this model organism was chosen in the present study.

We performed our experiments with two strains of *A. macleodii* isolated from contrasting environments and maintained in the Microbial Observatory Laboratoire Arago (MOLA) culture collection. The oceanic strain, MOLA377 (GenBank accession number: AM990654), was isolated from surface waters (5 m) of the oligotrophic South Pacific gyre (Sta. GYR4 of the Biogeochemistry and Optics South Pacific Experiment transect; 114°12'W, 26°24'S) where concentrations of nitrate ( $< 0.01 \mu\text{mol L}^{-1}$ ), phosphate ( $0.11 \mu\text{mol L}^{-1}$ ), and chlorophyll *a* ( $0.029 \mu\text{g L}^{-1}$ ) were particularly low in surface waters. The concentration of DFe was  $0.10 \text{ nmol L}^{-1}$  at 5 m (Blain et al. 2008). The coastal strain, MOLA60 (GenBank accession number: AM990835), was isolated from surface waters (3 m) at the Service d'Observation du Laboratoire Arago (Sta. SOLA) located about 1 km off the coast in the northwest Mediterranean Sea (42°29'N, 03°08'E). The MOLA60 strain was isolated from seawater sampled in July 2003 under oligotrophic conditions (concentrations of nitrate and phosphate  $< 0.1 \mu\text{mol L}^{-1}$  and concentrations of chlorophyll *a*  $< 0.5 \mu\text{g L}^{-1}$ ; Obernosterer et al. 2010). DFe concentrations were not measured at Sta. SOLA, but during summer they were expected to be of several  $\text{nmol L}^{-1}$  in surface waters of the northwest Mediterranean Sea (Bonnet and Guieu 2006).

*Culture conditions*—*A. macleodii* strains were cryopreserved in glycerol (35%) at  $-80^\circ\text{C}$  (<http://collection.obs-banyuls.fr/>). Cultures were initiated from a single colony that grew on a Marine Agar (Difco) plate at  $37^\circ\text{C}$  for 2 d. Bacterial cells were collected and transferred into liquid Marine Broth medium (Difco) and then allowed to grow for 24 h at  $37^\circ\text{C}$ . Cells were then inoculated in batch cultures containing the trace metal-buffered ( $0.1 \text{ mol L}^{-1}$  of ethylenediaminetetraacetic acid [EDTA]) Aquil medium (Morel and Rueter 1979; Price et al. 1989) modified by the addition of organic compounds (*see below*). Unlike traditional bacterial growth media, Aquil buffers free metal ion concentrations in culture experiments (Sunda and Huntsman 2003; Sunda et al. 2005). Strains were acclimated

to two concentrations of total Fe–EDTA ( $5.4 \mu\text{mol L}^{-1}$  and  $5.4 \text{ nmol L}^{-1}$ , denoted +Fe and –Fe, respectively) by daily transfer in either +Fe or –Fe fresh media for a period of 7–10 d. Finally, roughly  $1 \times 10^8$  acclimated cells were inoculated in 250 mL of Aquil with +Fe and –Fe treatments at a dilution of 1:1000. For experiments, cultures were conducted in triplicate at  $20^\circ\text{C}$ , in the dark, and they were continuously aerated with  $0.2 \mu\text{m}$ -filtered air (Acrodisc filter, Pall Corporation). The modified Aquil medium contains inorganic and organic nutrients in excess to ensure that Fe is the limiting factor for bacterial growth.

*Culture medium preparation*—All culture work and subsampling were conducted in a clean room (class 10,000) equipped with a laminar flow hood (class 100). We used polycarbonate bottles and plasticware soaked in 10% HCl for 24 h and subsequently soaked overnight with ultrapure water (18.2 Mohm resistivity, Elga). All the labware was systematically sterilized three times by microwaving (5 min, power 750 W) and then dried under the laminar flow hood before use.

Synthetic ocean water (SOW; Price et al. 1989) and solutions of inorganic nutrients ( $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$ ) and organic compounds (bacto-peptone and casein) were separately purified by removing trace metals using a Chelex 100 ion exchange resin (Bio-Rad). All solutions were filtered through metal-free  $0.2 \mu\text{m}$  syringe Acrodisc filters (Pall Corporation) before use. For modified Aquil preparation, the SOW was supplemented with organic compounds (bacto-peptone and casein, both at final concentration of  $0.2 \text{ g L}^{-1}$ ) and then sterilized by microwaving three times for 5 min at 750 W. The solutions were then cooled down to room temperature (RT) under the laminar flow hood, and vitamins (B12, thiamin, and biotin), phosphate, and nitrate were added in the same proportions as detailed in Price et al. (1989). Trace metal solutions were buffered with  $0.1 \text{ mol L}^{-1}$  of EDTA to result in free-ion concentrations similar to those calculated in Granger and Price (1999). Fe-replete (+Fe) and Fe-limited (–Fe) conditions were obtained by adding 300  $\mu\text{L}$  premixed solutions of Fe–EDTA (1:1) to 100 mL of modified Aquil, yielding final total Fe concentrations of  $5.4 \mu\text{mol L}^{-1}$  and  $5.4 \text{ nmol L}^{-1}$ , respectively. The premixed solution and the final medium were equilibrated for 24 h in the dark at  $20^\circ\text{C}$ . Granger and Price (1999) demonstrated that the labile inorganic Fe concentrations in the modified Aquil were directly proportional to the total Fe added. According to the work of Granger and Price (1999), the labile inorganic Fe concentrations in the resulting modified Aquil medium were  $35.8 \text{ pmol L}^{-1}$  and  $58 \text{ nmol L}^{-1}$  in –Fe and +Fe conditions, respectively. The concentrations of total Fe used in our study covered the range of concentrations in coastal and offshore marine environments.

