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Algae induce siderophore biosynthesis in the freshwater bacterium *Cupriavidus necator* H16

Colette Kurth · Ina Wasmuth · Thomas Wichard ⬚ · Georg Pohnert ⬚ · Markus Nett ⬚

Abstract Cupriachelin is a photoreactive lipopeptide siderophore produced by the freshwater bacterium *Cupriavidus necator* H16. In the presence of sunlight, the iron-loaded siderophore undergoes photolytic cleavage, thereby releasing solubilized iron into the environment. This iron is not only available to the siderophore producer, but also to the surrounding microbial community. In this study, the cupriachelin-based interaction between *C. necator* H16 and the freshwater diatom *Navicula pelliculosa* was investigated. A reporter strain of the bacterium was constructed to study differential expression levels of the cupriachelin biosynthesis gene *cucJ* in response to varying environmental conditions. Not only iron starvation, but also culture supernatants of *N. pelliculosa* were found to induce cupriachelin biosynthesis. The transcription factors involved in this differential gene expression were identified using DNA–protein pulldown assays. Besides the well-characterized ferric uptake regulator, a two-component system was found to tune the expression of cupriachelin biosynthesis genes in the presence of diatom supernatants.

Keywords Siderophore · *Cupriavidus necator* · Diatom · Interaction · Freshwater

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Introduction

Iron is involved in many biologically vital processes and is indispensable for virtually all forms of life (Andrews et al. 2003). It is highly abundant, yet hardly bioavailable in most ecosystems due to the formation of insoluble ferric oxides and hydroxides. Therefore, iron often represents a limiting factor for microbial growth. Microorganisms have evolved different strategies to overcome this challenge, one of the most well-known being the production of siderophores (Hider and Kong 2010; Kurth et al. 2016a). Siderophores are small molecular weight compounds that have a strong affinity towards ferric iron (Fe^{3+}). Once

secreted from the producing cell, they rapidly coordinate the metal and are then taken back up into the cell by active transport. In the cytoplasm, the iron is usually released from the siderophore by a reductive or hydrolytic mechanism (Schalk and Guillon 2013).

A different mode of siderophore-mediated iron acquisition was described from marine organisms. Some marine bacteria produce siderophores, of which the iron-bound complexes are prone to photolytic cleavage in the presence of sunlight. The complexed iron is thereby reduced and released into the environment (Barbeau et al. 2001). The production of photoreactive siderophores appears to be widely distributed in marine bacteria (Barbeau et al. 2001, 2002; Ito and Butler 2005; Martin et al. 2006; Homann et al. 2009; Amin et al. 2009b; Butler and Theisen 2010; Robertson et al. 2018). A characteristic of these molecules is the presence of an α-hydroxy-carboxylate function that confers the photoreactivity (Barbeau et al. 2001). Furthermore, many of these siderophores feature a fatty acid side chain. The latter was proposed to increase the residence time of the siderophore and to concentrate iron around the cell, which seems plausible in aquatic environments, where diffusion plays a significant role (Martinez 2000). Recently, photoreactive acyl siderophores have also been reported from freshwater bacteria. The finding of the cupriachelins (Kreutzer et al. 2012) and the variochelins (Kurth et al. 2016b) indicates that photoreactive siderophores are even more widespread in the environment than previously thought.

The light-induced release of soluble and, thus, bioavailable iron has a major impact on environmental iron cycling. Since aquatic bacteria often live in tight relationships with phytoplankton species (Kouzuma and Watanabe 2015; Seymour et al. 2017), siderophore-mediated biotic interactions are highly likely as well. Indeed, Amin et al. (2009b) demonstrated that the dinoflagellate *Scrippsiella trochoidea* utilizes iron, which is released from a photoreactive siderophore produced by associated bacteria. They further found that the bacterial siderophore producers receive exudates from the dinoflagellate, which serve as sources of carbon and energy. The described interaction was referred to as "carbon-for-iron" paradigm (Amin et al. 2009a) and can be regarded as mutualistic. It is conceivable that such iron sharing is a common phenomenon amongst plankton. Because iron represents a major limiting factor for the growth of

phytoplankton species (Blain et al. 2007), an association with producers of photoreactive siderophores would represent an important ecological advantage.

In this study, the cupriachelin-based interaction between the freshwater bacterium *Cupriavidus necator* H16 and the diatom *Navicula pelliculosa* was investigated. The two organisms were chosen, because members of the genera *Cupriavidus* and *Navicula* are known to co-occur in the same freshwater environments (Ferrero et al. 2004; Swanepoel 2015). Co-cultures of *C. necator* and *N. pelliculosa* can be maintained under laboratory conditions for at least two weeks. Furthermore, we had observed that the bacterium positively influences the growth of the diatom under iron deficiency (Fig. S1). We thus hypothesized that *N. pelliculosa* could trigger the production of cupriachelin and that the concomitant communication could rely on signal molecules secreted by the diatom (Fig. 1). To probe this scenario, we constructed a reporter of cupriachelin biosynthesis gene expression. We show that not only iron starvation but also culture supernatants of *N. pelliculosa* induce cupriachelin biosynthesis. Furthermore, we analyzed the transcription factors involved in the regulation of cupriachelin biosynthesis by DNA–protein-pulldown assays. In addition to the well-known ferric uptake regulator (Fur) a two-component system was found to tune the expression of cupriachelin biosynthesis genes.

