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Siderophores in marine coastal waters and their relevance for iron uptake by phytoplankton: experiments with the diatom *Phaeodactylum tricornutum*

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ABSTRACT: Natural marine bacteria populations collected from nearshore waters produce different types of siderophores depending on the degree of iron limitation. These siderophores can facilitate iron uptake in the marine diatom *Phaeodactylum tricornutum*. Water samples from 15 stations along the Italian coast of the northwest Adriatic Sea were collected and filter fractionated (3.0, 0.8 and 0.2 µm). Siderophore production in the fractions was determined using cross-feeding experiments with siderophore-auxotrophic bacteria. At most stations sampled, bacteria collected in the 3.0 and 0.8 µm filters produced siderophores which stimulated growth in *Morganella morganii*, the indicator strain for α-keto/α-hydroxy acids. The results suggest that MGF ('Morganella-Growth Factor') production is common among filamentous and appendaged bacteria or strains associated with particles. Natural bacteria populations grown in iron-deficient media stimulated growth of all the indicator strains in the cross-feeding tests. Examples of known MGF which supply iron to *M. morganii* were tested for their ability to act as iron source for the marine diatom *P. tricornutum*. Iron uptake from ^{55}Fe-MGFs was measured in *P. tricornutum* cells grown in Fe-sufficient and Fe-deficient media. Unchelated iron ($^{55}FeCl_3$) and ^{55}FeEDTA were used as controls. The uptake of iron from the ^{55}Fe-MGF and $^{55}FeCl_3$ by Fe-deficient cells was higher (109 to 150 pgFe mg^{-1}) than from ^{55}FeEDTA (34 pgFe mg^{-1}). Similarly, Fe-sufficient *P. tricornutum* took up iron from the ^{55}Fe-MGF and $^{55}FeCl_3$ to the same extent (~50 pgFe mg^{-1}) while minimal uptake (8 pgFe mg^{-1}) was measured from FeEDTA. In growth experiments where iron-deficient diatom cells were incubated in media containing different sources of iron, e.g. $FeCl_3$, Fe-MGF and FeEDTA, a greater increase in number was observed in cells supplied with Fe-MGF. Further experiments also show that the uptake of Fe from MGF was enhanced by light and that a reduction step was involved in the uptake process. MGF also promoted the uptake of colloidal ferrihydrites. This study gives further evidence that siderophores produced by bacteria can be utilized by phytoplankton as an iron source. We therefore suggest that these substances play an important role in increasing the availability of iron to phytoplankton in coastal waters and thus are major factors defining the chemistry of iron in the marine environment.

KEY WORDS: Phytoplankton · Bacteria · *Phaeodactylum tricornutum* · Siderophores · Morganella-Growth Factor · Iron-limitation · α-keto/α-hydroxy acids

INTRODUCTION

Due to rich contributions from land and rivers, it is often assumed that there is always enough iron for primary production in coastal regions. However, the biological availability of iron in the marine environment is markedly decreased due to the tendency of ferric ion to be strongly hydrolyzed at the pH of seawater, making it inaccessible for algal growth. Moreover, coastal phytoplankton species exhibit higher iron requirements than oceanic forms (Brand et al. 1983, Murphy et al. 1984). Thus, despite the higher total iron concentra-

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tions in nearshore waters, phytoplankton and bacteria are potentially iron limited in these regions (Wells et al. 1995). The question of which species of iron in seawater are available for algal uptake has not yet been resolved. Colloidal iron, which is believed to be the form of iron in the dissolved pool, is not readily taken up by phytoplankton (Anderson & Morel 1982, Rich & Morel 1990, Wells et al. 1991). Colloidal iron must first be photoreduced or solubilised to become available for phytoplankton growth (Rich & Morel 1990, Johnson et al. 1994). Recent studies however have shown that about 99% of the dissolved iron in surface water is organically bound and that the concentration of organic iron chelators far exceed the concentration of dissolved iron (Gledhill & van den Berg 1994). The nature of these organic ligands is still largely unknown, but it has been proposed that their major source are marine microorganisms (van den Berg 1995): siderophores from bacteria and porphyrins from phytoplankton (Rue & Bruland 1997).

