

The interplay between Fe-limitation, carbon and light in a (photo)heterotrophic bacterium

Coco Koedooder

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THE INTERPLAY BETWEEN FE-LIMITATION, CARBON AND LIGHT IN A (PHOTO)HETEROTROPHIC BACTERIUM

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voor wie dit leest

•••

"... en goed kauwen he? - dz belangrijk- goed kauwen, dat je eten gelijkmatig in je bloed komt..."

- van Kooten en de Bie

SUMMARY (ENG)

* For the 10 page summary in French please go to Section VI

Iron (Fe) is an essential element for marine microbial growth but is present in trace amounts (<0.1 nM) in surface waters of the ocean. In heterotrophic bacteria, Fe-limitation particularly impacts ATP production as Fe is an essential co-factor for enzymes involved in the electron-transport chain as well as the tricarboxylic acid (TCA) cycle. Fe-limitation therefore drastically reduces intracellular energy production which subsequently affects both bacterial growth and respiration. At a more global scale, the impact of Fe-limitation on heterotrophic bacteria affects the efficiency of organic carbon remineralization within the ocean.

Heterotrophic bacteria have been shown to implement various strategies to cope in the presence of Fe-limitation. In this thesis manuscript genetic approaches were used to gain insight into two potential mechanisms, namely the role of the glyoxylate shunt may and proteorhodopsin using the gammaproteobacterium, *Photobacterium angustum* S14.

Proteorhodopsin is a light activated proton pump found in several heterotrophic bacteria which, if coupled to ATP synthase, could ultimately generate a protein gradient for the production of ATP. We hypothesized that proteorhodopsin will be upregulated under Fe-limiting conditions in order to partially recover energy loss within the cell.

The glyoxylate shunt is a two-step metabolic pathway found in aerobic bacteria that serves as an alternative route within the classic tricarboxylic acid (TCA). Its primary function is to circumvent the carbon dioxide (CO₂) production step within the TCA cycle in order to drive the metabolism of fatty-acids or two carbon-compounds such as acetate toward the production of oxaloacetate, which can further serve as a precursor for gluconeogenesis. Recently, several studies have shown an additional role of the glyoxylate shunt in mitigating stress-conditions including both oxidative and iron stress. Because the efficiency of the electron transport chain is severely hindered under Fe-limitation, we hypothesized that redirecting a cell's metabolism away from Fe-limiting steps by means of the glyoxylate shunt would be an important strategy to increase metabolic efficiency under Fe-limitation. A recombinant bioluminescent reporter and gene knockout of *P. angustum* S14 was constructed for proteorhodopsin (ptr-luc, Δpr), and isocitrate-lyase, a key enzyme within the glyoxylate shunt (icl-luc, Δicl).

The genetic tools enabled us to test two key questions:

- 1. Is the gene of interest (isocitrate lyase, proteorhodopsin) upregulated under Felimiting conditions?
- 2. If so, does this result in an improved growth and respiration rate?

A strong and significant increase in the expression of isocitrate lyase was observed when subjecting the bioreporter icl-luc to strong Fe-limiting conditions. Although the growth and respiration rates decreased for both the wildtype and the knockout Δ icl under strong Felimitation, they were ±30% lower for Δ icl as compared to the wildtype (wt). Complementation of Δ icl with isocitrate lyase restored the growth and respiration rates to those observed in the wt, further confirming the importance of the glyoxylate shunt under strong Fe-limitation. Due to the ubiquitous nature of the glyoxylate shunt within marine bacteria, our results lead us to propose this pathway as an important acclimation strategy for marine heterotrophic bacteria that are subjected to Fe-limitation, although this upregulation may be species specific.

The upregulation of proteorhodopsin in Fe-replete conditions occurred as cells approached the stationary phase, but was absent during the exponential phase. In the stationary phase, a significant difference was also observed between continuous light and dark conditions. Under Fe-limitation, the upregulation of proteorhodopsin also occurred but no further differences in its expression were observed between light and dark conditions. Fe-limitation caused a decrease in both the growth and cell yield when *P. angustum* S14 was subjected to a range of Fe-concentrations, but no significant differences were found in relation to the presence (wt) or absence (Δpr) of proteorhodopsin either in constant light or constant dark conditions. Our results therefore show that in the exponential growth phase, proteorhodopsin is not upregulated in *P. angustum* S14 subjected to Fe-limitation and therefore does not affect the growth and respiration rate. Future work in elucidating the role of proteorhodopsin should focus on the stationary phase of a bacterial cell.

The results from this thesis manuscript contributed to a culminating body of work surrounding the versatility of marine heterotrophic bacteria in coping with Fe-limitation and is an appropriate addition to the literature surrounding the role of proteorhodopsin and the glyoxylate shunt within the marine environment. The use of genetic tools enabled us to address the specific impact of a gene towards a heterotrophic bacterium cell subjected to Fe-limitation but future work is required to further elucidate the exact mechanisms in which both genes can do so.

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DEHYDROGENASE, $CS = CIIRATE SYNTHASE, ACN = ACONITASE HYDRATASE, IDH = ISOCITRATEDEUVDROGENASE ICI ISOCITRATE I VASE OCDC OVOCUTARATE DEUVDROGENASE COMPLEX SCS$
DEFITION DEPARTMENT A SUCCENTRATE-LIASE, OUDC – UNOCLUTARATE DEFITIONOUTINASE COMPLEX, SCS SUCCENTRATE A SUNTHETASE SDH SUCCENTRE DEFITIONOUTINASE (COMPLEX II) FS
- SUCCINEL COENCLEWE A SENTILE IASE, SDH - SUCCINALE DER EDKOUENASE (COMPLEX II), \mathbf{r} - Fumadate hydratase MS - mai ate synthase MDH - mai ate deuvddogenase and the doman
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I. INTRODUCTION

Iron (Fe) is an essential trace element in organisms due to its ability to harbour several oxidation states, which can be utilized as a catalyst for various enzymatic reactions within the cell. Fe is most commonly found in either the ferrous (Fe²) or ferric (Fe³) form which together make up the following redox couple:

$$Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$$

As shown in the equation above, the transfer of electrons, as ferric Fe transitions to ferrous iron and vice versa, forms the basis behind the functioning of the metalloproteins that require Fe as a cofactor within the cell. Fe can be incorporated into metalloproteins in a mono- or binuclear form (non-heme proteins) or it can be part of a more complex structure such as haem (hemeproteins) or Fe-sulfur clusters (Fe-S proteins). The metalloproteins that require Fe for their functioning are responsible for major cellular processes such as photosynthesis, respiration, DNA synthesis and nitrogen fixation (Ilbert and Bonnefoy, 2013; Merchant and Helmann, 2012).

Despite the biological importance of Fe for cellular growth, the low productivity in vast regions of the ocean has been well documented and, in particular, high-nutrient low-chlorophyll (HNLC) sites have been attributed to Fe-limitation (Chisholm et al., 1991; Martin and Fitzwater, 1988a; Tagliabue et al., 2017). Fe concentrations within the ocean are often found in the pico- or nanomolar range and, as major HNLC sites, including the North Pacific Ocean, the Equatorial Pacific Ocean, and the Southern Ocean make up ±30% of the ocean, the effect of Fe-limitation on oceanic productivity is of importance at both a regional and global scale (Martin and Fitzwater, 1988b; Moore et al., 2013). Even in regions where the total Fe concentrations are considerably higher, such as the coastal ocean, Fe can still be deemed a limiting factor towards marine microorganisms (Cabaj and Kosakowska, 2009; Mackey et al., 2015; Soria-Dengg et al., 2001; Wells et al., 1995; Zhang et al., 2019). This is partially because the Fe that is deposited into the water column comes in various chemical forms and redox states, experiencing a multitude of transformations mediated by chemical, physical and biological processes that will ultimately determine both its solubility but also its bioavailability

towards the microorganisms present throughout the water column (Blain and Tagliabue, 2016; Lis et al., 2015; Tagliabue et al., 2017).

Within this introduction, we will attempt to briefly describe several concepts and components surrounding the complex and dynamic oceanic-Fe-cycle including the role and importance of heterotrophic bacteria. This is followed by a brief introduction regarding the overall effect of Fe-limitation on heterotrophic bacteria with respect towards cellular growth and metabolism followed by an overview of the various known strategies heterotrophic bacteria can utilize in order to cope with Fe-limitation. Finally, we will introduce 2 strategies that we hypothesize bacteria may utilize when encountering Fe-limitation which concerns the main body of work behind this thesis manuscript.

1.1 The Iron Cycle

In 1997, the publication of 30 vertical dissolved Fe profiles concerning the different water basins of the Pacific, Atlantic and Southern Ocean by Johnson et al (1997) allowed one of the first insights into the various processes that governed the oceanic-Fe-cycle. The profiles revealed a general Fe-distribution pattern despite considerable variability amongst the different water basins (Baar and Jong, 2001) and followed a nutrient-like profile which is characterised by lower concentrations at the surface and higher concentrations in deeper waters. More specifically, the Fe-profiles confirmed the presence of low dissolved Fe concentrations (<0.1 nmol L⁴) in the surface waters of the open ocean (Boyd and Ellwood, 2010; Tagliabue et al., 2017), followed by an increase to higher Fe-concentrations (\pm 0.76 nmol L⁴) below 500m water depths and relatively constant Fe-concentrations after 1000m (Chester, 2000, Figure 1). Altogether, the profiles reflect the both the sources (e.g. external input, Fe-regeneration) and sinks (e.g. biological Fe-uptake, chemical removal) of Fe within the water column. Altogether, the combination of these factors composes the oceanic Fe-cycle which is described in more detail below.



Figure 1. Vertical dissolved Fe-distribution profile from (a) the North Pacific central gyre and (b) the HNLC region of the subarctic Pacific. Higher Fe-concentrations at the surface waters implicate a large external Fe-source at (a) which does not occur at (b). Fe-concentrations slowly increase at lower depths of ± 500 m and remains relatively stable below 1000 m. Taken from Bruland et al., 1991; Martin et al., 1989 and presented in Chester, 2000.

The Fe-cycle (Figure 2) consists of several key processes, and its description typically starts with the external Fe-sources that enter the water column as a heterogenous pool of Fe. Based on the chemical Fe-speciation, Fe can further interact and bind to various organic and inorganic components present within the water column. The bioavailable fraction of this Fe pool can be taken up and incorporated into cellular components by the microbial community and hereby reach higher trophic levels. While a part of this Fe-fraction is recycled and remains within the water column through various remineralization processes, a part of the Fe-pool can also precipitate out of solution or form aggregates that sink to lower water depths. The vertical transport of Fe due to various processes such upwelling events will eventually bring Fe from deeper layers back into circulation and towards the upper water column.



Figure 2. A schematic and general overview of the various components that make up the Fe-cycle. Fe enters the ocean column where part of it is dissolved (Fe₄) and a fraction ends up as particulate inorganic Fe (Fe₄). The bioavailable fraction can be taken up by both phytoplankton and bacteria and incorporated within the cell to form a part of the particulate Fe pool. Particulate Fe can sink to lower water depths where a portion is brought back to the dissolved Fe pool through various remineralization processes including the presence of bacteria. A fraction of the particulate Fe sinks further down and is sequestered to deeper layers while vertical transport such as upwelling events can bring a part of this pool back into resuspension.

1.1.1 External Fe-sources

Variations in Fe-concentrations across the water column can partially be explained by the presence or absence of various external Fe-sources. There are several different sources of "new" Fe that enter the water column at various depths and the most important include continental dust deposits (Aeolian dust), dissolution of continental margins, hydrothermal vent emissions, glacial deposits and river, iceberg and submarine-groundwater discharge. It is important to note that the various sources of "new" Fe occur at a regional, rather than at a global scale resulting in both spatial and temporal heterogeneity of Fe-deposits within the ocean. In general, dust deposits are the dominant Fe-source in the low latitudes such as the subtropical North Atlantic gyre, while the regional HNLC sites such as the Southern Ocean,

coincide with a low flux of aerial dust deposition (Anderson et al., 2005; Tagliabue et al., 2017b, Figure 1). In contrast, the major external Fe-sources of the Southern Ocean constitutes the continental margin and upwelling from hydrothermal sources (Ardyna et al., 2019; Tagliabue et al., 2014).

1.1.2 Fe-sinks and internal recycling of Fe

As Fe-deposits from various sources enter the water column, part of the dissolved Fe-fraction is taken up and incorporated by marine microorganisms forming the biological particulate Fepool. On the other hand, both physical and chemical processes (e.g. adsorption, flocculation or precipitation events) can scavenge the dissolved Fe-fraction from solution and form inorganic aggregates of particulate Fe that sinks to lower water depths or the seafloor (Boyd and Ellwood, 2010). Vertical Fe-profiles depict increasing Fe-concentrations with depth, which implies that the ocean circulation and the presence of mixing events can supply Fe back to the surface. Part of the particulate Fe-fraction at lower depths can therefore be recycled within the water column through a combination of upwelling events and remineralization processes. An upwelling event is a physical process describing the wind-driven motion of water that brings deeper water layers back to the surface. The Fe that has been sequestered in deeper layers are hereby brought back into resuspension and made available for the marine microorganisms present within the upper water column (Blain and Tagliabue, 2016). Remineralization processes, on the other hand, largely refers to the biologically-mediated recycling of organic matter by zooplankton (Barbeau et al., 1996), viruses (Poorvin et al., 2004), and heterotrophic bacteria (Strzepek et al., 2005) which degrade a fraction of the particulate Fe pool (ie. organic detrital matter) back into dissolved-Fe that can then be reutilized by the microorganisms present within the water column.

1.1.3 Fe-stocks and Fe-fluxes

There are still large uncertainties when attempting to elucidate how the various components of the Fe-cycle play into the calculation of Fe-stocks and Fe-fluxes (Anderson et al., 2016; Tagliabue et al., 2017). Due to its reactivity within the water column, Fe is considered to have a relatively short residence time in comparison to other nutrients such as phosphorous

^{*} including wind-driven processes of Eckman Transport and the Coriolis Effect

and nitrogen^{*}, but predictions can range from a few to a few hundred years (Tagliabue et al., 2016; Völker and Tagliabue, 2015). This uncertainty is largely due to the difficulty with which one can measure Fe-speciation (and ligands) as well as its reactivity (both biotic and abiotic) at various scales which will impact calculations on the rates of the various mechanisms regarding the supply and internal cycling of Fe.

1.1.4 Operational definitions of the Fe-pool



Figure 3. A classic separation of the Fe-pool fraction according to size (soluble, colloidal and particulate) and its basic Fe-chemistry (inorganic, organic) with examples given within each fraction. The particulate Fe-fraction is separated from the dissolved Fe-fraction, the latter of which concerns the Fe-fraction that passes through a 0.2 μ m filter and is composed of both inorganic and organic Fe-species that can further be separated into the soluble (<0.02 μ M) and colloidal (0.02 μ M) fraction. Take note that definitions are arbitrary and that in reality Fe-speciation is a fluid continuum of a heterogenous pool where certain fractions can change into another and vice versa. (Derived and modified from Tagliabue et al., 2017).

Vertical Fe-profiles within the water column differ from the profiles that are generally observed with nutrients such as nitrogen or phosphorous because of the multiple sources, chemical forms and redox states that make up the Fe-pool, which alter its reactivity within the water column. Several factors and processes determine the exact speciation of Fe within the ocean, such as its

^{*} Nitrogen is reported to have a residence time of a few thousand years (Gruber, 2004) and phosphorous is reported to have a residence time an order of magnitude higher (10,000-17,000 years; (Delaney, 1998).

redox state⁻⁻, the pH, and temperature but also the presence and absence of organic and inorganic ligands which interact with Fe to form a myriad of Fe-complexes (Blain and Tagliabue, 2016; Sunda and Huntsman, 2003). The presence of light is known to further affect Fe-speciation within the surface waters of the ocean (Barbeau, 2006; Sunda and Huntsman, 2003) and exemplified by photochemical reactions that reduce Fe:ligand complexes into the more bioavailable form of Fe²⁺.

The complex and heterogenous nature of Fe within marine waters can be separated according to several operational definitions based on size as well as its chemical properties (Figure 3). Despite, the presence of such definitions, the separation within the Fe-pool is not necessarily fixed and should generally be viewed as a fluid continuum of Fe-species which may further evolve as they are cycled between and across their defined boundaries (Tagliabue et al., 2017).

Fe is primarily separated into a 'dissolved' and 'particulate' Fe-fraction according to a specific filter size. The selected filter size can vary, but GEOTRACES[•], operationally defines the "dissolved" Fe fraction as Fe that passes through a 0.2 μ m filter. Particulate Fe encompasses the retained fraction and includes Fe-aggregates from organic and inorganic material but also includes biological entities such as phytoplankton and bacterial cells (Tortell et al., 1996). The "dissolved" Fe-pool can further be divided into the smaller soluble fraction (<0.02 μ m) and the larger colloidal form (0.02 μ m – 0.2 μ m) each of which displays a different vertical distribution pattern (Wu et al., 2001; Figure 4). In fact, only a small fraction of the dissolved Fe-fraction, exists in the true unchelated and "free" inorganic ferrous or ferric iron form (Fe⁺) and in natural seawaters (pH 8), the dissolved Fe-fraction predominantly exists in the ferric form (Fe⁺) which can form complexes with both inorganic and organic ligands which can potentially form larger aggregates through colloid aggregation.

^{**}Ferrous and ferric iron have different chemical bonding affinities

^{*} GEOTRACES constitutes an international research program founded in 2006 that specializes in the marine biogeochemical cycles of trace elements and their isotopes with the aim to identify processes and quantify fluxes that control the distributions of these key trace elements and isotopes in the ocean, and to establish the sensitivity of these distributions to changing environmental conditions (Schrope, 2010).



Figure 4. The (a) soluble ($<0.02 \ \mu m$), and (b) colloidal ($0.02 - 0.4 \ \mu m$) Fe-fraction which makes up the total (c) dissolved Fe fraction ($<0.4 \ \mu m$). Note the similarity between the dissolved Fe-fraction and the colloidal Fe-fraction, which led to the conclusion that the majority of the dissolved Fe-fraction was bound to organic ligands that make up the colloidal Fe-fraction. Derived from (Wu et al., 2001).

The solubility of inorganic Fe-species in marine water (e.g. OH, F and SO_{4^2}), is extremely low and will often precipitate out of solution (Liu and Millero, 2002). Inorganic Fe-species (ie. Fehydroxides) also tend to be 'scavenged' from the medium by aggregates to form larger particles of particulate Fe that can sink to lower layers (Honeyman and Santschi, 1989).

The majority (99%) of the "dissolved" Fe fraction within the water column is complexed to organic ligands and assumed to be in the colloidal size range (Gledhill and Buck, 2012; Wu et al., 2001). Organic ligands that form complexes with Fe include small soluble ligands such as siderophores but also larger organic complexes such as metalloproteins, exopolymeric-substances (EPS) and even viruses (Bonnain et al., 2016). The composition of organically bound Fe-ligands is largely unknown and it is therefore unclear which fraction of the organic compounds plays an important role in Fe-biogeochemistry (Hassler et al., 2017).

Through electrochemistry, organic Fe-ligands are typically separated according to their binding strength to Fe (Gledhill and Buck, 2012) and organic ligands can hereby be classified into class L1, and L2-L4 ligands (Hunter and Boyd, 2007). The class of L1 ligands concerns ligands with a strong bonding affinity for Fe (log K value of 12-13) and siderophores make up an important component of L1 ligands. L2-L4 ligands, on the other hand, have a weaker affinity for Fe (log K value of 12-10 or lower) and are often composed of organic matter released through the degradation of organisms within the water column. Recently, the vertical distribution of these ligand classes, where L1 is prevalent in the mixed layer while L2-L4 is dominant in the deep

ocean, has been disputed (Buck et al., 2015; Gerringa et al., 2015). In an attempt to understand (and predict) what the dominant type of ligand within a particular region may be, Hassler et al (2017) has proposed to distinguish organic ligands according to their key sources, production and loss pathways. Organic ligands are hereby separated into three main groups, namely, humic-substances, siderophores and general biological degradation and excretion products. Although overlap between these groups is likely and, as of yet, difficult to quantify, a more holistic view on Fe-bound ligands is necessary to further understand their distribution and interactions across the water column.

1.1.5 Fe-bioavailability

Another important characterisation of Fe is its bioavailability towards microorganisms. Bioavailability can be defined as the 'degree to which an Fe-species can be taken up and utilized' by a particular organism. In practice however, the notion of bioavailability is not as straight-forward as several chemical, biological and physical factors are intrinsically linked and influence the bioavailability of an Fe-species for any particular organism (Shaked and Lis, 2012).

The study of Fe-availability has particularly focused on phytoplankton due to their role as carbon fixators within the marine environment. In general, the free unchelated form of Fe' is considered to be the most bioavailable form and can be taken up by the cell using high-affinity Fe-transporters (Hudson and Morel, 1993). More recently, however, laboratory and field experiments have demonstrated that phytoplankton can also access the more dominant and organically complexed Fe-pool via reductive Fe-uptake pathways and siderophore mediated Fe-acquisition (Lis et al., 2015). The reductive uptake pathway is a reduces ferric iron (Fe⁻) and Fe:ligands to ferrous iron (Fe⁻) through "generalist" reductases prior to its uptake within the cell by a ferrous Fe-transporter and is found to be widespread amongst phytoplankton (Morel et al., 2008a; Shaked et al., 2005). When the bioavailability of an Fe-substrate in phytoplankton is dependent on its reducibility, it can further be described in terms of a 'bioavailability envelope' (Figure 5) which is based on the uptake rate of two commonly used Fe-substrates in culture work which represent two extreme forms of bioavailability (Lis et al., 2015): Fe bound to ethylenediaminetetraacetic acid (Fe:EDTA) and Fe bound to deferoxamine-B (Fe:DFOB).

The bioavailability of other Fe:ligands is considered to fall between these two extremes.

 $Fe^{3+}:EDTA \Rightarrow Fe^{3+} + EDTA$ $Fe^{3+}:DFOB \Rightarrow Fe^{3+} + DFOB$

EDTA is a chelator with a known dissociation constant so that it is theoretically possible to calculate the amount of Fe' in the medium at any given time. Because EDTA is a synthetic chelator in marine waters, Fe is not bioavailable as the entire Fe:EDTA complex and the availability of Fe:EDTA is therefore determined by its dissociation rate into Fe' and EDTA (Shaked et al., 2005). On the other hand, Fe:DFOB forms an extremely stable complex so that any uptake of Fe as Fe' becomes negligible. In this bioavailability model, all Fe forms are taken up as Fe^{3,} so that the limiting step is the rate of the formation of Fe^{3,} (i.e. Fe-reducibility) which can be aided by the presence of extracellular reduction reductases (Lis et al., 2015; Shaked et al., 2005). The presence of transporters and Fe-reductases across the cell membrane is hereby an important factor influencing Fe-uptake and is proportional to the cell surface area (Lis et al., 2015). The current proposed Fe-bioavailability model may further be complicated by the recent finding that certain diatom species can utilize non-reductive high-affinity uptake mechanisms such as the utilization of the Fe-concentrating phytotransferrin ISPI2A or the acquisition of siderophore-bound Fe-complexes (Kazamia et al., 2018; McQuaid et al., 2018).

^{*} changes in the dissociation rate and exact concentration will also depend on temperature, pH and light.



Figure 5. The 'bioavailability envelope' which depicts Fe-substrate bioavailability as Fe-uptake rate (k_m) within different eukaryotic phytoplankton per surface area. The envelope's boundaries are delineated by straight lines, with unchelated inorganic iron (Fe') as its upper boundary and Fe:DFOB at its lower boundary. Each data point is a measurement from a different study. Derived from Shaked, 2015.

While similar factors are likely to play a role in the Fe-uptake for heterotrophic bacteria, as is predicted to be the case for cyanobacteria (Lis et al., 2015), heterotrophic bacteria are known to contain a wide variety of Fe-transporters which include the direct uptake of Fe' as well as the production and uptake of siderophores and other Fe-binding ligands in its entirety before being reduced inside the cell (Hopkinson and Barbeau, 2012). Various studies have shown that the genomic potential for Fe-uptake mechanisms can vary amongst bacterial species (Debeljak et al., 2019; Hogle et al., 2016; Hopkinson and Barbeau, 2012) but the exact mechanisms remain to be elucidated and are generally described for pathogenic bacteria (Braun, 2001). While both pathogenic- and cyanobacteria have been shown to contain Fe reductases, similar to phytoplankton Fe acquisition mechanisms (Braun, 2001), this has not yet been confirmed in marine heterotrophic bacteria.

Due to the specificity in which a bacterium can take up various Fe species, the true bioavailability of Fe-species is species-specific and difficult to fully elucidate. The majority of heterotrophic bacteria are gram-negative meaning that, in order for a bacterium to successfully take up Fe within its immediate environment, it must transport Fe from both the outer and inner cell membrane before it can be utilized within the cytoplasm (Figure 6). Free ferrous and ferric

iron can enter the cell through diffusion or by means of selective porins before being taken up by the inner cell membrane through ABC transporters which require ATP. When the molecular free weight of an Fe-ligand pair exceeds that of a porin (<600 Da), TonB dependent uptake mechanisms are put in place to import Fe within the cell. These transport systems are highly specific due to the presence of receptor enzymes on the outer membrane. Upon recognition of a particular Fe:ligand pair, the energy from a proton motive force produced within the inner membrane is transferred to the outer cell membrane and utilized by a TonB box to bring the Fe:ligand pair into the periplasmic space. Once in the periplasmic space, Fe:ligands are transported across the inner membrane through different ABC transporter systems. In the cytoplasm (or within the periplasmic space) Fe:ligands are reduced to release free ferric or ferrous forms of Fe to be utilized within the cell (Braun, 2001).



Figure 6. A schematic and general overview of the various Fe-uptake mechanisms found in heterotrophic bacteria. Free F^{2+} and Fe^{3+} or Fe^{3+} bound ligands smaller than 600 dalton can be taken up through porins into the periplasmic space. Several ferric bound ligands (including siderophores) are larger than the molecular cut off weight for porins (>600) and require the TonB transporter complex to be imported into the periplasmic space. Fe^{3+} iron is then complexed to periplasmic binding proteins (PBP) and passed through the inner membrane using ABC transporters which require ATP. Fe^{3+} can also be reduced to F^{2+} and taken up by the cell through the ABC transporter FeoB, using a ferric reductase in the inner or outer membrane. (Derived from Krewulak and Vogel, 2008; Velayudhan et al., 2002; Jones and Niederweis, 2010).

1.1.6 Impact of Iron on the Carbon Cycle

Half of the primary production on Earth occurs in the ocean through the photosynthetic activity of both phytoplankton and cyanobacteria (Field et al., 1998) and its fixation and drawdown to deeper water layers forms the basis of the biological carbon pump. The efficiency of carbon sequestration can vary (Henson et al., 2019) as a large majority of this fixed organic carbon is recycled through remineralization processes and the presence of viruses, zooplankton and heterotrophic bacteria (Boyd and Ellwood, 2010; Falkowski et al., 2000; Worden et al., 2015). All microorganisms require the uptake of Fe to grow and develop so that the effect of Fe-limitation within the global ocean has important implications towards the global carbon cycle as Fe-limitation can control the biomass and productivity of phytoplankton in certain regions such as HNLC sites (Boyd and Ellwood, 2010).

The impact of Fe on the drawdown of carbon to the seafloor resulted in the now infamous Fefertilization experiments that were originally proposed by Martin in 90's (Martin, 1990). Several open-ocean experiments were conducted in order to test whether Fe could indeed stimulate carbon fixation by phytoplankton which was predicted to increase the eventual drawdown of CO₂ within a natural environmental setting. In order to avoid the exclusion of several important factors such as a grazing response or bacterial remineralization processes⁻ (Cullen, 1991), the HNLC sites of the Southern and Pacific Ocean quickly became the backdrop for the setup of these experiments (Tagliabue et al., 2017). The resulting observations revealed that although Fe was indeed shown to trigger phytoplankton blooms in HNLC waters, the drawdown of carbon was far from efficient or systematic and stressed the importance of processes that limit carbon sequestration such as grazing and remineralization processes.

1.1.7 Fe-limitation and the impact of Heterotrophic Bacteria

In light of the Fe-hypothesis and considering the role of phytoplankton in the fixation of CO_2 to organic carbon, research on Fe-limitation has primarily focused on phytoplankton due to their major contribution to the biological carbon pump (Legendre et al., 2015). By contrast, less attention has been given to the role of Fe-limitation on heterotrophic bacteria despite playing a significant role in the biogeochemical cycles of both carbon and Fe through their role as principal remineralizers of dissolved organic matter (Azam. F. et al., 1983; Tagliabue et al.,

^{*} processes that cannot accurately be reproduced in batch culture experiments

2017). Heterotrophic make up a substantial fraction of the total particulate Fe pool (Tortell et al., 1996) and in the HNLC site of the subarctic Pacific, heterotrophic bacteria can even amount up to $\pm 50\%$ of that particulate iron fraction. Heterotrophic bacteria seem to have a higher Fe requirement (Fe:C) in comparison to phytoplankton, and are predicted to be responsible for $\pm 20-45\%$ of the total biological Fe-uptake. This suggests that heterotrophic bacteria are in direct competition with phytoplankton in order to meet their cellular iron requirements (Fourquez et al., 2015; Tortell et al., 1996). Furthermore, heterotrophic bacteria have the ability to transform Fe-complexes through the release of siderophores which binds to Fe with a high affinity further impacting its accessibility to the residing phytoplankton (Soria-Dengg et al., 2001). As mentioned, previously, heterotrophic bacteria are also an important component of the carbon and Fe-cycle as principle remineralizers degrading a substantial portion of the particulate organic carbon fraction and recycling Fe back into the water column. Regardless of spatial and temporal variability, the above-mentioned factors highlight the scale and the various processes by which heterotrophic bacterial can impact the biogeochemical cycling of Fe.