Materials and methods

Growth conditions of algal and bacterial strains

Navicula pelliculosa was obtained from the SAG culture collection as an axenic culture (SAG 1050-3, Göttingen, Germany). The diatom was grown in static, non-shaking culture in cell culture flasks (50 mL/125 mL, Sarstedt) at 18 °C in modified WC medium (36.8 mg/L $CaCl_2 \times 2H_2O$, 37 mg/L $MgSO_4 \times 7H_2O$, 12.6 mg/L $NaHCO_3$, 11.4 mg/L $K_2HPO_4 \times 3H_2O$, 85 mg/L $NaNO_3$, 21.2 mg/L $Na_2O_3Si \times 5H_2O$, 115 mg/L TES, 4.36 mg/L $EDTANa_2$, 0.01 mg/L $CuSO_4 \times 5H_2O$, 0.022 mg/L $ZnSO_4 \times 7H_2O$, 0.01 mg/L $CoCl_2 \times 6H_2O$, 0.18 mg/L $MnCl_2 \times 4H_2O$, 0.006 mg/L $Na_2MoO_4 \times 2H_2O$, 1 mg/L H_3BO_3, 0.1 mg/L thiamine HCl, 0.0005 mg/L biotin, 0.0005 mg/L cyanocobalamin). Ferric chloride was
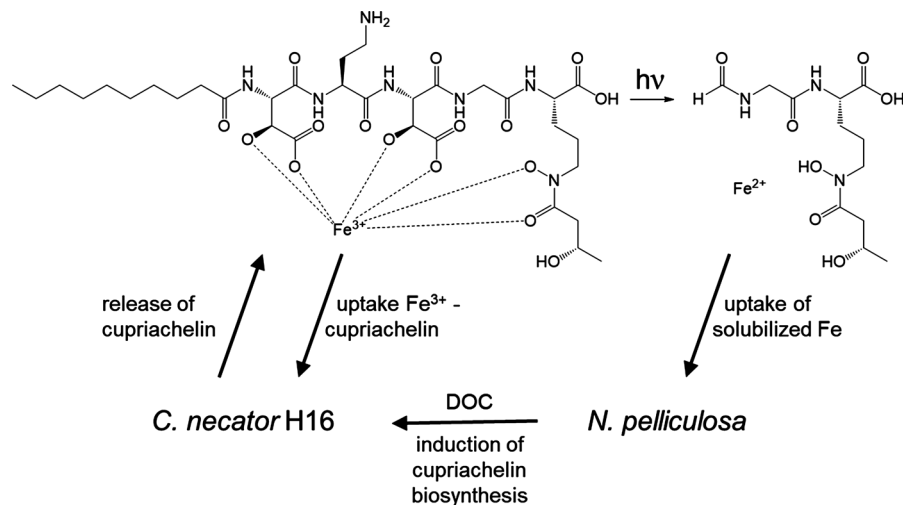
Fig. 1 Proposed mutualistic photoreactive siderophore-based iron sharing between bacteria and algae. *C. necator* H16 secretes cupriachelin under iron-deficient conditions. The ferrisiderophore will likely be taken up by *C. necator* H16 in the dark. In the light, however, the ferrisiderophore will undergo photolytic cleavage, thereby releasing Fe^{2+} into the environment. The solubilized iron is then also available to the surrounding planktonic community (e.g., *N. pelliculosa*). In exchange for the iron, the alga may provide the bacterium with dissolved organic carbon (DOC)

added to the medium at different concentrations corresponding to iron-limited (0.15 μM) and iron-replete (10 μM) conditions. The light was provided by a "JBL Solar Reptil Sun T8" tube covering the UV–Vis range from 200 to 800 nm. A light regime of 12:12 (light/dark) with 30–50 μmol/m^2/s light intensity was used. Growth was monitored by daily cell counts. For this purpose, micrographs were taken every day on ten randomly chosen spots of every cell culture flask, and the number of cells per monitored area (72230 μm^2) was counted using ImageJ (Schindelin et al. 2012). Seed cultures consisted of *N. pelliculosa* cells in their exponential growth phase and constituted 2% (v/v) of the final culture volume. Under these conditions, *N. pelliculosa* reached stationary phase after ~ 2 weeks. For cultivation of *C. necator* H16, H-3 mineral medium (2.3 g/L KH$_2$PO, 2.57 g/L Na$_2$HPO$_4$, 1.0 g/L NH$_4$Cl, 0.5 g/L MgSO$_4 \times$ 7H$_2$O, 0.5 g/L NaHCO$_3$, 0.01 g/L CaCl$_2 \times$ 2H$_2$O and 5 mL/L SL-6 trace element solution (0.1 g/L ZnSO$_4 \times$ 7H$_2$O, 0.03 g/L MnCl$_2 \times$ 4H$_2$O, 0.3 g/L H$_3$BO$_3$, 0.2 g/L CoCl$_2 \times$ 6H$_2$O, 0.01 g/L CuCl$_2 \times$ 2H$_2$O, 0.02 g/L NiCl$_2 \times$ 6H$_2$O, 0.03 g/L Na$_2$MoO$_4 \times$ 2H$_2$O)) supplemented with 1 g/L aspartate was used as standard growth medium. When required, iron was added as FeCl$_3$ to different concentrations. Cultures were vigorously shaken at 28 °C. For cultivation of *C. necator* H16 with culture supernatants of *N.*