Siderophores are strong iron-binding ligands, the production of which has been demonstrated for many oceanic bacterial strains grown in iron-deficient media (Trick 1989, Reid & Butler 1991, Haygood et al. 1993). Bacteria associated with typical nearshore ecosystems have also been shown to produce hydroxamic acids (Estep et al. 1975). Trick et al. (1983) reported the release of hydroxamate-type siderophores by several marine eukaryotic phytoplankton, most notably *Prorocentrum minimum*. They found Csaky positive material (hydroxamate-type siderophores) in iron-limited media where phytoplankton was grown, after concentration in a ratio of 1:200. Neilands (1984) however pointed to a possible enhancement of the Csaky test when appropriate amino acids for cyclization are present. Furthermore, the hydroxamate compound was not tested with other chemical or microbiological methods to confirm this as a hydroxamate-type siderophore. We also tested several species of freshwater and marine microalgae for siderophore production using cross-feeding tests commonly employed in microbiology (Reissbrodt & Rabsch 1988) with siderophore auxotrophic bacteria. For all 20 species tested, including *P. minimum*, no evidence of siderophore production was found (Horstmann et al. unpubl.). We applied the same cross-feeding method to natural coastal bacterial assemblages and found evidence of the production of substances which promote the growth of the enterobacteriaceae *Salmonella typhimurium* as well as of *Aureobacterium flavescens* and *Morganella morganii* grown under iron-limited conditions. Each of these bacterial strains functions as an indicator for a specific type of siderophore, namely catecholates, hydroxamates and keto/hydroxy bidentate ligands, respectively. In this study we report on the production of substances which promote the growth of *M. morganii*. These substances are synthesized by the deamination of amino acids like leucine, valine, tryptophan and phenylalanine to produce α-ketoacids and, after reduction, α-hydroxy acids. Along with other substances like phenylpyruvate and indolpyruvate, α-keto and α-hydroxy acids enhance the growth of *M. morganii* by supplying it with iron. We did not attempt to characterize the exact nature of the substances which promoted growth of *M. morganii* in our experiments: hence we arbitrarily adopted the term 'Morganella-Growth Factor' (MGF) for these substances.

Many terrestrial and pathogenic bacteria strains have lost the ability to produce siderophores but have retained the ability to recognize and utilize siderophores produced by other microorganisms. The cross-utilization of siderophores among marine bacteria has been demonstrated (Trick 1989). The utilization of the microbial hydroxamate-type siderophores, ferrioxamine B and E, as an iron source by iron-deficient cells of the marine diatom *Phaeodactylum tricornutum* has been shown (Soria-Dengg & Horstmann 1995). Hutchins et al. (1999) reported the utilization of Fe from siderophores by marine eukaryotic phytoplankton, although to a lesser extent compared to the uptake of Fe from porphyrins. In the terrestrial environment, bacteria associated with the roots of some higher plants release siderophores capable of facilitating iron uptake by the rhizomes (Crowley et al. 1988, Barness et al. 1991). To investigate the ecological relevance of MGF produced by bacteria in seawater, we tested the ability of some known MGF to supply iron to the diatom *P. tricornutum* in uptake experiments using ^{55}Fe-labelled MGF.

Our results provide further evidence that microbial activity modifies the chemistry of iron in the marine environment by producing iron chelating substances capable of supplying iron to phytoplankton. Furthermore, a possible mechanism of how these substances facilitate Fe uptake by *Phaeodactylum tricornutum*, or generally by diatoms, is discussed.

METHODS

Collection of samples. During 2 separate samplings on board the RV 'Poseidon' in the northern Adriatic Sea, seawater was collected from the stations shown in Fig. 1. For the preliminary experiments (first sampling) natural bacteria populations were collected by filtering 500 ml of pre-filtered (10 µm) water samples onto 0.45 µm sterile nitrate cellulose filters. The filters were placed face down onto nutrient broth agar for marine bacteria and incubated for 24 h at 20°C. The natural populations which grew on the plates were transferred into sterile filtered (0.2 µm) seawater

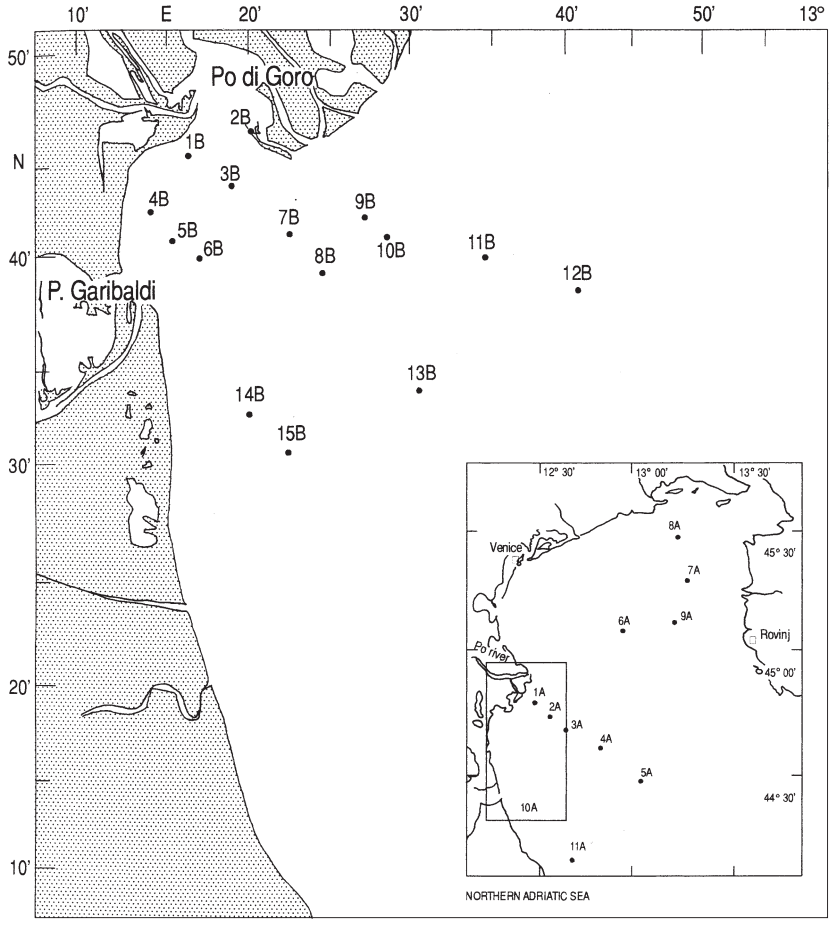

Fig. 1. Map of the northern Adriatic Sea showing the sampling stations for the cross-feeding experiments: (A) (inset) for the preliminary experiments where the bacteria were first grown in iron-deficient medium and (B) for the subsequent experiments where the bacteria were placed directly on the cross-feeding plates