1.2 Effect of Fe-limitation on heterotrophic bacteria

Fe-limitation has been shown to result in a decline in bacterial growth (Table 2) that is further coupled to a lower carbon growth efficiency and a decline in the overall ATP production within the cell (Tortell et al., 1996). In general, Fe-limitation will particularly impact processes that require large amounts of Fe-containing enzymes such as photosynthesis, respiration, ROS protection, nitrate fixation and DNA synthesis and repair. It is predicted that around 90% of the total cellular uptake of Fe within heterotrophic bacteria is utilized within the I, II, III, and IV complexes of the electron transport chain which contain large amounts of Fe in the form of Fe-sulfur proteins (Tortell et al., 1999; Winge, 2012, Table 1). Although some ATP is produced through glycolysis (and fermentation), the majority of ATP is produced through ATP synthase which requires the proton gradient produced by the electron transport chain. While the impact of Fe-limitation on a heterotrophic bacterium is dependent on the degree of Fe-limitation, the effect of Fe-limitation on a heterotrophic bacterium has also been shown that it is strain specific as certain bacterial species were even reported to have higher growth rates under Fe-limitation than under replete conditions (Tortell et al., 1996; Granger and Price, 1999; Fourquez et al., 2014, Table 2). Furthermore, the stoichiometry (ie. carbon, nitrogen and phosphorous) of heterotrophic bacteria is known to be flexible and can change depending on the surrounding environmental conditions (Godwin and Cotner, 2015; Worden et al., 2015). Similarly, the Fequota of heterotrophic bacteria (Tortell et al., 1996, 1999) as well as their capacity to take up different Fe-sources has been shown to differ (Debeljak et al., 2019; Hopkinson and Barbeau, 2012) according to the species, their cell size and whether they are grown in Fe-replete or Fe-limiting conditions.

In nature, the effect of Fe-limitation on bacterial populations is more complex in part due to the interaction of bacteria with other organisms within their immediate surrounding environment. Heterotrophic bacteria, for example, are known to consume and rely on the production of DOM by phytoplankton (Amin et al., 2012; Ducklow). As Fe-limitation will affect both phytoplankton and bacterial populations, it is difficult to effectively discern whether a decline in the overall bacterial growth rate is a direct effect of Fe-limitation, carbon-limitation or both (Church et al., 2000; Jain et al., 2015; Obernosterer et al., 2015).

Table 1. Theoretical Fe-concentrations based on the Fe-components of the respiratory Fe-complexes in E. coli bacteria (derived from Tortell et al., 1999)

Enzyme Complex	Complex #	Fe-content	[Fe]
		(atom/complex)	(mol Fe cell ¹ x10 ²⁰)
NADH-Q reductase	Complex I	2 x 2Fe-2S	
		6 x 4Fe-4S	89.4
Succinate-Q	Complex II	1 x 2Fe-2S	
dehydrogenase			
		1 x 3Fe-4S	
		1 x 4Fe-4S	46.7
Cytochrome b1	Complex III	1	5.9
Cytochrome oxidase	Complex IV	2	3.84

Bacterial strain	Isolate	Fe-replete: growth (d^{-1})	Fe-limited- growth (d^{-1})	Carbon Source	Publication
Photobacterium angustum	S14	$9.4^{\rm a}$	$6.2^{\rm b}, 3.2^{\rm c}$	Glucose (100 μ M[C])	This publication
Alteromonas macleodii	Jul88	8^{d}	$4^{ m e}$	Mixed*	Tortell 1996
n/a	LMb1	6.5^{d}	3.8 ^e	-	-
n/a	Puh3a	$10^{\rm d}$	4 ^e	-	-
n/a	Tef2	6^{d}	$10^{\rm e}$	_	-
Pseudoalteromonas rubra	Lmg1	$7.8^{\rm d}$	10 ^e	-	-
Pseudomonas haloplanktis	Neptune	12.9^{f}	$6.74^{ m g}$	Mixed*	Granger, 1999
n/a	P20pac	$4.27^{ m f}$	1.21^{g}	-	-
Pseudomonas sp	Iso15	3.59^{f}	$3.77^{ m g}$	-	-
Alteromonas macleodii	Jul88	$7.37^{ m f}$	$2.18^{ m g}$	-	-
Pseudoalteromonas rubra	Lmg1	15.7^{f}	$9.95^{ m g}$	-	-
Pseudoalteromonas piscicida	PWF3	11.9^{f}	$5.9^{ m g}$	-	-
Vibrio natriegens	PWH3a	20.9^{f}	$5.55^{ m g}$	-	-
Alteromonas macleodii	MOLA377	11.5^{h}	9.6^{i}	Mixed*	Fourguez, 2014
Alteromonas macleodii	MOLA60	12.2^{h}	6.1^{i}	_	-

a: Fe-replete conditions: 5.4 μ M Fe

b: Fe-limiting conditions: 5.4 μ M Fe and 10 μ M DFOB

c: Fe-limiting conditions: 5.4 μ M Fe and 100 μ M DFOB

d: Fe-replete conditions: $40\mu M$ Fe

e: Fe-limiting conditions: 25μ M Fe

f: Fe-replete conditions: 8.4 μ M Fe

g: Fe-limiting conditions: 12.5 nM Fe

h: Fe-replete conditions: 5.4 μ M Fe

i: Fe-limiting conditions: 5.4 nM Fe

* Mixed Carbon Source: bactopeptone and caseine

angustum S14 **Table 2.** A non-exhaustive list of bacterial growth rates subjected to Fe-replete and Fe-limiting conditions under various carbon sources including what was conducted for our model organism, P.

1.2.1 Fe-homeostasis

In general, bacteria tightly regulate Fe concentrations within the cell. As explained previously, Fe-limitation results in a decline in cellular growth due to the inability to form important Fecontaining enzymes. In oxic environments, an excess of intracellular Fe can become toxic due to the enhanced formation of reactive oxygen species (ROS)^{*}, and in particular the ability to form hydroxyl free radicals HO[•] through the Fenton reaction, which can interact with DNA and lipids and cause severe cellular damage (Dixon and Stockwell, 2014; Mullarky and Cantley, 2015; Touati, 2000). The Fenton reaction is a component within the Haber-Weiss Cycle which describes a series of reactions where superoxide (O_2^{*}) interacts with the presence of Fe to form H₂O₂:

$$O_2^{-*} + Fe(III) \rightarrow O_2 + Fe(II)$$

Where O_2^{-*} acts as a reductant

$$O_2^{-*} + Fe(II) \rightarrow H_2O_2 + Fe(III)$$

Where O_2^{-*} acts as an oxidant

$$H_2O_2 + Fe(II) \rightarrow HO^{-} + HO^{-} + Fe(III)$$

Where the formation of H_2O_2 together with free intracellular Fe can go on to form HO· (and ROS) through the Fenton reaction (Blain and Tagliabue, 2016; Rose, 2012).

A bacterial cell can detect an excess or limitation of intracellular Fe through metal-sensing regulatory transcription factors which result in the activation and suppression of several genes (Hantke, 2001). Fe-homeostasis is often achieved through the ferric-uptake regulator (FUR) although other regulators such as sigma factors are known to exist (Cornelis et al., 2009). FUR is a negative repressor that binds to free ferric Fe present within the cell (Figure 7). An excess of Fe will result in an increase in ferric-bound FUR which undergoes a conformational change

^{*} ROS is a general term used to describe a variety of reactive oxygen-containing molecules, including O_2^{-*} , H₂O₂, and the highly reactive HO, all of which, if accumulated to high enough concentrations within the cell, can cause oxidative stress.

allowing it to bind to FUR-binding sites located within the promoter region of a DNA segment. The binding of FUR to these regions prevent polymerase from transcribing genes downstream from these promoter regions. Interestingly, FUR can indirectly influence the positive upregulation of several genes as well (Crichton, 2008). This is achieved by preventing the transcription of non-coding RNAs which acts as an antisense regulator of RNA by binding to mRNA transcripts and targeting them for degradation (Silva and Faustino, 2015). The exact genes that are regulated under the Fe-limiting conditions by FUR and other Fe- regulators will vary between bacterial species and is species specific. In *E. coli*, for example, FUR is responsible for the regulation of 90 genes including genes with non-Fe functions (Lee and Helmann, 2007).



Figure 7. Schematic overview of the regulation of the ferric uptake regulator (FUR) in Fe-replete and Fe-limiting conditions. Under high Fe-concentrations, Fe binds to FUR and undergoes a conformational switch to allow it to bind to FUR-binding sites in DNA which prevents the transcription of various genes downstream. Under low Fe-concentrations, the absence of FUR prevents the binding of FUR to these FUR-binding sites which turns on translation downstream of these sites. It is important to note that while FUR is technically a negative regulator, FUR can also act as a positive regulation when inhibiting the translation of non-coding RNA which in turn inhibits the translation of other proteins.

1.2.2 Strategies in heterotrophic bacteria to cope with Fe-limitation

In theory, there are two strategies by which a bacterium can rearrange its metabolism in order to cope with Fe-limitation; a bacterial cell can either increase its effort in the uptake of Fe or it can reduce its intracellular Fe-requirements.

The uptake of Fe from siderophores has been well established (Boiteau et al., 2016, 2019) and hundreds of different siderophores have so far been isolated from different bacterial strains (Hider and Kong, 2010). As described previously, siderophores are bacterial-produced ligands that bind to Fe with a high affinity (L1). Its production is often regulated under FUR so that in the presence of Fe-limitation, siderophore-producing strains upregulate its synthesis and excretion⁻ into the surrounding environment (Neilands, 1995). The production of siderophores is costly, as a specific uptake mechanism using TonB-dependent transporters is required and a fraction of the excreted siderophores are lost to the environment (Granger and Price, 1999). Therefore, a trade-off between the production cost and the efficiency of this Fe-acquisition mechanism occurs and although the ability to take up siderophores seems to be relatively common amongst heterotrophic bacteria, the capacity to synthesize siderophores is rare (Hopkinson and Barbeau, 2012). In fact, it has been shown that various non-siderophore producing strains can indeed coexist with siderophore-producing bacterial species by taking advantage of the excreted siderophores released into the immediate environment whilst upregulating the necessary TonB-dependent transporters to take them up (Rankin et al., 2007; Sexton and Schuster, 2017).

The upregulation Fe-uptake mechanisms for other forms of Fe are also known to exist and recently, heme has been postulated to be an important Fe-source for marine bacteria in association with phytoplankton (Hogle et al., 2014, 2016). Heme-like molecules are ligands that are released into the water column through the degradation of cellular proteins from lysed cells such as the photosystems found in phytoplankton and are taken up by certain bacteria through specific Ton-B dependent transporters. Roseobacter strains are commonly referred as phytoplankton-associated bacteria and around $\pm 50\%$ of 153 studied Roseobacter strains were shown to contain putative heme uptake mechanisms. Competition studies between a wildtype Roseobacter strain with a heme-uptake mechanism and its gene knockout further confirmed a

^{*} albeit some aquatic bacteria have been shown to produce membrane-bound siderophores (Wandersman and Delepalaire, 2004).

selective advantage under Fe-limiting conditions in the uptake of Fe from diatom cell lysate (Hogle et al., 2016).

Another example of an Fe-uptake mechanism is the production and excretion of citrate which, when bound to Fe, forms the weak complex ferric-dicitrate which can be taken up in the presence of a ferric-citrate transport system. Citrate is produced in most bacteria as an intermediate within the TCA cycle. Under Fe-limitation, however, an overproduction of endogenous citrate occurred within a *Photobacterium damselae* strain which then appeared to be excreted, acting as a siderophore by scavenging Fe from the cell's immediate environment (Balado et al., 2017).

Although it is currently well established that heterotrophic bacteria contain a large diversity and specificity in Fe-uptake molecules few species, when present in Fe-limiting conditions, the majority of bacteria appear to invest in one rather than multiple uptake mechanisms (Debeljak et al., 2019). Employing one, rather than several, Fe-uptake mechanisms may partially be explained by a 'membrane occupancy constraint' as a bacterial cell membrane must also harbour sufficient space for the uptake of other nutrients whilst maintaining sufficient ATP concentrations from the electron transport-chain (Szenk et al., 2017; Zhuang et al., 2014).

Heterotrophic bacteria are known to harbour a certain physiological plasticity when subjected to environmental stressors and, under Fe-limitation, a bacterium can opt to reduce its Fe-quota by decreasing its dependency on Fe-containing enzymes. There are several ways in which this can be achieved; by downregulating metabolism and/or certain Fe-containing enzymes, by replacing various Fe-containing enzymes with non-Fe containing enzymes or by remodelling bacterial metabolism away from Fe-rich pathways. The reduction of a cell's Fe:quota was first shown in a study where the cellular Fe:C ratio for several heterotrophic bacterial isolates changed depending on the Fe-concentrations present in the environment (Tortell et al., 1996). While several studies have reported modifications in the expression of several enzymes and changes in the regulation of metabolic pathways in response to Fe-limitation, the majority of these studies focused on eukaryotic and phytoplankton cells (Oexle et al., 1999; Shakoury-Elizeh et al., 2010; Strzepek et al., 2011; Thimm et al., 2001) while less is known about heterotrophic bacteria (Fourquez et al., 2014) where the majority of studies have focused on
Fe-uptake strategies and concern pathogenic bacteria as Fe-limitation within the host cell is a common occurance (Andrews et al., 2003; Chandrangsu et al., 2017).

Several examples can be given where Fe-dependent enzymes are replaced by their non-Fecontaining counterparts. Escherichia coli have been shown, for example, to exchange the Fecontaining fumarase A/B of the TCA cycle with the Fe-independent fumarase C (Park and Gunsalus, 1995), a strategy that has also been shown for the marine bacterium Altermodas macleodii (Fourquez et al., 2014). Another example includes replacing the Fe-containing enzymes with Mg-homologs as has previously been observed for superoxide dismutases and ribonucleotide reductases (Chandrangsu et al., 2017). Another way to reduce the cellular Fequota is the downregulation of enzymatic activity either as a whole or for specific Fecontaining enzymes as has been observed for several key metabolic enzymes of the TCA cycle such as aconitase and succinate dehydrogenase (Fourquez et al., 2014; Smith et al., 2010). Finally, several bacterial strains have been shown to remodel their metabolism away from Fecontaining pathways. The presence of Fe-limitation in a Pseudomonas putida strain, for example, resulted in the rerouting of carbon flux towards gluconate production thereby maintaining cellular metabolism but decreasing the production of reducing agents (NADH) used in an otherwise inefficient electron transport chain (Sasnow et al., 2016). Interestingly, the overproduction of gluconate was also secreted into the immediate environment which may serve a purpose similar to what was predicted for *P. damselae* and the excess production of citrate (Balado et al., 2017; Sasnow et al., 2016). Another example can be seen in a coastal strain of Alteromonas macleodii which remodelled glycolysis by upregulating the Entner-Duodoroff pathway which reduces the consumption of ATP. The presence of Fe-limitation was further coupled with an increase in gluconeogenesis and thus carbon demand (Fourquez et al., 2014) which is in stark contrast to what was observed in eukaryotic cells where metabolism was redirected away from the Fe-rich TCA cycle and electron transport chain whilst gaining ATP from glycolysis (Oexle, 1999).

Taken altogether, while it is likely that general strategies exist to counter the effects of Felimitation, it is also apparent that bacterial cells harbour strain specific strategies when subjected to Fe-limitation. The limited work that has been conducted on marine heterotrophic bacterial strains therefore makes it difficult to gage the presence of general and specific responses within marine heterotrophic bacteria subjected to Fe-limitation.

1.3 The rise in "omics"

Culturable strains do not necessarily reflect natural bacterial populations, and the rise in "omics" (metagenomics, transcriptomics and proteomics) became an important tool to study the natural bacterial populations which mostly encompassed unculturable strains (Handelsman, 2004; Staley and Konopka, 1985). Over the years, technological advances and the rise of "omics" have improved several uncertainties around the Fe-cycle and the various components within it (Tagliabue et al., 2017). These tools have improved our current understanding towards the bioavailability of Fe in terms of the discovery of new transporter molecules but also has enhanced our understanding regarding Fe-chemistry present within the ocean (Tagliabue et al., 2017). A typical example is the finding that heme makes up a substantial component within the marine biogenic Fe-pool (Hogle et al., 2014). The rise in "omics" can further aid researchers in the search for previously unknown pathways or strategies heterotrophic bacteria may employ under various environmental conditions including Fe-limitation (Kraus, 2015; Morrissey and Bowler, 2012). The complexity of such data sets, however, can sometimes be limiting in providing concrete evidence that the observed upregulation or downregulation of certain metabolic pathways are truly an effect of Fe-limitation. For example, in HNLC sites, heterotrophic bacteria, are both directly and indirectly affected by the presence of Fe-limitation as lower primary production also implies a decrease in phytoplankton-derived dissolved organic matter (DOM) which bacteria are known to utilize (Kirchman et al., 2000; Obernosterer et al., 2015). We hereby come full circle where field observations as a result of "omics" can lead to new hypotheses that can be tested through lab experiments and culture work in order to clarify these observations. Culture studies must therefore be implemented in order to confirm the relevance of metabolic changes under Fe-limitation and how the regulation of various metabolic pathways can affect the survivability and overall fitness of a marine heterotrophic bacterium.

1.4 Thesis Objectives

The objective of this thesis manuscript was to use a culturable representative to discern and test whether the upregulation of specific metabolic pathways can be utilized by a heterotrophic bacterium cell when subjected to Fe-limitation. Specifically, this thesis focuses on the potential advantage of two such mechanisms. Firstly, the observed induction of the Glyoxylate Shunt

under Fe-limitation as an alternative metabolic-pathway, and secondly, the notion that proteorhodopsin may be upregulated when (photo)heterotrophic bacterial cells encounter lower energy levels in the presence of Fe-limitation. A heterotrophic bacterium strain was chosen whose genome had already been sequenced and which allowed the implementation of genetic tools in order to establish both a bioreporter of the gene(s) of interest as well as a gene knockout for comparison experiments. The two mechanisms that were studied and tested in this thesis manuscript are briefly described below.

1.4.1 The Glyoxylate Shunt

The glyoxylate shunt is an alternative metabolic pathway within the cell which allows bacteria to circumvent the NADH and CO₂ producing steps within the TCA cycle. Typically, the role of the glyoxylate shunt has been to allow anabolic reactions to take place when bacterial cells are utilizing 2-carbon compounds and fatty-acids. In Fe-limiting conditions, the upregulation of the glyoxylate shunt has been observed in several marine bacteria including the abundant SAR11 (Beier et al., 2015; Debeljak et al., 2019; Fourquez et al., 2014; Smith et al., 2010). Although, these observations suggest that the upregulation of the glyoxylate shunt is a possible strategy implemented by heterotrophic marine bacteria in order to cope with Fe-limitation, the strong association of the glyoxylate shunt towards the metabolism of specific carbon compounds makes it difficult to directly ascertain its induction under Fe-limitation. More recently, the induction of the glyoxylate shunt has been observed under both oxidative stress and Fe-limitation in the non-marine bacterium Pseudomonas aeruginosa (Ahn et al., 2016; Ha et al., 2018). Altogether, these results imply that the glyoxylate shunt is utilized for roles beyond the utilization of specific carbon sources such as 2-carbon compounds and fatty acids. In this thesis manuscript, experiments were designed to test whether the induction of the glyoxylate shunt could aid a marine bacterial cell subjected to Fe-limitation.

1.4.1.1 Chapter contributions

Frederique le Roux and François-Yves Bouget oversaw the creation of the knockout mutant and bioreporter of P. angustum S14 which were conducted by Remi vanGeersdaele and Valerie Vergé. Yannick Labreuche and Coco Koedooder constructed the complement strain. Audrey Gueneuges conducted the oxygen (respiration and growth) and expression experiments and Coco Koedooder performed the complementation experiments. Coco Koedooder was further responsible for the interpretation and analysis of the data, and writing the manuscript.

The results were submitted and published as:

Koedooder, C., Guéneuguès, A., Van Geersdaële, R., Vergé, V., Bouget, F.-Y., Labreuche, Y., et al. (2018). The Role of the Glyoxylate Shunt in the Acclimation to Iron Limitation in Marine Heterotrophic Bacteria. Front. Mar. Sci. 5, 435. doi:10.3389/fmars.2018.00435.

1.4.2 Proteorhodopsin

Because Fe-limitation particularly affects heterotrophic bacteria within the respiratory electron transport chain, Fe-limitation has a strong impact on the overall intracellular energy production. The indirect effect of Fe-limitation on ATP-production has led to the search of potential mechanisms by which a marine heterotrophic bacterium can maintain ATP production. Proteorhodopsin is a light activated proton pump found in several heterotrophic bacteria which, if coupled to ATP synthase, could ultimately generate a protein gradient that may be utilized in the production of ATP. The potential role of proteorhodopsin in Fe-limitation has previously been postulated for the diatom *Pseudo-nitzschia granii* and *Fragilariopsis cylindrus* studies (Marchetti et al., 2012; 2015) as well as from the genome analysis of several Roseobacter strains (Sun et al., 2017). In this thesis manuscript, a bioreporter and gene knockout was constructed to test the idea that proteorhodopsin could be utilized under Fe-limiting conditions to improve bacterial growth.

1.4.2.1 Chapter Contributions

Frederique le Roux and François-Yves Bouget oversaw the creation of the knockout mutant and bioreporter of P. angustum S14 which were conducted by Rémy van Geersdaële and Valérie Vergé. Coco Koedooder was responsible for the experimental work, the analysis and interpretation of the data.

The work is in preparation for submission to a peer-review journal.

II. EXPERIMENTAL APPROACH

In order to elucidate whether the glyoxylate shunt and proteorhodopsin are important metabolic pathways that can be utilized when a (photo)heterotrophic bacteria is subjected to Fe-limiting conditions, we opted for the construction of several genetic mutants of *P. angustum* S14 that were developed in order to address the following question(s):

"Is our gene of interest upregulated under Fe-limitation and if so, does its upregulation result in a measurable difference such as an improved growth and respiration rate?"

The experimental setup was conducted under trace metal clean conditions in order to control and avoid Fe-contamination as much as possible. Briefly, the regulation of the glyoxylate shunt and proteorhodopsin was determined using a bioreporter grown under both Fe-replete and Felimiting conditions. Growth and respiration rates were chosen as two key parameters in order to determine whether the induction of the glyoxylate shunt and/or proteorhodopsin resulted in an advantage to cells subjected to Fe-limitation. In this chapter we explain in more detail the choices regarding our experimental setup including the construction and use of genetic tools, working under trace metal-controlled conditions, defining Fe-replete and Fe-limiting conditions and measuring several parameters such as growth and respiration rates.

2.1 Genetic Tools

The construction of genetic tools, such as a bioreporter or a loss of function mutant, is an important and powerful tool that can be used to demonstrate the predicted role of a particular gene of interest. Several studies have, for example, successfully used gene knockout mutants in order to test the functioning of the glyoxylate shunt (Ahn et al., 2016; Ha et al., 2018) and proteorhodopsin (Akram et al., 2013; Gómez-Consarnau et al., 2016) under different environmental conditions. Despite the specificity in which genetic mutants can elucidate gene regulation and functioning, the use of such genetic tools is limited by its accessibility as not all bacterial strains can successfully be transformed.

2.1.1 Model Organism

A protocol has successfully been developed for the transformation of bacteria from the *Vibrionaceae* family (Lemire et al., 2015; Roux et al., 2007; Val et al., 2012) and was applied for the model organism *Photobacterium angustum S14*.

P. angustum S14 is a gram-negative gammaproteobacterium of the *Vibrionaceae* family that was originally isolated at 1m surface depths in Botany Bay, Australia in 1981 (Humphrey, 1983). The genome of *P. angustum* S14 has been sequenced previously and an extensive physiological data set has been constructed (Lauro et al., 2009). *P. angustum* S14 is often classified as an extreme copiotroph due to several distinctive traits including its ability to quickly respond to changes in the environment, containing a wide diversity of transporter genes as well as having the ability to evoke a specific starvation response under certain stress conditions (Boyd et al., 2015; Humphrey et al., 1983; Lauro et al., 2009; Nyström et al., 1992). Most importantly for this manuscript, however, is that *P. angustum* S14 contains both the glyoxylate shunt and proteorhodopsin within its gene repertoire.

Proteorhodopsin is a single gene/enzyme whereas the glyoxylate shunt is composed of two genes, namely isocitrate lyase and malate synthase. Therefore, for the construction of a bioreporter and gene knockout strain that tested the role of the glyoxylate shunt a choice had to be made between these two genes. Isocitrate lyase was chosen as our gene of interest as it is the first enzyme by which bacterial cells "enter" the glyoxylate shunt and will not result in the build-up of a potential toxic substrate (i.e. glyoxylate) if malate synthase was chosen (Puckett et al., 2017).

2.1.2 Bioreporter strains

Whole-cell bioreporters (Figure 8) are genetically engineered living-cells where a "reporter" gene, which produces an easily measurable signal (e.g. luminescence, fluorescence), is fused to a gene of interest either downstream of a promoter (i.e. transcriptional reporter) or in frame to a protein of interest (i.e. translational reporter). In both cases the reporter gene produces a measurable signal over time that respectfully reflects a sample's promoter/operon activity in response to various culture conditions (Sylvia Daunert et al., 2000).

Unlike qRT-PCR and western-blot, which respectfully measure transcript/protein expression levels at a given time point, the use of whole-cell bioreporters is a non-invasive technique that allows repetitive measurements on the same biological sample over time and does not require cell extractions and sample processing which would further increase sample variability between measurements (Corellou et al., 2009; Thommen et al., 2015). For this study, translational reporters were used as they give a more complete expression pattern of the gene of interest in comparison to the use of transcriptional reporters which are not further influenced by the presence of any potential post-transcriptional regulation.

There are various different reporter genes that can be used in the construction of a whole-cell bioreporter. The choice amongst different "reporter" genes is often between the use of green-fluorescent protein (GFP) or luciferase. For this study, luciferase was chosen as the reporter gene because its measurement does not require an external light source and it produces a much lower background noise in comparison to GFP (Choy et al., 2003). Briefly, luciferase as a reporter gene requires the presence of luciferin which is added to the medium and utilizes ATP and O_2 to produce light which can be monitored by a luminometer.



Figure 8. Schematic drawing of the functioning of a bioreporter. Briefly, a bioreporter fuses a gene of interest with the "reporter" gene luciferase. The "reporter" gene is hereby controlled by the same promoter/operon so that when the gene of interest is translated, luciferase will be translated in parallel. With the addition of luciferin, ATP and O_3 , luciferase produces a measurable signal that can be detected with a luminometer.

2.1.3 Gene knockout and complementation strains

A gene-knockout (Figure 9) allows one to study the function of a gene of interest by inactivating (i.e. by insertion of a sequence) or deleting that specific gene of interest and measuring the resulting change in phenotype in comparison to what was observed in the wildtype (i.e. respiration rate or growth rate). A clear limitation when constructing a gene-knockout is that they cannot be performed on "essential" genes which, if removed, would result in a non-viable bacterium cell.

Once a change in phenotype between the wildtype and the gene-knockout has been observed, these results are further confirmed with the construction of a complementation strain. A complementation strain consists of the gene-knockout mutant with the gene of interest reintroduced in the form of a plasmid containing a constitutively expressed promoter region (ie. P_{twe} operon). The gene of interest is hereby continuously upregulated within the plasmid. Therefore, if the complement strain is placed in an environment where the gene of interest is known to be upregulated, the complementation strain should regain the phenotype that was originally observed within the wildtype and was lost in the gene-knockout mutant.



Figure 9. Schematic drawing of the functioning of a gene knockout. Briefly, a gene knockout results in the loss of gene function by replacing the gene of interest with the regions that flank it (500 bp upstream and downstream) resulting in the deletion of that gene of interest.

2.1.4 Construction of the bioreporter and gene knockout

In total, 8 genetic constructs were generated (Table 3).

Table 3. List of constructed genetic mutants concerning the genes proteorhodopsin, isocitrate lyase and the combination of the two.

Proteorhodopsin (693 bp)		
1.	Luciferase bioreporter (pr-luc)	
2.	Gene knockout (Δpr)	
3.	Gene complement (pr [.])	
Isocitrate l	yase (1305 bp)	
4.	Luciferase bioreporter (icl-luc)	
5.	Gene knockout (Δicl)	
6.	Gene complement (icl·)	
Combinati	on	

- 7. Knockout-bioreporter (Δpr icl-luc)
- 8. Knockout-bioreporter ($\Delta icl pr-luc$)

The construction of a luciferase bioreporter for the expression of isocitrate lyase (icl-luc) proteorhodopsin (ptr-luc) as well as the construction of the knockout of either gene was conducted using a modified suicide plasmid constructed by Val et al. (2012) and implementing the transformation protocol adapted from Le Roux et al (2007) for members of the Vibrionaceae family (Figure 10). Briefly, the protocol describes the transformation of a plasmid construct into an *E. coli* host strain through electroporation. The *E. coli* host propagates the plasmid construct to sufficient quantities which is then extracted from its host cell, purified and subsequently transferred via electroporation to another *E. coli* host which will act as the donor cell. The plasmid is finally transferred to *P. angustum* S14 by bacterial conjugation, a form of horizontal gene transfer. After the plasmid is successfully transferred into *P. angustum* S14, homologous recombination will further result in the integration of the plasmid into the genome of *P. angustum* S14 at the targeted locus.

In general, the plasmid construct used to transform a bacterial cell must contain the gene insert (Figure 11), the ability for that gene to be integrated within the genome and several counterselection markers in order to ensure that, once the transformation process is complete,

one is solely left with a successfully transformed mutant of P. angustum S14 that is able to maintain its transformation. Importantly, the use of a suicide plasmid prevents its own propagation as a plasmid to daughter cells and is eventually lost over time. Daughter cells containing the plasmid that has been integrated into the genome are hereby the only cells to retain the successful transformation of P. angustum S14 into either a bioreporter or gene knockout. The protocol for the formation of each genetic mutant is described in more detail below.



Figure 10. Schematic representation and overview of the transformation of P. angustum S14 using the protocol developed by Le Roux et al., 2007 and the pSW7848 plasmid (Val et al., 2012). Briefly, the plasmid construct containing the gene insert is taken up by an E. coli host through electroporation where the plasmid is amplified. After plasmid extraction and purification, the plasmid is transferred into a secondary E. coli host through electroporation which will insert the plasmid into P. angustum S14 through bacterial conjugation. The gene insert is integrated within the genome through homologous recombination.

