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Heavy metal resistance in *Marinobacter adhaerens* HP15 supports colonization of transparent exopolymer particles during its interaction with diatoms

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ABSTRACT: Marine diatoms and heterotrophic bacteria play a crucial role in the oceanic carbon cycle as they form aggregates that sink and transport biomass down in the water column. Polysaccharides are a major component of these aggregates and can bind heavy metal cations. Therefore, both organism groups might also be important for the vertical or micro-scale distribution of heavy metals in the ocean. Our model organism for diatom–bacteria interactions, *Marinobacter adhaerens* HP15, possesses 2 operons coding for heavy metal efflux pumps of the *czcCBA* type that are responsible for zinc resistance in this bacterium. The goal of this study was to elucidate the role of zinc resistance in *M. adhaerens* HP15 during its interaction with the diatom *Thalassiosira weissflogii.* For this, an HP15 mutant deficient in both *czcCBA* gene clusters was used in diatom co-cultivation experiments with or without zinc stress. When zinc was added to the co-cultures, the release of Alcian Blue-stainable particles increased 10-fold compared to assays without zinc. Both *M. adhaerens* HP15 wild-type and ΔczcCBA.1/2 mutant colonized the aggregates, and the percentage of bacteria attaching to transparent exopolymer particles (TEP) increased significantly under zinc stress. In the assays with zinc, HP15 wild-type had a higher cell density inside the aggregates than the mutant, although the zinc concentrations inside TEP were comparable. As TEP can bind and thus enrich heavy metals, resistance against them may help *M. adhaerens* HP15 to colonize aggregates. These results suggest that heavy metal resistance can be beneficial for bacteria during TEP- and aggregate-associated lifestyles.

KEY WORDS: *Marinobacter adhaerens* HP15 · *Thalassiosira weissflogii* · Diatom–bacteria interactions · Marine aggregates · Transparent exopolymer particles · *czcCBA* · Zinc

1. INTRODUCTION

Contributing 20% to the global primary production, marine diatoms play a crucial role in the marine carbon cycle (Field et al. 1998). Heterotrophic bacteria, mainly α- and γ-*proteobacteria* and members of the *Flavobacteria–Bacteroides* group, interact with diatoms and use the organic matter provided by photosynthesis as carbon source (Azam et al. 1983, Grossart & Ploug 2001, Schäfer et al. 2002). By interacting with bacteria, diatoms increase the release of transparent exopolymer particles (TEP) (Gärdes et al. 2011) that enhance aggregate formation (Grossart et al. 1997, Engel et al. 2004). Believed to consist mainly of acidic polysaccharides (Alldredge et al. 1993), TEP are rich in various nutrients, making them hotspots for bacterial respiration (Ploug et al. 1999).

In order to study diatom–bacteria interactions at a laboratory scale, a bilateral model system consisting of the γ-proteobacterium *Marinobacter adhaerens* HP15 and the diatom *Thalassiosira weissflogii* was established. Isolated from particles in the German Wad-

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den Sea (Grossart et al. 2004), *M. adhaerens* HP15 was shown to attach to diatom cells and enhance TEP production and aggregate formation (Gärdes et al. 2011, 2012). Its genome is sequenced and fully annotated (Gärdes et al. 2010), and the organism is genetically accessible, which allows site-directed mutagenesis (Sonnenschein et al. 2011). Thus, *M. adhaerens* HP15 is excellently suited for in-depth studies of the role of single genes or particular enzymatic functions potentially involved in its interaction with diatoms.