which was collected from the sampling stations and had been previously deferrated by passing through a Chelex 100 column. The bacteria were allowed to grow for 24 h before the cross-feeding experiments were performed.

For the subsequent experiments (second sampling), surface water samples were collected from several coastal stations in the northern Adriatic Sea (Fig. 1).

The samples were collected by hand in acid-washed sterilized Nalgene bottles. To remove bigger sized particles, the water samples were pre-filtered (10 µm) prior to filter fractionation (3.0, 0.8, 0.2 µm) using nitrate cellulose filters. To allow the bacteria to grow, the filters were placed in sterile petri dishes at 20°C for at least 6 h before inoculating onto the cross-feeding plates.

Bacterial strains and growth medium. *Morganella morganii* SBK3, *Salmonella typhimurium* enb-7 and *Aureobacterium flavescens* JG-9 were obtained from the stocks of the Robert Koch-Institute in Wernigerode, Germany. *Aureobacterium flavescens* JG-9 was maintained in AM agar medium containing per liter: 2 g K_2HPO_4, 0.5 g $(NH_4)_2HPO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract, 10 g sucrose and 12 g Oxoid agar no. 1 (washed 5 times with Milli-Q water). Desferrioxamine B (obtained as Desferal® from Novartis Ltd, Basel, Switzerland) was added at a final concentration of 1 µg ml^{-1} and pH was adjusted to 7 by adding 1 N NaOH or 1 N HCl. *Salmonella typhimurium* enb-7 and *Morganella morganii* SBK3 were maintained in nutrient agar.

The cross-feeding plates were prepared according to Reissbrodt & Rabsch (1988). They were seeded with 3 ml of the indicator strains mentioned above.

Cross-feeding tests. For the preliminary experiments, the bacteria were concentrated by centrifugation and then introduced onto the cross-feeding plates seeded with specific indicator strains. For the subsequent experiments (second sampling), the filters with bacteria were placed face down onto the cross-feeding plates. The plates with *Salmonella typhimurium* enb-7 and *Morganella morganii* SBK3 were incubated for 24 h at 37°C and those with *Aureobacterium flavescens* JG9 at 30°C for 48 h. As controls for *S. typhimurium* enb-7 and *A. flavescens* JG9, ferrioxamine E was introduced onto a plate and for *M. morganii* SBK3 α-ketoisocaproic acid (0.1 µg $disc^{-1}$) was used.

Siderophore production by the test organisms was indicated by a growth zone around the filter or inoculum. The diameter of the growth zone was measured.

Siderophores and preparation of labelled complexes. The siderophores, α-hydroxyisocaproic acid, α-hydroxycaproic acid, α-hydroxyisovaleric acid and α-ketoisocaproic acid were purchased from Sigma Chemicals, St. Louis, MO, USA. $^{55}FeCl_3$ in 0.1 mM HCl was obtained from Amersham Co, Braunschweig, Germany. The procedure described by Müller & Raymond (1984) for labelling siderophores was employed. The siderophores were dissolved in sterile Milli-Q water to give a 1 mM stock solution. For the uptake experiments, 0.01 mM $^{55}Fe^{+3}$-siderophore and $^{55}Fe^{+3}$-EDTA were prepared with an activity of 5 µCi ml^{-1} at pH 7. The labelled complexes were prepared 12 h before the actual experiments to ensure complete reaction of Fe with the siderophores.

Preparation of algal cultures. *Phaeodactylum tricornutum* was obtained from the culture collection of

Table 1. Results of the cross-feeding tests of the preliminary experiments. Natural bacteria populations were collected from the field and grown in natural seawater medium previously deferrated by passing through a Chelex 100 column. Signs indicate the diameter of the growth zone around the inoculum (+:10–15 mm; ++:16–20 mm; –: no growth)

Stn	Indicator strain		
	Salmonella typhimurium enb-7	*Aureobacterium falvescens* JG-9	*Morganella morganii* SBK3
1A	++	++	++
2A	++	++	+
3A	++	++	++
4A	+	++	–
5A	++	++	++
6A	+	++	++
7A	++	++	++
8A	++	++	++
9A	+	++	++
10A	++	++	++
11A	++	++	++

the University of Göttingen, Germany. The cells were immediately transferred to a low iron (10 nM) AQUIL medium (Morel et al. 1979). The cells were transferred twice to low iron media and the last transfer was performed before the actual experiments to a medium with no added iron. Iron-sufficient cells were prepared in a medium containing 450 nM Fe bound to EDTA.