2.1.4.1 The pSW7848T Plasmid construct

Transformation of *P. angustum* S14 into either a bioreporter or gene-knockout was conducted using the pSW7848T plasmid (Val et al., 2012; Figure 11). For the construction of the bioreporter, the pSW7848T plasmid will contain the gene insert of *pr* or *icl* that has been fused with the "reporter" gene luciferin. The construction of a gene knockout, on the other hand, requires the replacement of *pr/icl* with a gene insert consisting of the DNA regions 500 base pairs upstream and downstream from the original gene of interest.

The pSW7848T plasmid is a suicide plasmid which is based on the pSW family of *pir*dependent R6K replicative origin (oriR6K) containing plasmids (Demarre et al., 2005; Val et al., 2012). A plasmid is an autonomous unit and its replication is independent from the cell. A suicide plasmid, is therefore a plasmid that cannot be replicated within a host cell and will consequently not be passed on to daughter cells resulting in its eventual loss over time. The pSW7848T plasmid contains the *pir*-dependent R6K replication origin (OriR6K) which can only be replicated when placed in a host cell that contains the *pir*-encoded π protein. When a host organism does not contain the *pir*-encoded π protein, the host is not capable of replicating pSW7848T which in turn acts as suicide plasmid (Kolter et al., 1978). As the genome of *P*. *angustum* S14 does not contain the *pir-encoded* π protein, the only way *P*. *angustum* S14 can maintain its transformation is if the plasmid is successfully incorporated within the genome.

The pSW7848T plasmid also carries an RP4 OriT replication origin ($OriT_{RM}$) which allows the plasmid to be transferred to a broad range of hosts by the process of bacterial conjugation. Bacterial conjugation is the transfer of genetic material from one donor to a recipient cell by a process requiring cell-to-cell contact. The successful transfer of a plasmid requires the use of sex pili which are appendages grown on the donor cell surface that can interact with specific sites on the surface of the recipient cell.

Lastly, the pSW7848T plasmid contains a series of counterselection markers. For both the construction of the bioreporter and gene-knockout, the resistance marker towards the antibiotic chloramphenicol (Cm) is used to select for cells containing the plasmid which are able to grow in medium containing Cm. For the construction of the gene knockout, the pSW7848T plasmid additionally contains the inducible CcdB toxin which is under the control of the arabinose P_{BAD} promoter (Roux et al., 2007; Val et al., 2012). In the presence of glucose, the P_{BAD} promoter is

repressed. In the absence of glucose, but in the presence of arabinose, the CcdB promotor is induced which allows for the transcription of the CcdB toxin that will kill the plasmid containing bacterium.



Figure 11. Schematic representation of the plasmid construct for (a) the bioreporter pr-luc and iclluc and (b) the gene knockout Δpr and Δicl to be inserted into the bacterial genome of P. angustum S14 through homologous recombination, hereby exemplified by the exchange of 2 identical DNA regions between the plasmid construct and the regions which flank the gene of interest (red and blue boxes). Both plasmids contain their respective gene insert, the origin of replication allowing plasmid replication (oriR6K), and bacterial conjugation (oriT) as well as an antibiotic resistance marker for the antibiotic chloramphenicol (cm). The key difference between the plasmid construct of the gene knockout and the bioreporter is that the plasmid construct of the gene knockout contains an additional selection marker: the CcdB toxin which is induced by the P_{bal} promoter in the presence of arabinose.

2.1.4.2 Genetic Transformation

The cloning and assembly of the gene insert into the pSW7848T plasmid was performed according to the Gibson assembly method, which allows multiple DNA fragments (i.e. the gene insert and plasmid) to be joined within a single, isothermal reaction (Lemire et al., 2015). Briefly, the pSW7848T plasmid is amplified using an inside-out PCR (Val et al., 2012) and the gene of interest is amplified using specific primers (Table 4). After purification and

quantification, the PCR fragments were subsequently cloned into the pSW7848T plasmid as described previously (Lemire et al., 2015).

P. angustum S14 cultures were grown out of cryopreservation and maintained in either ZoBell medium and ZoBell agar (Oppenheimer, 1952) or in Luria-Bertani (LB) medium and LB agar (Bertani, 1951) with the addition of NaCl (0.5 M), at 20°C. The *E. coli* strains Π 3813 and β 3914, that are respectively, used as plasmid hosts for the amplification and transformation of *P. angustum* S14 were grown in LB medium and LB agar at 37°C.

The plasmid construct is inserted into *E. coli* π 3813 via electroporation (Figure 10). A selection is made for *E. coli* π 3813 harbouring the plasmid by growing the cells on an LB + NaCl 0.5 M agar plate containing Cm. Cloning of the pSW7848T plasmid construct occurs in *E. coli* π 3813 because it contains the *pir*-encoded π protein which allows for plasmid replication via oriR6K. The amplified plasmid is isolated and purified before it is inserted into the second host, *E. coli* B3914, via electroporation. *E. coli* B3914 serves as the donor cell by transferring the pSW7848T plasmid construct to the recipient *P. angustum* S14 through bacterial conjugation from OriT_{BN} by a filter mating procedure as described previously (Biskri et al., 2005; Roux et al., 2007) with a donor/recipient ratio of 1:10 mL. *E. coli* B3914 are auxotrophic for the amino acid diaminopimelic acid (DAP), which must be supplemented to the medium in order for *E. coli* B3914 to grow. Growth on LB + NaCl 0.5 M plates without DAP and with the addition of Cm allows for the selection of *P. angustum* S14 containing the plasmid insert.

In order for *P. angustum* S14 to maintain the transformation within the cell, the plasmid insert must be integrated within the genome. Genome integration of the plasmid occurs through the first homologous recombination which is the exchange of two similar or identical molecules of DNA. In this case, homologous recombination occurs through the recognition of regions which flank the gene of interest that are present in both the plasmid construct and the genome of *P. angustum* S14 (Figure 11).

Homologous transformation and plasmid integration



Figure 12. Schematic representation of the homologous transformation of a plasmid and its integration into the genome of P. angustum S14. Briefly, homologous transformation occurs between identical components of the plasmid and the gene of interest (blue and red squares). The homologous components are designed to be the region that flanks the gene of interest (500bp upstream and downstream).

Selection for *P. angustum* S14 containing the integrated plasmid insert hereby occurs with the resistance towards Cm. As the plasmid acts as a suicide vector within *P. angustum* S14, only the integration of the plasmid into the genome of *P. angustum* S14 will allow its transfer into future daughter cells. In order to maintain the integrated plasmid, cells must always be grown in the presence of Cm (5 μ g/mL). In the case of the bioreporter, both the original gene and the gene-fusion product is maintained within the genome of the cell (Figure 12). This gene-fusion product is within the same reading frame as the original gene and will be upregulated in parallel to the original gene which can be measured upon the addition of luciferin (250 μ M final concentration) to the medium.

The construction of a gene knockout on the other hand requires the additional step of removing the plasmid backbone together with the gene of interest through a secondary homologous recombination step (intrachromosomal integration; Figure 13). An additional selection marker

must therefore be used to ensure that the removal of the plasmid has indeed taken place. The inducible CcdB toxin hereby acts as a second counterselection marker that is under the control of the arabinose P_{BAD} promoter (Roux et al., 2005). Growth in LB + NaCl 0.5 M containing 0.2% arabinose, selects for *P. angustum* S14 that has successfully removed its plasmid. As this second homologous recombination step can also cause the restitution of the wildtype a PCR of each colony forming unit (CFU) growing on the agar plate must therefore confirm the presence or absence of proteorhodopsin.

Secondary homologous transformation: removal of the plasmid backbone plasmid insert: gene of interest + Cm resistance + CcdB toxin inducing gene CcdB toxin restitution of gene of interest (wildtype)



a) restitution of wildtype



b) formation of gene-knockout: select using PCR

removal of gene of interest (knock-out)



Figure 13 Schematic representation of the secondary homologous transformation required for the formation of the gene knockout. The plasmid inserted in P. angustum S14 contains the CcdB toxin which is activated in the presence of arabinose. The second homologous recombination will remove the plasmid backbone in favour of either the wildtype (restitution) or the gene knockout. Growth on arabinose will hereby eliminate the P. angustum S14 where the secondary homologous transformation has not occurred and have retained the plasmid insert within its genome. Selection for the gene knockout occurs by plating the bacteria on an agar plate and testing each colony forming unit (CFU) by PCR in order to verify which CFU contains the presence or absence of the gene of interest.

2.1.5 Complementation

The construction of a complementation strain, where the missing gene within a gene knockout is reinserted as a constitutively expressed plasmid was done using the pMRB plasmid (Le Roux et al., 2011). A pMRB plasmid is a marine RNA-based plasmid family known to be stable within bacteria of the Vibrionaceae family and importantly does not function as a suicide plasmid. Briefly, the *pr* or *icl* gene was amplified from the wildtype strain of *P. angustum* S14 using primers containing Apa1/XhoI restriction sites within their tails. The *pr* and *icl* gene fragments (containing the additional Apa1/XhoI restriction site at the start and end) are consequently cloned into the ApaI/XhoI sites of the pMRB plasmid according to the Gibson assembly method and are subsequently controlled by the P_{Lxc} promoter which constitutively expresses the gene insert. Amplification and transformation of the pMRB plasmid into *P. angustum* S14 was performed as described previously (Roux et al., 2007).

Table 4. List of primers used for the amplification of the pSW7848T- and pMRB plasmid, and the primers for the gene of interest to be inserted into these plasmids through the Gibson Assembly Method and the verification of the presence or absence of these gene constructs.^{*}

Complementation	Primer name	Primer Sequence $(5' \rightarrow 3')$
Plasmid PMRB-PLAICL	For 300518-1	CTCGAGCTGCAGACGCGTCG
	Rev 300518-2	GGGCCCTTCCTTTGTTATCCGCTCAC
Pr-gene-apa1xho1	For PRapa1	GCCCGGGCCCATGCTAAATCCTAGTGACTTTGTAG
	Rev PRxho1	GCCCCTCGAG TTAAGCTCTTGTGTTGTCTGCATC
Icl-gene- apa1xho1	For ICLapa1	GCCCGGGCCCATGACATTAACACGCCAACAGC
	Rev ICLxho1	GCCCCTCGAG TTACTTAAACTGCTCTTCTTCTGTTG
Gene Knockout	Primer name	Primer Sequence $(5' \rightarrow 3')$
Plasmid pSW7848T	For 190613-22	GAATTCCTGCAGCCCGGGGG
1	Rev 190613-21	GAATTCGATATCAAGCTTATCGATAC
icl Upstream-fragment	For 111013-3	GTATCGATAAGCTTGATATCGAATTCGATGAGTTATTAGCGCCGTG
	Rev 111013-4	CATTGCTCCCCGATGATAACGTCTCTGTCCGCATTAATCC
icl Downstream-fragment	For 111013-5	GGATTAATGCGGACAGAGACGTTATCATCGGGGAGCAATG
C C	Rev 111013-6	CCCCCGGGCTGCAGGAATTCCCCGCGTACTTTGTCACGTAA
Icl-luc	Primer name	Primer Sequence $(5' \rightarrow 3')$
Plasmid pSW7848T*	190314-psw-noccdB-	GAATICICCAGCITIIGIICCCITIAGIG
Plasmid pSW78481*	190314-psw-noccdB- for	GAATICICCAGCITIIGIICCCIITAGIG
Plasmid pSW78481*	190314-psw-noccdB- for 070114-psw-rev	GAATICICCAGCITIIGIICCCIITAGIG GAATICGATATCAAGCITATCGATAC
Plasmid pSW7848'I* Promoter region	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for	GAATTCTCCAGCTTTTGTTCCCTTTAGTG GAATTCGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG
Plasmid pSW7848'I* Promoter region	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev	GAATICICCAGCITIIGTICCCITIAGIG GAATICGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC
Plasmid pSW7848'I* Promoter region isocitrate lyase	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for	GAATICICCAGCITIIGIICCCITIAGIG GAATICGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCAACAGC
Plasmid pSW7848'1* Promoter region isocitrate lyase	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev	GAATICICCAGCTITIGTICCCTITAGIG GAATICGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCCAACAGC CTTTATGTTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTTCTGTTG
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-luc-for	GAATTCGATATCAAGCTTATCGATAC GAATTCGATAAGCTTGATATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCAACAGC CTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTGTTG CAACAGAAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAG
Plasmid pSW7848'I* Promoter region isocitrate lyase luciferase	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-ILC-for 190314-luc-for 190314-luc-rev	GAATICICCAGCTITIGFICCCTITAGIGGAATICGATATCAAGCTTATCGATACGTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGGGCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAACGTTACAAGGATGGGGCGTTATGACATTAACACGCCCAACAGCCTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTGTTGCAACAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAGCACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase Ptr-luc	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-luc-for 190314-luc-rev Primer name	GAATICICCAGCTITIGITCCCTITAGIG GAATICGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCCAACAGC CTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTTCTGTTG CAACAGAAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAG CACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC Primer Sequence (5' → 3')
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase Ptr-luc Plasmid pSW7848T*	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-luc-for 190314-luc-rev Primer name 190314-psw-K7-fw	GAATTCCCAGCTTTTGTTCCCTTTAGTG GAATTCGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCCAACAGC CTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTTCTGTTG CAACAGAAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAG CACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC Primer Sequence (5' → 3') GAATTCTCCAGCTTTTGTTCCCTTTAGTG
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase Ptr-luc Plasmid pSW7848T*	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-luc-for 190314-luc-rev Primer name 190314-psw-K7-fw 070114-psw-rev	GAATTCCCAGCTTTTGTTCCCTTTAGTGGAATTCGATAAGCTTGATATCGATACGTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGGGCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAACGTTACAAGGATGGGGCGTTATGACATTAACACGCCCAACAGCCTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTTCTGTTGCAACAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAGCACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCCPrimer Sequence (5' \rightarrow 3')GAATTCTCCAGCTTTTGTTCCCTTTAGTGGAATTCCCAGCTTTTGTTCCCTTTAGTGGAATTCCCAGCTTTAGATAC
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase Ptr-luc Plasmid pSW7848T* Proteorhodopsin	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-luc-for 190314-luc-rev Primer name 190314-psw-K7-fw 070114-psw-rev 060514-pPR-for	GAATTCCCAGCTTTTGTTCCCTTTAGTGGAATTCGATAAGCTTGATATCGATACGTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGGGCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAACGTTACAAGGATGGGGCGTTATGACATTAACACGCCAACAGCCTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTGTTGCAACAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAGCACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC Primer Sequence (5' \rightarrow 3') GAATTCTCCAGCTTTGTTCCCTTTAGTGGAATTCGATATCAAGCTTATCGATACGTATCGATAAGCTTGATATCGAATTCGGTGTGCTTTATAGTGATACCG
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase Ptr-luc Plasmid pSW7848T* Proteorhodopsin	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-ILC-rev Primer name 190314-psw-K7-fw 070114-psw-rev 060514-pPR-for 060514-pPR-rev	GAATICICCAGCTITIGTICCCTITAGIG GAATICGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCAACAGC CTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTTGTTG CAACAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAG CACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC Primer Sequence (5' → 3') GAATTCTCCAGCTTTTGTTCCCTTTAGTG GAATTCGATAAGCTGATATCGATAC GTATCGATAAGCTTGATATCGAATTCGGTGTGCTTTATAGTGATACCG CTTTATGTTTTTGGCGTCTTCCATAGCTGTGTGTCTGCATCTC
Plasmid pSW7848'1* Promoter region isocitrate lyase luciferase Ptr-luc Plasmid pSW7848T* Proteorhodopsin luciferase	190314-psw-noccdB-for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-ILC-for 190314-ILC-rev 190314-ILC-rev 190314-ILC-rev 190314-ILC-rev 190314-ILC-rev 090314-luc-rev Primer name 190314-psw-K7-fw 070114-psw-rev 060514-pPR-for 060514-pPR-rev 060514-luc-forPR	GAATICICCAGCTITIGTICCCTITAGIG GAATICGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCAACAGC CTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTGTTG CAACAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAG CACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC Primer Sequence (5' → 3') GAATTCTCCAGCTTTTGTTCCCTTTAGTG GAATTCGATAAGCTGATATCGATAC GTATCGATAAGCTTGATATCGATATC GTATCGATAAGCTTGATATCGAATTCGGTGTGTTGTCTGCATCTC GAGATGCAGACAACACAAGAGCTATGGAAGACGCCAAAAACATAAAG
Plasmid pSW7848T* Promoter region isocitrate lyase luciferase Ptr-luc Plasmid pSW7848T* Proteorhodopsin luciferase	190314-psw-noccdB- for 070114-psw-rev 190314-p-aceBA-for 190314-p-aceBA-rev 190314-ICL-for 190314-ICL-rev 190314-luc-for 190314-luc-rev Primer name 190314-psw-K7-fw 070114-psw-rev 060514-pPR-for 060514-pPR-rev 060514-luc-forPR 190314-luc-re	GAATTCGATATCAAGCTTATCGATAC GTATCGATAAGCTTGATATCGATAC GTATCGATAAGCTTGATATCGAATTCTGTCTTTTGGGTGTAAGGGG GCTGTTGGCGTGTTAATGTCATAACGCCCCATCCTTGTAAC GTTACAAGGATGGGGCGTTATGACATTAACACGCCCAACAGC CTTTATGTTTTTGGCGTCTTCCATCTTAAACTGCTCTTCTGTTG CAACAGAAGAAGAAGAGCAGTTTAAGATGGAAGACGCCAAAAACATAAAG CACTAAAGGGAACAAAAGCTGGAGAATTCTTACACGGCGATCTTTCCGCC Primer Sequence (5' → 3') GAATTCTCCAGCTTTTGTTCCCTTTAGTG GAATTCGATAAGCTTGATATCGAATC GTATCGATAAGCTTGATATCGAATTCGGTGTGTCTTATAGTGATACCG CTTTATGTTTTTGGCGTCTTCCATAGCTGTGTGTCTGCATCTC GAGATGCAGACAACACAAGAGCTATGGAAGACGCCAAAAACATAAAG CACTAAAGGGAACAACACAAGAGCTATGGAAGACGCCAAAAACATAAAG

^{*} The plasmid pSW7848 in both icl-luc and pr-luc has the ccdB toxin gene removed.

Lastly, all constructed mutants were genetically sequenced to verify that they were correctly modified and were placed in cryopreservation using glycerol (35%) at -80° C. Prior to use, a simple PCR was conducted to verify the presence or absence of the acquired/lost gene insert (Table 4).

2.2 Trace Metal Controlled Conditions

When working with bacterial cultures subjected to Fe-limiting conditions, it is necessary to work in a controlled environment which prevents the contamination of Fe throughout the duration of the experiment. Furthermore, considering the ability of Fe to form complexes with a large variety of organic and inorganic ligands within seawater, which will further affect its availability, we attempted to control the Fe speciation within our experimental setup as much as possible (Morel et al., 2008b). Briefly, all experiments were conducted in a clean room (class 10000) under a laminar flow hood (ADS class 100). All used material was made trace metal free by soaking them for ± 24 hours in 10% HCl followed with a miliQ water (18.2 M Ω) bath for another 24 hours. Additionally, flasks were sterilized by adding miliQ water and microwaved 3x at 750W until boiling. All items were placed under the laminar flow hood to dry and subjected to UV light (± 20 min).

2.2.1 Aquil Medium

The majority of marine studies testing the role of Fe-limitation require the preparation and use of Aquil medium which offers a well-controlled speciation of Fe. Aquil is a chemically well-defined marine medium that has been designed for studying trace metal physiology in algae (Morel et al. 1979) and updated (Price et al., 1989). The preparation of Aquil medium requires a stringent procedure where synthetic ocean water (SOW) along with all other nutrients except vitamins and trace metals are purified (i.e removal of trace metals) is passed through a Chelex 100 column (200-400 mesh; Sigma) in order to remove any metal contaminants. The Chelex 100 is a resin composed of a styrene-divinyl benzene matrix containing iminodiacetate ions that will form a chelate with transition metals, thereby effectively removing them from solution as the medium passes through the column. Chelex 100 was prepared according to the manufacturer's instructions (Bio-Rad) and rinsed until a stable pH of 8.5 has been reached. The pH is carefully controlled and monitored because the chelating strength and ionic form of Chelex 100 will vary significantly with pH. The chelexed SOW was further sterilized by

microwaving it 3x at 750W until boiling and cooled prior to the addition of metals including Fe (5.4 μ M) and chelexed carbon, nutrients and vitamins filtered through a 0.2 μ M syringe Acrodisc filter (Pall Corporation).

2.2.2 Fe-conditions:

Fe-speciation was controlled in the medium by chelating Fe with EDTA. EDTA is a synthetic chelator that is widely used to complex trace metals. Chelating Fe to EDTA controls the Fe speciation because it prevents the Fe from binding with inorganic ligands present in seawater (ie. hydrous oxides) which has a low solubility and can precipitate out of solution. As EDTA is a chelator which isn't naturally present in seawater, it is assumed that the complex Fe-EDTA will not be taken up by phytoplankton (Sunda and Huntsman, 2003) and we further assume that this is also the case for *P. angustum* S14 although it is possible that free EDTA may serve as an additional carbon source for these bacteria. In our experimental setup, we hereby assume that *P. angustum* S14 takes up Fe from Fe:EDTA in the free Fe form noted as Fe'.

The dissociation constant of Fe:EDTA can be affected by the temperature, pH and light. When testing the effect of proteorhodopsin on Fe-limitation, the sensitivity of EDTA to light may become problematic because our experimental setup requires us to compare light and dark conditions. The presence of light can lead to the photolysis of Fe:EDTA which releases Fe' within the medium resulting in an increased bioavailability of Fe in light compared to dark conditions. Comparisons between the wild type and the gene knockout can hereby help us to distinguish whether an observed increase in growth or respiration rate in the presence of light is due to the gene of interest or due to photolysis of Fe:EDTA in the presence light. However, if photolysis occurs it is possible to account for the impact of light intensity in the calculation of Fe speciation (Sunda and Huntsman, 2003, 2011). Alternatively, the chelating agent NTA (nitriloacetic acid) can be used which is known to be less affected by the presence or absence of light.

As Fe concentrations within the water column are within the nanomolar range we chose to grow P. angustum S14 in a range of Fe concentrations that would obtain cell concentrations high enough to measure gene expression, growth and respiration rates above their respective detection limits while still attempting to reflect relatively natural conditions. We therefore

chose to work with Fe-replete concentrations of 5.4 μ M of total Fe and decreased the total Fe concentration towards Fe-limiting concentrations of 5.4 nM.

As bioreporters are grown with the addition of luciferin (250 μ M, final concentration) and chloramphenicol (5 μ g/mL), Fe contamination is automatically introduced within the medium. When Fe-concentrations could not be defined due to the presence of Fe-contamination, Fe-limitation was achieved with the addition of the Fe chelator desferrioxamine B (DFOB). DFOB has a much stronger association constant in comparison to Fe:EDTA and therefore will bind to any additional Fe forms within the medium.

2.2.3 Fe-acclimation steps:

Prior to the start of the experiment, all strains (wildtype (wt), Δ icl, icl+, and icl-luc) were taken out of cryopreservation and plated on ZoBell medium at 20°C for a period of 48 h at room temperature. Colonies were subsequently picked and transferred in 5 mL of liquid Aquil medium containing 6 mM[C] glucose for 24 h before being grown in larger batch cultures of 12 mL. Batches were refreshed at least 3× (25 µL of culture was transferred to a fresh 12 mL batch of Aquil medium every 2 days) in order to remove any traces of Fe and carbon that could be carried over from the ZoBell medium before being subjected to Fe-replete and Fe-limiting conditions and the presence or absence of light. Bioreporters and complement strains are grown with the addition of chloramphenicol (5 µg/mL) to maintain their gene constructs within the cell.

2.3 Measuring growth and respiration rates

Having constructed several bacterial mutants from *P. angustum* S14 and having defined our medium, including our choice of Fe-replete and Fe-limiting conditions, the effect of Fe-limitation was determined by measuring such as the growth curves and calculating the growth and respiration rates. Growth and respiration rates represent common parameters that can be used to describe and indicate the physiological state of the cell and are described in more detail below.

2.3.1 Growth Curves

The growth curve (Figure 14) of a bacterial culture represents the sequential division of bacterial cells to form two identical daughter cells. Bacterial growth curves are classically

divided into 4 phases: the lag phase, the exponential phase, the stationary phase and the death phase (Pletnev et al., 2015). Additionally, a 5^{*} phase may occur which can be described as the long-term stationary phase. The lag phase typically represents the time that is required for a bacterial cell culture to adapt to its new cultivation environment (Rolfe et al., 2012). Once bacterial cell cultures start to divide exponentially, they will enter into the logarithmic growth phase. The rate at which a bacterial cell divides gives an indication about the efficiency in which this is done. Bacterial cell cultures will continue to divide until a nutrient becomes limiting and is eventually exhausted and the division rate of a bacterium decline in cell division. As cells reach the stationary phase one can calculate the total cell yield, here defined as the maximum number of cells within a given experimental condition. Cells may enter the stationary phase for a variety of reasons such as the exhaustion of a particular nutrient but also the accumulation of toxic products.

P. *angustum* S14, like other members of the Vibrionaceae family, has the ability to induce a starvation state which enables its long-term survival upon entering the stationary phase (Boyd et al., 2015; Gómez-Consarnau et al., 2010). The starvation state is a series of transcriptional changes that are controlled by the presence of a sigma-factors within the cell. A sigma-factor is a protein that is required for the transcription of proteins. Under certain environmental conditions a sigma-factor will bind to polymerase and initiate the transcription downstream of a specific set of promoters. The presence of several different sigma-factors within the cell allows a bacterium cell to dramatically change its transcriptome in response to various environmental signals (Boyd et al., 2015; Pletnev et al., 2015). The induction of a starvation state remodels the cellular metabolism of *P. angustum* S14 and is tightly regulated by the RNA polymerase sigma factor RpoSA sigma-factor. Some bacterial species can further enter a viable-but-not-culturable (VBNC) state where cells remain metabolically active but are uncapable of forming colonies when plated out on a plate (Oliver, 2005; Wu et al., 2016).

2.3.2 Exponential growth phase

When studying the effect of Fe-limiting conditions, our primary focus was the exponential phase in *P. angustum* S14 as it would reflect the impact proteorhodopsin or the glyoxylate shunt may have on the efficiency at which a cell could grow under Fe-limitation. From the exponential phase, the growth rate (μ) was derived from the following equation:

$$\frac{dN}{dt} = \mu N$$

Where N is the number of bacterial cells at time *t*. The change in the bacterial cell abundance (ΔN) over time (Δt) can be derived from the integral of the rate of change in the cell abundance between t₀ and t, results in the following equation below:

$$ln\frac{N_t}{N_0} = \mu \left(t - t_0\right)$$

where Nt and N0 are respectively the abundance at t and t0.

The μ can be calculated from the slope of ln N over t. Based on this equation, the growth rate was hereby calculated from the slope of the ln transformed cell abundance. Cell abundance was obtained by measuring cell counts using a Becton Dickinson Accuri C6 flow cytometer (Marie et al., 1997). Bacteria were fixed in formaldehyde (2%) and stained with SYBR Green I (15 min).



Figure 14. (a) The bacterial growth curve of P. angustum S14 separated according to the classical division of the lag phase, the exponential phase, and the transition towards the stationary phase followed by its decline. (b) The growth rate (μ) can hereby be determined from the slope of the linear section (true exponential phase) of the cell count after a natural logarithm (lnx) transformation.

2.3.3 Cell size measurements

Bacterial cell size, was measured as the mean biovolume (μ m³) using a Cell counter and Analyser (CASY Model TTC, Roche Diagnostics). Bacterial cells are suspended in an electrolyte solution (CASY ton solution) and is passed through a small capillary. Cells (and other particles) displace the electrolyte solution resulting in the generation of an electrical pulse which can be measured in order to determine the exact volume of the cell passing through the capillary. Bacterial cells were analysed through a 45 µm capillary which can analyse a cellular size range between 0.7 and 30 µm according to the manufacturer's instructions. Briefly, a subsample of cells was diluted and processed in 10 mL of CASY ton buffer medium to obtain a concentration between 2 x10⁷ and 3 x10⁸ cells mL⁻¹. The mean volume (fl) was calculated from the measured particles that had passed within each sample whilst selecting a size range which excluded the measurement of debris and particles (<0.7 µm).

2.3.4 Oxygen measurements: Growth and Respiration Rates

When bacterial cells are in exponential growth, both the bacterial respiration rate and growth rate are assumed to be constants. Therefore, if the initial bacterial cell abundance (N_0) is known, both the growth (μ) and respiration rates per cell (R_{cel}) can be derived from continuous dissolved oxygen concentration (O_2) measurements (Figure 15). While the relevance in the growth rate has been explained in the previous paragraphs, the respiration rate is an additional important parameter which provides an indication of the effect of Fe-limitation as 99% of Fe is expected to reside within the electron transport chain of heterotrophic bacteria and the majority of O_2 is consumed through the electron transport chain where O_2 acts as the final electron acceptor.

 O_2 measurements were taken from batch cultures using a SensorDish reader (Presens) equipped with 24 glass vials of 4.8 mL containing non-invasive O2 sensors (OxoDish). All growth and respiration experiments were conducted in triplicate (where one replicate represents one O_2 sensor vial). The entire setup was placed in an incubator at a constant temperature of 20°C. O_2 concentrations were derived from 10-min averages of quenched fluorescence measurements taken every 30 s over a period of 24 h. Each vial was calibrated using a two-point protocol. A

^{*} other oxygen consuming entities are also known to occur such as NADH-oxidases but can be considered to be almost negligible in comparison to the electron transport chain (...).

 $0 \ \mu M O_2$ solution was prepared with a 1% Na₂SO₂ in Milli-Q water. A 100% oxygenated solution was prepared by bubbling air at a constant temperature for 2 h in Milli-Q water and left overnight with the lid open to allow exchange with air. O₂ concentrations in Aquil medium were further corrected for by taking into account the salinity of the medium (S = 35) using the solubility equation of Garcia and Gordon (1992). Blanks consisting of filtered Aquil medium (0.2 µm) were used to monitor for any significant drift from the O₂ sensors for the entire duration of the experiments (±24 h). A time window was carefully chosen in order to capture the exponential phase. In order to meet the assumption of a constant growth and respiration rate, care was taken to avoid the inclusion of any metabolic switches (Wolfe, 2005) that would result in a change in the oxygen consumption rate.