The *M. adhaerens* HP15 genome possesses 2 operons coding for *czcCBA* heavy metal efflux pumps in close proximity to each other in its genome (Stahl et al. 2015). Heavy metal resistance pumps of the *czcCBA*-type were previously described in *Alcaligenes eutrophus*, where they confer resistance against cobalt, zinc and cadmium (Nies et al. 1989). However, in *M. adhaerens* HP15 both *czcCBA* operons were found to be responsible for resistance against zinc only as demonstrated by generation and characterization of a mutant, HP15 ΔczcCBA.1/2, which lacks both heavy metal resistance operons and became zinc sensitive as compared to the wild-type (Stahl et al. 2015). Efflux pumps of the *czcCBA* type belong to the resistance-nodulation-cell division protein family and consist of 3 protein units: the efflux pump CzcA, the membrane fusion protein CzcB and the outer membrane factor CzcC (Tseng et al. 1999, Nies 2003). Although *czcCBA* was first described in the genome of a bacterium isolated from a terrestrial, heavy metal-contaminated environment (Vandamme & Coenye 2004), it has also been detected in many bacterial species from marine habitats (Wu et al. 2015, 2018).

Marine habitats potentially enriched in heavy metals are hydrothermal vents (Varnavas & Cronan 2005, Sander & Koschinsky 2011), manganese nodules, naturally enriched deep-sea sediments (Hein et al. 2013) as well as industrially or otherwise anthropogenically impacted areas (Buccolieri et al. 2006). Marine aggregates can also harbor elevated heavy metal concentrations (Decho 1990, Puig et al. 1999). Due to their capability to bind heavy metals, exopolymer particles can remove them from the surrounding water and be enriched in heavy metals. Metal concentrations in the water may play a role in the control of TEP formation by regulating TEP stickiness by changing the surface charge distribution due to metals binding to the exopolymers (Mari et al. 2007). Members of the genus *Marinobacter* have been reported to occur in different environments rich in heavy metal such as marine sediments and hydrothermal vents (Handley & Lloyd 2013). First indications that heavy metal resistance is important for *M. adhaerens* HP15 during its interaction with diatoms have recently been shown. In an *in vivo* expression technology (IVET)-based study with *M. adhaerens* HP15 we identified genes that are regulated by a promotor that is specifically active during the interaction with *T. weissflogii*. One of the identified genes was the *czcC* gene (HP15_108), suggesting that heavy metal resistance plays a role during the interaction (Torres-Monroy & Ullrich 2018).

The aim of the present study was to elucidate a potential functional role of heavy metal resistance for *M. adhaerens* HP15 in potentially heavy metal-enriched aggregates. For this, the interaction of *M. adhaerens* HP15 wild-type and its mutant ΔczcCBA.1/2 with *T. weissflogii* cells and TEP was analyzed in co-culture experiments with and without zinc stress. We used 0.3 mM $ZnSO_4$ as heavy metal stress, as this concentration leads to an upregulation of both heavy metal resistance operons (Stahl et al. 2015) and is sub-lethal for both bacterial organisms in f/2 medium.

2. MATERIALS AND METHODS

2.1. Microorganisms and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *Marinobacter adhaerens* HP15 was routinely grown in marine broth (MB) medium at 37°C unless otherwise stated (Sonnenschein et al. 2011). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C. When necessary for the selection of plasmids, growth media were supplemented with 50 mg l^{-1} 5-aminolevulinic acid (5-ALA), 25 mg l^{-1} chloramphenicol (Cm) and 50 mg l^{-1} ampicillin (Amp).

Thalassiosira weissflogii was received from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (East Boothbay, Maine, USA) and cultured under axenic conditions in f/2 medium (Guillard 1975) at 15°C, a photoperiod of 12 h and a light intensity of 115 µmol photons m^{-2} s^{-1}. Aliquots of the cultures were transferred to fresh medium every 2 to 3 wk and regularly checked for bacterial contamination by microscopy.

2.2. DNA manipulations and cloning

Enzymes were purchased from Thermo Fisher Scientific. Plasmid digests were separated via 1%

Table 1. Bacterial strains and plasmids used in this study. *M. adhaerens*: *Marinobacter adhaerens*; *E. coli*: *Escherichia coli*; Amp^R: ampicillin resistant; Cm^R: chloramphenicol resistant; Gm^R: gentamycin resistant