The axenic nature of the cultures was regularly monitored by inoculating onto nutrient plates for marine bacteria or by acridine orange staining (Hobbie et al. 1977).

Fe uptake experiments. Uptake experiments were done in 15 ml sterile polystyrol tubes with screw caps. These were filled with 5 ml iron-free AQUIL medium to which $^{55}Fe^{+3}$-siderophore, $^{55}Fe^{+3}$-EDTA or $^{55}FeCl_3$ was added to a final concentration of 0.4 µM. The iron-sufficient or iron-deficient cells were concentrated by centrifugation at 4000 rpm (3500 × *g*) for 15 min and resuspended in a small amount of iron-free AQUIL. These were introduced to the tubes and incubated under cool fluorescent light for 3 h at 20°C.

After incubation, the cells were filtered onto nitrate cellulose filters (0.45 µm) which were pre-soaked for 24 h in 1 mM EDTA with a pH of 5. To remove extracellularly adsorbed Fe, the citrate wash suggested by Hudson & Morel (1989) was employed. The filters were placed in vials and soaked in filter count scintillation fluid. Activity was counted with a Hewlett-Packard scintillation counter.

Iron uptake from $^{55}FeCl_3$ and $^{55}Fe^{+3}$-α-ketoisocaproic acid was also measured in the presence and absence of light and by using colloidal ferrihydrites. Two forms of colloidal ferrihydrite were prepared by heating $FeCl_3$ solution at 50°C (50°C FERR) and 20°C (20°C FERR) and then rapidly cooled (Wells et al. 1991). The prepared ferrihydrites were then bound to α-ketoisocaproic acid and allowed to stand overnight. The preparation of the experiments was the same as described for the uptake experiments. However, since the results showed that iron-sufficient cells also incorporated iron bound to α-ketoisocaproic acid, the cells used for the later experiments were not previously starved of iron. For the uptake experiments in the absence of light the incubation vials were individually wrapped in dark foil and were placed in a rack which was also covered with a dark non-transparent foil. The darkened (dark treatment) and non-darkened (light treatment) vials were incubated for 3 h in simulated sunlight.

Table 2. Results of cross-feeding tests from representative stations during the second sampling. Natural bacteria populations were collected from the field and filter fractionated. The bacteria were allowed to grow at ambient temperature for 6 h before incoculation onto the bioassay plates. Signs indicate the diameter of the growth zone around the inoculum (+: 10–15 mm; ++: 16–20 mm; +++: 21–25 mm; –: no growth)

Stn	Salinity (‰)	Temp. (°C)	Indicator strain	Filter pore size (µm) 3.0	0.8	0.25
1B	22.6	17.4	SBK3	++	–	–
			enb-7	+	–	–
			JG-9	–	–	+
2B	14.5	18.0	SBK3	–	–	–
			enb-7	–	–	–
			JG-9	–	–	–
5B	12.3	18.9	SBK3	+++	–	–
			enb-7	+	+	–
			JG-9	–	–	–
7B	17.0	20.9	SBK3	+	–	–
			enb-7	–	–	–
			JG-9	–	–	–
9B	26.5	19.2	SBK3	+++	+	–
			enb-7	–	–	–
			JG-9	–	–	–
11B	30.2	18.9	SBK3	+	–	–
			enb-7	–	–	–
			JG-9	–	–	–
12B	15.7	20.4	SBK3	+	–	+
			enb-7	–	–	–
			JG-9	–	–	–
13B	18.6	21.2	SBK3	–	–	–
			enb-7	–	–	–
			JG-9	–	–	–
14B	10.9	21.9	SBK3	+++	–	–
			enb-7	++	+	–
			JG-9	–	–	–
15B	15.9	22.9	SBK3	+++	+	–
			enb-7	+	–	–
			JG-9	–	–	–

Ferric reduction. We used the strong Fe^{+2} binder bathopenanthroline disulfonic acid (BPDS) to test if reduction of Fe from α-ketoisocaproic acid is a prereq-

uisite for cellular uptake. In addition to *Phaeodactylum tricornutum*, we also used the marine green alga *Tetraselmis chuii*. To non-iron-limited cultures of the 2 microalgae species, ^{55}Fe bound to α-ketoisocaproic acid was added. Then BPDS was added at a ratio of 1:1 with the Fe-siderophore complex. The rest of the experiment was conducted as described above for the uptake set up.

The reduction of Fe bound to MGF was quantified spectrophotometrically with the ferrous color reagent BPDS as decribed by Allnutt & Bonner (1987). Iron-starved *Phaeodactylum tricornutum* cells were concentrated by centrifugation at 4000 rpm (3500 × *g*) for 10 min. The cells were collected and resuspended in 10 ml of culture medium without the iron source. The cells were allowed to equilibrate in the tubes to dark or light conditions for 12 h prior to the addition of 400 µM BPDS. An equilibration time of 3 min was allowed after the BPDS addition. The iron source with a concentration of 2 µM was added as Fe^{+3}-α-ketoisocaproic acid or $FeCl_3$. For the dark treatment all the steps were done in the dark, with the tubes always covered in dark foil. The absorbance was measured after 2 h for color development at 535 nm, with 610 nm as reference wavelength.