Figure 15. O_2 consumption curves for P. angustum S14 grown in either Fe-replete (a) and Fe-limiting (b) conditions. Notice that two respiration curves can be observed for P. angustum S14 grown in Fe-replete conditions. Horizontal lines depict the window that was chosen to calculate the growth and respiration rate.

An average of every 10 min was taken to derive the growth and respiration rates from the selected O_2 measurements. Changes in ΔO_2 concentrations over time were derived from the integral of the rate of change in O_2 over time using the following equation below:

$$\frac{dO_2}{dt} = -N_0 R_{cell} \exp\left(\mu t\right)$$

The integration of the above equation gives:

$$\Delta O_2 = [O_2]_{ti} - [O_2]_{tf} = A (1 - \exp(\mu t))$$

with

$$A = \frac{-N_0 R_{cell}}{\mu}$$

where $[O_2]_{ti}$ and $[O_2]_{tf}$ are the respective O₂ concentrations at the starting time point (ti) and at the final time point (tf). $\Delta O_2 = f(t)$ was fitted to the equation using sigmaplot software to derive μ and $A = \frac{-N_0 R_{cell}}{\mu}$. A was then used to calculate R_{eell} .

Under Fe-limiting conditions, ΔO_2 was small and therefore the equation leads to an approximately linear relationship:

$$\Delta O_2 = N_0 R_{cell} t$$

where R_{cell} is derived from the slope of $\Delta O_2 = f(t)$ measurements which in this case can be defined from the following equation:

$$\mu = \frac{\ln (N_f - N_0)}{(N_0 t_f)}$$

Where N_f is the final cell abundance at t_f derived from flow cytometry measurements. The agreement between experimental data and model fitting was then plotted in order to verify that the main assumptions of the model concerning a constant growth and respiration rate were met (Figure 16).



Figure 16 ΔO_2 consumption curves derived from the selected time window for P. angustum S14 grown in Fe-replete (left) and Fe-limiting (right) conditions. Fitting of the model (blue) with the experimental values shows the model's prediction for both the growth and respiration rates. The curves meet the assumption of a constant growth and respiration rate.

2.3.5 ATP measurements

While O₂ measurements provides information on the proton gradient produced by the electron transport chain over time, a large portion of this gradient is expected to be coupled with the production of ATP through ATP synthase which, in the presence of Fe-limitation, is expected to decrease. ATP concentrations within the cell, can therefore provide information about the metabolic potential of the cell and is an additional measurement on the effect of Fe-limitation.

ATP measurements can be conducted using a luciferin-luciferase enzyme reaction (ATP Biomass Kit HS, BioThema) using a luminometer and following the manufacturer's instructions (Lundin, 2000). Briefly, ATP is extracted from the cell using the Extractant B/S. In the presence of luciferase, the addition of D-luciferin reacts with the extracted ATP releasing a measurable signal according to the following equation:

$$ATP + D - luciferin + O_2 \rightarrow AMP + PPi + oxyluciferin + CO_2 + light$$

Where the intensity of the light emitted is proportional to the amount of ATP that is measured using a luminometer. Luminescence is calculated from a 2-point calibration through the

addition of a known amount of ATP standard in order to calculate the amount of ATP within the samples in pmol from the following equation:

$$ATP_{smp} = \frac{I_{smp}}{(I_{smp+std} - I_{smp})}$$

Where I_{smp} is the measured light emission of the sample and $I_{smp + std}$ is the measured light emission of the sample with the addition of the known standard.

III. THE ROLE OF THE GLYOXYLATE SHUNT

3.1 Introduction

3.1.1 The Glyoxylate Shunt and Anabolic metabolism

Basic cellular metabolism can be defined as the uptake of precursors (e.g. glucose) in order to realize a series of biochemical reactions that can be split, according to their function, into either catabolic or anabolic pathways (Mathews et al., 2000). Catabolism concerns the pathways which breakdown molecules into smaller molecules and enables the production of energy (e.g. ATP) while anabolism consists of pathways for the production of larger molecules and the formation of cellular biomass in the form of lipids, proteins and nucleic acids. Together these reactions allow cellular growth and radiate from a core central metabolic pathway known as the tricarboxylic acid (TCA) cycle. Simply put, the primary function of the TCA cycle is to channel fluxes towards catabolic pathways and the production of ATP in order to meet the energy requirements within the cell (Roller and Schmidt, 2015). Once the basic cellular energy requirements have been met, the flux of components can shift within the TCA cycle towards anabolic pathways and the production of cellular biomass.

Briefly, when glucose is taken up by the cell (Figure 17), it is converted through glycolysis into pyruvate from which acetyl-CoA moieties can be formed through the pyruvate dehydrogenase reaction. Acetyl-CoA can then enter the TCA cycle by combining with oxaloacetate to form citrate which is then oxidised into CO₂, allowing the production of reducing agents (e.g. NADH, FADH₂) which will serve as electron donors for the respiratory electron transport chain. The respiratory electron transport chain produces a proton gradient which, when coupled to ATP synthase is used to generate ATP within the cell. Simultaneously, intermediates within the TCA cycle (e.g. oxaloacetate) are used for anabolic reactions for the formation of sugars, lipids, proteins and nucleic acids.



Figure 17. Simplified overview of the TCA cycle and electron transport chain. Dotted boxes indicate major metabolic processes, indented text are molecules and metabolites produced by enzymes signified by single lined boxes using the following abbreviations: **PDH** - Pyruvate dehydrogenase, **CS** - citrate synthase, **ACN** - aconitase hydratase, **IDH** - isocitrate dehydrogenase, **ICL** - isocitrate-lyase, **OGDC** - oxoglutarate dehydrogenase complex, **SCS** - succinyl coenzyme A synthetase, **SDH** - succinate dehydrogenase (Complex II), **FS** - Fumarate hydratase, **MS** - malate synthase, **MDH** - malate dehydrogenase, and the roman numerals **I**, **II**, **III**, **IV** indicate the 4 complexes of the electron-transport chain. Asterisks indicate enzyme (complexes) containing Fe. Take note that the conversion of pyruvate to acetyl-coA by PDH is an irreversible reaction.

As mentioned previously, entry into the TCA cycle starts with citrate synthase through the condensation of acetyl-CoA with oxaloacetate to form citrate. The metabolism of fatty acids and 2-carbon sources (e.g. acetate) hereby represents a special case because it surpasses the glycolysis step and enters the TCA as a moiety of acetyl-coA. While glycolysis is an important step in the formation of acetyl-coA from glucose, it is also required to provide the oxaloacetate that condenses with acetyl-coA to form citrate. Oxaloacetate can be produced from pyruvate through pyruvate carboxylase but the production of pyruvate form acetyl-coA does not occur because the pyruvate dehydrogenase reaction is irreversible. Therefore, as 2-carbon sources and fatty acids enter the TCA cycle as acetyl-coA, a dilemma is created because the formed acetyl-coA moieties are completely oxidized into CO₁units and cannot yield any net carbon assimilation in the form of oxaloacetate. Simply put, the consumption of carbon sources such as acetate and fatty-acids does not allow for the regeneration or increase in TCA cycle intermediates (e.g. oxaloacetate) and in the absence of a mechanism to conserve carbon towards pathways concerning biomass production (e.g. gluconeogenesis) no growth under these carbon substrates is possible.

Growth on 2-carbon sources and fatty acids can occur by means of the glyoxylate shunt which is a two-step metabolic pathway found in aerobic bacteria that serves as an alternative route within the TCA cycle (Kornberg, 1966; Kornberg and Krebs, 1957; Figure 16). Its primary function is to circumvent the carbon dioxide (CO₃) production steps within the TCA cycle in order to drive the metabolism of fatty-acids or 2-carbon compounds such as acetate towards the production of oxaloacetate, which can increase the production of cellular respiration or serves as a precursor for gluconeogenesis. During this process, the glyoxylate shunt also bypasses the NADH producing steps which are required within the respiratory electron transport chain.

The glyoxylate shunt consists of the two enzymes isocitrate lyase (ICL) and malate synthase (MS). The first step of the glyoxylate shunt is the cleavage of isocitrate by the enzyme ICL into succinate and glyoxylate. Malate synthase combines succinate with another acetyl-coA moiety to form malate. Entry into the glyoxylate shunt occurs as ICL competes with the TCA molecule isocitrate dehydrogenase (IDH) for the substrate isocitrate. Because ICL has a lower affinity for isocitrate in comparison to IDH, the flux of isocitrate towards IDH must either be

inhibited or slowed down to allow for the accumulation of isocitrate to levels high enough to channel isocitrate towards the glyoxylate shunt (LaPorte and Koshland, 1982).

The glyoxylate shunt is widely distributed in aerobic bacteria (Ahn et al., 2016). Although its prevalence is ubiquitous amongst aerobic bacteria, it is particularly found in the phyla of Proteobacter, Actinobacteria and Firmicutes suggesting that this bypass is not randomly distributed across known bacterial species. Furthermore, since its discovery, the role of the glyoxylate shunt in the regulation of carbon flux from 2-carbon substrates towards anabolic reactions is an oversimplification and the glyoxylate shunt has additionally been implicated in a range of other cellular functions (Dolan and Welch, 2018). Recently, the glyoxylate shunt has been upregulated under a multitude of stresses including oxidative stress and Fe-limitation although its exact regulation has been shown to vary amongst species (Ahn et al., 2016; Ha et al., 2018; Meylan et al., 2017).

3.1.2 Glyoxylate shunt and Oxidative Stress

In several pathogenic bacteria, the glyoxylate shunt was upregulated under antibiotic-induced oxidative stress (Dolan and Welch, 2018). Under O₂-limitation, M. tuberculosis strains were shown to induce the glyoxylate shunt enabling the cell to redirect its metabolism towards glycolysis. The upregulation of the glyoxylate shunt increased succinate concentrations and replenished NAD+ which were then utilized for the production of ATP through glycolysis (Eoh and Rhee, 2013; Wayne and Lin, 1982; Wayne and Sohaskey, 2001). Its upregulation was also observed in Pseudomonas aeruginosa strains harbouring a resistance towards antibiotics targeting the electron transport chain (Meylan et al., 2017). The presence of several antibiotics (e.g. isoniazid, rifampicin, and streptomycin) can induce oxidative stress in *M. tuberculosis* by enhancing the formation of respiratory-derived reactive oxygen species (ROS). In response, M. tuberculosis was shown to channel its carbon flux towards the glyoxylate shunt (Nandakumar et al., 2014) and upon deletion of the glyoxylate shunt, bacterial persistence towards the same antibiotics could still be maintained upon the addition of antioxidants. This led to the assumption that the glyoxylate shunt was able to improve the ability of a bacterial cell to cope in the presence of antibiotics by reducing the formation of ROS within the cell. One possible explanation is that the glyoxylate shunt channels carbon flux away from the production of NADH. NADH serves as the electron donor for Complex I of the respiratory electron transport chain and the oxidation of NADH at Complex I is known to be a major site for ROS generation

(Vinogradov and Grivennikova, 2016). Interestingly, removal of the glyoxylate shunt improved the growth rate of *P. aeruginosa* under oxidative or antibiotic stress as cells switched to aerobic denitrification (Ahn et al., 2016). The glyoxylate shunt therefore seems to play a regulatory role in aerobic respiration (and the suppression of aerobic denitrification) and the management of ROS. Lastly, experiments conducted in *P. aeruginosa* showed the importance of the glyoxylate shunt in the presence of the antibiotic tobramycin, whose uptake is influenced by the membrane potential within the cell. Decreasing cellular respiration through the induction of the glyoxylate shunt reduced the uptake of tobramycin and resulted in a tolerance towards this antibiotic (Meylan et al., 2017).

3.1.3 Glyoxylate shunt and Fe-limitation

A recent study has linked the upregulation of the glyoxylate shunt in *P. aeruginosa* to both oxidative stress (H_2O_2) and Fe-homeostasis (Ha et al., 2018). The presence of the glyoxylate shunt in *P. aeruginosa* resulted in a lower intracellular Fe-concentration in comparison to when the glyoxylate shunt was absent. Deletion of the glyoxylate shunt improved the respiration rate of the cell but also made cells more susceptible towards oxidative stress. The absence of the glyoxylate shunt under oxidative stress therefore resulted in an enhanced formation of ROS which negatively affected *P. aeruginosa*. Under Fe-limiting conditions, the glyoxylate shunt was upregulated, and increased as cells approached the stationary phase although it remained to be elucidated why this occurred.

The upregulation of the glyoxylate shunt under Fe-limitation has further been observed in several marine heterotrophic bacteria. Fourquez et al (2014) has revealed its upregulation in the coastal strain, the gammaproteobacterium *Alteromonas macleodii*. The increased expression of *icl* was also noted in experiments conducted by Smith et al (2010) using various strains of "Candidatus" Pelagibacter ubique and in the field studies conducted by Beier et al (2015) for members of the SAR11 clade as well as heterotrophic bacteria within the HNLC site of the Southern Ocean (Debeljak et al., 2019). Although, these observations suggest that the glyoxylate shunt is a possible acclimation strategy for heterotrophic marine bacteria when dealing with Fe-limitation, the strong association of the glyoxylate shunt towards the metabolism of certain carbon compounds makes it difficult to directly ascertain its induction with Fe-limitation. Combined these findings have sparked interest in whether the induction of

the glyoxylate shunt could indeed aid a bacterium cell coping with Fe-limitation within a marine context.

It is known that under Fe-limitation the efficiency of the electron transport chain is severely hindered including the ability for cells to produce ATP (Tortell et al., 1999). Fe-limitation can therefore lead to the accumulation of NADH (Wu et al., 2001) which cannot be utilized due to an inefficient respiratory complex. Entrance into the glyoxylate shunt, on the other hand, can redirect metabolism towards the electron transport chain at Complex II which requires succinate as the electron donor rather than NADH (Rutter et al., 2010). Complex II is also known to require significantly less Fe than Complex I (Table 2). We hereby hypothesized that redirecting a cell's metabolism away from Fe-limiting steps by means of the glyoxylate shunt could be an important mechanism to enhance the metabolic efficiency of a heterotrophic bacterium cell subjected to Fe-stress (Figure 18).



Figure 18. Schematic representation of our hypothesis comparing glucose metabolism in (a) Fe-replete conditions and (b) in the presence of Fe-limitation. Under Fe-limitation, the glyoxylate shunt is hypothesized to be upregulated enabling P. angustum S14 to redirect its metabolism away from key Fe-limiting steps resulting in a lower CO; and NADH production but decreasing the utilization of the Fe-rich respiration complex I.

3.1.4 Objectives

The objective was to investigate, in a marine context, whether the induction of the glyoxylate shunt is indeed directly related to Fe-limitation and whether its induction can affect bacterial growth and respiration rates. Through the use of genetic tools, the role of the glyoxylate shunt under Fe-limitation was elucidated via the construction of both a luminescent bioreporter (icl-luc), a knockout (Δ icl) and its complement (icl+) of the isocitrate lyase gene in the model organism *P. angustum* S14. Isocitrate lyase (icl) was the chosen gene of interest as it is one of two key enzymes within the glyoxylate shunt.

3.2 Material and Methods

3.2.1 Experimental setup:

Experiments were conducted using the bioreporter icl-luc and the gene-knockout Δ icl and gene complement icl+. Icl-luc tested the expression of *icl* under various levels of Fe-limitation and different sources of organic carbon, while comparative experiments between the wildtype, Δ icl and its complement (icl+) aimed to determine whether icl induction allowed a physiological advantage in terms of growth or respiration rates.

All experiments were conducted under trace metal clean conditions. *P. angustum* S14 (wt, Δ icl, icl+, and icl-luc) was taken out of cryopreservation and plated on ZoBell medium at 20°C for a period of 48 h at room temperature. Colonies were subsequently picked, transferred and maintained in 12 mL batches of liquid Aquil medium containing the single-carbon glucose (6 mM[C]) in Fe-replete conditions (5.4 μ M). Batches were refreshed at least 3× (25 μ L of culture was transferred to a fresh 12 mL batch of Aquil medium every 2 days) prior to the start of the experiment, in order to remove any traces of Fe and carbon that could be carried over from the ZoBell medium. When necessary, chloramphenicol (Cm), 5 μ g/mL was added to the Aquil medium.

3.2.1.1 ICL-luc expression experiments

Aquil medium was prepared containing Fe-replete conditions and different single carbon sources (acetate, serine and alanine) that were added at a final concentration of 100 μ M[C]

while glucose was added at both 100 μ M[C] and 6 mM[C]. Fe-limitation was obtained through the addition of DFOB due to the unknown possibility of Fe-contamination resulting from the usage of luciferin and Cm so that it was not possible to quantitatively calculate the total bioavailable Fe (Fe') within the medium. Aquil medium was prepared containing 6mM[C] of glucose and 5.4 μ M of Fe and a range of DFOB concentrations (0, 10, 50, 100, and 500 μ M). The addition of DFOB was left to equilibrate in the medium for a period of 24 h prior to its use. Experiments testing different carbon sources or glucose concentrations were conducted using six replicates, while the experiment testing different concentrations of DFOB was conducted in triplicate (one replicate representing one 200 μ L well).

Icl-luc was diluted to starting concentrations ($1.8 \times 10^{\circ}$ cells mL⁻¹) within their respective media (see before) and pipetted into 200 µL wells of a clear-bottom 96-well microplate (Greiner Bio-One) containing 5 µL of luciferin (final concentration of 250 µM) and covered with a clear diamond film seal (Thermo Fisher ScientificTM). Coinciding with the late-exponential phase, luminescence was measured at 16 h (5 s per well) using a luminometer (Berthold Centro LB 960). Samples, for the determination of cell abundance by flow cytometry, were taken simultaneously in order to measure expression of icl per cell. The expression of the icl was measured as luminescence (arbitrary units, a.u.) normalized to 1000 cell counts.

3.2.1.2 Δicl gene knockout experiments

Growth and respiration rates of wt, Δ icl, icl+ in Aquil medium containing Fe-replete (5.4 μ M) and a range of Fe-limiting (5.4 μ M + 10 and 100 μ M DFOB) conditions were derived from continuous dissolved oxygen (O₂) measurements in batch cultures using a SensorDish reader (Presens) equipped with 24 glass vials of 4.8 mL containing non-invasive O₂ sensors (OxoDish). The entire setup was placed in an incubator at a constant temperature of 20°C. O₂ measurements were taken for a total of 16 hours and cell counts were taken when *P. angustum* S14 were grown under Fe-limiting conditions.

3.2.1.3 Statistical analysis:

All statistics were conducted in R-studio (Version 1.0.136)[•]. Data was analysed accordingly using a parametric Analysis of Variance (ANOVA) test after having respectfully met the assumptions of normality and homoscedasticity using a Levene and Shapiro-Wilk test. A Tukey analysis was subsequently used as a post hoc analysis. In order to account for a skewed and unbalanced dataset obtained for both growth and respiration measurements (i.e. unequal numbers of observations for each level of a factor), data were trimmed and log transformed before conducting a type III ANOVA with contrasts of both factors set to "contr.sum" and "contr.poly" (Wilcox, 2012).

3.3 Results

In order to determine the optimal growth conditions while maintaining a low initial iclexpression level due to carbon utilization, icl-luc was grown under Fe-replete conditions (5.4 μ M) and in the presence of different single carbon sources glucose, acetate, serine and alanine (100 μ M[C], Figure 19a)

^{*} All analysis from this chapter can be found in a markup file using the following github link https://github.com/cocokoedooder/Stats_ICLP.angS14



Figure 19. a) expression of icl as luminescence per 1000 cells of icl-luc grown under different carbonsources (100 μ M[C]). *b)* expression of icl as luminescence per 1000 cells of icl-luc grown under 2x *different glucose concentrations (100 \muM[C] and 6mM[C]). Outliers are marked as dots.*

Acetate as a single carbon source, served as a control due to its well-established association with the induction of the glyoxylate shunt (Kornberg and Krebs, 1957). In Fe-replete conditions, the luminescence, as a proxy for icl expression, was especially induced when bacteria were grown in acetate with a luminescence of 23.4 a.u cell⁻¹ and a standard error of ± 0.9 , while an intermediary level of expression was observed when icl-luc was grown in either serine (8.5 ± 0.7 a.u cell⁻¹) or alanine (8.0 ± 0.4 a.u cell⁻¹), two amino acids. Luminescence was lowest in glucose, where almost no expression (1.1 ± 0.1 a.u cell⁻¹) was detected. An ANOVA and subsequent Tukey Test confirmed that luminescence was significantly different between all carbon sources (p < 0.0005), except between alanine and serine (p = 0.937). The above results led us to confirm the use of glucose for the acclimation of our bacterial strains while consequently subjecting them to Fe-limitation.

Luminescence was further compared between two different glucose concentrations under Fereplete conditions (5.4 μ M, Figure 19b) in order to determine whether the carbon concentration can also induce the glyoxylate shunt. Luminescence remained low (2.3 ± 0.1 a.u cell⁻¹) when cells were grown in 60× more carbon (6 mM[C]) despite the signal being twofold higher
compared to cells grown in 100 μ M[C] (p < 0.0005). A concentration of 6 mM[C] was therefore chosen for our acclimation conditions as it provided the best conditions for growth while maintaining the lowest induction of the glyoxylate shunt as a direct result of carbon source utilization.

Icl-luc was subsequently grown under a range of DFOB concentrations. When cells were subjected to Fe-limitation a gradual increase in the upregulation of isocitrate lyase was observed over time which increased with higher concentrations of DFOB (Supplementary Figure 1). From a range of DFOB concentrations, cell counts were further sampled at ±16 hours which coincided with the time cell counts were obtained from previous experiments in order to derive the luminescence per cell (Figure 20). Similar to previous experiments, in glucose and under Fe-replete conditions, luminescence was low $(3.6 \pm 0.5 \text{ a.u cell}^{-1})$. The luminescence, however, quickly increased to 14.6 ± 1.4 a.u cell- with the addition of 10 μ M DFOB. Luminescence peaked around 100 μ M DFOB (27 ± 3.1 a.u cell⁻¹) and slightly decreased with the addition of 500 μ M DFOB (22.7 ± 2.6 a.u cell⁻¹). For the statistical analysis, due to a skewed distribution of the data set, a natural log (ln) transformation was conducted in order to, respectively, meet the assumptions of normality and homogeneity of variance required by an ANOVA test. A post hoc Tukey analysis revealed significant differences in icl expression between Fe-replete and gradually increasing levels of Fe-limitation (p < 0.0005). No significant differences in *icl* expression were found between the high levels of Fe-limitation (addition of 50, 100, and 500 µM final concentrations of DFOB).



Figure 20. expression of icl as luminescence per 1000 cells of icl-luc grown in glucose 6mM[C]) under *Fe-limitation through the addition of increasing DFOB concentrations. Measurements were taken at* $\pm 16hrs$, coinciding with the exponential phase. Outliers are marked as dots.

Growth and respiration rates were derived from O_2 measurements for the wt and the Δ icl grown under different levels of Fe-limitation (0, 10, and 100 μ M DFOB) in 6 mM[C] glucose (Figures 21 and 22).



Figure 21. Growth rates of WT (black) and Δicl (white) under different levels of Fe-limitation through the addition of 0 μ M, 10 μ M and 100 μ M DFOB performed in triplicate. Then natural logarithm (Ln) was used in order to account for a skewed data set. Black dots represent outliers while significant differences between WT and Δicl are marked with an asterisk, where: *p<0.05, **p<0.005, **p<0.0005.



Figure 22. Respiration rates of wt (black) and Δicl (white) under different levels of Fe-limitation (Fereplete, $10\mu M$ DFOB and $100\mu M$ DFOB). The natural logarithm (Ln) was used in order to account for a skewed data set. Significant differences between wt and Δicl are marked with an asterisk, where: * p<0.05, **p<0.005, **p<0.005.

For the statistical analysis of our data, growth rates were ln transformed in order to account for the skewed data set and to meet the assumptions of an ANOVA test. In Fe-replete conditions, growth rates were roughly the same between the wt $(10.4 \pm 0.4 \text{ d}-1)$ and $\Delta icl (9.8 \pm 0.9 \text{ d}-1)$ and a Tukey test confirmed them to be statistically insignificant (p = 0.9998). A clear decrease in growth rate was observed with increasing Fe-limitation for both the wt and the Δ icl (Figure 21). However, under extreme Fe-limitation (100 µM DFOB), an 85% reduction in the growth rate was seen for $\Delta icl (1.4 \pm 0.4 d-1)$ while only a 70% reduction was observed for wt (3.2 ± 0.2 d–1). A Tukey test confirmed this difference as significant (p < 0.0005). The respiration rate, like the growth rate, decreased with increasing Fe-limitation for both the Δ icl and the wt (Figure 22). In replete conditions, a higher respiration rate was observed in $\Delta icl (4.4 \pm 0.4 \text{ fmol})$ h-1 cell-1) compared to the wt $(3.2 \pm 0.3 \text{ fmol h}-1 \text{ cell}-1)$ although this was not significantly different (p = 0.2109). Under Fe-limitation, the respiration rate decreased to around 1.4 ± 0.3 fmol h–1 cell–1 for the wt and was even lower for $\Delta icl (0.9 \pm 0.1 \text{ fmol h}-1 \text{ cell}-1)$. A Tukey test further revealed that the respiration rate of the wt was significantly higher than the respiration rate of Δicl (p = 0.012) under extremely low Fe-levels (100 μ M DFOB), while no differences were found under intermediate Fe-levels (10 μ M DFOB, p = 0.9038).

To confirm the role of icl in the glyoxylate shunt under Fe-limitation, the gene was constitutively expressed in trans in the form of a plasmid (icl+). Growth and respiration rates were obtained from O_2 measurements from wt, Δ icl and icl+ grown under strong Fe-limitation (100 μ M DFOB) and conducted using eight replicates (Figure 23, Table 5).



Figure 23. ΔO_2 consumption curves derived from the selected time window for P. angustum S14 wt (black), Δicl (white) and icl+(grey) grown in 6mM[C] and subjected to Fe-limitation (100 μM DFOB).

For this experiment, the wt expressed a growth rate of $5.21 \pm 0.04 \text{ d}^{-1}$ and a respiration rate of $0.81 \pm 0.02 \text{ fmol } \text{O}_2 \text{ h}^{-1} \text{ cell}^{-1}$. The removal of the glyoxylate shunt (Δicl) caused a significant overall decrease of $\pm 30\%$ in both the growth and respiration rates (p < 0.0005). The growth rate was reduced to around $2.9 \pm 0.04 \text{ d}^{-1}$, while the respiration rate decreased to 0.4 ± 0.01 fmol $\text{O}_2 \text{ h}^{-1} \text{ cell}^{-1}$. A complete restoration of both the growth ($5.95 \pm 0.03 \text{ d}^{-1}$) and respiration rate ($0.85 \pm 0.02 \text{ fmol } \text{O}_2 \text{ h}^{-1} \text{ cell}$) occurred when the gene was provided in trans (icl+). The growth rate was slightly higher for icl+ ($5.95 \pm 0.03 \text{ d}^{-1}$) compared to the wt ($5.21 \pm 0.03 \text{ d}^{-1}$) but was not considered to be significantly different (Tukey test, p = 0.334).

Table 5. Growth and respiration rates obtained from ΔO_2 consumption curves derived from the selected time window for P. angustum S14 wt, Δicl , and icl + grown in 6mM[C] and subjected to Fe-limitation (100 μM DFOB).

Bacterial strain	Growth rate (day-)	Respiration rate (fmol hr ¹ cell- ¹)
P. angustum S14 (wt)	5.21 ± 0.04	0.81 ± 0.02
Δicl	2.91 ± 0.04	0.42 ± 0.01
icl+	5.95 ± 0.03	0.85 ± 0.02

3.4 Discussion

An overall decrease in the growth rate is observed when marine heterotrophic bacteria are subjected to Fe-limitation. The magnitude of this decrease, depends on the degree of Fe-limitation and is strain specific (Table 2) as certain bacterial species were even reported to have higher growth rates under Fe-limitation than under replete conditions (Fourquez et al., 2014; Granger and Price, 1999; Tortell et al., 1996).

Within *P. angustum* S14, we observed that, under extreme limitation (DFOB 100 μ M), the growth rate can decrease to as low as 70% for the wt and 85% for Δ icl in comparison to their respective growth rate under Fe-replete conditions. This decrease in growth rate has previously been coupled to a decrease in intracellular Fe-concentrations and thus the Fe-quota of the cell (Fourquez et al., 2014; Hubbard et al.; Kaplan et al., 2006). As over 90% of intracellular Fe is predicted to reside within the enzymes of the electron-transport chain within marine heterotrophic bacteria, the reduction of a cell's Fe-quota due to Fe-deficiency can directly be linked to a decline in ATP production as well as a cell's respiration rate (Kirchman et al., 2003; Tortell et al., 1996). The observed decline in respiration rate, in *P. angustum* S14 and other strains (Fourquez et al., 2014; Hubbard et al., 1986; Oexle et al., 1999) subjected to Felimitation, confirms this prediction and is frequently derived from oxygen consumption measurements. Due to the various different culturing and trace metal conditions, further comparisons between the respiration rates amongst studies proves difficult but comparable conditions were present between our results and the study of Fourquez et al (2014). Although P. angustum S14 had similar growth rates as those observed in both strains of A. macleodii under the same Fe-replete conditions (Table 2), the respiration rate of *P. angustum* S14 was

lower and more similar to the respiration rate of *A. macleodii* under Fe-limiting conditions. This is likely due to variations amongst bacterial strains and differences in both the carbon source(s) and concentrations that were utilized in each experiment.