Strain or plasmid	Characterization	Reference
Bacteria		
M. adhaerens HP15	Wild-type	Grossart et al. (2004)
M. adhaerens HP15 ΔczcCBA.1/2	Deletion mutant lacking both czcCBA operons, involved in heavy metal resistance	Stahl et al. (2015)
E. coli DH5α	*subE44* Δ*lacU169* (ϕ*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook & Russell (2001)
E. coli ST18	λ*pir*Δ*hemA* pro thihsd R+ TprSmr chromosome::RP4-2 Tc::Mu-Kan::Tn7	Thoma & Schobert (2009)
Plasmids		
pBBR1MCS-4	Broad host range cloning vector, Amp^R	Kovach et al. (1994)
pBBR-4-*DsRed*	*DsRed* under control of constitutive *LacZ*promoter $P_{A1/04/03}$, Amp^R, Cm^R	This study
pBluescript II SK	Broad host range cloning vector, Amp^R	Stratagene, San Diego, CA, USA
pBlue-*DsRed*	Intermediate plasmid, *DsRed* under control of constitutive *LacZ*promoter $P_{A1/04/03}$, Amp^R, Cm^R	This study
miniTn7(Gm) $P_{A1/04/03}$ *DsRedExpress*-a	Source of *DsRedExpress*gene, Amp^R, Cm^R, Gm^R	Lambertsen et al. (2004)

agarose gel runs. DNA fragments ligated into plasmid backbones were purified from agarose gels using a 'GeneJET Gel Extraction Kit' (Thermo Fisher Scientific). Plasmid extractions were carried out with a 'GeneJET Plasmid Miniprep Kit' (Thermo Fisher Scientific).

2.3. Fluorescent labelling of bacterial strains

Plasmids carrying the gene for a DsRed fluorescence protein were introduced into *M. adhaerens* HP15 strains via bi-parental conjugation. Plasmid pBBR1MCS-4 (Kovach et al. 1994) has previously been shown to replicate in *M. adhaerens* HP15 (Sonnenschein et al. 2011) and was used as a backbone for the fluorescent protein-coding gene *DsRed.* The miniTn7 delivery plasmid as described by Lambertsen et al. (2004) — miniTn7(Gm) $P_{A1/04/03}$ *DsRedExpress*-a (Table 1) — served as source for the fluorescent protein-coding gene. A fragment of ~2 kb was obtained by *Not*I digestion of the plasmid. This fragment contained the gene encoding for the fluorescent protein under control of promoter $P_{A1/04/03}$ and a chloramphenicol resistance cassette (*cat*). As pBBR1MCS-4 harbors 2 *Not*I restriction sites, the excised 2 kb fragment was first cloned into the exclusive *Not*I site of pBluescript, resulting in plasmid pBlue-*DsRed* (Table 1). Next, insert fragments were re-excised and cloned into the final backbone pBBR1MCS-4. The *DsRed*/*cat* gene fragment was excised by *Eco*RI digestion and correspondingly ligated into the *Eco*RI site of pBBR1MCS-4. Plasmids were routinely transformed into chemically competent *E. coli* strains via heat-shock. The plasmid encoding the fluorescent protein (pBBR-4-*DsRed*, Table 1) was conjugated into *M. adhaerens* HP15 wild-type and ΔczcCBA.1/2 by bi-parental conjugation using *E. coli* ST18 (Thoma & Schobert 2009). Briefly, *M. adhaerens* HP15 wild-type and mutant were grown for 2 d on MB agar. *E. coli* ST18, previously transformed with the fluorescent protein encoding plasmid, was grown overnight on LB-Cm-5-ALA. Biomass was removed from the agar plates using an inoculation loop, re-suspended in MB (*M. adhaerens* HP15) or LB medium (*E. coli* ST18), respectively, and the optical density $(OD)_{600}$ was adjusted to 1.0. Of each cell suspension, 200 µl were combined, spotted on non-selective MB-5-ALA agar, and incubated at 28°C. Cell biomass was harvested after 20 h of incubation, re-suspended, and re-streaked on selective MB-Cm or MB-Amp agar for *M. adhaerens* HP15 wild-type or ΔczcCBA.1/2, respectively. *M. adhaerens* HP15 transformants appeared within 2 d of incubation at 37°C.