*Measurements of intracellular Fe quota and elemental composition*—The Fe quota (mol Fe per cell) was measured using the radiotracer  $^{55}\text{Fe}$  (specific activity  $1.86 \times 10^3 \text{ Ci mol}^{-1}$ , Perkin Elmer). Following the period of acclimation cells were transferred into modified Aquil medium containing total Fe concentrations of  $5.4 \text{ nmol L}^{-1}$  (–Fe,

100%  $^{55}\text{Fe}$ ) or  $5.4 \mu\text{mol L}^{-1}$  (+Fe, 10%  $^{55}\text{Fe}$ ). The Fe cell quota was determined in the late exponential phase, after at least five generations, to ensure uniform labeling of cells with the radiotracer  $^{55}\text{Fe}$ . For radioactivity measurements, cells were filtered onto  $0.2 \mu\text{m}$  pore size, 25 mm diameter, nitrocellulose filters (Nucleopore) and subsequently washed with 1.5 mL of titanium–citrate–EDTA solution for 2 min to dissolve ferric species not incorporated by cells (Hudson and Morel 1989). The washing step was completed with 1 mL of  $0.2 \mu\text{m}$ -filtered SOW sitting on the filters for 1 min, repeated 10 times before filtration (Fourquez et al. 2012). Filters were placed in scintillation vials with 10 mL of Filter Count cocktail (Perkin Elmer). Finally, the vials were agitated overnight and  $^{55}\text{Fe}$  radioactivity counted by liquid scintillation (Beckman Coulter LS 6500). Fe cell quotas were determined for biological triplicates. For each biological replicate, three filters were prepared and counted. Controls were provided by incubating killed cells (formaldehyde, 2% final concentration) with the same amount of  $^{55}\text{Fe}$  as the +Fe and –Fe treatments, and then treated in the same way as the live treatments.

*Cellular C and N content*—To determine the organic C and N content of bacterial cells, 10 mL of the culture was filtered onto combusted ( $450^\circ\text{C}$ , 5 h) Whatman GF/F filters at the end of the exponential phase. The filters were rinsed with 10 mL of SOW and dried overnight at  $50^\circ\text{C}$ . Particulate organic C and N analyses were performed on a 2400 Perkin Elmer analyzer. For the blank determination,  $0.2 \mu\text{m}$ -filtered modified Aquil medium (10 mL, in triplicate) was treated as described above. Subsamples for the enumeration of bacterial cells were taken in parallel and processed as described below. Sampling for the cellular C and N content was done at the end of the exponential phase, simultaneously to the collection of cells for proteomics analyses.

*Respiration rates*—Bacterial respiration rates were measured using a Clark-type oxygen microelectrode (Unisense). The oxygen Clark microelectrode was calibrated before each experiment, as reported by the manufacturer (Unisense). Bacterial respiration rates were derived from the slope of the linear decrease of dissolved dioxygen ( $\text{O}_2$ ) over time. At the beginning of the exponential growth phase, subsamples of the bacterial cultures ( $\sim 5 \text{ mL}$ ) were transferred into clean, gas-tight chambers. Triplicate subsamples were collected from each biological replicate, and three consecutive respiration measurements, each lasting for 10 min, were done on every subsample. The concentration of  $\text{O}_2$  was monitored continuously over time (one measurement every 5 s) during the 10 min and data were subsequently visualized by the software MicOx (Unisense). Between measurements in one set of replicate subsamples, the electrode was transferred for 15 min into a control chamber with  $0.2 \mu\text{m}$ -filtered modified Aquil saturated in  $\text{O}_2$  (medium was bubbled during 10 min before sealing the chamber with the cap). This protocol allows drift correction of the electrode if any drift occurs. During the respiration measurement, the subsamples were stirred with small, glass-coated magnets (180 rpm) and submerged



in a water bath that was maintained at 20°C. Subsamples for the enumeration of cell abundances were taken before and after the respiration rate measurements.

**Total proteome analyses**—For the determination of total protein content, bacterial cells were collected from roughly 150 mL of culture (cell abundance was about  $1 \times 10^8$  cell mL<sup>-1</sup>) by centrifugation at  $15,000 \times g$  for 15 min at 4°C. Cell pellets were resuspended in 1 mL of 0.2 µm-filtered SOW and centrifuged twice at  $10,000 \times g$  for 10 min at 4°C. Pellets were conserved at -80°C until protein extraction.

For protein extraction, cell pellets were suspended in 2 mL of lysis buffer (urea 7 mol L<sup>-1</sup>, thiourea 2 mol L<sup>-1</sup>, tributyl phosphine 2 mmol L<sup>-1</sup>, dithiothreitol [DTT] 10 mmol L<sup>-1</sup>, C7BZ0 0.5%, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate 2%) and agitated until total dissolution. Cells were disrupted by freezing and defreezing steps followed by sonication at 4°C with pulses of 2 s for 2 min. Samples were then centrifuged at  $10,000 \times g$  for 30 min at RT and the supernatants (protein extract) were stored at -20°C until protein quantification using the Bio-Rad protein assay.

**Two-dimensional gel electrophoresis**—The first-dimension gel separation was carried out with a 18 cm long pH 4.7 ReadyStrip (Bio-Rad), rehydrated overnight at RT with 350 µL lysis buffer, supplemented by 0.2% Coomassie Blue R-250. Proteins (80–100 µg) were resuspended in lysis buffer supplemented by carrier ampholytes 1% v : v (pH 3–10, GE Healthcare), and loaded onto rehydrated strips. Isoelectric focusing was performed using the IPG-Phor III (GE Healthcare) system with the following parameters: 500 V for 1 h, 1000 V for 1 h, 10,000 V for 3 h, and 10,000 V constant for 1.5 h, for a total of 31,500 V × h. After focalization, strips were first reduced in 2% DTT and alkylated in 2% iodoacetamide, contained in a solution composed of urea 7 mol L<sup>-1</sup>, Tris-HCl 50 mmol L<sup>-1</sup> pH 6.8, glycerol 30%, sodium dodecyl sulfate (SDS) 2%, and Coomassie blue 1%.

The second dimension consisted of SDS-polyacrylamide gel electrophoresis using 10% (w : v) acrylamide and bisacrylamide resolving gels in a Protean II XL cell (Bio-Rad). Strips were laid on the separation gel and maintained with 0.5% agarose in migration buffer. Migration was carried out at 4°C using the following parameters: 30 min at 5 mA per gel, 1.5 h at 10 mA per gel, and 3.5 h at 20 mA per gel. After migration, the protein spots were revealed by silver nitrate staining.