pelliculosa, a 1:1 mixture of H-3 mineral medium and filter-sterilized (0.2 μM) algal culture supernatant supplemented with 2% fructose was used. The control consisted of modified WC medium supplemented with 2% fructose. The described cultivation conditions were used for β-galactosidase assays, as well as for DNA–protein pulldown assays. *Escherichia coli* strains were cultivated at 37 °C in lysogeny broth (LB). When required, antibiotics were added to the following concentrations: 100 μg ampicillin/mL and 25 μg chloramphenicol/mL.

Construction of reporter and control strains

Plasmids used in this study are listed in Table 1. The broad host range expression vector pRANGER-BTB-3 was used as the backbone for all constructed plasmids. First, *araC* was removed from the plasmid by *Bsa*XI digestion and religation yielding pCK01. The β-galactosidase gene *lacZ*, obtained from *Xmn*I digestion of pMLB1034 (ATCC 37222), was introduced into the *Eco*RV restriction site of the plasmid resulting in pCK02. Three different promoters were subsequently cloned in front of *lacZ*. (i) The arabinose-inducible promoter *araBp* was PCR-amplified along with *araC* from pRANGER-BTB-3 using the primers *araBp*_F (5′-cccgggtataggaacttcgaagcagctc-3′) and *araBp*_R (5′-cccgggttctcctttactcatgctagcc-3′).

Table 1 Plasmids used in this study and their features and origin

Plasmid	Features	Origin
pRANGER-BTB-3	pBBR1, *cat*, *araC*	Lucigen
pMLB1034	pMB1, *bla*, *lacZ*	ATCC
pGEX-6P-2	pBR322, *bla*, *tacp*	GE Healthcare Life Sciences
pCK01	pBBR1, *cat* (pRANGER-BTB-3 without *araC*)	This study
pCK02	pBBR1, *cat*, *lacZ* (without promoter)	This study
pCK03	pBBR1, *cat*, *lacZ* with arabinose-inducible promoter *araBp*	This study
pCK04	pBBR1, *cat*, *lacZ* with artificial promoter *tacp*	This study
pCK05	pBBR1, *cat*, *lacZ* with cupriachelin biosynthesis gene promoter *cucJp*	This study

It was then cloned into pJET1.2/blunt (Thermo Fisher Scientific), introduced into chemically competent *E. coli* DH5α and sequenced. The genes *araBp* and *araC* were cut out from pJET1.2/blunt using *Sma*I and cloned into the *Sma*I site of pCK02, resulting in pCK03. (ii) The constitutive promoter *tacp* (Fukui et al. 2011) was amplified via PCR from pGEX-6P-2 with the primers *tacp*_F (5′-cccgggttggccgattcattaat-gcag-3′) and *tacp*_R (5′-cccgggtagtataggggacatgaat-actg-3′). Cloning of *tacp* into pCK02 was performed as previously described for *araBp*, resulting in pCK04. (iii) *cucJp* is the 1037 bp upstream region of *h16_b1683*, which encodes an enzyme involved in cupriachelin biosynthesis (Kreutzer et al. 2012). It was amplified via PCR from *C. necator* H16 genomic DNA using the primers *cucJp*_F (5′-cccgggctaggtgc-cgacggctttac-3′) and *cucJp*_R (5′-cccgggcataggct-gcgtcattgggtc-3′). Cloning of *cucJp* into pCK02 was performed as previously described for *araBp*, result-ing in pCK05. The plasmids pCK02, pCK03, pCK04 and pCK05 were introduced into *C. necator* H16 via electroporation. Electrocompetent cells were prepared by growing *C. necator* H16 in LB medium to an OD_{600} of 0.4. The cells were then placed on ice for 20 min, harvested by centrifugation (5 min, $2627 \times g$, 4 °C), washed twice with 10% glycerol and resuspended in 10% glycerol. After electroporation (2500 V, 200 Ω, 25 μF), the transformed cells were allowed to regen-erate for 150 min shaking at 30 °C in super optimal broth with catabolite repression (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose). *C. necator*:pCK03 was plated on LB agar containing 10 mM arabinose and 0.1 mg/mL X-Gal to confirm the production of β-galactosidase in the host strain. The *C. necator*

reporter strain harboring pCK05 was subsequently used for β-galactosidase assays, along with the control strains harboring pCK02 (negative control) and pCK04 (positive control).