2.4. Attachment assays

To analyze the attachment behavior of *M. adhaerens* HP15 wild-type and mutant ΔczcCBA.1/2 to *T. weissflogii* cells with and without zinc, stress attachment assays were performed. Both types of

organisms were grown into mid to late exponential phase. *M. adhaerens* HP15 cells were harvested to obtain a cell density of 10^7 CFU ml^{-1} in 300 ml culture volume. The respective culture volume was harvested by centrifugation (15 min, 3000 × *g*, 4°C). The cell pellets were washed twice in 4 ml 75% North Sea water (NSW) and re-suspended in 3 ml fresh f/2 medium. *T. weissflogii* cells were counted with a Sedgewick-Rafter Cell (SPI Supplies) and harvested by centrifugation (15 min, 1250 × *g*, 4°C) to obtain a cell density of 10^4 cells ml^{-1} in a final volume of 300 ml, and re-suspended in 3 ml fresh f/2 medium. In the attachment assay, both organisms were combined in 300 ml freshly prepared f/2 medium. As controls, only one organism or plain f/2 medium were used. All assays were done in triplicate. Assays were incubated for 24 h at 15°C, a photoperiod of 12 h and a light intensity of 115 µmol photons m^{-2} s^{-1}. After the incubation, free-living and attached bacteria, were separated by filtration of the assay volume through a plankton sieve with a pore size of 10 µm. For ease of explanation, the filter residue will be referred to as 'attached fraction' even if no bacteria were present in the assay. The attached fraction was washed off the filter and re-suspended in 10 ml NSW.

Bacterial CFU were enumerated by serial dilution plating at the start of the incubation and after filtration for each of the separated fractions. Ultimately, CFU numbers of the individual fractions were presented as percentages of the total number of CFU for easier comparison of the aggregate colonization under different conditions. Microscopy was done after filtration, and samples from the attached fraction were used for the quantification of TEP. The zinc concentration was measured in both the free-living and the attached fraction.

A concentration of 0.3 mM $ZnSO_4$ was chosen to be representative of zinc stress. This concentration proved to be sub-lethal for both *M. adhaerens* HP15 wild-type and mutant ΔczcCBA.1/2 as shown by the minimal inhibitory concentration (MIC) determination for $ZnSO_4$ in f/2 medium (Table S1 in the Supplement at www.int-res.com/articles/suppl/m658p047_supp.pdf).

2.5. Microscopy

Microscopy was performed on samples of the attached fraction after filtration. For sample preparation, 10 µl sample and 10 µl Alcian Blue solution (1:100 diluted from 0.02% stock solution in 0.06% acetic acid) were mixed directly on a microscopy slide. Microscopy was done at 1000× magnification with an AxioPlan 2 imaging microscope (Carl Zeiss). Pictures were taken with an AxioCam HRc camera (Carl Zeiss) and processed with the AxioVision software (release 4.8.2.0, Carl Zeiss MicroImaging).

2.6. Quantification of TEP

TEP in the attached fractions of the attachment assays were quantified using the dye-binding spectrophotometric assay (Passow & Alldredge 1995). Two ml of each sample were filtered onto polycarbonate filters (pore size 0.4 µm, Sartorius) by low constant vacuum. Filters were stained with pre-calibrated Alcian Blue solution for 30 s (Fig. S1), and excess dye was rinsed with double-distilled water (ddH_2O) and vacuum filtered. Subsequently, the Alcian Blue dye was dissolved in 80% sulfuric acid and spectrophotometrically measured at 787 nm. Samples from assays with zinc addition were diluted 1:10 to fit into the range of the calibration curve. All samples were analyzed with 3 technical replicates. TEP concentrations were given as gum Xanthan equivalents (µg Xeq. l^{-1}).