**Gel analysis**—For each biological replicate, one protein extraction was performed, and for each protein extraction, two gels were realized. In total for the two strains, 27 gels were realized: six gels for each condition (i.e., +Fe and -Fe) plus three additional gels for spot excision. After staining, the gels were scanned with the fluorimager ProXpress from Perkin Elmer, and images were analyzed by the software SameSpots 4.0 (Nonlinear Dynamics) for normalization of the gels and statistical analyses. The two-dimensional (2D) gels were superimposed to form a standard image. The different standard gels for +Fe and

-Fe conditions were then compared for differential expression analysis. Volumes of spots were automatically normalized by the software and a statistical analysis was performed to compare the corresponding reference gels of the two conditions (+Fe and -Fe). In this study, the statistical criteria used were as follows: analysis of variance (ANOVA) significance and *q* value < 0.05, *P* (power analysis) > 0.8, and a volume difference  $\geq 1.6$ . For the ANOVA, only spots whose average normalized volume was  $\geq 65,000$  were considered.

**Database analysis**—The protein spots that had a significantly different expression following 2D gel analysis were manually excised and subjected to in-gel digestion with MultiPROBE II (Perkin Elmer) using trypsin, as previously described in Sulaeman et al. (2012), to be then analyzed in liquid chromatography coupled with tandem mass spectrometry (MS<sup>2</sup>), and OrbiTrap. The peak lists from the MS<sup>2</sup> spectra obtained were compared to the National Center for Biotechnology Information database in 2010 and 2011 (restricted to bacteria) using the Mascot Daemon search program (<http://matrixscience.com>).

Identified proteins were localized into metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes pathway database (<http://www.genome.jp/kegg/pathway.html>) and were linked together using the Search Tool for the Retrieval of Interacting Genes/Proteins database (<http://string-db.org>).

**Bacterial abundance and biovolume**—Bacterial cell abundance was monitored by flow cytometry. Cells were fixed (2% formaldehyde, final concentration) for 15 min at 4°C and stored frozen at -80°C until processed. For enumeration, bacteria were stained with SYBR<sup>®</sup> Green I and counted using a Becton Dickinson fluorescence-activated cell-sorting Calibur flow cytometer (Marie et al. 1997). Specific growth rates were calculated from the slope of log-linear regression over time during the exponential phase.

For the determination of the biovolume, formaldehyde-fixed cells were filtered onto 0.2 µm polycarbonate filters (Millipore) and stained with 4',6-diamidino-2-phenylindole (2 µL mL<sup>-1</sup> in a 4 : 1 mixture of Citifluor : Vectashield, Vector Laboratories). The bacterial cell volume was determined by epifluorescence microscopy (Olympus Provis AX70), using an image analysis system (Microbe Counter software; Cottrell and Kirchman 2003). Ten fields of view, each containing on average 150 cells, were counted per filter. Mean biovolumes of each biological replicate for Fe-replete and Fe-limited cultures were determined.

**Statistical analysis**—All statistical comparisons between +Fe and -Fe treatments were performed using a two-tailed, unpaired Student's *t*-test (Microsoft Excel Analysis ToolPak). Differences were considered statistically significant at  $p \leq 0.05$ .

## Results

**Biovolumes and Fe quotas**—Fe limitation affected bacterial biochemical composition and metabolism in several

Table 1. Biochemical composition, respiration, and growth rates of the oceanic strain (MOLA377) and the coastal strain (MOLA60) of *Alteromonas macleodii* grown under Fe-replete (+Fe) and Fe-limited (–Fe) conditions. Values are means of biological triplicate  $\pm$  standard deviation (SD). The Student's *t*-test compares the difference between +Fe and –Fe treatment.

	Oceanic strain			Coastal strain		
	+Fe	–Fe	<i>p</i>	+Fe	–Fe	<i>p</i>
Fe quota ( $10^{-20}$ mol Fe $\mu\text{m}^{-3}$ )	99.2 $\pm$ 5.8	6.2 $\pm$ 0.8	10 $^{-5}$ *	227 $\pm$ 25.5	7.2 $\pm$ 2.2	10 $^{-2}$ *
C quota (fmol C $\mu\text{m}^{-3}$ )	62.2 $\pm$ 6.1	145 $\pm$ 19.8	2 $\times$ 10 $^{-3}$ *	17.2 $\pm$ 2.25	125 $\pm$ 3.7	2 $\times$ 10 $^{-6}$ *
N quota (fmol N $\mu\text{m}^{-3}$ )	13.8 $\pm$ 1.5	33 $\pm$ 4.3	2 $\times$ 10 $^{-3}$ *	3.36 $\pm$ 0.33	28.3 $\pm$ 1.1	2 $\times$ 10 $^{-6}$ *
C:N ratio (mol mol $^{-1}$ )	4.5 $\pm$ 0.1	4.4 $\pm$ 0.1	2 $\times$ 10 $^{-1}$	5.1 $\pm$ 0.2	4.4 $\pm$ 0.2	10 $^{-2}$ *
Fe:C ratio ( $\mu\text{mol mol}^{-1}$ )	16.1 $\pm$ 2.3	0.43 $\pm$ 0.1	3 $\times$ 10 $^{-4}$ *	141 $\pm$ 24.7	0.56 $\pm$ 0.19	10 $^{-2}$ *
Respiration rate (fmol O <sub>2</sub> cell $^{-1}$ h $^{-1}$ )	11.1 $\pm$ 0.4	5.3 $\pm$ 0.1	3 $\times$ 10 $^{-3}$ *	11.2 $\pm$ 2.6	6.2 $\pm$ 1.4	4 $\times$ 10 $^{-2}$ *
Growth rate (d $^{-1}$ )	11.5 $\pm$ 0.2	9.6 $\pm$ 0.1	10 $^{-3}$ *	12.2 $\pm$ 0.1	6.1 $\pm$ 0.5	2 $\times$ 10 $^{-5}$ *