Measuring of iron concentration in media and algal culture supernatants

The iron concentration in media and supernatants was determined on a contrAA 700 high-resolution contin-uum source atomic absorption spectrometer (AAS, Analytik Jena, Germany) using graphite tubes (Her-menau et al. 2018).

Chrome azurol S (CAS) assay

The presence of iron-chelating compounds in culture supernatants of *N. pelliculosa* was investigated using the CAS assay (Schwyn and Neilands 1987). For this, 40 μL of the supernatant were mixed with 200 μL CAS assay solution and incubated for 60 min. A mixture of WC medium and CAS assay solution served as negative control.

β-Galactosidase assay

The reporter strain was grown in 50 mL volumes under the respective conditions. A culture volume of 2–4 mL was harvested by centrifugation (1 min, $15{,}700 \times g$, room temperature) for each assay and resuspended in 200 μL phosphate-buffered saline pH 7.4 (8 g/L NaCl, 0.2 g/L KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4). The β-galactosidase assay was performed as previously described by Griffith and

Wolf (2002), with the exception that microplates were incubated at 28 °C instead of room temperature.

Statistical analyses

For β-galactosidase assays, reporter stains were cultivated as triplicates for every treatment. *N. pelliculosa* was cultivated in biological triplicates. Every supernatant of the diatom culture was split into three samples and added to the reporter strains, resulting in nine replicates for each data point. Outliers were identified according to Dixon's Q test at the 95% confidence interval and eliminated from the analysis. Statistical significance of results was assessed via one-way ANOVA with Tukey's post hoc for pairwise comparisons ($P < 0.05$) using VassarStats for statistical computation (Lowry 2018).

DNA–protein pulldown assay

Cupriavidus necator H16 was cultivated in 500 mL cultures under the respective conditions. After having reached stationary growth phase, cells were harvested by centrifugation (10 min, $9500 \times g$, 4 °C), washed with 20 mL washing buffer (10 mM Tris–HCl pH 7.5, 5% glycerol, 2 mM EDTA, 120 mM NaCl) and resuspended in 2 mL BS/THES buffer (Jutras et al. 2012). Cells were lysed via sonication ($7 \times 40\%$ pulsed cycles of 1 min with 20% power and 1 min break in between the cycles) using a Bandelin Sonopuls HD 2070. Cell debris was separated from the soluble protein by centrifugation (30 min, $30{,}000 \times g$, 4 °C). The DNA fragment containing *cucJp* was amplified using *cucJp*_R and 5′-biotinylated *cucJp*_F. The DNA–protein pulldown assay was then performed as previously described (Jutras et al. 2012). Briefly, the DNA fragment containing *cucJp* was attached to Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) by mixing 200 μL of beads resuspended in $2\times$ B/W buffer (Jutras et al. 2012) and 200 μL PCR product for 20 min. The bead-probe complex was then washed three times with TE buffer (1 mM EDTA, 10 mM Tris–Cl, pH 8.0), two times with BS/THES buffer und once with BS/THES buffer supplemented with Poly(dI-dC) (i.e., poly(deoxyinosinic-deoxycytidylic) acid). The supernatant of the bacterial cell lysate was added to the beads resuspended in BS/THES buffer supplemented with Poly(dI-dC) and mixed for 30 min at RT. This step

was repeated at 4 °C. The bead-probe-protein complex was then washed five times with BS/THES buffer supplemented with Poly(dI-dC) and two times with BS/THES buffer in order to remove unspecifically bound proteins. Target proteins were finally eluted using increasing NaCl concentrations (200–1000 mM).

Protein analysis

Proteins were initially analyzed via SDS-PAGE using 4–20% Mini-PROTEAN® TGX™ Gels (Bio-Rad). Electrophoresis was performed in Mini-PROTEAN® Tetra Cells (Bio-Rad) at 180 V for 30 min. Coomassie staining of the gel was conducted following the protocol of Dyballa and Metzger (2009). Protein bands of interest were excised from the gel and treated after the protocol of Shevchenko et al. (1996) with slight modifications. Briefly, protein bands were destained, and proteins were reduced with dithiothreitol and iodoacetamide before in-gel digestion with trypsin. The resulting peptides were analyzed on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics).

Results

Generation and testing of reporter strains

To investigate the effect of varying environmental conditions on cupriachelin biosynthesis, we constructed *C. necator* H16 reporter strains harboring plasmids with the β-galactosidase gene *lacZ*. Initially, an arabinose-inducible promoter was cloned in front of the reporter gene and the corresponding plasmid, pCK03, was introduced into *C. necator*. When plated on LB agar containing arabinose and X-Gal, *C. necator*:pCK03 formed blue colonies, confirming the production of recombinant β-galactosidase (Fig. S2). In contrast, the negative control strain *C. necator*:pCK02, which does not harbor any promoter in front of *lacZ*, formed white colonies on the same medium. The latter strain was also used as negative control for the β-galactosidase assay. *C. necator*:pCK04, which harbors the constitutive promoter *tacp* (Fukui et al. 2011), served as positive control. For the actual cupriachelin biosynthesis reporter strain, *cucJp* was inserted in front of *lacZ*, yielding pCK05.