2.7. Quantification of zinc

Zinc was quantified in samples of the free-living and attached fractions of attachment assays with and without zinc. Zinc was quantified using the Zinc assay kit (Sigma Aldrich) according to the manufacturer's instructions. For the assays with $ZnSO_4$ supplementation, 15 µl of samples of the free-living fraction and 10 µl of the attached fraction (1:20 diluted) were brought to 50 µl with ddH_2O and combined with 200 µl zinc reagent mix. For the assays without $ZnSO_4$ supplementation, 50 µl of all samples were used. Different volumes and dilutions of samples were necessary to be in the detection range of the assay from 0 to 5 nmol zinc per well. The zinc assays were incubated at room temperature in the dark for 10 min, and the absorbance at 560 nm was measured. All samples were measured in duplicates. In order to see if the wild-type and mutant ΔczcCBA.1/2 of *M. adhaerens* HP15 faced comparable zinc stress in the aggregates, the zinc concentration in the attached fraction was presented relative to TEP.

2.8. Statistical analysis

Statistically significant differences between bacterial cell numbers, TEP concentrations and zinc concentrations were determined using the Student's *t*-test in Microsoft Excel 2013. Differences with a p-value below 0.05 were regarded as significant.

3. RESULTS

3.1. Microscopy

DsRed-labeled transformants of *Marinobacter adhaerens* HP15 wild-type and ΔczcCBA.1/2 emitted an intense fluorescence, allowing easy microscopic observation (Fig. S2). Detection of bacterial fluorescence signals inside TEP was made possible because Alcian Blue-stained particles did not express fluorescent signals as shown in the *Thalassiosira weissflogii* and the cell free f/2 medium controls (Fig. S3). Autofluorescence was only emitted by living diatom cells.

In samples of the attached fraction (diatom and bacterial cells and TEP retained on the 10 µm filter surface), TEP was stained with Alcian Blue, and aggregates were subsequently observed by light and fluorescent microscopy. In the attached fractions of the co-cultures of *M. adhaerens* HP15 and *T. weissflogii*, cells of both wild-type and mutant were mainly found inside the TEP particles but to a much lesser extent on the surface of diatom cells (Fig. 1, Fig. S4), suggesting that *M. adhaerens* prefers to attach and reside in TEP rather than on the direct surface of the diatom cell. As the bacterial cells directly attached to the diatom surface accounted for only around 1 % of the total cell number in all assays, they were included into the total attached fraction. In order to detect potential differences in cell densities and TEP formation not observable microscopically, TEP and bacterial cell numbers were subsequently quantified.

3.2. TEP

TEP were quantified in all attached fractions of the attachment assay samples. The TEP concentration in the cell-free f/2 medium control was taken as background noise and subtracted from all other sample values because f/2 medium has occasionally the tendency to form small particles over time as a result of undefined and unspecific precipitation reactions (data not shown).

When $ZnSO_4$ was added to the attachment assays, TEP production was enhanced compared to the assays without $ZnSO_4$. In the co-cultures, TEP production increased by a factor of around 30; in the control mono-cultures the increase was lower at a factor between 3 and 10 (Fig. 2). With $ZnSO_4$ addition, the highest TEP concentrations were measured in the co-cultures with *T. weissflogii* and either *M. adhaerens* HP15 wild-type or mutant ΔczcCBA.1/2. The TEP concentration in both bacterial and the *T. weissflogii* controls were lower than in the co-cultures. These differences were significant with the exception of the comparison between the diatom control and the co-culture with *M. adhaerens* HP15 wild-type ($p < 0.05$, Table S2). In the co-cultures as well as in the bacter-