\* Difference is statistically significant at 95% confidence level ( $p \leq 0.05$ ).

ways, and some pronounced differences between the two strains were detectable. For the oceanic strain, biovolumes of cells grown under Fe-limited conditions ( $0.19 \pm 0.04 \mu\text{m}^3$ ) were, on average, 2.5-fold higher than biovolumes of cells grown under Fe-replete conditions ( $0.08 \pm 0.01 \mu\text{m}^3$ ). These results were obtained for the late exponential phase and the stationary phase. To our best of our knowledge, only one study has thus far reported similar results in *E. coli*, where Fe limitation led to an increase by a factor of two of the biovolume (Hubbard et al. 1986). By contrast, biovolumes of the coastal strain were not affected by Fe limitation ( $0.10 \pm 0.01 \mu\text{m}^3$  for both treatments). Because of these differences in the biovolumes between strains and treatments, the Fe cell quota is presented on a volumetric basis. For both strains, Fe quotas decreased in response to Fe limitation. The reduction was most pronounced for the coastal strain, with the Fe quota 31-fold lower in –Fe than in +Fe treatment (Table 1). For the oceanic strain, the Fe quota decreased by a factor of 16. Under Fe limitation, Fe quotas were similar between the two strains (Table 1), and these values are in good agreement with those reported elsewhere (Granger and Price 1999).

**Biochemical composition**—Concentrations of C and N were two to eight times higher in Fe-limited cells compared to Fe-replete cells. By contrast, ratios of C:N were not affected by Fe limitation. At the end of the exponential phase, the C:N ratio equaled 4.5 mol mol $^{-1}$  (mean value of the two strains; Table 1), which is consistent with the estimated 4.4 mol mol $^{-1}$  as reported in the literature (Fagerbakke et al. 1996). To better understand the increase in C and N with a constant ratio, we investigated several potential mechanisms. We compared the protein content of +Fe and –Fe cells in the oceanic strain. When normalized to biovolume, the protein content was not significantly different between treatments ( $4.8 \pm 0.1$  and  $5.3 \pm 0.5 \times 10^{-10}$  mg  $\mu\text{m}^{-3}$  for +Fe and –Fe cells, respectively; Student's *t*-test,  $t = 0.33$ , degrees of freedom = 4,  $p = 0.76$ ). Thus, the increase in C and N contents does not seem to be related to higher protein levels in Fe-limited cells. We also investigated the presence (or absence) of exopolysaccharides (EPS) and storage of polysaccharides using transmission electron microscopy, according to the staining

techniques of Heissenberger et al. (1996) and Thiery (1967), respectively. We detected storage of polysaccharides only in the coastal strain of *A. macleodii*, grown under +Fe conditions. Through these approaches, we established that the strains MOLA377 and MOLA60 of *A. macleodii* do not produce EPS or store polysaccharides in response to Fe limitation. These bacterial strains may store C and N in other forms for subsequent utilization when Fe limitation is elevated, but this point deserves additional study.

**Bacterial metabolism**—Fe limitation affected respiration and growth rates in different ways. For both strains, respiration of cells grown under Fe-limited conditions was reduced by twofold compared to those grown under Fe-replete conditions (Table 1). By contrast, growth rates of the oceanic strain were less affected (reduced by 1.2-fold) than those of the coastal strain (reduced by 2-fold) by Fe limitation (Table 1).

Bacterial growth efficiency is an important parameter to evaluate the fate of organic C in marine environments that can be calculated on the basis of respiration rate, growth rate, and C content in cells. However, in our experiments, it was not possible to measure C content and respiration rates at the same time. For this reason, we address the effect of Fe limitation on respiration rates and C content separately in this manuscript.

**Proteins**—To better understand these differences in the overall responses to Fe limitation, we used a proteomic approach and investigated variations in total proteins by 2D gel analysis. From gel analyses, a total of 1719 and 1408 spots (whose average was  $\geq 65,000$ ) were discriminated on 2D electropherograms obtained from MOLA377 and MOLA60, respectively. Gel comparison among treatments (i.e., –Fe and +Fe) allowed us to determine that in MOLA377 152 spots (102 overexpressed and 50 underexpressed) and in MOLA60 98 spots (44 overexpressed and 54 underexpressed) were affected by the growth conditions. MS<sup>2</sup> analysis followed by sequence matching on the protein database resulted in the identification of 88 and 99 of these differentially expressed proteins and protein isoforms by the oceanic (MOLA377) and the coastal strain (MOLA60), respectively (see Web Appendix, Tables A1–A4, Figs. A1 and A2, [www.aslo.org/lo/toc/vol\\_59/issue\\_2/0349a.html](http://www.aslo.org/lo/toc/vol_59/issue_2/0349a.html)).