The gene *cucJ* encodes a nonribosomal peptide synthetase, which is required for the assembly of cupriachelin (Kreutzer et al. 2012), and its transcription level was thus expected to correlate with the level of cupriachelin biosynthesis.

Effect of iron concentration on transcription levels of *cucJ*

The iron concentration in the growth medium measured by AAS was found to have a major effect on transcription levels of *cucJ* in *C. necator* (Fig. 2). On the second day after inoculation, reporter strain cultures grown under iron deficiency ($\leq 1 \mu M$ Fe) showed significantly higher β-galactosidase activities compared to cultures supplied with higher iron concentrations. The iron concentration in the growth medium correlates with the measured β-galactosidase activity. It became apparent that iron concentrations below 1 μM highly induce siderophore biosynthesis, while β-galactosidase activities of cultures grown with 10 μM to 500 μM iron are comparatively low. Interestingly, the increased expression of *cucJ* in low-iron media was short-lived. On the third day after inoculation, the cultures showed only negligible differences in β-galactosidase activity.
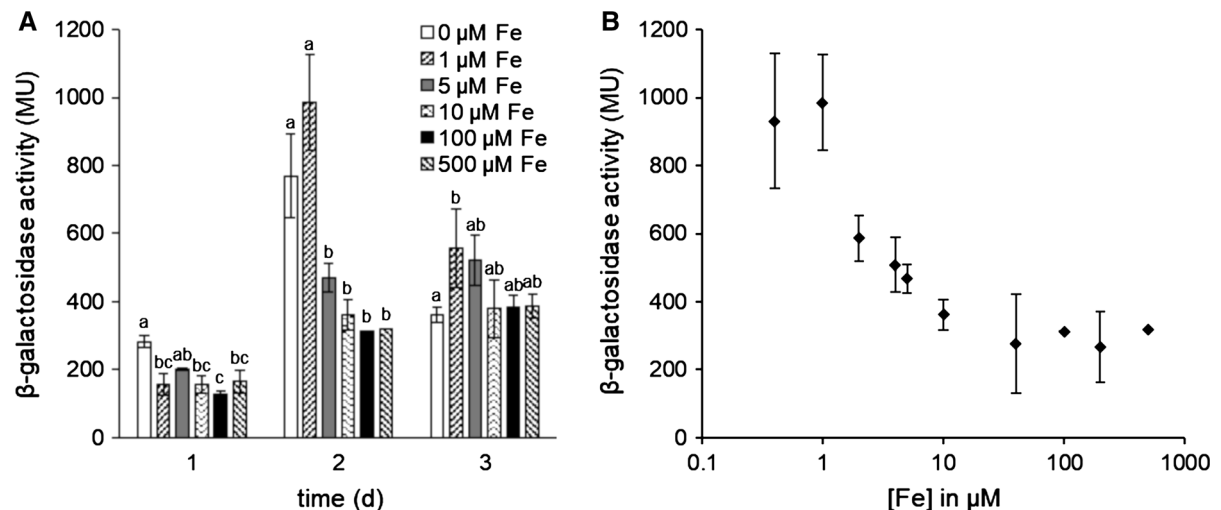
Effect of *N. pelliculosa* culture supernatants on transcription levels of *cucJ*

To test a possible effect of *N. pelliculosa* supernatants on the expression level of *cucJ*, the diatom was grown axenically under iron starvation (0.15 μM Fe) and iron repletion (10 μM Fe) to stationary phase (Fig. S3). AAS measurements revealed that *N. pelliculosa* sequestered most iron present in the medium during that time. The remaining iron concentrations in the culture supernatants after cultivation were 0.07 μM $\pm$ 0.003 μM and 0.56 μM $\pm$ 0.090 μM for iron starvation and iron repletion conditions, respectively. CAS assays demonstrated the absence of iron-chelating substances in the culture supernatants. For the β-galactosidase assay, *C. necator* reporter strains were inoculated into a 1:1 mixture of fresh H-3 mineral medium and *N. pelliculosa* supernatant. The iron concentration in the latter had been adjusted to 0.65 μM (0.56 μM + 0.090 μM). In doing so, the effect of varying iron concentrations on *cucJ* expression levels could be eliminated, and the observed differences in reporter gene transcription levels could be ascribed to metabolites secreted by the diatom. Since *C. necator* grew slowly in the given media composition (data not shown), β-galactosidase assays