Serving as an alternative pathway within the classic TCA cycle, the glyoxylate shunt allows for the conservation of carbon atoms that would otherwise have been utilized for the production of CO₂. The glyoxylate shunt is frequently associated with its role in the metabolism of twocarbon substrates, such as acetate, and the replenishment of TCA cycle intermediates essential for the production of biomolecules (Dolan and Welch, 2018; Vanni et al., 1990). Although its upregulation under Fe-limitation has previously been reported in several marine field studies (Beier et al., 2015; Debeljak et al., 2019), it is not possible under that setting to verify whether the upregulation of the glyoxylate shunt is a direct effect of Fe-limitation or an indirect effect of the quantity and quality of organic matter provided by phytoplankton (Kirchman et al., 2000; Obernosterer et al., 2015). Furthermore, culture studies using comparative proteomics or transcriptomics worked with mixed carbon sources so that it could not be ruled out that the glyoxylate shunt was upregulated as a result of bacteria utilizing different carbon sources (Fourquez et al., 2014; Smith et al., 2010). As shown in our study, the presence of acetate, serine, alanine or glucose as sole growth substrate, resulted in significant differences in the upregulation of icl. Growth of a bioreporter using glucose as a sole carbon-source was hereby essential in confirming that the upregulation of the glyoxylate shunt was a direct result of Felimitation as opposed to carbon substrate.

Comparison experiments between the wildtype, the gene knockout mutant (Δ icl) as well as its complement strain (icl+) verified that the induction of the glyoxylate shunt can indeed be utilized as a strategy to improve cell growth and respiration rates under strong Fe-limitation for P. angustum S14. Recently, it has been hypothesized that the glyoxylate shunt could have a role in the metabolic rearrangement and maintenance of various processes surrounding the respiratory chain but the majority of this work has focused on oxidative stress and the presence of ROS (Ahn et al., 2016; Dolan and Welch, 2018; Eoh and Rhee, 2013; Ha et al., 2018; Meylan et al., 2017; Nandakumar et al., 2014). An increase in intracellular Fe concentrations is often associated with increased levels of oxidative stress due to the Fenton reaction. Oxidative respiration, by means of the electron transport chain, is also inevitably linked to the formation of ROS due to the concentrated presence of Fe-S clusters and the formation of O_2^* (Lesser, 2006). An inefficient electron transport chain may hereby increase the production of ROS within the cell. On the other hand, the production of ROS can partially be neutralized by Fe-containing enzymes such as ferrous superoxide dismutase and peroxidase/catalases, which are, respectively, used to convert ROS molecules to H₂O₂ which in turn is converted to the non-reactive molecules of H₂O and O₂ (Tiwari et al., 2002). The maintenance of Fe-homeostasis is therefore crucial and its disruption has been shown to increases susceptibility towards oxidative stress (Ha et al., 2018). It is therefore possible that the upregulation of the glyoxylate shunt in *P. angustum* S14 under Fe-limitation could be due to an increase in ROS concentrations within the cell. This was previously shown in the phytoplankton strain Anabaena sp. and has also been observed in the heterotrophic bacterium Caulobacter crescentus, but not in E. coli bacteria (Latifi et al., 2005; Leaden et al., 2018). On the other hand, reducing both growth and respiration rates, as was observed in P. angustum S14 under Fe-limitation, has also been argued to result in a lower production of ROS and improved tolerance towards oxidative stress (Brauner et al., 2016; Gray et al., 2019). Furthermore, it is important to note, that P. angustum S14 in particular is known for its efficient resistance to high levels of H₂O₂ and ROS due to several oxidative response enzymes including glyoxalase, AhpC, recA as well as ferrous superoxide dismutase and peroxidase (Matallana-Surget et al., 2012, 2013; Ostrowski et al., 2001).

Under Fe-limitation, the induction of the glyoxylate shunt itself does not have the direct capability of replacing the loss of ATP. Instead, the glyoxylate shunt seems to serve as a pathway that redirects a cell's metabolism away from the electron transport chain through the conservation of carbon while preventing both the unnecessary production of NADH and lowering the possibility of a cell to encounter oxidative stress due to an inefficient electron transport chain. This could explain why, although, the growth and respiration rates of the wt improved in comparison to Δ icl under strong Fe-limitation, these rates still remained significantly lower than those found under Fe-replete conditions. Furthermore, the induction of the glyoxylate shunt is also known to occur as the enzyme ICL competes with the metabolic enzyme isocitrate dehydrogenase (IDH) for the substrate isocitrate (Dolan and Welch, 2018) and the induction of the glyoxylate shunt can only be maintained after a build-up of isocitrate with concentration above that of 0.6 mM (El-Mansi et al., 1985) and it is likely that this occurred when *P. angustum* S14 was grown under Fe-limitation.

Under strong Fe-limitation, a lower respiration rate was observed in bacterial strains without the ability to induce the glyoxylate shunt (Δ icl). A similar observation was made between an oceanic strain which did not harbour the glyoxylate shunt and a coastal strain of A. macleodii that did (Fourqez et al., 2014). In contrast, the inhibition of the electron transport chain through antibiotics (and not Fe-limitation), induced the glyoxylate shunt in a P. aeruginosa strain but was coupled with a lower respiration rate (Meylan et al., 2017). When considering the link between the activity of the glyoxylate shunt and the respiration rate, carbon flux is redirected towards succinate dehydrogenase (SDH), an Fe-S containing enzyme that is required in both the TCA cycle and the electron transport chain (Rutter et al., 2010; Figure 17). SDH harbours less Fe in comparison to Complex I and unlike Complex I, SDH does not participate in the formation of a proton gradient but does carry-over electrons towards Complex III and Complex IV which do serve as proton pumps. Therefore, under Fe-limitation, the induction of the glyoxylate shunt may further reduce the production of NADH, but our results also suggest that this can be compensated by an enhanced electron flow provided by an increase in SDH activity supplied by isocitrate lyase. It is thus possible, that the induction of the glyoxylate shunt under Fe-limitation, helps rearrange bacterial metabolism in a way which increases the efficiency and ultimately the oxygen consumption rate within the cell.

Interestingly, the proteorhodopsin-containing *Dokdonia* sp. MED134 was also shown to induce the glyoxylate shunt upon the presence of light (Palovaara et al., 2014). Proteorhodopsin, a light-driven proton pump can be linked towards ATP synthesis whilst redirecting the TCA cycle towards the glyoxylate shunt in order to increase biosynthesis. It is known that *P. angustum* S14 contains a proteorhodopsin gene. Whether, in the presence of light, the combined use of the glyoxylate shunt and proteorhodopsin in *P. angustum* S14 serves as an additional strategy in coping with Fe-limitation would deserve further investigation (see Supplementary Data).

Our results with a model marine heterotrophic bacterium lead to a better understanding of bacteria and their ability to acclimate under strong Fe-limitation. For *P. angustum* S14, Fe-limitation significantly inhibits both growth and respiration rates, however, its impact is countered by the induction of the glyoxylate shunt. Deletion of the glyoxylate shunt led to a further 30% decline in both growth and respiration rates within our model organism. The glyoxylate shunt is widely distributed amongst aerobic marine bacteria (Ahn et al., 2016;

Palovaara et al., 2014) including SAR11 (Beier et al., 2015; Smith et al., 2010) but its upregulation under Fe-limiting conditions, however, was shown to be taxa specific as the majority but not all species containing the glyoxylate shunt upregulated it within an HNLC site in the Southern Ocean (Debeljak et al., 2019). Ultimately, this form of plasticity can have consequences for biogeochemical cycling when bacteria with a glyoxylate shunt tend to have a more efficient metabolism coupled with an improved respiration rate when subjected to Fe-limitation as compared to a bacterial strain that does not.

3.5 Supplementary Data: The Glyoxylate Shunt in the presence of light

3.5.1 The Glyoxylate Shunt in the presence of light.

When *Dokdonia sp. MED134* was grown in either alanine or a mixed carbon source (yeast extract and bactopeptone), the presence of light resulted in the upregulation of proteorhodopsin (Palovaara et al., 2014) and was coupled with significant differences in the growth rate and cell yield between light and dark conditions that were more pronounced upon growth in the presence of alanine compared to YEP. Interestingly, upon growth in alanine, the presence of light resulted in the upregulation of the glyoxylate shunt and in that particular study, it was postulated that if proteorhodopsin resulted in the production of ATP, the glyoxylate shunt may serve as an important pathway to redirect carbon towards anabolic pathways, such as gluconeogenesis, and stimulate growth in carbon-limited but sunlit marine environments.

P. angustum S14 contains a proteorhodopsin gene, and while it is not constitutively expressed as was observed in *Dokdonia* sp. MED134 (See Ch.3 Proteorhodopsin), the work by Palovaara et al (2014), sparked interest in whether the upregulation of the glyoxylate shunt under Felimitation may change in the presence of light. We hypothesized that under Felimitation, the upregulation of proteorhodopsin in the presence of light could provide an additional proton gradient in parallel to the gradient produced by the electron transport chain, particularly if the glyoxylate shunt redirects the flux of electrons towards Complex II rather than Complex I. We therefore hypothesized that the addition of light could further stimulate growth and cell yield in Fe-limited cells.

Icl-luc was grown in Aquil medium and 6mM[C] glucose with a range of DFOB concentrations (0, 10, 50, 100, and 500 µM of DFOB) and subjected to the presence or absence of blue light (±30 µmol photons m⁻² s⁻¹). Hourly luminescence measurements were taken to follow the expression pattern of *icl* when cells were in the exponential growth phase. Cell counts were not measured for these preliminary experiments and the total expression per cell could therefore not be derived from these results. Our results showed no significant differences in the upregulation of the glyoxylate shunt between light and dark conditions (Supplementary Figure 1).



Supplementary Figure 1. Expression of icl as total luminescence in icl-luc grown under a range of Fe-limitation (0, 10, 50, 100 μ M DFOB) respectively shown in white, light grey, grey and dark grey in (a) the presence or (b) the absence of blue light.

3.5.1.1 The addition of light in Fe-replete conditions



Supplementary Figure 2. Total luminescence of icl-luc grown in the presence of Fe-replete conditions (5.4 μ M) in either constant blue light (white points) or constant darkness (black points).

In Fe-replete conditions using glucose as carbon source, isocitrate lyase was not further upregulated in the presence of light during the exponential growth phase (Supplementary Figure 2). As cells approached the stationary phase, isocitrate became highly upregulated in both the presence and absence of light and the addition of light resulted in a higher luminescent signal compared to the dark conditions. It is important to note that cell counts were not taken to confirm that the upregulation was a result of regulation rather than differences in cell counts. The upregulation of the glyoxylate shunt as cells approached the stationary phase is similar to what was observed for proteorhodopsin in *P. angustum* S14 (see Ch. 3 Proteorhodopsin) as well as *V. campbelli* (Wang et al., 2012) and *Vibrio sp.* AND4 (Akram et al., 2013). The expression of proteorhodopsin in *V. campbelli* was shown to be regulated by the starvation-induced sigma factor RpoS1 as cells entered the stationary phase, and it is therefore possible that the glyoxylate shunt may also be upregulated by this process.

Within *P. angustum* S14, the induction of a starvation-induced stress response is induced by the sigma factor RpoE. When cells approach the stationary phase, the induction of RpoE results in the alteration of protein profiles and a decline in cell size coupled as a result of reductive cell division (Hild et al., 2000). Changes in the upregulation of several proteins would predominately include anabolic reactions and may explain the upregulation of the glyoxylate shunt. On the other hand, work on *E. coli* has also shown that the highest level of intracellular reactive oxygen species (ROS) occurs during the transition period from the exponential to the stationary phase (Noor et al., 2009; Nyström et al., 1992) so that the upregulation of the glyoxylate shunt might occur in relation to oxidative stress. Interestingly, the starvation response in *P. angustum* S14 by RpoE has also been linked to the ability of *P. angustum* S14 to tolerate high levels of oxidative stress within the cell (Nyström et al., 1992). More work would be required to further elucidate these responses.

3.5.1.2 The addition of light in Fe-limiting conditions



Supplementary Figure 3. Total luminescence of icl-luc grown in the presence of Fe-limiting conditions (50 μ M DFOB) in either constant blue light (white points) or constant darkness (black points).

Under Fe-limiting conditions, an upregulation can be seen in the glyoxylate shunt although less pronounced in comparison to Fe-replete conditions. Interestingly, the addition of light did not result in a higher luminescent signal in the presence of light in comparison to dark conditions (Supplementary Figure 3). Instead, as Fe-limited cells started to approach the stationary phase the observed increase in total luminescence was lower in the presence of light in comparison to dark conditions. This is exactly the reverse of what was observed for the expression of isocitrate lyase in Fe-replete conditions. Although it is likely that the lower luminescence that was observed in the presence of light is a result of an increased mortality due to the presence of photooxidative stress, this would require verification through cell counts.

IV. THE ROLE OF PROTEORHODOPSIN

4.1 Introduction

4.1.1 Discovery and mechanism of proteorhodopsin.

Proteorhodopsin is a light-driven proton pump that was first discovered through the genomic analyses of an uncultivated γ -proteobacterium strain of the ubiquitous SAR86 clade (Beja et al., 2000). The discovery of proteorhodopsin is often used as a classic textbook example of the power of metagenomics which has enabled the discovery of new functional genes whilst bypassing the use of the limited availability and representability of cultivatable bacteria (DeLong, 2005; Overmann and Lepleux, 2016).

Proteorhodopsin is part of the Type 1 Rhodopsin family which consists of a group of proteins present in all three domains of life that utilize the chromophore retinal (Sharma et al., 2006). In the presence of a specific wavelength of light, retinal undergoes a conformational change from all-trans to all-cis retinal which can be used to conduct a specific function within the cell (Table 7). Type 1 Rhodopsin molecules are hereby separated into the two functional categories of transporters or receptors and, as a proton pump, proteorhodopsin molecules are therefore typically considered to have a transport function (Sharma et al., 2006). Interestingly, proteorhodopsin molecules have also been harvested in deep-ocean layers that were shown to display a lower photocycle rate and are predicted to have been formed when proteorhodopsin transitioned from a transporter function towards a sensory function (Bielawski et al., 2004; Wang et al., 2003, Table 6).

Table 6. Brief overview of the functional descriptions of Type I Rhodopsin family (derived from Sharma et al., 2006). Proteorhodopsin is classified as a transporter protein due to its ability to transport proteins across the cell membrane in the presence of either blue or green light.

Transpo	ort function	
i.	Proton transport (H ⁺)	Light energy is used to create a proton gradient for
		ATP production, flagellar rotation, TonB dependent
		uptake and other energy-requiring processes.
ii.	Chloride transport (Cl)	Chloride ions are pumped into the cytoplasm and
		hyperpolarizing the cell membrane (e.g.
		halorhodopsin)
Recepto	r function	
i.	Phototaxis	Sensory function of rhodopsin-mediated movement
		towards or away from a specific wavelength of light
		(e.g. sensory rhodopsin)
ii.	Photoadaptation	The use of light to regulate gene expression or protein
		function (e.g. triggering differential biosynthesis of
		light-absorbing pigments based on the quantity and
		quality of light).

Proteorhodopsin is a relatively small transmembrane protein with an amino acid chain of ± 247 amino acids that folds to form seven alpha-helices and contains retinal as a cofactor (Béja et al., 2001). The full functioning of proteorhodopsin is relatively simple, and requires the proteorhodopsin gene, the genes for the complete pathway for retinal biosynthesis (*blh*) and finally the genes encoding beta-carotene from isoprenoid precursors (*crt*) (Pinhassi et al., 2016; Sabehi et al., 2005).

Proteorhodopsin can further be subdivided into 2 groups according to their ability to absorb either blue λ_{max} (490nm) or green λ_{max} (525nm) light (Béja et al., 2001; Man et al., 2003). This phenomenon in proteorhodopsin, also known as spectral tuning, is primarily due to the amino acid present in position 105 of the amino acid chain. The presence of glutamine (Gln) or leucine (Leu) respectively results in a blue-light or green-light absorbing proteorhodopsin molecule. According to the light availability at different water depths (Figure 24), proteorhodopsin molecules displayed different distribution patterns within the surface waters of the ocean where green-absorbing proteorhodopsin molecules were mostly present within the shallow surface waters while blue-absorbing molecules were abundant at lower water depths (Béja et al., 2001; Sabehi et al., 2007). It is important to note that the exact blue- and green-light spectral tuning is not clear-cut and that their differential distribution across the water column are also presumed to be correlated by as yet unknown environmental parameters other than the light absorption spectrum (Burr et al., 2017; Nguyen et al., 2015; Sabehi et al., 2007).



Figure 24. Visible light spectrum of the (a) clear open ocean and the (b) more turbid coastal waters. Notice that blue light (455-492) penetrates to lower depths in the open ocean in comparison to green light (492-577) but that green light is highly available, in comparison to blue light, in coastal waters. Derived from Chandler et al (2017) and taken from data of NOAA Ocean Explorer.

In the presence of either blue or green light, proteorhodopsin will undergo a conformational change that allows for the absorption and release of a proton across a membrane. The formation of a proton gradient, if coupled to ATP synthase, could in theory generate energy (Figure 25). The low production costs, unlike other light harvesting complexes, coupled with the widespread prevalence of proteorhodopsin within the surface waters of the ocean, including its presence in the important SAR11 and SAR86 clades, suggested the presence of a new form of photoheterotrophy that could potentially signify a previously unknown but important microbial

process within the surface waters of the ocean (Beja et al., 2000; Béja et al., 2001; Pinhassi et al., 2016; Sabehi et al., 2005).

4.1.2 Diversity and function of proteorhodopsin

Theoretically, the construction of a proton gradient in the presence of light can be utilized in a wide variety of functions such as ATP synthesis (when coupled to ATP synthase), the direct or indirect use in the transportation and uptake of nutrients through TonB-dependent transporters (which require a proton gradient) and ABC transporters (which require ATP) or the motility of bacteria by the powering of bacterial flagella (Fuhrman et al., 2008; Figure 25). Indeed, the transformation of an *E. coli* bacterium with a proteorhodopsin molecule has shown both an increase in cellular ATP production but also flagellar movement (Martinez et al., 2007; Walter et al., 2007).



Figure 25. Schematic representation of a proteorhodopsin molecule (left) producing a proton gradient which can coupled with various other proteins and thus be utilized for several different functions (right) such as ATP synthesis, flagellar movement, and uptake and transport using TonB dependent complexes. Note that proteorhodopsin can indirectly be utilized by ABC transporters that require ATP which can be synthesized from ATP synthase which requires a proton gradient.

Although the construction of a proton gradient due to proteorhodopsin was initially linked to a light-dependent energy harvesting function through ATP synthesis, a back of the hand

calculation of the energy maintenance costs by Kirchman and Hanson (2013) estimated that, unless there was a high investment of proteorhodopsin along the cell membrane towards energy production and coupled with sufficient light intensities, proteorhodopsin would not sufficiently cover the maintenance costs within an average heterotrophic bacterium. The recent publication of Gomez-Consarnau et al (2019), however, calculated that the cellular energy yield captured by PR within the Mediterranean Sea were either within or below that minimum energy threshold required for survival in heterotrophic bacteria. Several attempts to distinguish whether certain marine bacterial clades could benefit from the presence of light, were conducted by measuring bacterial cell counts from field experiments over a period of several days (Lami et al., 2009; Schwalbach et al., 2005). While only a select few members within certain phylogenetic groups (ie. SAR11 and Flavobacteria) showed minor or mixed responses to light, the majority of heterotrophic bacteria, with the exception of cyanobacteria, did not seem to rely on light over the course of the experiment. It is important to note that for the above experiments, the collected water was not filtered and that the presence of photochemical degradation and release of nutrients cannot be excluded from these different culture conditions. These results also suggested that proteorhodopsin may have alternative, yet undetermined ecological benefits besides phototrophy. It was suggested that factors unrelated to phototrophy coupled with a slight energetic benefit within an average bacterium would likely explain the distribution and persistence of proteorhodopsin across the surface waters of the ocean and it is currently predicted that proteorhodopsin may serve bacteria in a wide variety of functions (Fuhrman et al., 2008; Pinhassi et al., 2016).

Thus far, no uniform response towards light, has been found amongst several culture work studies (Table 8) and a key factor for this variation is the relative ease in which proteorhodopsin can undergo horizontal gene transfer (Sharma et al., 2006). Proteorhodopsin containing strains within the *Vibrionaceae* family, for example, include proteorhodopsin genes with both ancestral alphaproteobacteria and gammaproteobacteria origins despite being classified as gammaproteobacteria according to their 16s rRNA gene (Gómez-Consarnau et al., 2010, Figure 26).



Figure 26. Α Neighbour-joining-tree of 16sRNA (a) for proteorhodopsin-containing Gammaproteobacteria (green) and Alphaproteobacteria (blue) and a neighbour-joining-tree of proteorhodopsin *(b)* with gammaproteobacterial-derived proteorhodopsin (green) and alphaproteobacterial-derived (blue) proteorhodopsin genes. Notice that in this figure, Vibrio sp. AND and BAA-11164 are classified as gammaproteobacteria according to their 16sRNA profiles, but that their proteorhodopsin genes are both classified as alphaproteobacterium-derived proteorhodopsin genes. P. angustum S14 on the other hand contains a proteorhodopsin gene derived from gammaproteobacterium. Derived from (Gómez-Consarnau et al., 2010).

The ability to laterally transfer proteorhodopsin across phyla and domains does not imply the simultaneous transfer of that particular function nor the transfer of its metabolic and genetic regulation. However, in order for the lateral transfer of a gene (or group of genes) to remain integrated within its newfound host, the recipient must benefit from the newly acquired gene within its living environment (Ochman et al., 2000). The prevalence of lateral gene transfer of proteorhodopsin strengthens the possibility that one can expect fundamentally different modes of regulation and function depending on whether light energy is indeed harvested by proteorhodopsin and utilised under different environmental conditions (Fuhrman et al., 2008; Pinhassi et al., 2016)

4.1.3 Cultivatable proteorhodopsin strains

While the role of proteorhodopsin activity in its native state still remains to be elucidated, several cultivatable strains have helped gain insight into the possible use and benefit of proteorhodopsin (Table 7). Currently, the function of proteorhodopsin appears to be strain specific and a wide range of functions have been found ranging from the promotion of growth to its use as a survival mechanism under stressful conditions such as periods of carbon starvation and other forms of nutrient limitation (Pinhassi et al., 2016). Below we will briefly describe a few key studies surrounding the role of proteorhodopsin within different bacterial culture-studies.

A SAR11 strain ('*Pelagibacter ubique'*) represented the first cultivatable strain to contain proteorhodopsin (Giovannoni et al., 2005). SAR11 is a streamlined bacterium ubiquitously present within the surface waters of the ocean including oligotrophic conditions (Giovannoni, 2016), which strengthened the notion that proteorhodopsin could have an important function for these bacteria. Interestingly, no differences in SAR11 was observed between dark conditions and the presence of light:dark cycles (Giovannoni et al., 2005) regarding growth rates, cell yields, respiration rates, ATP, and taurine uptake (Steindler et al., 2011).

The work of Gomez-Consarnau et al on different *Dokdonia* sp. strains (2010, 2016) offered a concrete explanation behind the light-enhanced growth of 2 out of 3 proteorhodopsincontaining strains. In this particular case-study, proteorhodopsin contained a light-dependent transport function and the different responses of three *Dokdonia* strains were the result of auxotrophy for vitamin B1. Two of the three strains (MED134 and DSW-1) were not able to synthesize vitamin B1 and in the presence of light a significant increase in both the growth and cell yield was observed. As uptake of vitamin B1 requires the use of both TonB-dependent transporters (TBDT) and ATP-dependent transporters, their results therefore suggested that proteorhodopsin was utilized to enhance the uptake of vitamin B1 in the medium (Gómez-Consarnau et al., 2016).

Cultured bacterial isolates containing PR	Taxonomy (phylum)	PR type	PR enhancement	Additional findings	Reference
HTCC2207 sp001438605 (SAR92)	Gammaproteobacteria	green	N^*	No differences in growth or cell yield	Stingl et al., 2007
Vibrio campbellii AND4	Gammaproteobacteria	green	Y	Longterm survival in sterile seawater	Gómez-Consarnau et al., 2010
	1			PR expression is determined by nutrient limitation (dilutions)	Akram et al., 2013
				Sigma factor RpoS plays key regulatory role	
Vibrio campbellii BAA-1116	Gamma proteo bacteria	green	Y	Longterm survival under respiratory stress (sodium azide)	Wang et al., 2012
		0		Enhanced ATP production	
				Sigma factor RpoS1 plays key regulatory role	
Candidatus Pelagibacter ubique HTCC1062	Alphaproteobacteria	green	N	Constitutively expressed	Giovannoni et al., 2005
(SAR11)				no differences in growth or cell yield	
Dokdonia sp. MED134 , DSW-1	Bacteroidetes	green	Y	Upregulated in light	Gómez-Consarnau et al., 2007;
		1		Enhanced growth rate and cell yield under low (0.6 mM[C]) and,	2016;
				intermediate (1.10 mM[C]) concentrations of carbon	Palovaara et al., 2014
				Enhanced Vitamin B1 acquisition	Kimura et al., 2011
				Upregulation of the Glyoxylate Shunt in light	
				Upregulation in transporters Na+/alanine and Na+ phosphate and the	
				NA+-Complex I of the electron transport chain in light	
Nonlabens marinus S1-08	Bacteroidetes	n/a	Y^*	Enhanced growth rate and cell yield under low carbon concentrations	Yoshisawa et al., 2014
				(0.14 mM[C])	
				Contains 3 rhodopsin types $(H+, Na+ and Cl- pumps)$	
Polaribacter sp. MED152	Bacteroidetes	n/a	N^*	Enhanced bicarbonate fixation and uptake	González et al, 2012
Psychroflexus torquis ATCC 700755	Bacteroidetes	green	Y	Enhanced growth and cell yield under hyposalinity stress	Feng et al., 2013; 2015
				Constant expression but post transcriptional upregulation by light and	Burr et al., 2017
				salinity	
				Increased yield in natural Antarctic sea ice in the presence of blue	
				compared to green light (!)	
Hymenobacter nivis P3T	Bacteroidetes		Y^*	Enhanced growth and cell yield	Terashima et al., 2018
P. angustum S14	Gammaproteobacteria	blue	Y^*	No observed difference in growth and cell yield	Courties et al., 2016
				Decrease in Pirt's maintenance coefficient in light and under high	
				carbon concentrations	
				Lower maintenance energy and Increased carbon yield	

Table 7. A non-exhaustive list of culture-based studies testing the function of proteorhodopsin present in various strains of heterotrophic bacteria and a summary of their findings.

Several culture studies have further been conducted on proteorhodopsin-containing members of the Vibrionaceae family and within several *Vibrio* bacterial strains the proteorhodopsin has been described in the context of a light-dependent survival response (Akram et al., 2013; Courties et al., 2015; Gómez-Consarnau et al., 2010; Wang et al., 2012). Under carbon starvation, the presence of light enabled the long-term survival of Vibrio sp. AND4 (Gómez-Consarnau et al., 2010). Further work on *Vibrio* sp. AND4 also revealed that proteorhodopsin was tightly regulated and its upregulation was absent in actively growing cells (Akram et al., 2013). Instead, upregulation of proteorhodopsin occurred as cells entered the stationary phase and under nutrient limitation achieved through dilution experiments. Similar expression results were observed in *Vibrio campbellii* and it was demonstrated that the regulator of the adaptive stress response (RpoS1) was responsible for regulating proteorhodopsin expression (Wang et al., 2012). When subjected to the respiratory chain inhibitor sodium azide (NaN₃), the presence of light showed an enhancement of survival measured by the number of colony-forming units.

Antarctic sea ice is considered to be a fluctuating environment that harbours wide temperature and salinity gradients (Kottmeier and Sullivan, 1987; Petrich and Eicken). The Antarctic seaice bacterium *Psychroflexus torquis* contains a blue-tuned proteorhodopsin gene which was shown to be constitutively expressed in the cell when comparing the early, middle and late exponential growth phase as well as the transition from the exponential to the stationary phase (Feng et al., 2013). In the presence of light, *P. tourquis* showcased an enhanced protonpumping activity which declined when subjected to dark conditions. Interestingly, in the presence of osmotic stress (increased salinity concentrations) but not nutrient limitation, higher cell yields were observed when *P. tourquis* was subjected to light compared to dark conditions suggesting that proteorhodopsin may play an important role in the adaptation of *P. torquis* to grow and survive within Antarctic sea-ice. These results were further supported when enhanced growth rates and cell yields were observed when *P. torquis* was grown in natural sea ice conditions in the presence and absence of blue and green light (Burr et al., 2017).

4.1.4 Proteorhodopsin and Fe-limitation

The comparison of three closely related Roseobacter strains showed that the strains isolated from an environment characterised by Fe-limitation (Antarctic), contained proteorhodopsin whereas the other 2 Rposeobacter strains from Fe-replete regions contained the more Fe-rich bacteriochlorophyll a (Sun et al., 2017). The presence of proteorhodopsin was hereby

postulated to represent an adaptive strategy that replaced the more Fe-rich bacteriochlorophyll albeit this was not further tested.

Proteorhodopsin genes were also identified in several marine eukaryotic protists, including diatoms and, in comparison to prokaryotes, the proteorhodopsin genes within eukaryotes seem to form a monophyletic clade which suggests a common ancestor and perhaps a common function within the cell (Marchetti et al., 2012, 2015). Interestingly proteorhodopsin was upregulated under Fe-limitation within the oceanic diatom *Pseudo-nitzschia granii* and the polar sea-ice diatom *Fragilariopsis cylindrus* which suggested its role as a strategy under Fe-limitation through its potential ability to produce ATP for continued survival until Fe becomes available again to allow cellular growth (Cohen et al., 2018; Marchetti et al., 2012, 2015). Although proteorhodopsin was shown to be upregulated in both light and dark conditions in the presence of Fe-limitation, no clear differences were observed in the growth rate of *P. granii*.

Whether a similar prediction can be made for proteorhodopsin-containing bacteria subjected to Fe-limitation has thus far, to our knowledge, not been tested. Because Fe-limitation particularly affects cells in their efficiency to produce ATP (Tortell et al., 1996) and since proteorhodopsin has already been shown to increase the survival of a cell under limiting nutrient conditions (Akram et al., 2013; Gómez-Consarnau et al., 2010), and improve the uptake rate of a limiting substrate (Gómez-Consarnau et al., 2016) we hypothesize that bacterial cells can upregulated and utilise proteorhodopsin either as a growth enhancement or survival strategy under the presence of Fe-limitation. We predict that the presence of proteorhodopsin has the potential to supply cells with a proton gradient which could be utilized in the energy production in the form of ATP, whilst surpassing the Fe-rich protein complexes of the respiratory electron transport chain (Figure 27).