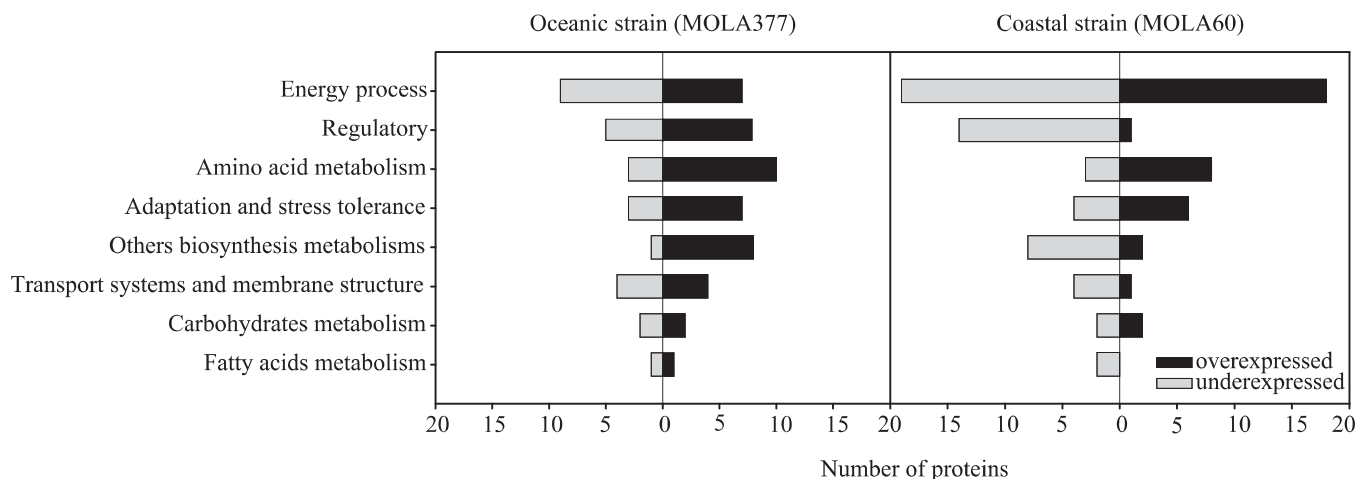


Fig. 1. Functional category distribution of proteins differently expressed in response to Fe limitation in the oceanic (MOLA377) and the coastal strain (MOLA60) of *Alteromonas macleodii*. Black bars denote proteins that are overexpressed in the  $-Fe$  treatment as compared to the  $+Fe$  treatment. Gray bars denote proteins that are underexpressed in bacteria grown in  $-Fe$  medium compared to  $+Fe$  medium. Note that for both strains, energy process category regroups the most of proteins that are differently expressed under Fe limitation.

In the present study, the terms “overexpressed” and “under-expressed” are used to refer to statistically significant changes in protein abundance in the  $-Fe$  treatment relative to the  $+Fe$  treatment. For the oceanic strain, 32 proteins and protein isoforms were underexpressed and 56 proteins and protein isoforms were overexpressed in response to Fe limitation (Fig. 1). For the coastal strain, 41 proteins and protein isoforms were overexpressed and 58 proteins and protein isoforms were underexpressed in response to Fe limitation (Fig. 1). Changes in the expression of individual enzymes in response to Fe limitation were overall specific to one strain. Only nine and six enzymes were concurrently overexpressed or underexpressed in both strains. These differently expressed proteins were classified into the functional categories amino acid metabolism, fatty acid metabolism, carbohydrate metabolism, regulatory pathways, transport systems and membrane structure, adaptation and stress tolerance, other biosynthesis metabolism, energy process, and unknown or multiple functions (see Web Appendix, Tables A1–A4). Proteins belonging to the energy process category accounted for the highest fraction of differently expressed proteins in response to Fe limitation: 21% and 39% for the oceanic and coastal strain, respectively. In the present study, the energy process category includes pathways that participate in energy production, such as glycolysis, the CAC, and oxidative phosphorylation.

The key glycolytic enzymes phosphopyruvate hydratase (or enolase) and glyceraldehyde-3-phosphate dehydrogenase were underexpressed in response to Fe limitation in the coastal strain (Fig. 2). By contrast, the expression of gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK, both strains) and fructose-1,6-bisphosphatase (oceanic strain) increased. In the CAC, several enzymes were differently expressed in the coastal strain. Of the three Fe-containing proteins in CAC, only the enzyme aconitase was differentially detected by 2D gel

analysis. Aconitase was underexpressed in Fe-limited compared to Fe-replete cells. It was, however, interesting to note that fumarase C, the only non-Fe-containing fumarase, was overexpressed in response to Fe limitation. Fe limitation also affected the expression of isocitrate dehydrogenase (IDH), an enzyme that catalyzes the consecutive step in the CAC. This is likely a consequence of the reduced aconitase expression that should lead to a decrease in the formation of isocitrate, the substrate of IDH (Fig. 2). Seven isoforms of the main enzyme in the glyoxylate shunt, isocitrate lyase, were overexpressed in Fe-limited cells of the coastal strain. These protein isoforms catalyze the formation of glyoxylate from isocitrate and play a regulatory role in the glyoxylate cycle. In the oceanic strain, proteins involved in energy pathways using alternative sources of C, such as acetate or fatty acids, were overexpressed in Fe-limited cells. The increased expression of these enzymes is consistent with the decreased expression of enzymes involved in the CAC, because these pathways are closely coupled. For the oceanic strain, several enzymes involved in the catabolism of amino acids were overexpressed under Fe limitation. The degradation of amino acids leads to the formation of compounds such as succinyl-coenzyme A (CoA) and acetyl-CoA, which are part of an intermediary metabolism to produce energy or for the synthesis of carbohydrates and lipids. For the coastal strain, we also notified the overexpression of the enzyme acetyl-CoA synthetase, which catalyzes the conversion of acetate into acetyl-CoA.

Another energy process affected by Fe limitation is oxidative phosphorylation. Two proteins related to electron transport and containing Fe were underexpressed. These proteins included the Fe-sulfur subunit in the  $Na^+$ -nicotinamide adenine dinucleotide (NADH) quinone reductase complex for the coastal strain, and the Fe-sulfur subunit in the *bcl* complex for the oceanic strain (see Web Appendix, Tables A2 and A4). The expression of the



electron transfer flavoprotein beta subunit also decreased in response to Fe limitation in the coastal strain. Finally, in the respiratory chain, the electron transport ATP synthase complex was affected through the reduced expression of the subunit beta (both strains) and the subunit gamma (oceanic strain).

## Discussion

Our results demonstrate that Fe limitation induces significant alterations in biochemical properties, respiration, and C metabolism of heterotrophic bacteria, and that these modifications vary between closely related marine bacterial strains. The acclimation process is largely mediated by proteins. Differential expressions of enzymes, including Fe-containing proteins, involved in energy metabolism provide insights into the molecular mechanisms that underpin the contrasted responses observed.