Fig. 2 **A** β-Galactosidase activity of *C. necator*:pCK05 on three successive days after inoculation in H-3 mineral medium with different iron concentrations. The values represent the means and standard deviations for samples tested in triplicates. Statistical significance was assessed via one-way ANOVA with Tukey's post hoc test (P < 0.05) for every day comparison. Statistical differences are denoted by different letters within

each day. **B** β-Galactosidase activity of *C. necator*:pCK05 on the second day after inoculation in H-3 mineral medium plotted against iron concentration in the medium. The values represent the means and standard deviations for samples tested in triplicates. The β-glactosidase activity was significantly (*t* test, P < 0.05) different at low iron ($\leq 1 \mu M$) compared to replete conditions ($\geq 10 \mu M$)

were performed only on days 3 and 4 after inoculation. The results show that, regardless of iron concentration, the addition of diatom supernatants had a significant impact on the β-galactosidase activity in the reporter strain (Fig. 3). Supernatants from *N. pelliculosa* cultures that had been grown under iron-replete conditions strongly induced β-galactosidase activity in the reporter strains compared to the medium control on both days. The same increase in β-galactosidase activity, but 1 day delayed, was observed in the reporter strains incubated with culture supernatants grown under iron starvation (Fig. 3).

Transcriptional regulation of cupriachelin biosynthesis

Following the assessment of differential gene expression of *cucJ* in the β-galactosidase assay, we set out to identify the responsible transcription factors. For this purpose, a DNA–protein pulldown assay was performed. Briefly, a DNA fragment comprising *cucJp* was generated and incubated with cell lysates of *C.*
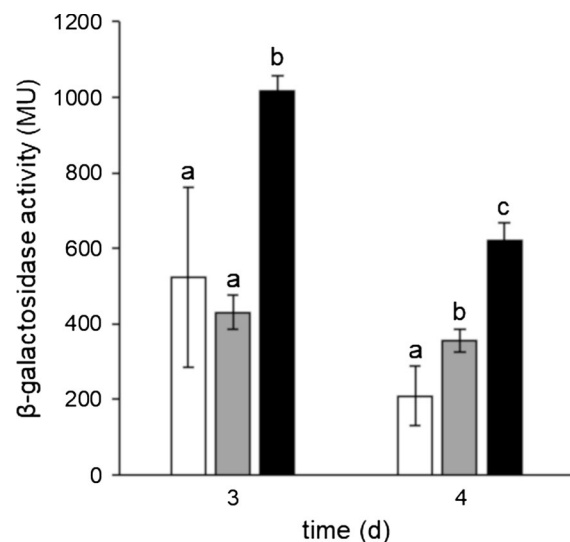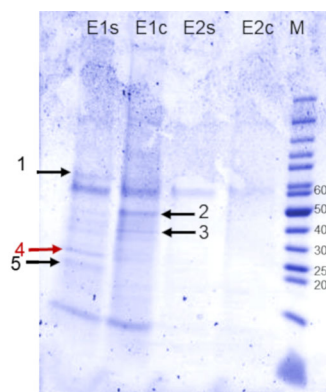
necator H16 to allow transcription factors to bind to *cucJp*. After several washing steps, bound proteins were eluted from the DNA and analyzed via SDS-PAGE and MALDI-TOF/MS. In this way, the Fur protein H16_A3143 was found to be involved in the transcriptional regulation of cupriachelin biosynthesis (Fig. S4). The protein was present in *C. necator* H16 cells grown under iron starvation (no added iron) and iron-replete conditions (190 μM Fe). Interestingly, when the bacterium was cultivated with diatom supernatants, the DNA–protein pulldown assay revealed a second transcription factor, H16_A1372, binding to *cucJp* (Fig. 4). H16_A1372 is annotated as a response regulator of the NarL family. NarL response regulators harbor a characteristic helix-turn-helix motif in their DNA-binding domain, which is also present in H16_A1372 (Kohlmann 2015). Besides the response regulator, four other proteins were identified in this DNA–protein pulldown assay (Fig. 4). They include proteins involved in energy metabolism (dihydrolipoyl dehydrogenase) and

Fig. 3 β-Galactosidase activity of *C. necator*:pCK05 on days 3 and 4 after inoculation in a 1:1 mixture of H-3 mineral medium and culture supernatant of *N. pelliculosa* grown under iron starvation (0.15 μM; grey bar) or iron replete conditions (10 μM; black bar) or modified WC medium (control; white bar). The values represent means and standard deviations of nine replicates (each of three algal culture supernatants distributed amongst triplicate reporter strains). Statistical significance was assessed via one-way ANOVA with Tukey's post hoc test (P < 0.05) for every day comparison. Statistical differences are denoted by different letters within each day

No.	Identified protein (accession no.)	MW [kDa]
1	Dihydrolipoyl dehydrogenase (WP_011615030.1)	62.1
2	Dihydroorotase (KUE90283.1)	45.3
3	Aspartate carbamoyltransferase (Q0K7N2.1)	35.3
4	Response regulator NarL family (AEI76745.1)	23.4
5	Hypothetical protein ASL20_18650 (KUE87334.1)	20.4