Figure 27. Schematic representation regarding the experimental hypothesis surrounding proteorhodopsin when P. angustum S14 is subjected to Fe-limitation. We hypothesize that in the presence of Fe-replete conditions, the electron transport chain produces enough proton motive force for ATP production through ATP synthase. In Fe-limiting conditions, the electron transport chain is severely hindered and the upregulation of proteorhodopsin can compensate (in part) for the loss of the proton motive force and enhance ATP production within the cell.

4.1.5 Objectives

In this chapter, we investigate whether the expression of proteorhodopsin is regulated as a result of Fe-limitation and whether its upregulation, if any, can further affect the cell in terms of growth, cell yield and cell size. The potential role of proteorhodopsin in the presence of Fe-limitation was elucidated through the construction of a luminescent bioreporter (ptr-luc), a knockout (Δpr) and its complement (pr⁻) in the model organism *P. angustum* S14.

4.2 Material and Methods

4.2.1 Experimental Setup

The bioreporter pr-luc was used to test the expression of proteorhodopsin under various light conditions, levels of Fe-limitation, and different sources and concentrations of organic carbon (glucose, acetate and serine). Comparison experiments between the wildtype and the gene knockout Δpr were carried out in the presence of a range of Fe and light concentrations in order to elucidate any potential differences in growth, cell yield and respiration rates as a result of proteorhodopsin expression. Additionally, cell volume, ATP and ROS measurements were taken.

All experiments were conducted under trace metal clean conditions. Prior to the start of an experiment, *P. angustum* S14 (wt, Δpr , and *ptrluc*) was taken out of cryopreservation and plated before being grown and acclimated in 6mM[C] of either glucose, serine or acetate in either Fereplete (5.4 μ M) or Fe-limiting (5.4nM) conditions. The bioreporter *pr-luc* was additionally grown in the presence of 5 μ g/mL of Cm. Cells were refreshed at least 3x prior to the start of every experiment. All cells were acclimated in the dark prior to the start of the experiment.

4.2.2 Pr-luc expression experiments

The bioreporter *ptr-luc* was used to measure the expression of proteorhodopsin over time via luminescence (arbitrary units, a.u.) in various wavelengths (white, red, green, blue) and concentrations (0, 15 μ E, 30 μ E, 60 μ E) of light further referred to in the text as 0%, 25%, 50% and 100% blue light, a range of DFOB concentrations (0 μ M, 10 μ M, 50 μ M, 100 μ M), and different single carbon sources (acetate, glucose, serine) in varying concentrations (60 μ M, 600 μ M, 6 mM).

Luminescence was measured every hour (5 s per well) using a luminometer (Berthold Centro LB 960). Unlike isocitrate lyase, proteorhodopsin is a membrane rather than a cytosolic enzyme and so the overall expression of proteorhodopsin is expected to be lower in comparison to isocitrate lyase (Spitzer and Poolman, 2013). Therefore, in order to obtain a luminescence signal with a high enough resolution for the observation of possible changes during the start of the experiment, a higher starting density of bacterial cells was required compared to the 1.8

x10^s cells mL⁴ starting density for *icl-luc*. Ptr-luc was diluted in Aquil media (in combination with various Fe and carbon conditions) to a starting concentration of ($\pm 1.0 \times 10^{\circ}$ cells mL⁻¹). 200 μ L was pipetted into each well of a clear-bottom black 96-well microplate (Greiner Bio-One) containing luciferin (final concentration 250 μ M) and covered with a clear diamond film seal (Thermo Fisher ScientificTM).

Cell counts were taken using flow cytometry in order to calculate the expression of proteorhodopsin per cell for a specific time point where luminescence was normalized to 10,000 cell counts. Bacteria cells were fixed in formaldehyde (2%) and stained with SYBR Green I (15 min) prior to measuring cell counts using a Becton Dickinson Accuri C6 flow cytometer (Marie et al., 1997). From the growth curves, growth rates and cell yields were determined as described previously. Briefly growth rates were derived from the slope in excel (LINEST function) after a natural log transformation of cell counts over time which provides a linear line when cells are within the exponential growth phase.

4.2.3 Δpr gene knockout experiments

The wildtype and Δpr were grown in a range of blue light (0%, 25%, 50%, 100%) and Fe (5.4 μ M, 0.54 μ M, 54 nM, 5.4 nM) concentrations. Growth curves were constructed from cell counts over time and used to calculate the growth rate (μ day⁴) and cell yield. In parallel, the mean cell volume (μ m⁴) was determined for several time points using a coulter counter (CASY Cell Counter, Roche) and analysed according to the manufacturer's instructions.

ATP measurements (fmol 1000 cells¹) were taken from the wildtype *P. angustum* S14 subjected to Fe-replete and Fe-limiting (50 μ M DFOB) conditions using the ATP Biomass Kit HS (BioThema) following the manufacturer's instructions (Lundin, 2000). Briefly, 50 μ L of B/S extractant was added to 50 μ L sample (1:1 ratio) and frozen at 20 °C prior to its analysis. During the analysis, samples were thawed and 40 μ L was added to 160 μ L of ATP Reagent HS (containing luciferase and luciferin). Light emission is measured from samples using a luminometer without the addition of ATP standard and immediately followed by the measurement of the same sample with the addition of 10 μ L ATP standard (10⁷ mol L⁴ ATP). The background noise was removed by subtracting luminescence of blank controls consisting of 40 μ L B/S extractant and 40 μ L Aquil medium.

4.2.4 Oxidative Stress (ROS)

Oxidative stress was measured in *P. angustum* S14 cells using the CellROX® Deep Red reagent according to the manufacturer's instructions (Lifetechnologies, Switzerland) and its verified use in prokaryotic cells (Manoil and Bouillaguet, 2018). Briefly, CellROX® Deep Red detects oxidative stress in cells by reacting to ROS within the cells and becoming brightly fluorescent (excitation/emission of 640/665 nm). Cells were grown within their respective media and pipetted into 200 μ L wells of a clear-bottom 96-well microplate (Greiner Bio-One) containing 5 μ L of luciferin (final concentration of 250 μ M) and covered with a clear diamond film seal (Thermo Fisher Scientific^m). Cells were incubated for 30 minutes with 5 μ M CellROX® Deep Red and then consequently fixed in formaldehyde (2%) and stained with SYBR Green I (15 min). Oxidative stress was derived from APC-H fluorescence using a BD FACS-Canto II and the geometric mean was expressed in relative fluorescence units (RFU).

4.2.5 Statistical Analysis

All statistical analysis was conducted in R software (Version 3.5.1)[•]. Statistical analysis was conducted using a One-way or Two-way Analysis of variance (ANOVA) once the assumptions of normality (Shapiro-Wilk) and homogeneity of variance (Levene Test) were met. A Tukey HSD test was conducted to further elucidate significant interactive results amongst the different conditions.

4.3 Results

4.3.1 Blanks

Blanks consisting of Aquil medium and luciferin $(5\mu M)$ were subjected to a range of DFOB concentrations $(0\mu M, 10\mu M, 50\mu M, 100\mu M)$ and blue light conditions (0%, 25%, 50%, 100%) in order to verify that luminescence measurements were not influenced by the presence of DFOB and/or light. The use of a white 96-well plate indicated that light had a clear and significant (p<0.0001) impact on luminescence where in the presence of light the background

^{*} All analysis from this chapter can be found in a markup file using the following github link https://github.com/cocokoedooder/STATS_PRP.angS14

luminescence increased from around ± 10.0 a.u to ± 80.0 a.u (Figure 28). Furthermore, in the presence of light, a stronger variance of luminescence measurements was also observed and resulted from a small but significant interactive effect of DFOB in blue-light (p=0.0016).



Figure 28. Blanks containing Aquil medium, luciferin (μM) and a range of DFOB concentrations (0, 10, 50, 100 μM) placed in a white 96-well plate and subjected to (a) blue light (50%) or (b) no light for a period of ±48 hours.

The use of a black 96-well plate resolved this issue by reducing cross-talks between wells and resulted in a much lower background noise of ± 25.0 a.u (Figure 29). A significant difference was still obtained between light and dark conditions (p=0.0065) but respectively concerned a difference between ± 2.6 a.u and ± 2.8 a.u. Therefore, the use of a black 96-well plate was used for the rest of the expression experiments and the obtained luminescence measurements were corrected for by subtracting the observed background noise of blanks from the sample measurements.



Figure 29. Blanks containing Aquil medium, luciferin (μM) and a range of light concentrations (0, 10, 50, 100 %) placed in a black 96-well plate for a period of 96-hours. The use of a black plate resulted removed the differences found in the presence or absence of light when using a white plate.

4.3.2 Preliminary Expression Experiments

The expression pattern of proteorhodopsin over time in *P. angustum* S14 has, thus far, not been shown experimentally. The proteorhodopsin molecule of *P. angustum* S14 (EBAC31A08) contains glutamine at position 105 of its amino acid sequence and is therefore predicted to be a blue-light absorbing proteorhodopsin molecule (Courties et al., 2015; Gómez-Consarnau et al., 2010). In order to verify that *P. angustusm* S14 contains a blue-type proteorhodopsin molecule, *ptrluc* cells were acclimated in Aquil medium containing 6mM[C] glucose and Fereplete conditions (5.4μ M) before being subjected to continuous blue, green and red light over time (Figure 30). A clear increase in luminescence was observed over time in the presence of continuous blue-light while green or red light had a similar luminescence signal compared to cells grown in the dark.



Figure 30. PTR-luc in Aquil medium and 6mM[C] glucose subjected to either (a) no light, (b) red-light, (c) green-light and (d) blue-light. An increase in luminescence occurs at 8 hours and continues to increase in blue light but not in the other light conditions, verifying that P. angustum S14 contains a blue-tuned proteorhodopsin molecule.

In order to further understand the sensitivity of proteorhodopsin towards blue-light, *ptrluc* was grown in the presence of different light conditions. *P. angustum* S14 was subjected to continuous blue light, continuous darkness and a range of 12-hour light:12-hour dark/12-hour dark:12-hour light cycles (Figure 31). The expression of proteorhodopsin in glucose became sensitive towards the presence of light after a \pm 24-hour period. Briefly, an increase in luminescence was observed in all conditions after \pm 8-hours which was followed by a further increase in the presence of light but not dark conditions. After \pm 24 hours, turning off or turning on the light respectively resulted in a decrease or increase of the luminescent signal.



Figure 31. PTR-luc in Aquil medium and 6mM[C] glucose, subjected to either (a) no light, (b) 12 h dark – 12 h light cycles, (c) 12 h light – 12 h dark cycles (12L:12D) and (d) constant blue light (50%). After 8 hours, an increase in luminescence is observed regardless of the presence or absence of light. After 24 hours, respective increase or decrease in luminescence is observed when light is either present or absent.

4.3.3 Fe-replete conditions: Expression per Cell

In order to obtain the luminescence per cell, luminescence measurements and cell counts were obtained from *ptrluc* grown in 6mM[C] glucose amended with $5.4\mu M$ Fe (Fe-replete) and subjected to either continuous blue-light or continuous darkness (Figure 32, Table 8, Table 9).



Figure 32. PTR-luc grown in Fe-replete conditions in Aquil medium and 6mM[C] glucose subjected to either dark (dark grey circles) or constant blue light (white circles). (a) Total luminescence where vertical dashed lines represent the time points when cell counts were taken, (b) cell counts and (c) luminescence per cell. Total luminescence shows an upregulation after ± 25 hours in the presence of both light and dark, which coincides with the time cells are approaching the stationary phase. In the presence of light, luminescence continues to increase and peaks at ± 60 hours which coincides with slightly elevated cell counts in the presence of light, albeit this is not significantly different.

During the exponential phase, cells initially expressed a low luminescent signal. Upon approaching the stationary phase at around ± 30 hours, the luminescence signal increased per cell in both light and dark conditions. The increased luminescent signal became significantly higher in the presence of blue light compared to the dark at ± 48 hours (p=0.0006) and ± 57 hours (p<0.0001). Luminescence peaked around ± 60 hours and then gradually declines over time in both light and dark conditions.

When comparing the growth rates of *P. angustum* S14 grown in Fe-replete conditions in the presence $(\pm 7.4 \ \mu \ day^{-1})$ or absence $(\pm 7.1 \ \mu \ day^{-1})$ of light (Table 8), no significant differences were observed (p=0.9081). The growth rates coincided with cell yields of ± 1.26 and ± 1.31 (x10^s cells mL⁻¹) respectively in dark and blue light (50%) conditions and also revealed no significant differences (p=0.2345).

Table 8. Growth rates and cell yield of PTR-luc grown in Fe-replete and Fe-limiting conditions and in the presence or absence of light (Figure 32, Figure 36).

Conditions	Growth (μ day- i)	Yield (x10 ^s cells mL ¹)
Fe-rep + dark	7.4 ± 0.1	1.26 ± 0.09
Fe-rep + light	7.1 ± 0.4	1.31 ± 0.29
Fe-lim + dark	4.0 ± 0.1	0.114 ± 0.08
Fe-lim + light	5.1 ± 1.0	0.107 ± 0.13

In order to verify that the upregulation of proteorhodopsin around ±30 hours was indeed related to the depletion of glucose we further grew *ptrluc* in a range of glucose concentrations (60 μ M[C], 600 μ M[C] and 6000 μ M[C]) in the presence and absence of light (Figure 33). Although cell counts were not measured, the upregulation of proteorhodopsin differed depending on the carbon concentration present within the medium. Upregulation of proteorhodopsin subsequently occurred earlier under low carbon concentrations and later under high carbon concentrations. Peak luminescence was also lower with decreasing carbon concentrations and interestingly, the clear distinction between light and dark in the presence of 6mM[C] glucose became less pronounced in lower carbon concentrations (600 μ M[C]) and was absent in the lowest carbon concentration (60 μ M[C]).

Table 9. Statistical analysis of luminescence per cell of PTR-luc grown in Fe-replete and Fe-limiting conditions and in the presence or absence of light (Figure 32, Figure 36) using a 2-way ANOVA followed by a post-hoc Tukey HSD analysis. Significant differences are shown with an asterisk where: *(p=0.01), **(p=0.001), ***(p=0.0001), ****(p<0.00001)

2-way Anova	4h	11h	20h	26h	30h	33h	48h	57h
Iron	p=0.0500*	p<0.0001****	p<0.0001****	p<0.0001	p<0.0001****	p=0.0004***	p<0.0001****	p<0.0001****
Light	p=0.0018**	p=0.0024**	p=0.8640	p=0.3860	p=0.6632	p=0.7147	p=0.0015**	p=0.0002***
Iron:Light	p=0.0872	p=0.0595	p=0.8450	p=0.4260	p=0.0207*	p=0.0169*	p=0.0035**	p<0.0001****
Tukey HSD	4h	11h	20h	26h	30h	33h	48h	57h
Fe-lim dark	+ p=0.3195	p=0.2585	p=0.9927	p=0.9999	p=0.3771	p=0.3162	p=0.0006***	p<0.0001****
Fe-lim light	+ p=0.0076**	p=0.0072**	p=1.0000	p=0.6208	p=0.1648	p=0.1557	p=1.0000	p=0.8866



Figure 33. PTR-luc grown in Fe-replete conditions in Aquil medium and (a) $(6mM[C] (b) 600 \mu M[C],$ and (c) $60 \mu M[C]$ glucose and subjected to either dark conditions (grey circles) or constant blue light (white circles).

4.3.4 Alternative Carbon Sources: Acetate and Serine

In order to establish whether the expression and upregulation of proteorhodopsin in *ptrluc* was present in other carbon sources and not specific to glucose metabolism, *ptrluc* was additionally acclimated in 6mM[C] acetate and 6mM[C] serine in the dark and then subjected to the absence or presence of constant blue light (Figure 34 and Figure 35).

Different luminescence profiles were observed when cells were grown in either serine and acetate in comparison to glucose. In the presence of light, a lower luminescence signal was observed upon growth in serine and the signal completely flatlined in the presence of acetate.


Figure 34. Ptr-luc grown in Fe-replete conditions in Aquil medium and acetate (6mM[C]) subjected to either dark conditions (black circles) or constant blue light (grey circles). The vertical striped line indicates the time point that cell counts and the measurement of ROS was taken.



Figure 35. Ptr-luc grown in Fe-replete conditions in Aquil medium and serine (6mM[C]) subjected to either dark conditions (black circles) or constant blue light (grey circles). The high standard deviation is likely a result of a high production of EPS which formed particles that interfered with the luminescence measurements and made the correction by cell counts not possible.

The acclimation and growth of *ptrluc* in serine resulted in the formation of visible EPS particles which was reflected in a large standard deviation in the luminescence signal (Figure 35). The presence of large EPS particles hereby prevented the further use of flow cytometry in order to measure cell counts. Although it was possible to take cell counts for *ptrluc* grown in the presence of acetate, the low luminescent signal prevented us from obtaining an accurate and reliable measurement of the luminescence per cell. Cell counts were taken instead at ± 96 hours when a clear difference in luminescence could be found (Figure 34 and Table 10). The low luminescent signal for cells grown in acetate in the presence of light was the result of the absence of cell growth. The presence of ROS was measured in *P. angustum* grown in acetate (Table 10), in order to test whether light had a detrimental effect on cellular growth through oxidative stress and whether this was dependent on the carbon source metabolized. Indeed, when measuring oxidative stress within the cells using the CellROX deep red dye cells grown in acetate showed a significantly higher presence of oxidative stress within the cell that doubled in the presence of blue light in comparison to cells grown in the dark.

Conditions	Cell Counts (mL ¹)	<i>ROS</i> (<i>r. f. u</i>)	ROS 1000 cells ¹
Acetate dark	8.29 x10 ^s	7716	0.93
	7.94 x10 ^s	8276	1.04
Acetate light	3.13 x10 ^a	15388	4.92
	3.53 x10 [,]	13919	3.94

Table 10. Cell counts and ROS measurements taken at ± 96 hours from ptrluc (2n) grown in Fereplete conditions in Aquil medium and acetate (6 mM[C) and subjected to either dark conditions or constant blue light).

These results hereby showed that the sensitivity of *ptrluc* grown in acetate and serine to the presence of continuous blue-light ultimately outweighed any potential benefit for the cells in terms of proteorhodopsin. Furthermore, this sensitivity towards light did not seem to improve when subjecting cells to lower light concentrations (25% blue-light) or 12:12 dark-light and 12:12 light-dark conditions (data not shown). Therefore, based on the above results, we therefore continued our proteorhodopsin expression tests in the presence of glucose (and not acetate and serine) in order to elucidate whether proteorhodopsin could improve the growth rate of a cell when subjected to Fe-limitation.

4.3.5 Fe-limiting conditions

Fe-limitation was achieved through the addition of DFOB (50 μ M DFOB) in order to assure that *ptrluc* were subjected to Fe-limiting conditions. Luminescence measurements, cell counts and the luminescence per cell were obtained from *ptrluc* grown in 6mM[C] glucose and 50 μ M DFOB and subjected to either continuous blue light (50%) or continuous darkness (Figure 36; Table 8, 9).

Growth rates significantly declined from ± 7.4 and ± 7.1 (μ day-1) in Fe-replete conditions to ± 4.0 and ± 5.1 (μ day-1) when subjected to either constant darkness or blue-light (p<0.0001). This further coincided with a significant decline in cell yields from ± 1.29 and ± 1.31 (x10° cells mL³) to ± 0.114 and ± 0.107 (x10° cells mL⁴) in dark or blue-light conditions (p<0.0001). Cells approached the stationary phase before ± 20 hours, that coincided with an increase in luminescence in both light and dark conditions. No significant differences in the luminescence per cell was observed between light and dark conditions under Fe-limitation. Taking these experiments into account, the expression of proteorhodopsin specifically seems to be upregulated in the presence of glucose as cells approach the stationary phase (in both Fe-replete and Fe-limiting conditions) while clear differences in expression between light and dark conditions but not Fe-limiting conditions.



Figure 36. Ptrluc grown in Fe-limiting conditions (50 μ M DFOB) in Aquil medium and 6mM[C] glucose subjected to either dark conditions (dark grey circles) or constant blue light (white circles). (a) total luminescence where vertical dashed lines represent the time points when cell counts were taken, (b) cell counts and (c) luminescence per cell. Total luminescence shows an upregulation after ±20 hours in the presence of both light and dark, which coincides with the time cells are approaching the stationary phase. No clear difference between light and dark conditions were observed.

4.3.6 Effect of light on the growth rates, cell yield and cell size of wildtype P. angustum S14.

As the addition of luciferin or Cm is not necessary for the growth of the wildtype and in order to establish an Fe-gradient in the absence of DFOB as a chelator (Farkas et al., 1997; Simionato et al., 2006) further tests were conducted on the wildtype *P. angustum* S14 to see whether the observed upregulation of proteorhodopsin could have an actual effect on the overall growth rate and cell yield when *P. angustum* S14 was grown in a range of different light (0%, 25%, 50%, 100%) and Fe concentrations (5.4 μ M, 0.54 μ M, 54 nM, 5.4 nM) (Figure 37, Table 11, Table 12 and their statistical analysis Table 13).



Figure 37. Growth curves of P. angustum S14 grown in Aquil with 6mM[C] glucose, subjected to(a) 5.4 μ M, (b) 0.54 μ M, (c) 54 nM, (d) 5.4 nM of F and 0, 25, 50, 100% blue light (dark grey, grey, light grey and white circles).

Table 11. Growth rate (day¹) of *P*. angustum S14 grown in a range of Fe-concentrations and subjected to either constant darkness (0%-blue) or 25, 50, 100% blue light in a white 96-well plate.

Fe-concentration	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	7.4 ± 0.05	7.1 ± 0.03	7.1 ± 0.05	7.1 ± 0.04
0.54 μM	6.7 ± 0.03	6.7 ± 0.08	6.5 ± 0.06	6.8 ± 0.08
54 nM	5.2 ± 0.05	5.3 ± 0.08	5.4 ± 0.01	5.6 ± 0.2
5.4 nM	3.8 ± 0.09	4.3 ± 0.18	4.3 ± 0.04	4.3 ± 0.06

Table 12. Cell yield $(x10^{\circ})$ of P. angustum S14 (\pm 74 hours) grown in a range of Fe-concentrations and subjected to either constant darkness (0% blue) or 25, 50, 100% blue light

Fe-concentration	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	1.39 ± 0.01	1.24 ± 0.03	1.26 ± 0.10	1.06 ± 0.14
0.54 μM	1.32 ± 0.02	1.24 ± 0.03	1.20 ± 0.08	1.15 ± 0.09
54 nM	0.66 ± 0.01	0.69 ± 0.03	0.77 ± 0.04	0.64 ± 0.12
5.4 nM	0.22 ± 0.01	0.27 ± 0.04	0.23 ± 0.01	0.19 ± 0.02

Overall, low Fe-concentrations resulted in a significant and gradual decline in both the growth rate (p<0.0001) and cell yield (p<0.0001) of *P. angustum* S14. In Fe-replete conditions, growth rates reached values between ± 7.1 and 7.4 (μ day⁴) and decreased to rates between ± 3.8 and 4.1 (μ day⁴) under severe Fe-limitation. Cell yields reached values between ± 1.06 and 1.39×10^{4} (mL⁴) and decrease to yields between ± 0.19 and 0.27×10^{4} (mL⁴). The bacterial cell yield obtained from cells grown under 5.4 μ M Fe:EDTA are similar to the cell yield obtained at 0.54 μ M Fe:EDTA indicating that, at these concentrations, Fe is not considered to be a limiting factor within the medium. The presence of light significantly affected both the growth rate (p=0.0004) and cell yield (p=0.0007) although differences were relatively small in comparison to the effect of Fe concentrations had on growth rate and cell yield. The presence of light, for example, decreased the growth rate of *P. angustum* S14 in Fe-replete conditions from $\pm 7.4 (\mu \, day^4)$ to $\pm 7.1 (\mu \, day^4)$ to $\pm 4.3 (\mu \, day^4)$ (Table 11, 13). Significantly lower cell yields (Table 12, Table 13) were observed for *P. angustum* S14 in Fe-replete conditions grown in the presence of high light intensities of 100% blue light (p=0.0013) but no further significant differences

were observed in Fe-limiting conditions. The effect of light on the growth rates of *P. angustum* S14 grown under different Fe-concentrations can further be summarized in Figure 38.

Table 13. 2-way ANOVA and post-hoc Tukey HSD analysis comparing the growth rate and cell yield of *P*. angustum S14 in different Fe and light concentrations. Significant differences are shown with an asterisk where: *(p=0.01), **(p=0.001), ***(p=0.0001), ****(p<0.00001)

2-way ANOVA	Growth		Cell Yield	
Iron	p<0.0001	****	p<0.0001	****
Light	p=0.0004	***	p=0.0007	***
Iron:Light	p<0.0001	****	p=0.0522	
	Cnowth		Coll Wield	
Tukey HSD	Growin		Cell Yleid	
5.4 µM [Fe]				
dark vs 25%	p=0.0945		p=0.9807	
dark vs 50%	p=0.0101	*	p=0.8075	
dark vs 100%	p=0.0850		p=0.0013	**
0.54 μM [Fe]				
dark vs 25%	p=1.0000		p=0.9807	
dark vs 50%	p=0.9860		p=0.8168	
dark vs 100%	p=0.9978		p=0.2831	
54 nM [Fe]				
dark vs 25%	p=0.9986		p=1.0000	
dark vs 50%	p=0.6149		p=0.9423	
dark vs 100%	p=0.0012	**	p=1.0000	
5.4 nM [Fe]				
dark vs 25%	P<0.0001	****	p=1.0000	
dark vs 50%	p=0.0003	***	p=1.0000	
dark vs 100%	p<0.0001	****	p=1.0000	



Figure 38. The relationship between the growth rate (day¹) of P. angustum S14 for a range of Feconcentrations and grown in either 0, 25, 50, 100% blue light (dark grey, grey, light grey and white circles). Under low Fe-concentrations, the presence of light positively influences the growth rate while under high Fe-concentrations, the presence of light seems to negatively influence the growth rate.

In order to verify whether these differences in growth rate and cell yield were a result of proteorhodopsin, the experiment was repeated using the gene knockout Δpr (Figure 39, Table 14, Table 15, Table 16). Similar growth curve patterns were observed for Δpr compared to the wt. Fe-limitation caused a gradual and significant decline in growth rate (p<0.0001) and cell yield (p<0.0001) and a significant interactive effect between light and Fe-concentrations was present on both the growth rate (p=0.0001) and cell yield (p=0.0029) which slightly increased in the presence of light. A Tukey Test revealed significant differences in the growth rate between dark and 100% blue-light at 54 nM Fe concentrations (p=0.0022) and significant differences in cell yields were observed between dark and 100% blue-light for 5.4 μ M Fe concentrations (p=0.0003).

Our results obtained from the experiments of Δpr hereby showed that the presence of proteorhodopsin does not seem to be the cause of the observed differences in growth and cell

yield in the presence of a range of light conditions. Interestingly for Δpr , no negative effect of light on cell growth and cell yield was observed under high light intensities (ie. 100% blue light). It is, however, important to note that the wt and Δpr experiments are not directly comparable with each other because the experiments were respectively carried out using white and dark coloured 96-well plates which would significantly alter the exact light concentrations that were present within the two experiments. This can explain why a gradual increase in the overall cell yield and growth rate in Δpr was observed with increasing concentrations of light while a negative impact on the growth rate and cell yield of the wt was observed under high light intensities.



Figure 39. Growth curves of Δpr grown in Aquil with 6mM[C] glucose, subjected to a range of Fe concentrations and 0, 25, 50, 100% blue light (dark grey, grey, light grey and white circles).

Table 14. Growth rate (day^{*}) of Δpr grown in a range of Fe-concentrations and subjected to either constant darkness (0%-blue) or 25, 50, 100% blue light in a 96-well black plate.

Fe-concentration	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	6.8 ± 0.22	7.0 ±0.159	6.9 ± 0.13	7.0 ± 0.16
0.54 μM	5.8 ± 0.13	6.2 ± 0.17	5.7 ±b0.26	5.4 ± 0.39
54 nM	4.6 ±0.1	$4.6 \pm \pm 0.2$	4.9 ± 0.2	5.5 ± 0.02
5.4 nM	3.3 ± 0.1	3.3 ±0.02	3.3 ± 0.04	3.2 ± 0.10

Table 15. Cell yield $(x10^{\circ})$ of Δpr (± 91 hours) grown in a range of Fe-concentrations and subjected to either constant darkness (0% blue) or 25, 50, 100% blue light.

Fe-concentration	0% Blue	25% Blue	50% Blue	100% Blue
5.4 µM	1.22 ± 0.10	1.29 ± 0.09	1.11 ± 0.06	1.52 ± 0.18
0.54 μΜ	1.23 ± 0.06	1.16 ± 0.08	1.08 ± 0.05	1.33 ±0.11
54 nM	0.31 ± 0.02	0.37 ± 0.02	0.33 ± 0.03	0.45 ± 0.03
5.4 nM	0.13 ± 0.03	0.16 ± 0.01	0.23 ±0.03	0.27 ± 0.03

Table 16. 2-way ANOVA and post-hoc Tukey HSD analysis comparing the growth rate and cell yield of Δpr in different Fe and light concentrations. Δpr was grown in a range of Fe-concentrations and subjected to either constant darkness (0%-blue) or 25, 50, 100% blue light. Significant differences are shown with an asterisk where: * (p=0.01), **(p=0.001), ***(p=0.0001), ****(p<0.00001).

2-way ANOVA	Growth		Cell Yield	
Iron	p<0.0001	****	p<0.0001	****
Light	p=0.0985		p<0.0001	****
Iron:Light	p=0.0001	***	p=0.0029	**
Tukey HSD	Growth		Cell Yield	
5.4 µM [Fe]				
dark vs 25%	p=0.9974		p=0.9887	
dark vs 50%	p=0.9999		p=0.7882	
dark vs 100%	p=0.9851		p=0.0003	***
0.54 µM [Fe]				
dark vs 25%	p=0.7058		p=0.9968	
dark vs 50%	p=1.0000		p=1.0000	
dark vs 100%	p=0.5415		p=0.3608	
54 nM [Fe]				
dark vs 25%	p=1.0000		p=0.9915	
dark vs 50%	p=0.8871		p=0.3210	
dark vs 100%	p=0.0022	**	p=0.9074	
5.4 nM [Fe]				
dark vs 25%	p=1.0000		p=0.9999	
dark vs 50%	p=1.0000		p=0.8919	
dark vs 100%	p=0.9982		p=0.3035	

The findings of the above experiments were further supported by growth and respiration experiments derived from O_2 measurements taken for both the wt and Δpr grown in Fe-limited conditions (100 μ M DFOB) in the presence or absence of light (Table 17).