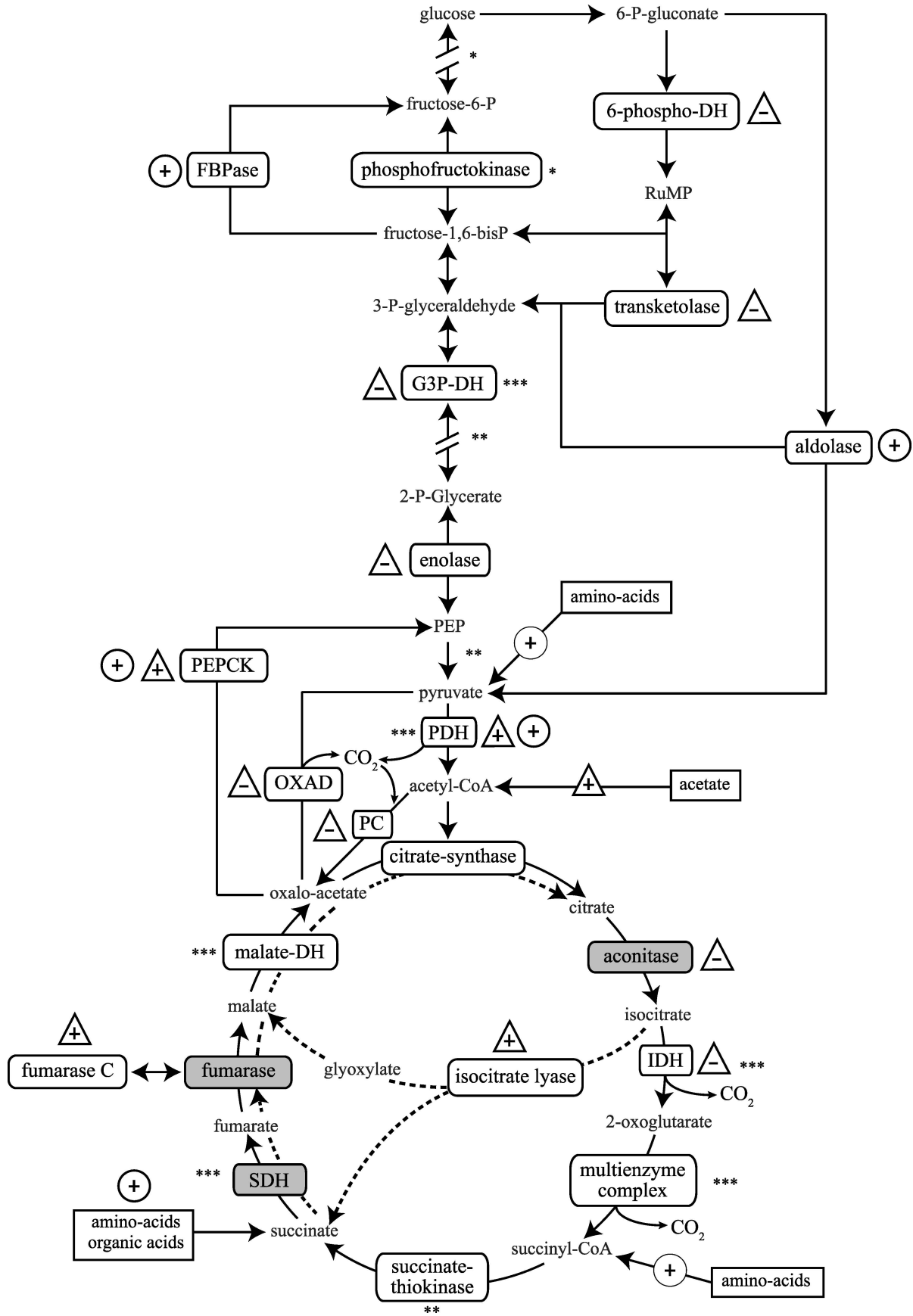
The reduction of the Fe quota is a physiological response to different levels of Fe stress that has been noted in culture and field observations and for various marine autotrophic microorganisms (Sunda and Huntsman 1995; Berman-Frank et al. 2010; Strzepak et al. 2011). Investigations at the molecular level have provided explanations for these modifications. Rearrangements of the molecular architecture of the photosynthetic apparatus could lead to significant economy of Fe under limited conditions (Strzepak and Harrison 2004; Saito et al. 2011), whereas the capability for Fe storage could result in luxury uptake under replete conditions (Marchetti et al. 2009). Our results are consistent with previous observations on heterotrophic bacteria (Granger and Price 1999) and confirm that the cellular Fe quota is largely dependent on Fe concentrations leading to pronounced changes in metabolism. Here, using a whole-cell proteomic approach combined with bulk measurements, we report the first comprehensive interpretation on the biochemical mechanisms responsible for these responses.

The reduction in the Fe cell quota should result in an underexpression of Fe-containing proteins with major modifications in the associated metabolic rates. For example, Fe deficiency sets a limit to the formation of Fe-containing prosthetic groups such as cytochromes and Fe-sulfur proteins, which could cause an overall reduction in the respiration rate (Hubmacher et al. 2003; Morrissey and Bowler 2012). Experimental evidence for a decrease in respiration induced by Fe limitation in marine bacteria is scarce. Two previous studies provide indirect evidence demonstrating that the ETS is markedly lowered by Fe limitation (Tortell et al. 1996; Kirchman et al. 2003). Our study is the first to provide direct measurements, and we show that under Fe-limited conditions respiration is reduced by 50% in both strains (Table 1; Fig. 3). The decrease in respiration was accompanied by the modification of the expression of several enzymes involved in oxidative phosphorylation, but also in glycolysis and the CAC. These results demonstrate the effect of Fe limitation on enzymes located in the respiratory chain and also on C metabolism. We provide here insights into the regulation of these pathways, and how they differ between a coastal and an oceanic strain of *A. macleodii* (Fig. 2).