Fig. 4 SDS-PAGE of proteins of the DNA–protein pulldown assays performed with *C. necator* H16 grown with culture supernatant of *N. pelliculosa* (s) or with medium (c). E1 and E2 refer to eluates with increasing NaCl concentrations (200 mM and 300 mM, respectively). Protein bands clearly visible in the supernatant samples (s), but less pronounced medium samples (c), or vice versa were subjected to MALDI-TOF/TOF MS analysis (see arrows). The identified proteins are given in the table below. PageRuler Unstained Protein Ladder (Thermo Fisher Scientific) was used as marker (M). The numbers indicate protein masses in kDa

nucleotide biosynthesis (dihydroorotase, aspartate carbamoyltransferase). Since an unspecific or indirect binding with *cucJp* cannot be ruled out, these proteins were not further considered in this study.

Discussion

The production of siderophores by microorganisms depends on several environmental conditions, one of the most important being the availability of iron. Siderophore biosynthesis is generally upregulated under low iron stress, which was also confirmed for the production of cupriachelin in this study. Iron concentrations ≤ 1 µM increased the transcription level of the reporter gene compared to higher iron concentrations (Fig. 2). Interestingly, β-galactosidase activity was also observed with iron concentrations as high as 500 µM. Since iron concentrations > 190 µM are known to abolish cupriachelin production (Kreutzer et al. 2012), it is possible that the biosynthesis is also regulated post-transcriptionally. Such control at the mRNA level has been recognized to play a pivotal role in prokaryotic gene expression (Papenfort and Vogel 2009), but its identification remains challenging due to different constraints (Livny and Waldor 2007). Another observation was the short persistence of the increased expression of *cucJ* in low-iron media (Fig. 2). A possible explanation might be that cupriachelin is only produced under severe iron starvation. Once a sufficient intracellular iron concentration is reached, *C. necator* H16 may switch to the production of a metabolically less costly siderophore, as previously reported for *Pseudomonas* spp. (Dumas et al. 2013). A gene cluster for the biosynthesis of an auxiliary siderophore is indeed present in the genome of *C. necator* H16 (Schwartz et al. 2003).

Besides the environmental iron concentration, cell density has a major influence on siderophore transcription levels (Niehus et al. 2017). Siderophore production is generally most effective when iron concentrations are low, and cell densities are high. This is because higher cell densities entail higher siderophore production rates and, concurrently, higher chances for cells to encounter iron-siderophore complexes. Quorum sensing mediates this concentration-dependent mechanism of siderophore biosynthesis in *Pseudomonas aeruginosa* (Stintzi et al. 1998), and it is conceivable that this may be true also for other

bacteria. When iron availability is very low, cells grow poorly and benefit less from producing siderophores. The poor growth may explain why lower transcription levels of the reporter gene were measured when bacteria had been grown without any iron supplementation compared to the cases when small amounts of iron (0.4 µM, 1 µM) had been added to the cultures (Fig. 2).

Another factor influencing siderophore biosynthesis is the presence of iron-competing strains (Niehus et al. 2017). As secreted molecules, siderophores may be considered as public goods, especially when non-producers possess receptors and transporters for the uptake of the corresponding siderophore (Niehus et al. 2017). Photoreactive siderophores indeed are particular in this context. Their photolytic cleavage yields free Fe(II) that will rapidly reoxidize to a soluble, bioavailable form of iron, i.e. Fe(III)' (the sum of all inorganic Fe(III)-hydrolysis species), under aerobic conditions (Bruland and Rue 2001; Amin et al. 2012). To which extent iron is shared with other organisms or used by the producer is an important aspect that has to be considered. The cupriachelin gene cluster encodes a TonB-dependent receptor (Kreutzer et al. 2012), suggesting that the Fe-cupriachelin complex can be taken up by the bacterium, at least in the dark. The genome of *C. necator* H16 further exhibits a ferrous iron uptake system (H16_B0083-H16_B0085) (Cartron et al. 2006; Kreutzer et al. 2012) that may be used for the uptake of the photochemically released Fe(II). However, fast reoxidation to Fe(III)' under aerobic conditions and the apparent lack of a ferric iron uptake system in the bacterial genome, reduce the chance for cupriachelin-based iron uptake in sunlight. This leads to the assumption that cupriachelin will deliver iron to *C. necator* H16 mainly in the dark, while potentially providing iron to other organisms in the light (Fig. 1). Precedence for such a diurnal shifting in iron distribution comes from the photoreactive siderophore vibrioferrin (Amin et al. 2009a). It is evident that the production of a publically shared siderophore only makes sense, if the other organisms are mutualists, providing other benefits to *C. necator* H16. Considering the "iron-for-carbon" paradigm (Amin et al. 2009a), mutualistic iron sharing of *C. necator* H16 with phytoplankton seems possible.