Table 17. Growth and respiration rates derived from O_2 measurements of P. angustum S14 (wt) and Δpr grown in Fe-limiting conditions (100 μ M DFOB) and the presence of constant light or dark conditions.

Bacterial strain	Growth rate (day ⁻¹)	Respiration rate (fmol hr¹ cell- ¹)
WT + light	2.6 ± 0.1	0.80 ± 0.16
WT + dark	2.6 ± 0.1	1.07 ± 0.20
$\Delta PR + light$	2.8 ± 0.05	1.30 ± 0.15
$\Delta PR + dark$	3.0 ± 0.16	1.16 ± 0.25

4.3.7 Changes in cell volume

Changes in cell volume were measured for *P. angustum* S14 grown in both Fe-replete (5.4μ M) and Fe-limiting (addition of 50μ M DFOB) conditions in the presence of 6mM[C] glucose and subjected to either constant blue light or constant darkness (Figure 40, Table 18). The mean cell volume declined from over time in both Fe-limiting and Fe-replete conditions and was always lower in Fe-limiting conditions compared to Fe-replete conditions. Interestingly, the presence of blue light further influenced the mean cell volume, which was always lower in the presence of light. Therefore, in Fe-replete conditions, the presence of blue light caused a significant decline in cell-volume at ± 24 hours (p=0.0056), while in Fe-limiting conditions, a significant decline was observed after ± 50 hours (p<0.0001).



Figure 40. Mean cell volume (μm) of P. angustum S14 at several time points grown in either 6mM[C] of glucose in (a) Fe-replete or (b) Fe-limiting conditions and subjected to constant darkness or blue light. At 1 hour, cell size was measured from acclimated cells grown overnight in glucose and Fe-replete conditions. 24 hours coincides with the end of the exponential growth phase and start of the exponential phase. 50 hours coincides with peak proteorhodopsin expression and 75 hours when the expression of proteorhodopsin is in decline.

<i>Table 18.</i> Mean cell volume (μm) of P. angustum S14 at several time points grown in $6mM[C]$ of
glucose in either Fe-replete or Fe-limiting conditions and subjected to constant darkness or blue
light. At 1 hour, cell size was measured from acclimated cells grown overnight in glucose and Fe-
replete conditions. 24 hours coincides with the end of the exponential growth phase and start of the
exponential phase. 50 hours coincides with peak proteorhodopsin expression and 75 hours when the
expression of proteorhodopsin is in decline. Significant differences between light and dark conditions
for each time point are shown with an asterisk where: $(p=0.01)$, $**(p=0.001)$, $***(p=0.0001)$,
****(p<0.00001)

Conditions	1h	24h	50h	74h
Fe-rep + dark	1.44	1.09 ± 0.00	0.80 ±0.01	0.69 ± 0.00
Fe-rep + light	1.44	1.04 ± 0.02	0.78 ± 0.01	0.67 ± 0.00
		p= 0.0056**	p= 0.0707	p= 0.9860
Fe-lim + dark	1.35	0.90 ± 0.00	0.85 ± 0.00	0.76 ± 0.00
Fe-lim + light	1.35	0.88 ± 0.00	0.74 ± 0.01	0.59 ± 0.08
		p= 0.3343	p<0.0001 ****	p= 0.0573

4.3.8 ATP production

Lastly, ATP measurements were taken for *P. angustum* S14 grown in Fe-replete (5.4 μ M) and Fe-limiting (addition of 50 μ M DFOB) conditions at ±20 hours (Table 19). ATP measurements were significantly lower in Fe-limiting conditions compared to Fe-replete conditions but no significant differences were observed in the presence or absence of light under both Fe-conditions. Altogether, these results do not support the hypothesis that proteorhodopsin is implicated in enhancing the energy production of the cell under Fe-limitation.

Table 19. ATP measurements taken at 20hrs (coinciding with the exponential phase) for cells grown in Fe-replete and Fe-limiting conditions and p-values of a 1-way ANOVA comparing differences between light and dark conditions after an ln transformation in order to meet the assumptions of homogeneity of variance.

ATP (fmol 1000 cells ¹)	Dark	Light	p-value
Fe-replete	11.8 ± 1.47	8.2 ± 2.99	p = 0.1357
Fe-limiting	0.9 ± 0.04	0.9 ± 0.05	p = 1.0000

4.4 Discussion

4.4.1 Upregulation of proteorhodopsin in Fe-replete conditions

An extensive literature search on proteorhodopsin and its expression patterns (Table 7 and sources therein) reflects that the regulation of proteorhodopsin can differ amongst various bacterial strains. Several studies have been able to demonstrate that proteorhodopsin can both be facultatively or continuously expressed within the cell and our results show that *P. angustum* S14 facultatively expresses proteorhodopsin within the cell. Proteorhodopsin was shown to be downregulated when cells were in the exponential growth phase and upregulated upon entering the stationary phase in both Fe-replete and Fe-limiting conditions.

P. angustum S14 belongs to the *Vibrionaceae* family and within this family, the regulation of proteorhodopsin has previously been tested in two other model organisms: *Vibrio* AND4 and *Vibrio campbelli*, both of which contain green-tuned proteorhodopsin molecules (Akram et

al., 2013; Gómez-Consarnau et al., 2010; Wang et al., 2012). *P. angustum* S14, on the other hand, is predicted to be a blue-tuned proteorhodopsin molecule which was further verified within our experiments when its expression was shown to differ in blue-light while its expression in the presence of red or green light reflected dark conditions. *P. angustum* S14 hereby give us an opportunity to gain further insight into the expression of a proteorhodopsin molecule with a different ancestral origin within the *Vibrionaceae* family. Despite these differences, a very similar expression pattern was observed for the proteorhodopsin present in *P. angustum* S14 when grown in glucose in comparison to the work conducted on *Vibrio sp.* AND4 (Akram et al., 2013) and *Vibrio campbelli* (Wang et al., 2012). In both of those strains the expression of proteorhodopsin was shown to be either low or absent during the exponential growth phase and peaked when cells approached the stationary phase in both dark and light conditions. Our results additionally showed a significantly higher peak in the expression of proteorhodopsin in the presence of light which coincided with the expression pattern found in *V. campbelli* but not for *Vibrio sp. AND4* where no significant differences were seen between light and dark conditions.

Interestingly, when testing a range of different glucose concentrations, clear and significant differences in expression between light and dark conditions were only observed when cells were subjected to high glucose concentrations. These differences declined with decreasing carbon concentrations until no differences could be observed. Although it is possible that these observations are a result of lower cell counts which decreases the resolution to observe differences between light and dark conditions, considering the relative sensitivity of this assay, it is more likely that the observed differences in light and dark conditions are dependent on other factors. For example, one possible explanation is that the upregulation of proteorhodopsin is sensitive to the carbon concentration present within the medium. The observed growth differences between light and dark conditions in Dokdonia sp. MED 134, for example, were only observed within a specific window of DOC concentrations ranging between 140 μ M[C] and 1.1 mM[C] but not in lower carbon concentrations of 60μ M[C] and extremely high concentrations of 242 mM[C]. Although a range of carbon concentrations was not tested, the experiments conducted in V. campbelli showcasing similar differences in the expression of proteorhodopsin between light and dark conditions was conducted in carbon concentrations of $132 \,\mu M[C]$, which is 2x higher than the carbon concentrations (60 $\mu M[C]$) used where no differences between the light and dark conditions were observed in *P. angustum* S14.

P. angustum S14, like other members of the Vibrionaceae family, is defined as a copiotrophic bacterium which is known to thrive in nutrient rich environments, invoking a "boom or bust" as opposed to oligotrophic bacteria which typically grow in low carbon lifestyle, concentrations (Ho et al., 2017; Lauro et al., 2009). Proteorhodopsin is currently predicted to act as an important mechanism that enables a heterotrophic bacteria to maintain sufficient energy-levels to allow a continuation of the basal metabolic functioning within the cell under unfavourable and starved conditions (Gómez-Consarnau et al., 2019). This notion is supported by the observation that the upregulation of proteorhodopsin in both V. campbelli and Vibrio sp. AND4 appears to be linked with their ability to induce a stringently controlled starvation state upon energy and nutrient deprivation, a common phenomenon observed in several members of the Vibrionaceae family including P. angustum S14 (Lever et al., 2015). The induction of a starvation state specifically occurs under carbon starvation which is regulated by the alternative sigma factor RpoS1 and does not occur under other forms of limitation such as nitrogen or phosphorous. (Holmquist and Kjelleberg, 1993). Indeed, deletion of RpoS1 in Vibrio sp. Campbellii, resulted in a decrease in proteorhodopsin expression and confirmed its role in the upregulation of proteorhodopsin (Wang et al., 2012). Although we have not tested whether this is indeed the case for *P. angustum* S14, the similar expression patterns coupled with the known presence of the stress response in P. angustum S14 (Hild et al., 2000) makes it likely that a similar form of regulation is occurring for this bacterial strain.

Our results, together with prior work conducted on *P. angustum* S14 grown in glucose (Courties et al., 2015), as well as *Vibrio* sp. AND4 (Akram et al., 2013; Gómez-Consarnau et al., 2010) and *Vibrio campbelli* (Wang et al., 2012) observed that the presence and upregulation of proteorhodopsin did not improve either growth rates or cell yields for cells grown in continuous light compared to dark conditions. This seems logical as the expression of proteorhodopsin does not appear to be upregulated in the exponential growth phase of these cells. Furthermore, in Fe-replete conditions, the presence of light even lowered both the cell yield and cell counts and is likely, as noted by others, the result of photo-oxidative stress (Akram et al., 2013; Wang et al., 2012). This notion is further strengthened by our findings that similar cell yields were obtained in both 5.4 μ M Fe and 0.54 μ M Fe concentrations, which suggests a surplus of Fe in the medium containing 5.4 μ M Fe which may lead to an increase in the production of ROS in the presence of light and the Fenton reaction.

As mentioned previously, several experiments have shown that under carbon starvation the presence and upregulation of proteorhodopsin plays a role in the survival of various bacterial strains. It is known that under such starvation conditions, several strains of the Vibrionaceae family, including *P. angustum* S14 are characterized to undergo a reduction in cell size and is described as an attempt to optimize their energetic efficiency within the cell (Gómez-Consarnau et al., 2007; Kjelleberg et al., 1982; Lauro et al., 2009; Nyström and Kjelleberg, 1989). Over time, the presence of light resulted in a slower decline in cell counts compared to dark conditions in Vibrio sp. AND4, upon transfer of cells from rich medium to natural sea water containing low concentrations of organic and inorganic nutrients in a series of dilution experiments (Akram et al., 2013; Gómez-Consarnau et al., 2010). The upregulation of proteorhodopsin in Vibrio sp. AND4 was hereby coupled with a sharp increase in cell numbers within the first 2 days as cells underwent cell division by fragmentation which resulted in the formation of an ultramicro-cell. In P. angustum S14, the formation of these ultramicro-cells has further been linked to an enhanced resistance towards other stressors such as heat and UV light (Nyström et al., 1992). Biovolume measurements conducted on P. angustum S14 at several different time points coinciding with different growth phases of the cell supported the notion that *P. angustum* S14 undergoes a reduction in cell size as cells approach the stationary phase in both Fe-replete and Fe-limiting conditions. Although it is quite possible that the upregulation of proteorhodopsin can aid the survival of P. angustum S14 cells over time that have been starved for carbon and nutrients, this has yet to be tested and verified.

Another mechanism by which the upregulation or proteorhodopsin can aid a cell within the stationary phase is in the search and uptake of available nutrients. Copiotrophs are often characterized by a wide diversity in transporters that enables the uptake of various nutrients (Ho et al., 2017). Under carbon starvation, *P. angustum* S14 has the ability, for example, to induce a high-affinity uptake system for glucose (Lauro et al., 2009; Lever et al., 2015) and has also been shown to evoke a chemotaxis response towards several carbon sources (Malmcrona-Friberg et al., 1990). Both the observed chemotaxis response towards glucose in the presence of carbon-starvation and the ability of *P. angustum* to upregulate high-uptake mechanisms for glucose are all potential functions that could ultimately require the use of proteorhodopsin (Fuhrman et al., 2008). For *P. angustum* S14, the presence or absence of light

has not been tested thus far in these response mechanisms including Fe-limitation (see Supplementary Material).

4.4.2 Growth on different carbon sources: light and the oxidative stress response

All experiments testing proteorhodopsin-containing members of the Vibrionaceae family have been conducted in either glucose or mixed carbon sources (e.g. bactopeptone and yeast extract). Interestingly, experiments conducted on *Dokdonia* sp MED134 had a more pronounced effect from the presence of light when cells were grown in alanine compared to mixed carbon sources (Palovaara et al., 2014). For most bacteria, glucose is often considered to support the fastest and most efficient growth rate compared to other carbon-compounds such as alanine, serine or acetate and can even act as a catabolic repressor, preventing the growth on other carbon sources (Görke and Stülke, 2008). Furthermore, the entry of other carbon sources into the TCA cycle differs from that of glucose and can require the use of other metabolic pathways. Growth on acetate, for example, obligates a bacterium to enter the glyoxylate shunt (Kornberg, 1966; Kornberg and Krebs, 1957) while growth on serine surpasses a large part of glycolysis. It was hereby interesting to test whether the use of carbon sources such as serine or acetate which entered the TCA cycle at different points as opposed to glucose to see whether the benefit of proteorhodopsin and light could be more clearly observed in "poorer" carbon environments (Palovaara et al., 2014).

A different and contrasting expression pattern emerged when *P. angustum* S14 was grown in carbon sources other than glucose. The total expression of proteorhodopsin in the presence of light was lower compared to the dark conditions for both serine and acetate and upon further investigation, this was due to the absence of growth from bacteria grown in acetate in the presence of light. Additionally, these findings coincided with a significantly higher production of ROS in the presence of light for cells grown in acetate and a similar explanation is likely for serine, albeit less pronounced. This sensitivity towards light did not improve when cells were grown in the presence lower light concentrations or under 12:12 light-dark and 12:12 dark-light conditions. Furthermore, despite the small volume used within our experimental setup, the light intensities that were used in this experimental setup were within the lower-range of light intensities used in previous studies (Table 20).

Bacterial strains	Light (µE)	Volume (mL)	Reference
SAR11 + Flavobacter	830	30,000	Lami et al., 2009
Vibrio sp. AND4	130; 6	75	Gomez- Consarnau et al., 2010
Dodkonia sp. MED134	150	250	Kimura et al., 2011
Vibrio campbellii	42	50	Wang et al., 2012
C. Pelagibacter ubique	70	250	Steindler et al., 2008
Dokdonia MED 134	130	1,900	Gomez-Consarnau (2007; 2016)
C. Pelagibacter ubique	24; 250; 680	100	Giovanni et al., 2005
Psychroflexus torquis	27.7; 3.7	5	Feng et al., 2013
Dokdonia sp. PRO95	95	250	Riedel et al., 2013
Vibrio campbellii AND4	157		Akram et al., 2013
P. angustum S14	60; 30; 15	0.2	This study

Table 20. Non-exhaustive list of several culture-studies testing the role of proteorhodopsin and the light intensities and volume used for each experiment.

Based on these results we postulate that the carbon metabolism of P. angustum S14 in the presence of either acetate or serine as sole carbon source results in an inefficient mechanism towards countering oxidative stress within the cell and causes a build-up of ROS which increases in the presence of blue-light.

As explained previously, the formation of ROS is a natural occurrence within aerobic cellular metabolism due the production of by-products and the leakage of electrons within the respiratory electron transport chain as well as through the Fenton reaction when Fe is in excess (Tiwari et al., 2002; Wang et al., 2017). Blue-light is known to further contribute to the generation of ROS with its ability to destabilise certain ROS producing enzymes (Wang et al., 2017). For example, blue-light can enhance the production of ROS in complex II (succinate dehydrogenase) of the electron transport chain which requires the coenzyme flavin adenine dinucleotide (FAD). In the presence of blue-light, FAD can destabilize and undergo an electron transfer reaction leading to the leakage of electrons which enhances the formation of ROS (Yankovskaya et al., 2003).

(Photo)oxidative stress occurs when ROS concentrations exceed a cell's capacity to neutralize them or repair resulting damage (Cabiscol et al., 2000). There are several methods by which

bacteria can deal with oxidative stress and consists of either keeping ROS concentrations to a minimum or repairing the oxidative damage that has been done to the cell (Cabiscol et al., 2000). P. angustum S14 in particular is known for its efficient resistance towards oxidative stress (Matallana-Surget et al., 2012, 2013) including high levels of H₂O₂ (Ostrowski et al., 2001). This is in part because P. angustum S14 harbours several oxidative response enzymes including glyoxalase, AhpC, recA as well as ferrous superoxide dismutase and peroxidase (Matallana-Surget et al., 2012, 2013). A possible explanation of the enhanced sensitivity to light when P. angustum S14 cells are grown in acetate (and serine) can be explained through the ability for a cell to efficiently redirect metabolism towards the pentose phosphate pathway (PPP, Zhao et al., 2004). The PPP plays an important role in both DNA synthesis (i.e repair) and the production of NADPH which is required for glutathione metabolism, an important antioxidant and defence mechanism (Mullarky and Cantley, 2015). Entry into the pentose phosphate pathway occurs via glucose-6-phosphate, a key intermediate within glycolysis. Growth on glucose automatically produces glucose-6-phosphatase through glycolysis but its production upon growth in acetate is less efficient and requires the redirection of carbon flux towards the glyoxylate shunt and towards gluconeogenesis (Mullarky and Cantley, 2015; Zhao et al., 2004). The PPP can hereby serve as an example of how bacterial growth on different carbon sources may result in different sensitivities towards combating oxidative stress. Although the above hypothesis remains speculative and needs to be tested, our results nonetheless show that the potential benefit towards the upregulation of proteorhodopsin and light can be hampered by the carbon conditions that *P. angustum* S14 is grown in.

4.4.3 Proteorhodopsin and Fe-limitation

In *P. angustum* S14, the presence of Fe-limiting conditions did not result in the upregulation of proteorhodopsin during exponential growth phase. Similar to Fe-replete conditions, the upregulation of proteorhodopsin occurred as cells entered the stationary phase while no significant differences were observed in the presence and absence of light as was observed under Fe-replete conditions. While enhanced growth and cell yields were observed in the presence of light in the presence of Fe-limitation, it is important to note that these differences, are likely unrelated to the context of proteorhodopsin, as no upregulation was shown to occur during the exponential growth phase of *P. angustum* S14. This was confirmed when repeating the experiment using Δpr and observing a similar pattern. Our results, however, coincide with the work conducted on the oceanic diatom *P. granii* subjected to Fe-limitation although it is

important to note that Fe-limitation will additionally affect phytoplankton in their photosynthetic electron transport chain. Proteorhodopsin was upregulated when *P. granii* was subjected to Fe-limitation in the presence of both light and dark conditions, but no clear differences could be observed in the growth rate of *P. granii* (Marchetti et al., 2012, 2015).

As mentioned previously, biovolume measurements conducted on *P. angustum* S14 grown in Fe-limiting conditions showed a clear reduction in cell-size. This implies that division through fragmentation may also be occurring for cells subjected to Fe-limitation and are subsequently entering the stationary phase. Interestingly, in the presence of Fe-limitation, our results are in contrast to what was observed for a small oceanic *A. macleodii* strain (Fourquez et al., 2014) which resulted in a 2-fold increase in its biovolume. Similar results have also been shown for *E. coli* bacteria (Hubbard et al. 1986). The reduction in cell size coupled with the upregulation of proteorhodopsin suggests that *P. angustum* S14 is inducing a stringently controlled starvation state as cells enter the stationary phase both under Fe-replete and Fe-limiting conditions.

The exact mechanism in which proteorhodopsin can benefit *P. angustum* S14 in the presence of Fe-limitation is still unclear. We initially predicted that proteorhodopsin would improve a cell's ability to produce energy, but no clear differences were observed between light and dark conditions concerning ATP production, respiration rates, and cellular biovolume.

While carbon-limitation will directly affect energy production within the cell, and several studies have been able to discern the importance of proteorhodopsin under starvation conditions, Fe-limitation is inherently more complex. Fe-limitation indirectly affects energy production by inhibiting Fe-containing enzymes which include enzymes located within the respiratory electron transport chain but also includes other enzymes within the cell (ie. enzymes involved in the TCA cycle and DNA synthesis). Therefore, although energy-limitation is a measurable result when cells are subjected to Fe-limiting conditions (as shown through a decline in respiration rates and ATP production), the exact role of proteorhodopsin in the presence of Fe-limitation may be more difficult to discern in comparison to carbon limitation.

Recently, a field study conducted on *Vibrio* bacteria showed that, while the addition of dust leachate stimulates *Vibrio* growth, the addition of Fe alone did not and implies that other factors

such as the presence of organic carbon and phosphorous present in the dust leachate additions may directly or indirectly stimulate *Vibrio* growth (Zhang et al., 2019). Considering the divergent functions that have been proposed for proteorhodopsin (Fuhrman et al., 2008), it would therefore be relevant to test additional culturable strains other than members of the Vibrionaceae family (ie. SAR11) in order to discern the potential link between the upregulation of proteorhodopsin and Fe-limitation.

4.5 Supplementary Data: preliminary tests to study Fe-uptake

As mentioned previously, the upregulation of proteorhodopsin and corresponding proton gradient may be utilized for a function other than energy production. Studies have previously shown a link between proteorhodopsin and TonB dependent uptake mechanisms for vitamin B1 (Gómez-Consarnau et al., 2016) and taurine (Steindler et al., 2011) which requires a protonmotive force. It is therefore possible that proteorhodopsin could be utilised for Fe-uptake within the cell.

The method of Fe-uptake within a bacterial cell is highly dependent on the Fe form (Figure 6). While inorganic Fe can be taken up by the outer cell membrane through porins, most Fe is complexed to ligands which exceed the molecular cut off weight for those porins (600 kDa). Mechanisms to transport Fe-bound ligands in their entirety often requires the use of a TonB dependent transport system. Once in the periplasmic space, Fe can then be transported across the inner membrane via ATP-dependent transporters (Krewulak and Vogel, 2011; Noinaj et al., 2010; Shin et al., 2019). We hypothesized that proteorhodopsin, in the presence of light, could improve the uptake of Fe either by enhancing the production of ATP required for ATP-dependent transport or if the Fe:ligand complex requires a TonB dependent transport system.

4.5.1 Proteorhodopsin and Fe-uptake

4.5.1.1 Fe-uptake – Acclimation

When *P. angustum* S14 is depleted of nitrogen, phosphorus or carbon, recovery rates in nitrogen and phosphorous have been shown to exhibit a longer lag period of more than 3 hours in comparison to the fast recovery rates observed under carbon depletion and was linked to the ability to induce a high-affinity uptake system for carbon upon starvation (Holmquist and

Kjelleberg, 1993). Therefore, one possible method to test whether proteorhodopsin could improve Fe-uptake within the cell, *P. angustum* S14 was acclimated in either Fe-replete $(5.4\mu M)$ or Fe-limiting (5.4nM) conditions for a period of 24 hours before being grown in the presence of Fe-replete or Fe-limiting conditions in the presence or absence of light (Supplementary Figure 4). The short lag phase for cells acclimated in Fe-limiting conditions implies a fast and efficient Fe-uptake mechanism and that *P. angustum* S14 is quick to Fe when made available in order to regain growth. A faster growth rate was observed for cells grown in Fe-limiting conditions in comparison to cells acclimated in Fe-replete conditions (p=0.0002), but no significant differences were observed in the presence or absence of light regardless of the acclimation condition (p=0.7274). Higher cell yields were obtained for cells grown in Fe-limiting conditions that were acclimated in Fe-replete conditions in comparison to cells acclimated for cells grown in Fe-limiting conditions.



Supplementary Figure 4. P. angustum S14 acclimated either in Fe-limiting (top-left) or Fe-replete (top-right) conditions and grown in the presence (bottom) or Fe-limiting (top) conditions. Regardless of the acclimation strategy, growth rates and yield are very similar when subjected to Fe-replete or Felimiting conditions. Higher yields were observed when cells were acclimated in Fe-replete conditions and grown in Fe-limitation in comparison to cells that were acclimated in Fe-limited conditions and continued to grow in Fe-limiting conditions. This is likely a result of carry over Fe from the acclimated conditions.

4.5.1.2 Fe-uptake – Fe:EDTA vs Fe:Citrate

Ptr-luc was grown in the presence of two different forms of Fe (Fe:EDTA and Fe:citrate) which was predicted to reflect two theoretically different Fe-uptake mechanisms (Supplementary Figure 6). The total expression pattern of proteorhodopsin was monitored in order to discern whether any differences were present. No clear differences could be seen and the growth of ptr-luc in both Fe:citrate and Fe:EDTA seemed to display an almost identical proteorhodopsin expression pattern which suggests a similar uptake mechanism used for both Fe:citrate and Fe:EDTA in *P. angustum* S14. This is further supported when looking in the genome of *P. angustum* S14 which does not contain a TonB-dependent ferric-uptake system for citrate (*FecA*) within its genome. Instead, *P. angustum* S14 harbours a G(A)TP-dependent ferrous Fe-uptake gene (*feoB*) and it is likely that Fe:citrate is taken up by the cell in its free dissociated

form rather than taken up in its entirety (Marshall et al., 2009). Upon further investigation, the genome of *P. angustum* S14 contains the presence of a ferric TonB-dependent receptor for the siderophore achromobactin (EAS66744.1) as well as a receptor for heme-transport (EAS64785.1), a common ligand for Fe binding in seawater (Hogle et al., 2014). As achromobactin is not commercially available, it may be interesting to look further into the possibility of using Fe:heme as an Fe-source when testing TonB-dependent Fe-uptake mechanisms.

It is important to note that when testing various Fe sources, the presence of light, which is necessary for the proper functioning of proteorhodopsin, can also increase the dissociation of certain Fe-ligands which will impact the bioavailability of Fe within the medium (Amin et al., 2009a, 2009b). This effect can, however, be assessed through the use of Δpr where comparative experiments between the wildtype and Δpr are required to discern whether any observed differences are a direct result of proteorhodopsin or the influence light on the disassociation and bioavailability of Fe.



Supplementary Figure 5. Ptr-luc grown in the presence or absence of light in (a) Fe:Citrate or (b) Fe:EDTA. A similar expression luminescence pattern was observed over time for both Fe-types.

4.5.1.3 Fe-uptake – Radioactivity

In an attempt to establish whether Fe-uptake could be measured in *P. angustum* S14 in Fereplete and Fe-limiting conditions in the presence and absence of blue-light, preliminary experiments using "Fe were conducted. Fe-uptake rates can be obtained from short term experiments where *P. angustum* S14 is grown in the presence of "Fe in either Fe-replete or Felimiting conditions where the accumulation of radioactivity within the cell is measured over several timepoints (typically between 4-8 hours depending on the growth rate) in order to determine the slope of the intracellular "Fe concentration accumulating over time (Kranzler et al., 2011, 2014; Lis et al., 2015).

The Fe-uptake rate per cell (ρFe) can be determined from the following equation:

$$\rho Fe = \frac{A x {}^{55} \text{Fe}_{cell}}{t_{inc}}$$

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Where $t_{inc.}$ is the incubation time (h¹), where

$$A = \frac{\text{mol}^{55}\text{Fe added} + \text{mol Fe in the medium}}{\text{mol}^{55}\text{Fe added}}$$

and

$${}^{55}\text{Fe}_{cell} = \frac{dpm_{filter} - dpm_{blank}}{N_{filter}} x \frac{1}{specific \ activity \ {}^{55}\text{Fe}}$$

where dpm_{inter} is the disintegrations per minute measured for each sample and subtracted by the dpm measured from a blank, N_{inter} represents the number of cells filtered, and the specific activity (fmol cpm⁻¹). Fe-uptake rates can then be derived from the slope of several time points.

A preliminary experiment was conducted where radioactivity measurements were taken after a period of ± 12 and ± 24 hours in order to determine whether the resolution was high enough to clearly distinguish intracellular ⁵⁵Fe concentrations between conditions and in comparison, to the background signal given from the filter and scintillation counter. *P. angustum* S14 was acclimated in Fe-replete conditions and exponentially growing cells were transferred to fresh Aquil medium containing Fe-replete (5.4 μ M, 20% ⁵⁵Fe- solution) or Fe-limiting (5.4 nM, 100% ⁵⁵Fe- solution) conditions from an ⁵⁵FeCl₃ solution (2.09 x 10³ Ci mol⁻¹ specific activity, Perkin Elmer) amended with EDTA (1:1 ratio). After ± 12 and ± 24 hours, samples were fixed with glutaraldehyde (0.2%) and 2 mL was pipetted onto a 0.2 μ m filter (Nucleopore) and gently washed for 2 minutes with 4 mL of titanium (III) chloride-citrate-EDTA followed by 10x 1-minute rinse with SOW to remove extracellular Fe-concentrations whilst retaining the bacterial cells (Granger and Price, 1999; Price et al., 1989). The filter was then placed in a scintillation vial containing 10 mL Ultima Gold Scintillation Filter Cocktail (PerkinElmer) for 24 hours until the filter is completely dissolved. Radioactivity of the filter was measured and quantified using a scintillation counter (Beckman Coulter LS 6500). Blanks containing the Aquil medium without bacterial cells were used as control. In parallel, cell-counts were obtained and analysed through flowcytometry.

The low resolution of ⁵⁵Fe combined with the fast uptake rates of Fe in *P. angustum* S14 (particularly for Fe-replete conditions) prevented us from obtaining clear results that were distinguishable from the blanks in dark or light conditions. While ⁵⁵Fe uptake rates are still deemed one of the best methods to test whether proteorhodopsin can part aid in the uptake of Fe. Due to time constraints, further tests were not pursued. Continuing these tests using ⁵⁹Fe, however, can be an alternative choice of isotope when testing small differences in Fe-uptake.