The coastal strain was overall more affected, and several modifications of the expression of key enzymes in the CAC were noticeable. The expression of aconitase and IDH was reduced by Fe limitation, whereas that of fumarase C and isocitrate lyase was increased. The enzyme aconitase catalyzes the conversion of citrate into isocitrate through a [4Fe-4S] cluster, and its expression is linked to the cellular Fe status (Beinert et al. 1997). The reduced expression of IDH, a non-Fe-containing enzyme, could result from the limited availability of its substrate, isocitrate, which is produced by aconitase. Thus, the reduction of aconitase expression could indirectly affect the expression of IDH under Fe limitation, as suggested for eukaryotic cells (Oexle et al. 1999). On the other hand, the phosphorylation and dephosphorylation cycle of IDH is also known to regulate the activity of this enzyme (LaPorte 1993). When IDH is phosphorylated, its activity is inhibited, and consequently isocitrate would pass through the glyoxylate cycle, an alternative pathway that bypasses the step in the CAC where C is lost as CO<sub>2</sub>. Isocitrate lyase is a key enzyme in the glyoxylate cycle, and it was overexpressed in Fe-limited as compared to Fe-replete cells (Fig. 2). This enzyme recovers isocitrate from the CAC and catalyzes its transformation into succinate and glyoxylate. These findings support the idea that Fe limitation promotes C flux through the glyoxylate cycle. A similar observation was made for *Candidatus pelagibacter ubique* (SAR11 clade), where messenger ribonucleic acid transcripts for the gene encoding isocitrate lyase were at least 50% more abundant in the Fe-limited cultures compared to the control cultures (Smith et al. 2010). The glyoxylate cycle is essential for microorganisms to grow on certain sources of C such as acetate or fatty acids, which allows the conversion of acetyl-CoA into intermediate compounds without the loss of C as CO<sub>2</sub>.

Our results also suggest that the coastal strain of *A. macleodii* remodels its energetic metabolism to deal with low Fe availability and optimizes its metabolic activity with a reduced pool of Fe-containing proteins. The expression of fumarase C, the non-Fe-containing fumarase, could be favored by Fe limitation at the expense of fumarases A and B, thereby maintaining the functioning of central metabolic pathways independently of changes in extracellular Fe concentrations (Fig. 2). Glucose is a primary product that is used in many ways to generate energy in the cell. The Entner-Doudoroff pathway, or channel 2-keto-3-deoxy-6-phosphogluconate, is, together with glycolysis and the pentose phosphate pathway, one of the three routes of degradation of glucose into pyruvate. We showed a down-expression of several key enzymes involved in glycolysis and the pentose phosphate pathway, whereas the aldolase in the Entner-Doudoroff pathway was up-expressed (Fig. 2). The Entner-Doudoroff pathway converts glucose to pyruvate following a series of steps catalyzed by enzymes different from those used in either the pentose phosphate pathways or glycolysis. Redirection of glucose into this pathway may allow Fe-limited cells to bypass the first step of glycolysis, which consumes ATP while maintaining pyruvate production. Pyruvate then enters the CAC converted to acetyl-CoA through the enzymatic activity of





pyruvate dehydrogenase (PDH). This enzyme was up-expressed in Fe-limited cells (Fig. 2).

Glycolysis (glucose degradation) and gluconeogenesis (glucose synthesis from non-glucidic substrates such as amino acids) are other pathways markedly affected by Fe limitation. These pathways are regulated in such a way that glycolysis is relatively inefficient when gluconeogenesis is activated and vice versa. In Fe-limited cells, the expression of several enzymes involved in gluconeogenesis were overexpressed, such as PEPCK (both strains) and fructose-1,6-bisphosphatase (oceanic strain). Under Fe-limited conditions, the synthesis of PEPCK was also elevated in the pathogen *Mycobacterium tuberculosis* (Wong et al. 1999). By contrast, Oexle et al. (1999) reported that glycolysis was stimulated in Fe-limited eukaryotic cells following the impairment of aerobic ATP generation. These contrasting responses could be related to the nature of the C sources used in the experiments. C enters the CAC primarily as acetyl-CoA. The production of acetyl-CoA from carbohydrates is, therefore, a major control point of the cycle. Other catabolic processes provide substrate to the CAC. For example, the intracellular recycling through the increased amino acid catabolism in *A. macleodii* might be used to increase the availability of pyruvate and acetyl-CoA precursors of the CAC (Fig. 2). A similar “biomass recycling” response was revealed in the diatom *T. oceanica* when cells were exposed to Fe stress; this strategy was proposed to support cellular maintenance under stress conditions (Lommer et al. 2012).

For the experimental conditions used in our study, we propose that the decreased expression of enzymes in the CAC was compensated for by a high C demand, as suggested by Kirchman et al. (1990). In this case, gluconeogenesis may be used to promote glucose formation from non-glucidic substrates, and the catabolism of amino acids would be stimulated to supply the C demand (Fig. 2). This may involve potential interactions between the supply and quality of C and Fe availability for bacterial growth. This assumption suggests that the composition and availability of marine dissolved organic matter could affect the manner of metabolic remodeling under Fe limitation, with important consequences for the fate of organic C following bacterial processing. These changes in the expression of enzymes could also have important implications for the efficiency of bacterial growth, because the CAC and glycolysis produce NADH and flavin adenine dinucleotide (FADH<sub>2</sub>) and also small amounts of ATP. Thus, the effect of Fe limitation on these pathways that feed into the

oxidative phosphorylation may be associated with the decreased expression levels of enzymes that are part of the respiratory chain.

The production of ATP by the electron transport system of the respiratory chain depends on the electrochemical gradient established around the inner membrane. We have shown the reduced expression of transmembrane complexes involved in pumping of H<sup>+</sup> and Na<sup>+</sup> ions in Fe-limited *A. macleodii* cells. This modification should reduce the proton-motive force and therefore the associated ATP production. For the oceanic strain, the analysis of the total proteome reveals that the expression of the ubiquinol-Fe-sulfur subunit was reduced by Fe limitation. This protein is part of the cytochrome bcl complex of the respiratory chain. Similar results were also reported for *Halobacterium salinarum* (Hubmacher et al. 2003) where an alternative terminal oxidase (bd oxidase) was preferred under Fe-limited conditions. The gene coding for bd oxidase was identified in the genome of *A. macleodii* deep ecotype (Ivars-Martinez et al. 2008b). Many microorganisms possess an alternative pathway in the respiratory chain that is activated when the main cytochrome is oversaturated or inhibited (Richardson 2000; Andrews et al. 2003). A more thorough investigation of the respiratory chains in *A. macleodii* strains is, however, necessary to determine whether an alternative pathway exists.