To determine if the cell membrane is involved in the reduction of Fe^{+3} bound to MGF, the same procedure as described above was done in tubes not containing diatom cells.

Growth experiments. Growth stimulation of *Phaeodactylum tricornutum* by α-ketoisocaproic acid in iron-sufficient and iron-deficient conditions was investigated using cells previously grown in Fe-limited SOW (artificial seawater) medium containing 10 nM Fe and 100 mM EDTA. The cells were harvested after 14 d and transferred to polycarbonate bottles containing the prepared experimental medium (Price et al. 1989). This contained other metals at the following concentrations:

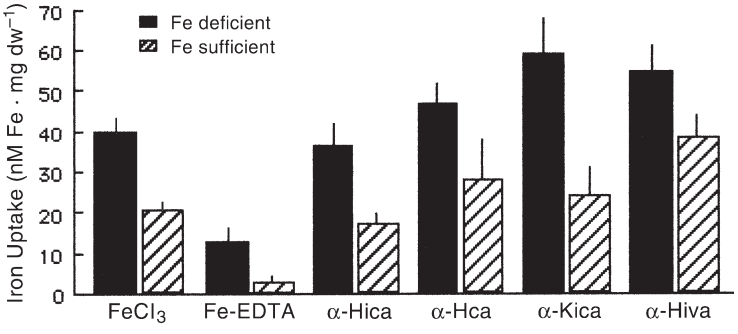

Fig. 2. ^{55}Fe uptake from different MGF (α-keto/α-hydoxy bidentate ligands) by *Phaeodactylum tricornutum* cells grown in iron-deficient and iron-sufficient media. $FeCl_3$ and FeEDTA were used as controls. The cells were incubated for 3 h at 20°C under cool fluorescent light. MGFs used were α-hydroxycaproic acid (Hca), α-hydroxyisovaleric acid (Hiva), α-hydroxy-iso-caproic acid (Hica) and α-ketoisocaproic acid (Kica) (6 replicates and 1 SD)

Table 3. Uptake of iron (nM mg dw^{-1}) from MGF in 2 species of microalgae (iron replete) with and without addition of BPDS (nd: not detected)

	No added BPDS	With BPDS
Phaedactylum tricornutum	12.3 ± 2.59	nd
Tetraselmis chuii	6.23 ± 2.58	nd

Mn = 23 nM; Zn = 4 nM; Co = 2.5 nM; and Cu = 1 nM. $FeCl_3$ was added at 500 nM for the iron-sufficient conditions and at 50 nM for the iron-deficient conditions. Iron was added either as $FeCl_3$, Fe-α-ketoisocaproic acid or Fe-EDTA, the latter prepared in stoichometric amounts. Fe-α-ketoisocaproic acid and Fe-EDTA were first prepared before addition to the culture medium. α-ketoisocaproic acid as a representative of the Keto-hydroxy bidentate ligands (KHBLs) was added at a concentration 20-fold to that of Fe, and EDTA concentration was 100 mM. The cultures were incubated at 18°C under continuous light. Growth was measured as the increase in the number of cells.

RESULTS

Cross-feeding experiments

In the preliminary experiments, positive bioassays for all indicator strains were obtained in the cross-feeding tests. Natural bacteria populations collected from all the stations, except for *Morganella morganii* SBK3 at Stn 4A (Table 1), produced growth zones around the inoculum when introduced onto the cross-feeding plates after being grown under iron-limiting conditions.

This suggests that natural bacteria populations are capable of producing catecholate-type, hydroxamate-type siderophores and α-keto-/α-hydroxy acids. In the subsequent experiments where the samples were collected from stations nearer the shore and the samples were immediately introduced onto the bioasay plates after fractionated filtration, positive results were obtained from 10 of the 15 stations for *Morganella morganii* SBK3. At 4 stations positive tests were obtained for *Salmonella typhimurium* enb-7 and at only 1 station for *Aureobacterium flavescens* JG-9 (results from representative stations are shown in Table 2). This indicates that α-keto and α-hydroxy acid production is common among coastal bacterial strains.

Most of the positive results for *Morganella morganii* SBK3 were obtained in the larger fractions (3.0 and 0.8 µm) and for *Aureobacterium*

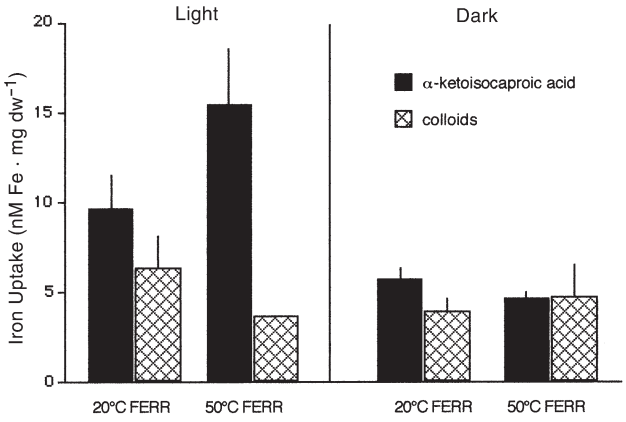

Fig. 3. ^{55}Fe from different colloidal ferrihydrites (20°C FERR and 50°C FERR) by iron-sufficient *Phaeodactylum tricornutum* cells in the presence and absence of light. The ferrihydrites were introduced either as colloids or bound to α-ketoisocaproic acid. The cells were incubated for 3 h at 20°C under cool fluorescent light (6 replicates and 1 SD)

flavescens JG-9 only in the smallest fractions (0.2 µm). No correlation was found between siderophore production and salinity (11 to 33 ppt) and temperature (17 to 23°C) as well as to the distance of the station from the shore (Table 2, Fig. 1).