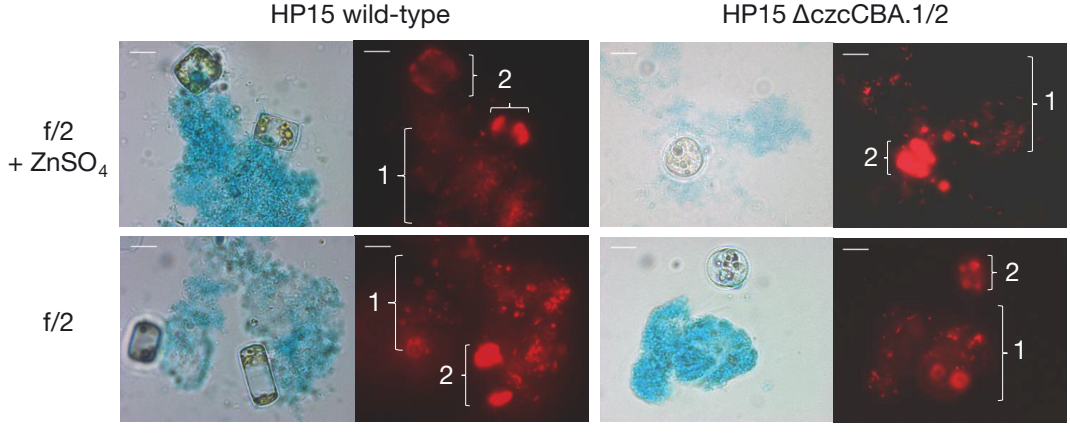

Fig. 1. Light and fluorescence microscopy of the attached fractions of co-cultures of *Marinobacter adhaerens* HP15 and *Thalassiosira weissflogii*. Transparent exopolymer particles (TEP) in the samples were stained with Alcian Blue. Pictures were taken at a 1000× magnification under phase contrast (light microscopy) and an Alexa546 filter (fluorescence microscopy). $ZnSO_4$ was added at a concentration of 0.3 mM. 1: fluorescence from *M. adhaerens* HP15 cells; 2: autofluorescence from diatom cells. Scale bars = 10 µm

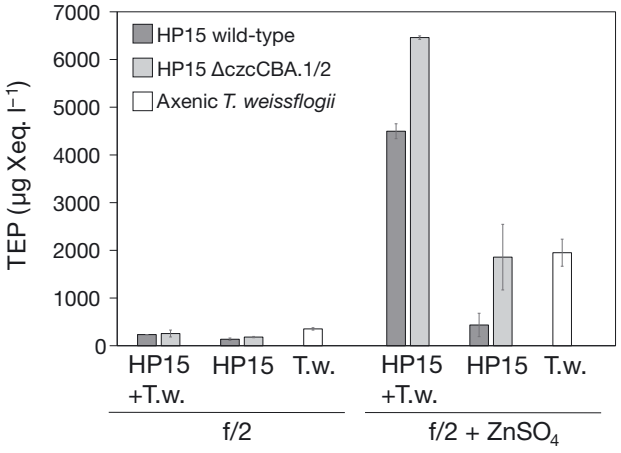

Fig. 2. Quantification of transparent exopolymer particles (TEP) in the attached fractions of the attachment assays. TEP were quantified using the dye-binding photometric Alcian Blue assay and given in gum Xanthan equivalents (Xeq.). Assays without $ZnSO_4$ supplementation on left; assays with supplementation of 0.3 mM $ZnSO_4$ on right. HP15 + T.w.: co-cultures of *Marinobacter adhaerens* HP15 and *Thalassiosira weissflogii*; HP15: *M. adhaerens* HP15 control; T.w.: *T. weissflogii* control. Results are shown as means ± SD, n = 3. A graph with the assays without $ZnSO_4$ supplementation alone can be found in Fig. S5

ial controls, TEP concentrations were higher, but not significantly different, in the assays with *M. adhaerens* HP15 ΔczcCBA.1/2 than in the assays with the wild-type strain ($p > 0.05$, Table S2) (Fig. 2).

In assays without $ZnSO_4$ addition, only small, not significant (Table S2) differences between the assays were observed. The highest TEP concentration was measured in the *T. weissflogii* control, followed by the co-cultures (Fig. 2, Fig. S5). The lowest concentrations of Alcian Blue-stained material were found in the bacterial controls without $ZnSO_4$, with a slightly higher concentration produced by mutant ΔczcCBA.1/2 than by the wild-type (Fig. 2, Fig. S5).