Especially diatoms, which have been coexisting with bacteria in aquatic environments for more than 200 million years, appear to form specific interactions

with bacteria (Amin et al. 2012). These encompass, e.g. the exchange of iron (Amin et al. 2009a), vitamins (Croft et al. 2005), nitrogen (Foster et al. 2011) and dissolved organic carbon (Haynes et al. 2007). Interactions typically take place within the phycosphere, i.e. the zone directly surrounding microalgae (Seymour et al. 2017) and macroalgae (Wichard 2016). Here, algae can "cultivate" their bacteria by secreting metabolites that will attract certain microorganisms. The phycosphere does not mix with the surrounding fluids and readily accumulates metabolites. The resulting accumulation of microorganisms in microscale patches throughout aquatic environments (Blackburn et al. 1998) can favor cooperation by keeping cooperators and metabolites together. This is reinforced by the production of metabolites with rather a low diffusivity. For siderophores, it was found that low habitat structuring and severe iron starvation, conditions that prevail in aquatic environments, favor the production of siderophores with low diffusivity (Kümmerli et al. 2014; Boiteau et al. 2016). All this leads to the assumption that aquatic photoreactive siderophores are public goods, but stay in the close vicinity of the producing organism and thereby share their iron only with a fine selection of mutualists. It seems plausible that the metabolically closely linked organisms will influence each other on different levels. The observed increase of reporter gene transcription levels after treatment with *N. pelliculosa* culture supernatants (Fig. 3) corroborates this assumption. The fact that culture supernatants of iron-replete algae had a greater inducing effect on cupriachelin biosynthesis than those of iron-starved algae, can be explained by the different growth behavior of the algal cultures. In the presence of 10 μM iron, *N. pelliculosa* generally reached higher cell densities compared to 0.15 μM iron (Fig. S3). This difference in growth is probably also reflected in the amount of secreted metabolites and, thereby, in the inducing effect on cupriachelin transcription levels. The induction of cupriachelin biosynthesis will likely maximize the amount of iron *N. pelliculosa* can obtain from *C. necator* H16 and result in an important growth advantage for the alga. It illustrates the importance of microbial interactions for the success of a species.

In this study, we used the promoter region of *cucJ*, a gene involved in cupriachelin biosynthesis, to determine cupriachelin transcription levels and regulation. *CucJp* harbors a Fur box

(AATGAGAATGATTATCA) (Fillat 2014), and it is therefore not surprising that we identified Fur (H16_A3143) to bind to the promoter in the DNA–protein pulldown assay. Fur is widely distributed in bacteria and regulates transcription of many genes related to iron metabolism. When iron concentration within the cell is high, a*po*-Fur binds Fe^{2+} and dimerizes. The resulting *holo*-protein then binds to Fur boxes of the corresponding promoters and, thereby, blocks the RNA polymerase binding site (de Lorenzo et al. 1987). In this classical model, Fur acts as a transcriptional repressor that prevents the transcription of siderophore biosynthesis genes, when the intracellular iron concentration is high. Fur is autoregulated, binding to its promoter and thus preventing its transcription when it is already abundant (Delany et al. 2002). This ensures a homogeneous distribution of the transcription factor and ensures an adequate response to environmental stimuli (Watnick et al. 1997). In our assays, Fur could be found in both iron-deplete and iron-replete cultures of *C. necator* H16, illustrating the constitutive expression of the *fur* gene (*h16_a3143*). There is increasing evidence that Fur does not only act as a transcriptional repressor but also as an activator (Massé et al. 2007). It can thus be regarded as a global regulator of gene expression in many bacteria.

When *C. necator* H16 was grown with culture supernatants of *N. pelliculosa*, we identified a second transcription factor binding to *cucJp*. This protein, H16_A1372, was previously characterized as a global regulator of energy and carbon metabolism in *C. necator* H16 (Kohlmann 2015). It is a response regulator of the NarL family. H16_A1372 seemed more abundant in *C. necator* H16 samples that had been grown with *N. pelliculosa* culture supernatants, rather than in the control samples grown with modified WC medium (Fig. 4). Since transcription levels of the reporter gene were upregulated under the former conditions, we hypothesize that H16_A1372 acts as a transcriptional activator. Regulation of siderophore biosynthesis and uptake by two-component systems has been reported before (Dean and Poole 1993; Liao et al. 1996), but seems relatively rare compared to the regulation by Fur. Also, this is the first report of a NarL-type response regulator involved siderophore biosynthesis regulation to our knowledge. The interplay between Fur and H16_A1372 may be crucial for the fine-tuning of cupriachelin transcription levels but

remains elusive so far. Further studies need to be conducted to unravel the underlying mechanisms.

In summary, cupriachelin biosynthesis was found to be highly dependent on environmental conditions. These include varying iron concentrations, but also the presence of other microorganisms, such as the diatom *N. pelliculosa*. To our knowledge, this is the first report of an alga manipulating siderophore biosynthesis in a bacterium. Photoreactive siderophores have a major influence on iron cycling in aquatic environments and have the potential to shape planktic communities. Considering the importance of plankton dynamics for climate change and the formation of toxic algal blooms (Hallegraeff 2010), the fundamental understanding of such interactions is of major interest.

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