We have not isolated and characterized the substances which induced growth in *Morganella morganii*; hence we used the general term Morganella-Growth Factor (MGF).

Fe uptake from the siderophores

We used different α-keto and α-hydroxy acids in the laboratory as iron source for the diatom *Phaeodactylum tricornutum*. α-hydroxycaproic acid, α-hydroxyisocaproic acid, α-hydroxyisovaleric acid and α-ketoisocaproic acid are known to promote growth of *Morganella morganii* SBK3 by supplying it with iron (Thieken & Winkelmann 1993). Iron-deficient and iron-sufficient *P. tricornutum* cells showed the ability to take up iron from all the MGFs used (Fig. 2). Iron uptake from α-ketoisocaproic acid and α-hydroxyisovaleric acid was significantly higher than from α-hydroxyisocaproic acid and α-hydroxycaproic acid as well as from $^{55}FeCl_3$. The uptake of iron from $^{55}FeCl_3$ was comparable to that from α-hydroxycaproic acid and α-hydroxyisocaproic acid. In the concentration used, FeEDTA acts as a poor iron source. The results also showed that iron-sufficient cells are capable of taking up iron from the MGF, but to a lower extent compared to the iron-deficient cells. This however may be explained by the lower iron requirement of the iron-sufficient cells than by activation of special iron uptake mechanisms by the iron-deplete cells.

Table 4. Ferric reduction (µm $FeBPDS_3$) as determined by BPDS method performed with and without cells and in the presence and absence of light. The cells were grown under either iron-replete or -deplete condition. Fe^{+3} reduction is expressed as amount of $FeBPDS_3$ generated (α-Kica: α-ketoisocaproic acid; nd: not detected)

Iron source	With cells		No cells	
	Light	Dark	Light	Dark
Fe-deplete				
α-Kica	3.26	1.65	0.55	0.40
$FeCl_3$	0.61	nd	nd	nd
Fe-replete				
α-Kica	2.60	1.51	0.0014	nd
$FeCl_3$	0.33	nd	nd	nd

Uptake in dark and light

Although Fe uptake from α-ketoisocaproic acid was also observed in the dark, light seems to enhance the uptake of iron from this siderophore (Fig. 3). The 2 forms of colloidal iron we used were taken up more readily by *Phaeodactylum tricornutum* when bound to α-ketoisocaproic acid and enhanced uptake was observed in the presence of light (Fig. 3). We did not attempt however to find out if the MGF acts as a reductant for iron.

Ferric reduction

No measureable uptake of Fe from the siderophore was found in *Phaeodactylum tricornutum* and *Tetraselmis chuii* if BPDS, a strong Fe^{+2}-binder, was added to the medium (Table 3). This implies that uptake occurs after extracellular reduction of the nutrient.

The results of the BPDS experiments show that the cell membrane is involved in the reduction of iron from the MGF. In the absence of cells, $FeBPDS_3$ was either not detected or was present only in negligible concentrations (Table 4). The importance of light in the reduction of iron bound to MGF can be seen in the results obtained from the experiments. More $FeBPDS_3$ was measured when the Fe was bound to the MGF than $FeCl_3$, which indicates that photoreduction of Fe^{+3} is enhanced by the presence of the siderophore.

Growth experiments

At low iron concentrations, MGF is an efficient iron supplier for *Phaeodactylum tricornutum* cells as

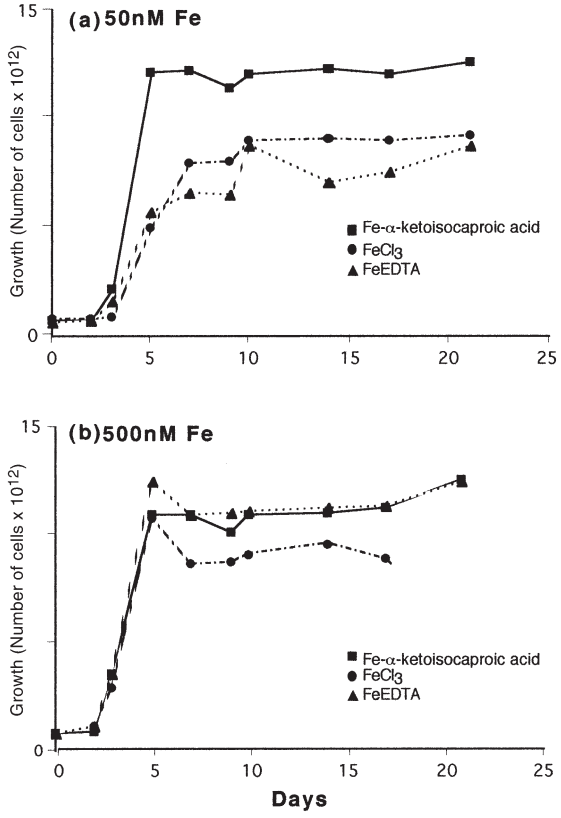

Fig. 4. Growth of *Phaeodactylum tricornutum* cells previously grown under iron-deplete conditions and then transferred to media containing either (a) low iron (50 nM Fe) or (b) high iron (500 nM). The nutrient was added either as $FeCl_3$, FeEDTA or Fe-α-ketoisocaproic acid. Incubation was at 20°C under cool fluorescent light