Finally, our data show that the reduction of the oxidative phosphorylation is linked to a decrease in several ATP synthase subunits. Consequently, this could alter the energetic status in the cells. Stored chemical energy, such as in the form of ATP, is used to drive many biochemical reactions in the cell, particularly those involved in biosynthesis pathways (e.g., production of amino acids or fatty acids). As a consequence, failures in energy processes that participate in ATP production can largely affect cellular growth.

In the present study, the reduction in growth rate induced by Fe limitation was greater in the coastal strain compared to the oceanic strain. Interestingly, the decrease in respiration was similar for both strains (Fig. 3). Fe influences respiration and growth rates, as previously observed (Tortell et al. 1996; Kirchman et al. 2003). The close coupling between respiration and growth is not surprising given that oxidative phosphorylation produces the major part of ATP in aerobic cells. However, the high flexibility of the respiratory chain in bacteria (Richardson 2000) and the existence of several pathways consuming ATP (del Giorgio and Cole 1998) do not allow the establishment of

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Fig. 2. Changes in the expression patterns of enzymes belonging to the CAC and glycolysis in response to Fe limitation of *Alteromonas macleodii*. Enzymes that are overexpressed and underexpressed in Fe-limited conditions compared to Fe-replete conditions are indicated by the symbols + and –, respectively. No symbol means that the protein was not determined to be statistically different between treatments. Triangles and circles stand for the coastal (MOLA60) and the oceanic (MOLA377) strain of *A. macleodii*, respectively. The dashed line represents the glyoxylate pathway. Enzymes are framed and Fe-containing enzymes are notified in gray boxes. \*\*\*, production of NADH or FADH<sub>2</sub>; \*\*, production of ATP or guanosine triphosphate (GTP); \*, consumption of ATP; G3P-DH, glyceraldehyde-3-phosphate dehydrogenase; OXAD, oxaloacetate decarboxylase; PC, pyruvate carboxylase; SDH, succinate dehydrogenase; PEP, phosphoenolpyruvate; 6-phospho-DH: glucose-6-phosphate dehydrogenase; RumP, ribulose monophosphate; malate-DH, malate dehydrogenase; fructose-6-P, fructose-6-phosphate; fructose-1,6-bisP, fructose-1,6-bisphosphate; FBPase: fructose-1,6-bisphosphatase.

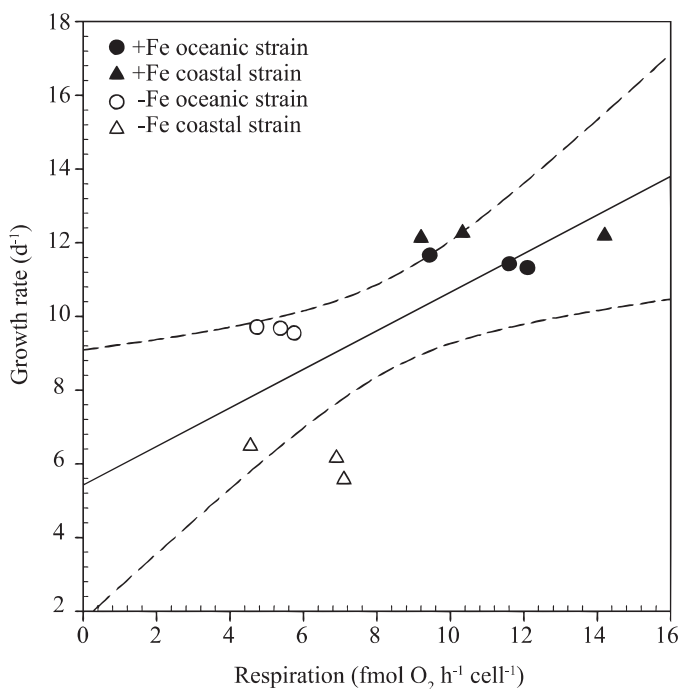


Fig. 3. Relationship between growth rate and respiration in *Alteromonas macleodii*, oceanic (MOLA377) and coastal (MOLA60) strain. Black symbols denote +Fe culture conditions and white symbols denote -Fe culture conditions. The plotted line (—) was obtained by least-square regression including all values ( $r^2 = 0.45$ ,  $p = 0.002$ ). Dashed line (---) represents 95% confidence intervals.

strict relationships between respiration, ATP production, and growth. In this regard, in *A. macleodii*, the statistical strength of the correlation between respiration and growth rate is lower ( $r^2 = 0.45$ ,  $p = 0.002$ ) than that reported by Tortell et al. (1996;  $r^2 = 0.65$ ,  $p = 0.005$ ). In fact, the growth rate is less affected by Fe limitation for the oceanic strain compared to the coastal strain, which creates high variability in the relationship (Fig. 3). The capability of the oceanic strain of *A. macleodii* to maintain a high growth rate is not explained by the efficiency to obtain more Fe, as both strains had similar intracellular Fe levels under Fe-limited conditions. Our results suggest that the oceanic strain requires less Fe for its metabolic activities, which would denote a different configuration of the cellular machinery. As respiration is the main way to produce ATP in aerobic cells, and as both strains had similar respiration rates under Fe-limited conditions, another explanation would be a different use of energy in the cell. This could imply that the oceanic strain takes advantages through its specific cellular configuration when Fe is scarce in the environment.

Fe limitation of natural bacterial communities has important implications for the cycling of C. Our study clearly shows a bacterial metabolic response to Fe limitation. The respiratory quotient may dramatically change because of the expression of alternative metabolic pathways, such as the glyoxylate cycle, which bypasses the two steps of CO<sub>2</sub> production in the CAC. These modifications of the C metabolism, together with the

decrease in O<sub>2</sub> consumption and in growth rate, suggest that the bioavailability of Fe for heterotrophic bacteria should not be overlooked when considering their key role in the biogeochemical cycle of C.

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