V. CONCLUSION AND PERSPECTIVES

When heterotrophic bacteria are included in global-scale simulations of ocean ecosystems and biogeochemical cycles, they are often depicted as one homogenous population (Worden et al., 2015). Understanding the biogeochemical contribution of heterotrophic bacteria at the single-cell level is necessary to make predictions of how ecosystems will respond to global change (Azam and Malfatti, 2007). Since the initial experiments of Martin and Fitzwater (1988) which have revealed that the constraint of microbial growth is dictated by the low bioavailable Felevels found in their surface waters, coupled with the work of Tortell in the late 90's which highlighted the impact of Fe-limitation on heterotrophic bacteria and emphasized their relevance towards Fe biogeochemical cycling (Tortell, 1999; Tortell et al., 1996), an increasing body of work has focussed on the various strategies heterotrophic bacteria can implement when coping with Fe-limitation.

This thesis manuscript took the model organism, *P. angustum* S14 and applied the construction of several genetic mutants (Ch. 2) in order to test two possible strategies that *P. angustum* S14 could implement, namely the glyoxylate shunt (Ch. 3) and proteorhodopsin (Ch. 4) when subjected to Fe-limitation.

While culture work enables us to work in a standardized and controlled experimental setting, a major challenge is to link the obtained experimental results of one model organism to *in situ* patterns and processes. This is especially true when considering the observed diversity of heterotrophic bacteria residing in Fe-limiting conditions (Debeljak et al., 2019). Despite this limitation, the power of culture work lies in the ability to untangle the direct and indirect effects of Fe-limitation and the resulting response within a heterotrophic bacterium. The results from this thesis manuscript hereby contributes to a culminating body of work that furthers our understanding towards the versatility of marine heterotrophic bacteria in coping with Fe-limitation and is an appropriate addition to the literature concerning the role of proteorhodopsin and the glyoxylate shunt within the marine environment.

This last and final chapter summarizes the results from previous chapters and discusses future perspectives that could help us improve our understanding within the field and the possibility of further extrapolating our results towards broader scales and biogeochemical cycling.

5.1 The Glyoxylate Shunt

Global summary

• Is the glyoxylate shunt upregulated under Fe-limiting conditions? Yes, within the exponential growth phase.

• If so, does this result in an improved growth and respiration rate?

Yes. The upregulation of the glyoxylate shunt resulted in an improved growth and respiration rate although it should be noted that the glyoxylate shunt is not a strategy that resolves the problem of Fe-limitation but improves the ability of a bacterium cell to cope with Fe-stress.

There is an increasing body of evidence that the glyoxylate shunt is not only associated with carbon metabolism but also plays a key role in bacterial persistence and antibiotic resistance in pathogenic bacteria (Dolan and Welcht, 2017). Several studies have previously shown the upregulation of the glyoxylate shunt under Fe-limitation and our results confirm the importance of the glyoxylate shunt within a marine bacterium in coping with Fe-limitation. We further speculate that the glyoxylate shunt is an important pathway that can redirect a cell's carbon flux away from the inhibitory effects of Fe-limitation on the electron transport chain. The upregulation of the glyoxylate shunt should theoretically rearrange the construction of the proton motive force within the electron transport chain away from Complex I, which is particularly affected under Fe-limitation, and towards Complex II (succinate dehydrogenase). This was not verified within our experimental setup and, to the best of our knowledge, the switch between the various respiratory enzyme complexes has not yet been tested within a marine heterotrophic bacterium subjected to Fe-limitation.

One possible way to determine the impact of the glyoxylate shunt on the ratio between Complex I and Complex II of the electron transport chain would be the isolation of membrane protein complexes through Blue-Native 2-dimensional non-denaturing Polyacrylamide Gel Electrophoresis (BN-PAGE; Wittig et al., 2006) followed by the immunological detection of Complex I and II through antibodies and Western Blotting. BN-PAGE has originally been developed to study the different respiratory enzyme complexes within the mitochondria (Schägger and von Jagow, 1991), but has also been used for a wide range of organisms including a marine gram-negative bacterium (Wöhlbrand et al., 2016).

The utilization of the glyoxylate shunt under Fe-limitation, altered the respiration rate within *P. angustum* S14 as *P. angustum* S14 moved its intracellular carbon flux away from the CO₂ producing steps. Given the extent of Fe-limiting regions within the ocean (ie. HNLC sites) and the prevalence of the glyoxylate shunt within the vast majority of marine bacteria (including SAR11), it would hereby be relevant to test the potential implications of the glyoxylate shunt on the variability of bacterial respiration both in terms of CO₂ production and O₂ consumption. Despite the knowledge that heterotrophic bacterial respiration plays a large component within the carbon cycle, the contribution of heterotrophic bacteria to the respiration of CO₂ is still poorly quantified (Robinson, 2019). Underpinning the mechanistic understanding of the glyoxylate shunt and its influence on bacterial respiration could further improve the current understanding and representation of heterotrophic bacteria in typical carbon-cycle studies and the subsequent calculation of carbon fluxes.

The respiration quotient (RQ) represents the ratio of moles of CO_2 produced per mole of O_2 consumed:

$$RQ = \frac{\Delta [CO_2]_{production}}{\Delta [O_2]_{consumption}}$$

As a carbon substrate is metabolised, CO_2 is released and the production of NADH and FADH₂ will serve as the electron donors at the start of the electron transport chain (respectively complex I and II) which are finally taken up by O_2 acting as the final electron acceptor within complex IV. As the electrons pass through the electron transport chain, the different enzyme complexes consequently produce a hydrogen gradient which is utilized for the production of ATP.

RQ values are known to vary according to the carbon substrate consumed which depends on the degree of oxidation as well as the metabolic pathways required for their consumption (Oschlies et al., 2018; Reinthaler et al., 2005). The RQ value has also been shown to vary according to the physiological state of the cell and differences have been observed when cells are in exponential growth or entering in the stationary phase (Berdalet et al., 1995; Romero-Kutzner et al., 2015; Roy et al., 1999). Under Fe-limitation, the electron transport chain is severely affected resulting in a sharp decrease in the O_2 consumption rate. As the upregulation of the glyoxylate shunt under Fe-limitation would bypass several CO₂ producing steps, we expect to see differences in the RQ value in comparison to the absence of the glyoxylate shunt.

The oxidation of 1 mole of glucose undergoing glycolysis, pyruvate dehydrogenase and the TCA cycle results in the production of 6 CO₂ and 10 NADH and 2 FADH₂ (Mathews et al., 2000; Figure 41). NADH or FADH₂, enters the electron transport chain either through complex I or complex II and it is known that complex I, III and IV respectively results in the translocation of 4, 4 and 2 H² across the membrane, while complex II does not result in the production of a proton motive force. Therefore, the oxidation of 1 mole of NADH or FADH₂, respectively results in the translocation of 10 or 6 protons (H²). Theoretically, for every 4 H², 1 ATP is produced so that NADH and FADH₂ contribute to the production of 2.5 and 1.5 molecules of ATP respectively. For 1 mole of glucose, 6O₂ are required for the uptake of the electrons donated by the produced NADH and FADH₂ so that a theoretical grand total of 32 molecules of ATP can be formed (Table 21). The RQ value for the metabolism glucose is hereby 1 (1:1).

Table 21. Summary of the total theoretical total ATP produced from 1 molecule of glucose separated for the different metabolic processes of glycolysis, pyruvate oxidation, the TCA cycle. The NADH and FADH₂ produced in these processes can donate electrons which pass at different stages of the electron transport chain to produce a total of 2.5 and 1.5 molecules of ATP by ATP synthase. It is important to note that in some texts, the total ATP produced from NADH and FADH₂ are rounded up to 3 and 2, which respectfully results in a grand total of 38 ATP molecules.

Metabolic cycle	output	ATP produced
Glycolysis	2 ATP	2
	2 NADH	5
Pyruvate oxidation	2 NADH	5
TCA cycle	2 ATP	2
	6 NADH	15
	2 FADH ₂	3
	Total:	32

When the TCA cycle is redirected through the glyoxylate shunt, a decline in the production of NADH and FADH₂ occurs within the TCA cycle, resulting in fewer electrons that can flow

through the electron chain resulting in a lower concentration of O_2 respired and less ATP (Figure 42, Table 22). Therefore, redirection of cellular metabolism through the glyoxylate shunt will result in only 25 ATP molecules and the complete oxidation of glucose in the presence of the glyoxylate shunt results in an RQ value of 0.4 (2:5).

Table 22. Summary of the total theoretical total ATP produced from 1 molecule of glucose separated for the different metabolic processes of glycolysis, pyruvate oxidation, the TCA cycle when going through the glyoxylate shunt.

Metabolic cycle	output	ATP produced
Glycolysis	2 ATP	2
	2 NADH	5
Pyruvate oxidation	2 NADH	5
TCA cycle (glyoxylate shunt)	4 NADH	10
	2 FADH ₂	3
	Total:	25

A concise way to test this hypothesis would be to apply a Membrane Mass Inlet Spectrometer (MIMS) which can simultaneously monitor, at high precision, changes in both CO₂ production and O₂ consumption in near real-time (Hoch and Kok, 1963). MIMS allows one to extract gases from (ie. Aquil) medium across a semi-permeable membrane into the ion source of the mass spectrometer and has successfully been applied to measure microbial respiration rates in various marine field studies (Tortell, 2005; Guéguen and Tortell, 2007). The MIMS has also successfully been applied to marine culture medium, albeit it was not used to measure microbial respiration rates (Casey et al., 2017).



Figure 41. Illustration of the metabolism of glucose and the theoretical ATP production and RQ quotient of P. angustum S14. The oxidation of glucose results in the respiration of 6CO₂ and the production of 10NADH and 2FADH₂ which donate their electrons to the electron transport chain resulting in the consumption of 6O2 and leads to an RQ value of 1 (6:6). The formed proton gradient, when coupled with ATP synthase, produces 28 ATP molecules and together with the ATP molecules produced during glycolysis and the TCA cycle results in a grand total of 32 ATP molecules. Dotted boxes represent major metabolic pathways, lined boxes represent total production from 1M of glucose.



Figure 42. Illustration of the metabolism of glucose and the theoretical ATP production and RQ quotient of P. angustum S14 when going through the glyoxylate shunt. Carbon is reserved and results in the respiration of 2CO₂ molecules and the production of 8NADH and 2FADH₂ which donate their electrons to the electron transport chain resulting in the consumption of 5O₂ and leads to an RQ value of 0.4 (2:5). The formed proton gradient, when coupled with ATP synthase, produces 23 ATP molecules and together with the ATP molecules produced during glycolysis results in a grand total of 25 ATP molecules. Dotted boxes represent major metabolic pathways, lined boxes represent total production from 1M of glucose. Note that the glyoxylate shunt splits citrate into both malate and succinate which each go on to produce 2 NADH with 4 NADH produced in total.

5.2 Proteorhodopsin

<u>Global Summary</u>

• Is proteorhodopsin upregulated under Fe-limiting conditions? No. Upregulation of proteorhodopsin occurs as cells approach the stationary phase under both Fe-replete and Fe-limiting conditions.

• If so, does this result in an improved growth and respiration rate?

No. Proteorhodopsin is not expressed during the exponential growth phase.

While the abundant distribution and concentration of proteorhodopsin in heterotrophic bacteria $(\pm 75\%)$ residing within the surface waters of the ocean strongly suggests its relevance and ecological importance (Gomez-Consarnau et al., 2019), the overall assumption that proteorhodopsin is utilized to produce energy and maintain basal metabolism within a heterotrophic bacterial cell remains to be verified within a global context.

Our work around proteorhodopsin in *P. angustum* S14 adds to the growing body of culturework that aims to resolve the function of proteorhodopsin under various environmental conditions. But while culture work has been instrumental in elucidating the genetic potential of heterotrophic bacteria, a major goal will be to extrapolate these results towards larger scales and analyse their implication towards biogeochemical fluxes of carbon and energy. The construction of a proton motive force from proteorhodopsin can theoretically impact the carbon cycle in two ways (Gomez-Consernau et al., 2016). If proteorhodopsin is utilized for the production of ATP, lower carbon requirements (respiration rates) will be required to maintain energy levels within the cell. Secondly, proteorhodopsin could be used to enhance the uptake of solutes and subsequently result in an increased contribution of heterotrophic bacterial populations towards global respiration rates.

Currently, the upregulation of proteorhodopsin and the resulting proton gradient, is predicted to provide a wide array of functions, rather than a single function (Fuhrman et al., 2008), and under this prediction, the exact function of proteorhodopsin in *P. angustum* S14, despite its

^{* *}determined from retinal concentrations

upregulation will become particularly difficult to discern. Furthermore, our results show that the upregulation of proteorhodopsin occurs as cells approach the stationary phase and is largely suppressed when cells were in the exponential growth phase in either Fe-replete and Felimiting conditions. This is an observation that seems to be consistent with several strains from the Vibrionaeceae family (Wang et al., 2012; Akram et al., 2013) and supports the notion that proteorhodopsin, at least for several bacterial strains, is facultatively expressed and thus tightly regulated instead of constitutively expressed in the presence of light. Our results further highlight that growth on carbon sources other than glucose (acetate and serine) resulted in an enhanced sensitivity to light and the presence of light had a negative impact on the growth and respiration rate of *P. angustum* S14. With regards to Fe-limitation, these results can be of particular interest as Fe-limitation is known to affect both the quantity but also the quality of the carbon that can be utilized by heterotrophic bacteria residing within the ocean (Obernosterer et al., 2015; Kirchman, 2000). Our results support the notion that the investment of proteorhodopsin must be high before it can sufficiently start covering maintenance costs within an average heterotrophic bacterium (Kirchman and Hanson, 2012) and further postulates that, for some bacterial strains, the potential benefit of proteorhodopsin will only occur under certain growth conditions (namely the stationary phase) and is only feasible in conditions where light does not become an inhibiting factor on growth.

Within the surface waters of the ocean, the majority of heterotrophic bacteria are considered to be present in a starvation (stationary) state until specific nutrients become available for them to grow (Morita, 1997). While our results show the upregulation of proteorhodopsin in *P. angustum* S14 entering the stationary phase in both Fe-replete and Fe-limiting conditions whether proteorhodopsin is utilized to increase the survivability of *P. angustum* S14 is still unknown. To verify this, starvation experiments similar to those conducted by Gomez-Consarnau (2010) under carbon starvation (Figure 42a) and recovery experiments conducted by Holmquist and Kjelleberg (1993) can be carried out in order to test the effect of proteorhodopsin in *P. angustum* S14 (Figure 42b). For these experiments, *P. angustum* S14 are exponentially grown and harvested (by centrifugation) before being subjected and grown in Aquil medium in the absence of carbon or Fe. *P. angustum* S14 is hereby forced to enter the stationary phase either as a result of Fe-limitation or carbon-limitation. Cells are then grown in continuous dark or light conditions and the decline in cell counts is measured over time. If proteorhodopsin is upregulated to cover maintenance costs of cells a higher percentage of *P.*

angustum S14 is expected to survive in the presence of light compared to dark conditions. To confirm that any differences observed between light and dark conditions is a direct result of proteorhodopsin, Δpr is grown in parallel. After several days, recovery experiments, where cells starved of Fe or carbon are reintroduced to Aquil medium containing both Fe and carbon concentrations can further discern whether proteorhodopsin can aid a cell.



a) survival assay

Figure 43. Predicted outcome of (a) survivability assay derived from experiments conducted by Gomez-Consarnau et al (2010) where exponentially growing P. angustum S14 and Δpr are harvested from Fereplete conditions, centrifuged and washed 2x in Aquil medium before subjecting cells to fresh Aquil medium without Fe or without carbon in the presence (light grey) or absence (black) of light. Cells are hereby forced to enter a stationary phase and the decline in bacterial cell counts is measured over a period of time. After several days, (b) recovery assays derived from experiments conducted by Holmquist and Kjelleberg (1993) are conducted where starved P. angustum S14 and Δpr are regrown in Aquil medium containing carbon and Fe-replete conditions in the presence (light grey) or absence (black) of light.
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VII. RESUMÉ (FR) 10pg

Le fer (Fe) est un élément essentiel à la croissance des microorganismes marin, mais il est présent à l'état de traces (<0,1 nM) dans les eaux de surface de l'océan. Compte tenu de son importance biologique dans la faible productivité de vastes régions océaniques à haute teneur en éléments nutritifs faibles en chlorophylle (HNLC) son rôle a été bien documenté. Toutefois l'étude de la disponibilité en fer s'est essentiellement concentrée sur le phytoplancton en raison de son rôle dans la fixation du carbone par la photosynthèse. Les recherches actuelles sur la limitation en Fe portent donc principalement sur le plancton photosynthétique, en raison de leur contribution majeure à la pompe à carbone biologique. En revanche, le rôle de la limitation du fer sur les bactéries hétérotrophes, qui joue un rôle important dans le cycle biogéochimique du carbone en tant que principal responsable de la reminéralisation de la matière organique dissoute, a été beaucoup moins étudié.

Chez les bactéries hétérotrophes, la limitation en Fe affecte particulièrement la production d'ATP car le Fe est un cofacteur essentiel pour les enzymes impliquées dans la chaîne de transport d'électrons ainsi que dans le cycle de l'acide tricarboxylique (TCA). La limitation en Fe réduit donc considérablement la production d'énergie intracellulaire, ce qui affecte à la fois la croissance bactérienne et la respiration. À une échelle plus globale, l'impact de la limitation en Fe sur les bactéries hétérotrophes affecte l'efficacité de la reminéralisation du carbone organique dans l'océan.

Les bactéries hétérotrophes sont connues pour présenter une certaine plasticité physiologique lorsqu'elles sont soumises à des facteurs de stress environnementaux. Dans le cas de la

limitation en Fe, une bactérie peut choisir de réduire son quota de Fe en diminuant sa dépendance aux enzymes contenant du Fe. Ceci peut être réalisé de plusieurs manières : En régulant négativement le métabolisme et / ou certaines enzymes contenant du Fe, en remplaçant diverses enzymes contenant du Fe par des enzymes ne contenant pas de Fe ou en remodelant son métabolisme pour l'éloigner des voies riches en Fe.

L'objectif de ce manuscrit de thèse était d'utiliser une bactérie modèle cultivable pour discerner et tester si la régulation à la hausse de voies métaboliques spécifiques peut être utilisée par une cellule bactérienne hétérotrophe lorsqu'elle est soumise à une limitation en Fe. Plus précisément, cette thèse met l'accent sur l'avantage potentiel de deux de ces mécanismes. Premièrement, l'induction observée du Shunt glyoxylate sous la limitation du Fe comme voie métabolique alternative, et deuxièmement, la notion selon laquelle la protéorhodopsine peut être régulée positivement lorsque les cellules bactériennes (photo)hétérotrophes rencontrent des niveaux d'énergie plus faibles en présence de la limitation du Fe. Nous avons choisi une souche de bactérie hétérotrophe dont le génome avait déjà été séquencé et qui a permis la mise en œuvre d'outils génétiques afin de produire à la fois un biorapporteur du ou des gènes d'intérêt et knock-out des mêmes gènes pour les expériences de comparaison. Les deux approches qui ont été étudiés et testés dans ce manuscrit de thèse sont brièvement décrits ci-dessous.

Etant donné que la limitation en Fe affecte particulièrement les bactéries hétérotrophes au sein de la chaîne de transport d'électrons respiratoire, la limitation en Fe a un impact important sur la production globale d'énergie intracellulaire. L'effet indirect de la limitation en Fe sur la production d'ATP a conduit à la recherche de mécanismes potentiels par lesquels une bactérie hétérotrophe marine peut maintenir la production d'ATP. La protéorhodopsine

est une pompe à protons activée par la lumière. Elle a été identifiée chez plusieurs bactéries hétérotrophes qui, si elle était couplée à l'ATP synthase, pourrait finalement générer un gradient de protons pouvant être utilisé dans la production d'ATP. Dans ce manuscrit de thèse, un biorapporteur et un knock-out de gène ont été construits pour tester l'idée que la protéorhodopsine pourrait être utilisée dans des conditions limitantes en Fe pour ainsi améliorer la croissance bactérienne.

Le shunt en glyoxylate est une voie métabolique alternative dans la cellule qui permet aux bactéries de contourner les étapes de production de NADH et de CO₂ dans le cycle du TCA. En règle générale, le rôle du shunt glyoxylate est de permettre à des réactions anaboliques de se produire lorsque des cellules bactériennes utilisent des composés à 2 atomes de carbone et des acides gras. Dans des conditions limitantes en Fe, la régulation à la hausse du shunt glyoxylate a été observée chez plusieurs bactéries marines, y compris l'abondante SAR11. Bien que ces observations suggèrent que la régulation à la hausse du Shunt glyoxylate soit une stratégie possible mise en œuvre par les bactéries marines hétérotrophes pour faire face à la limitation en Fe, la forte association du Shunt glyoxylate au métabolisme de composés spécifiques du carbone rend difficile l'identification directe de son induction en condition limitantes en fer. Plus récemment, l'induction du Shunt glyoxylate a été observée sous stress oxydatif et limitation en Fe chez la bactérie non marine Pseudomonas aeruginosa. Globalement, ces résultats impliquent que le shunt glyoxylate est utilisé pour des fonctions autres que l'utilisation de sources de carbone spécifiques telles que les composés à 2 carbones et les acides gras. Dans ce manuscrit de thèse, des expériences ont été conçues pour vérifier si l'induction du Shunt glyoxylate pouvait être favorable à une cellule bactérienne marine soumise à la limitation du Fe.

1. Approche expérimentaleh

Un rapporteur bioluminescent recombinant et un gène knock-out de P. angustum S14 ont été construits pour la protéorhodopsine (ptr-luc, pr) et l'isocitrate-lyase (icl-luc, icl), une enzyme clé du shunt glyoxylate

Les outils génétiques nous ont permis d'aborder deux questions clés:

1. Le gène d'intérêt (isocitrate lyase, protéorhodopsine) est-il régulé positivement dans des conditions limitantes en Fe?

2. Dans l'affirmative, cela entraîne-t-il une amélioration de la croissance et de la respiration?

Le dispositif expérimental a été réalisé dans des conditions typiques des expérience avec les traces de métaux afin de contrôler et d'éviter au maximum la contamination par le Fe.

2. Résultats

2.1. Le shunt glyoxylate

Le bioreporteur, Icl-luc, a été cultivé dans des conditions limitante en Fe par addition de concentrations croissantes de DFOB. Une augmentation forte et significative de l'expression de l'isocitrate lyase a été observée lors de la soumission du bioreporter icl-luc à des conditions limitant fortement le Fe. Bien que les taux de croissance et de respiration aient diminué à la fois pour le type sauvage et le knock-out Δ icl soumis à une forte limitation en Fe, ils étaient inférieurs de \pm 30% pour Δ icl par rapport au type sauvage (wt).



Figure 1. Expression de icl sous forme de luminescence pour 1000 cellules de icl-luc cultivées dans du glucose 6 mM [C]) en conditions limitantes en Fe par addition de concentrations croissantes de DFOB. Les mesures ont été prises à \pm 16 heures, coïncidant avec la phase exponentielle. Les valeurs aberrantes sont marquées par les points.

La complémentation de Δ icl avec l'isocitrate lyase a rétabli les taux de croissance et de respiration à ceux observés dans sauvage, confirmant en outre l'importance du shunt glyoxylate dans le cas d'une forte limitation en Fe. En raison de la nature omniprésente du shunt glyoxylate dans les bactéries marines, nos résultats nous ont amenés à proposer cette voie comme stratégie d'acclimatation importante pour les bactéries hétérotrophes marines soumises à la limitation du Fe, bien que cette régulation à la hausse puisse être spécifique à une espèce.



Figure 2. Les taux de croissance de WT (noir) et Δicl (blanc) sous différents niveaux de limitation de Fe par addition de DFOB 0 μ M, 10 μ M et 100 μ M ont été réalisés en triple. Ensuite, le logarithme naturel (Ln) a été utilisé afin de prendre en compte un ensemble de données asymétrique. Les points noirs représentent des points aberrants alors que les différences significatives entre WT et Acic sont marquées d'un astérisque, où: * p <0,05, *** p <0,005.



Figure 3. Les taux de respiration de wt (noir) et de Δicl (blanc) sous différents niveaux de limitation de Fe (Fe-rempli, 10 μ M DFOB et 100 μ M DFOB). Le logarithme naturel (Ln) a été utilisé afin de prendre en compte un ensemble de données asymétrique. Les différences significatives entre wt et Δicl sont marquées d'un astérisque, où:

* *p* <0,05, ** *p* <0,005, *** *p* <0,0005.

Proteorhodopsine

La régulation à la hausse de la protéorhodopsine dans des conditions de réplication de Fe s'est produite lorsque les cellules ont approché la phase stationnaire, mais était absente pendant la phase exponentielle. En phase stationnaire, une différence significative a également été observée entre des conditions de lumière continue et d'obscurité. En présence d'une limitation en Fe, la régulation à la hausse de la protéorhodopsine s'est également produite, mais aucune autre différence d'expression n'a été observée entre les conditions de lumière et d'obscurité. La limitation en Fe a entraîné une diminution à la fois de la croissance et du rendement cellulaire lorsque P. angustum S14 a été soumis à une plage de concentrations en Fe, mais aucune différence significative n'a été constatée en ce qui concerne la présence (wt) ou l'absence (Δpr) de la protéorhodopsine que ce soit avec une lumière constante ou dans des conditions d'obscurité constante. Nos résultats montrent donc que, dans la phase de croissance exponentielle, la protéorhodopsine chez P. angustum S14 n'est pas régulée positivement par la lumière dans des conditions non limitantes ou limitantes en Fe et n'affecte donc pas la vitesse de croissance et de respiration. Dans le cas de la croissance avec des sources de carbone autres que le glucose (acétate et sérine), P. angustum S14 était plus sensible à la lumière, la présence de lumière empêchant même la croissance de ces cellules avec l'acétate . Cela semble à une augmentation de la production d'espèces réactives de l'oxygène ROS en présence de lumière. Le bénéfice potentiel pour P. angustum S14 qui peut découler de la régulation à la hausse de la protéorhodopsine semble dépendre du carbone. Comme la protéorhodopsine est régulée positivement dans la phase stationnaire, les travaux futurs visant à élucider le rôle de la protéorhodopsine devraient se concentrer sur la phase stationnaire d'une cellule bactérienne.



Figure 4. Cellules PTR-luc cultivées dans des conditions non limitantes en Fe dans du milieu Aquil avec glucose [C] 6 mM, soumis à soit à l'obscurité (cercles gris foncé), soit à une lumière bleue constante (cercles blancs). (a) Luminescence totale où les lignes pointillées verticales représentent les pas de temps où le comptage des cellules a été effectué, (b) comptage des cellules et (c) luminescence par cellule. La luminescence totale montre une régulation à la hausse après ± 25 heures en présence de lumière et d'obscurité, ce qui coïncide avec le moment où les cellules se rapprochent de la phase stationnaire. En présence de lumière, la luminescence continue d'augmenter et atteint son maximum à ± 60 heures, ce qui coïncide avec un nombre de cellules légèrement élevé en présence de lumière, bien que cela ne soit pas significativement différent.



Figure 1. Cellules Ptrluc cultivées dans des conditions limitantes en Fe (50 μ M de DFOB) dans du milieu Aquil et du glucose 6 mM [C] soumis à des conditions d'obscurité (cercles gris foncé) ou à une lumière bleue constante (cercles blancs). (a) luminescence totale où les lignes pointillées verticales représentent les pas de temps où le comptage des cellules a été effectué, (b) comptage des cellules et (c) luminescence par cellule. La luminescence totale montre une régulation à la hausse après \pm 20 heures en présence de lumière et d'obscurité, ce qui coïncide avec le moment où les cellules se rapprochent de la phase stationnaire. Aucune différence claire entre les conditions de lumière et d'obscurité n'a été observée.

3. Discussion et conclusions

L'utilisation d'outils génétiques nous a permis d'aborder l'impact spécifique d'un gène sur une cellule bactérienne hétérotrophe soumise à une limitation en Fe. Les résultats de ce manuscrit de thèse ont contribué démontré la polyvalence des bactéries marines hétérotrophes pour faire face à la limitation en Fe et apportent de nouveaux résultats sur le rôle de la protéorhodopsine et du shunt glyoxylate dans l'environnement marin. Bien que les travaux sur des cultures aient contribué à élucider le potentiel génétique des bactéries hétérotrophes, un objectif majeur sera d'extrapoler ces résultats à plus grande échelle et d'analyser leur implication dans les flux biogéochimiques de carbone et d'énergie.

La régulation à la hausse du shunt glyoxylate dans des conditions limitantes en Fe devrait, en théorie, conduire à une réorganisation de la force motrice issue des protons produits dans la chaîne de transport d'électrons en évitant le complexe I, particulièrement affecté par la limitation en Fe, et privilegiant le complexe II (succinate déshydrogénase). En outre, l'utilisation différencié du shunt glyoxylate selon les conditions environnementales devrait également entraîner une variabilité de la respiration bactérienne, à la fois en termes de production de CO₂ et de consommation d'O₂. Cela n'a pas été vérifié dans notre cadre expérimental et, à notre connaissance, n'a pas encore été testé sur une bactérie hétérotrophe marine soumise à la limitation du Fe.

Dans les eaux de surface de l'océan, on considère que la majorité des bactéries hétérotrophes sont présentes en état de dormance en attendant que des nutriments spécifiques deviennent disponibles pour leur croissance. Nos résultats soulignent que le bénéfice potentiel de la protéorhodopsine ne se produira que dans certaines conditions de croissance (notamment la phase stationnaire) et n'est réalisable que dans des conditions où la lumière ne devient pas un

facteur inhibant la croissance. Afin de mieux élucider le rôle de la protéorhodopsine, des expériences de limitation et de récupération pourraient être menées pour voir si la protéorhodopsine peut être bénéfique à une cellule pour sa survie lorsqu'elle est privée de carbone ou de Fe et son aptitude à retrouver sa croissance lorsque ces éléments sont de nouveau présents.

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