shown by the significantly higher growth rate in the medium containing Fe^{+3}-α-ketoisocaproic acid than in the media with $FeCl_3$ or Fe^{+3}-EDTA (Fig. 4a). However, at high iron concentrations, the growth rate of the diatom was not influenced by the form of iron in the medium (Fig. 4b). The growth rates of *P. tricornutum* at 50 and 500 nM Fe^{+3}-α-ketoisocaproic acid do not differ significantly, which leads to the assumption that the lower iron concentration was enough to fulfill the requirement of the cells for growth. Thus when the concentration of iron in the medium becomes limiting, the presence of MGF could be allievating by making sufficient amounts of this nutrient available to sustain growth.

DISCUSSION

In this study we have presented evidence for the production of a siderophore, which we termed MGF, by natural bacteria populations in coastal waters. We have also shown that this siderophore type enhances the availabilty of iron for uptake by phytoplankton. The production of this type of siderophore by natural bacteria populations has not been reported. The results of our preliminary experiments give evidence that marine bacteria are capable of producing MGF as well as hydroxamate- and catecholate-type siderophores when grown under iron-deficient conditions. Although some freshwater bacterial strains are capable of surviving in marine media, the samples we collected were first incubated in iron-deficient marine media before inoculation onto the cross-feeding plates. The production of hydroxamate-type siderophores has been shown in several marine eubacteria strains (Goyne & Carpenter 1974, Trick et al. 1983, Reid & Butler 1991, Haygood et al. 1993).

In the subsequent cross-feeding experiments (second sampling), the bacteria directly collected from the sea produced MGF. There is only a minor possibility that these siderophores were produced by bacteria of freshwater or terrestrial origin which could have temporarily survived higher salinities. So far we have not encountered literature on the survival ability of the *Proteus-Providencia-Morganella* group in saline medium. However, other strains such as *Escherichia coli* show rapid mortality at salinities higher than 25 ppt (Carlucci & Pramer 1959, Ayres 1977), although some faecal coliforms are less affected by salinity ranges from 15 to 40 ppt than at 7 to 15 ppt (Solic & Krstulovic 1992). Recently Reissbrodt et al. (1997) has found *E. coli* and *Salmonella typhimurium* strains capable of producing α-keto and α-hydroxy acids under different iron-limited conditions. Palenik & Morel (1990) found that marine phytoplankton can produce α-keto acids as a by-product of amino acid utilization. However, not all α-keto acids have siderophore properties (Drechsel et al. 1993) and we have found in a separate study using several species of marine microalgae that these are not capable of producing such siderophores.

The positive assay we observed for *Salmonella typhimurium* enb-7 indicates catecholate-type as well as hydroxamate siderophore production. MGF production and utilization by *S. typhimurium* enb-7 has also been observed under certain conditions of iron deficiency (Reissbrodt et al. 1997). It is possible that coastal marine bacteria are capable of producing and utilizing the 3 types of siderophores we tested for.

The α-keto and α-hydroxy bidentate ligands, the MGF we used in the laboratory experiments, are weak iron binders compared to other types of siderophores produced by bacteria (Drechsel et al. 1993). This is consistent with our findings. MGF was mainly produced in samples from nearshore stations. Coastal bacteria may not find the necessity to produce stronger iron chelators because of the relatively higher con-

centrations of iron in the environment compared to oceanic waters. However, marine bacteria which were grown in iron-deficient media released hydroxamates, which are stronger iron chelators in addition to MGF, which was probably a reaction to an increase in iron deficiency. In spite of the lower affnity of MGF for iron compared to hydroxamate siderophores, the uptake of Fe in iron-deficient *Phaeodactylum tricornutum* cells was higher from MGF (150 pg mg^{-1}) compared to uptake from either ferrioxamine B (68 pg mg^{-1}) or ferrioxamine E (117 pg mg^{-1}) (Soria-Dengg & Horstmann 1995). This shows the remarkable efficiency of MGF in supplying iron to phytoplankton.

In bacteria, production and utilization of MGF are induced by low iron concentrations in the medium (Drechsel et al. 1993, Thieken & Winkelmann 1993, Reissbrodt et al. 1997). However, at least for *Phaeodactylum tricornutum*, utilization of MGF-bound iron is not influenced by low iron conditions. Thus, MGF may be the most relevant type of siderophore in coastal waters. It is possible that the ambient iron concentrations in nearshore environments are already limiting for natural bacteria populations. This induces siderophore production, which makes iron more available for uptake by the otherwise non-iron-limited phytoplankton.

It is interesting to note that MGF production is found mostly in the larger fractions (3.0 to 0.8 µm). This may indicate that filamentous and appendaged bacteria and some strains associated with particles are responsible for MGF production. Smaller free-living marine bacteria may not be capable of producing MGF but produce other types of siderophores, such as hydroxamic acids (Stn 1B). Trick (1989) found hydroxamic acid production in several strains of marine free-living bacteria. Drechsel et al. (1993) reported that the type of MGF produced by bacteria belonging to the *Providencia-Proteus-Morganella* group depends on the culture medium in which they were grown. In a 'minimal medium' preference for α-hydroxy acid synthesis was observed, whereas in a medium with high concentrations of amino acids, α-keto acids were produced. Bacteria attached to particles may preferably synthesize MGF of the α-keto form because of the availability of sufficient substrates. If this is the case, this would present an advantage to phytoplankton since, similar to *Morganella morganii* (Thieken & Winkelmann 1993), *Phaeodactylum tricornutum* cells also preferred iron bound to the α-keto form than from the α-hydroxy analog of isocaproic acid. This implies that in the coastal environment the availability of iron for phytoplankton uptake may be enhanced because there would be a higher concentration of substrate for the synthesis of α-keto acids. If the bacteria in coastal environments are equipped with the ability to synthesize different types of siderophores, the type of siderophore produced would depend on the iron conditions in the environment. MGF production may thus be preferred when iron limitation is not extreme and sufficient substrate for deamination is available. In oceanic waters, where substrates for MGF synthesis are rather scarce and iron concentrations are lower, siderophore types which possess higher affinity for iron like hydroxamic acids may be produced. A further study on the particle-associated bacteria community is necessary to determine the role of these strains in siderophore production in coastal waters.

The observations made in the iron uptake experiments demonstrate the relevance of MGF in supplying Fe not only to iron-deficient but also to iron-sufficient *Phaeodactylum tricornutum* cells. The results suggest that low-iron induceable uptake systems are not involved in the transport of iron from this source. This is different from the results obtained for other microbial siderophores, ferrioxamine B and E for the same alga species, where iron uptake from these chelators appears to be induced by low cellular iron levels (Soria-Dengg & Horstmann 1995).

It is possible that MGF transport iron into the cells via a similar mechanism as $FeCl_3$, i.e. by ligand exchange (Hudson & Morel 1990, 1993) after reduction by non-specific reductases on the cell surface (Weger 1999). However, MGF are more efficient suppliers of iron than $FeCl_3$. As our results indicate, MGF facilitates the reduction of Fe^{+3}, a necessary prerequisite for iron uptake in phytoplankton. The higher uptake observed in iron-deficient compared to iron-sufficient cells can be explained by the increase in the number of exchange ligands on the membrane of the cells grown under iron limitation (Hudson & Morel 1990) as a consequence of a higher Fe-demand of iron-deficient cells. In siderophore-producing bacteria like *Salmonella typhimurium*, iron bound to α-keto/α-hydroxy acids were actively taken up via *ton B* (Kingsley et al. 1996). Thus the mechanism for Fe uptake from MGF apparently differs in bacteria and in diatoms.

The exact mechanism by which iron bound to the MGF is taken up by *Phaeodactylum tricornutum* is not known although our results strongly point to a reduction step prior to internalisation. Light apparently plays an important role in this step. It seems that surface reductases as well as photoreduction are both involved in the reduction step since only a decrease and not a complete inhibition of uptake was observed in the dark. The involvement of surface reductases was indicated by Fe^{+2} production (measured as $FeBPDS_3$) only in the presence of cells. Even the not too available ferrihydrite was made labile by MGF and this is enhanced in the presence of light. Wells & Mayer (1991) reported an increase in the lability of colloidal

Fe oxyhydroxides with irradiation as a result of organic-dependent Fe photoreduction. In any case, MGF may well be one of the unknown organic ligands in seawater which were reported by Kuma et al. (1996) and Gledhill et al. (1998), which promote the photoreduction of Fe^{+3}. Siderophores produced by bacteria in the marine environment evidently play an important role in increasing the bioavailability of iron not only for the microorganism producing the siderophore but also for phytoplankton organisms which do not produce but can utilize siderophores (Soria-Dengg & Horstmann 1995). Our results show that α-keto and α-hydroxy bidentate ligands are the major type of siderophore produced by natural coastal bacterial assemblages and that these are produced even under the supposedly higher iron concentrations in coastal waters. The results of the uptake experiments suggest that phytoplankton are Fe 'pirates', enjoying the luxury of not investing energy for siderophore production but benefiting from those produced by bacteria.

Wells et al. (1995) proposed that biology influences the speciation of iron through the release of organic molecules which may bind iron or may mediate in redox processes on the cell surface. We have shown that siderophores are selectively released by bacteria in the environment most likely as a response to the iron conditions. The different siderophore types exhibit different properties (e.g. iron affinities and availability to phytoplankton) and thus may affect the transfer of iron from the dissolved to the particulate phase (Geider 1999). The development of quantitative methods for the determination of soluble iron-siderophore complexes in seawater is necessary for a better understanding of the role of bacteria in the chemistry of iron in the marine environment.

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