3.3. Quantification of bacterial cell numbers and CFU

Bacterial CFU ml^{-1} were determined at the beginning of the incubation and after filtration. The CFU ml^{-1} numbers determined at the beginning of the incubation confirmed that the assays were started with the aimed cell density of 10^7 CFU ml^{-1} (Table S3). In the attached fractions of the bacterial controls, the CFU numbers may reflect aggregated bacterial cells. Consequently, these CFU numbers were subtracted from the cell numbers in the diatom co-cultures. Assays with *T. weissflogii* only, as well as the f/2 medium controls, were always bacteria-free.

After filtration, the assays without $ZnSO_4$ addition still showed a total cell number of 10^7 CFU ml^{-1} (Fig. 3, Table S3), which was not significantly different between wild-type and mutant ($p > 0.05$). For both *M. adhaerens* HP15 wild-type and ΔczcCBA.1/2 the majority of bacterial cells was free-living, and only a small fraction of bacteria was found in the attached fraction. When no zinc was added, there were no significant differences observed for the percentage of attached bacteria (Fig. 3).

As expected, when $ZnSO_4$ was added, the total bacterial cell numbers after filtration dropped by one order of magnitude for both wild-type and mutant (Fig. 3, Table S3) but did not differ significantly between wild-type and mutant ($p > 0.05$). However, the fraction of attached bacteria increased for both wild-type and mutant, with a percentage of attached HP15 wild-type cells (45%) being twice as high as the percentage of attached mutant ΔczcCBA.1/2 cells (20%) (Fig. 3).

The bacterial cell density inside TEP was calculated as the ratio of bacterial cell numbers in the attached fraction to the respective TEP (Table S3). In the assays without $ZnSO_4$ addition, there was no difference observed between the 2 bacterial organisms, which both showed a cell density in the range of 10^6 CFU µg TEP (Xeq.)$^{-1}$. With addition of $ZnSO_4$, this cell density dropped for both wild-type and

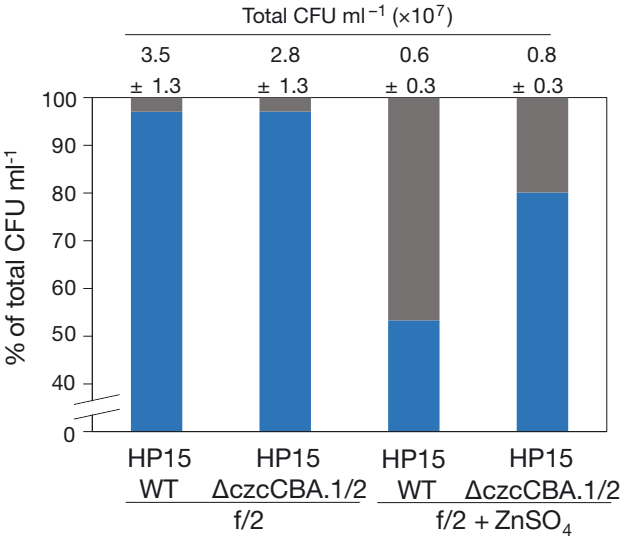

Fig. 3. Percentages of the free-living (blue) and attached (grey) bacterial fractions in co-cultures with *Thalassiosira weissflogii*. Bacterial CFU ml^{-1} were determined by serial dilution plating; percentages were calculated relative to the total cell number. Results shown as mean ± SD, n = 6. WT: wild-type

mutant by one order of magnitude. However, *M. adhaerens* HP15 wild-type still showed a significantly higher ($p < 0.05$) cell density than its mutant ΔczcCBA.1/2 with 5.5×10^5 CFU µg TEP (Xeq.)$^{-1}$ and 1.5×10^5 and CFU µg TEP (Xeq.)$^{-1}$, respectively (Table S3). For an approximation of how many attached *M. adhaerens* HP15 cells survived the zinc stress, the ratio of the cell densities in the attached fractions for samples with and without $ZnSO_4$ was calculated. A total of 20% of *M. adhaerens* HP15 wild-type but only 5% of mutant ΔczcCBA.1/2 survived the $ZnSO_4$ treatment.

3.4. Quantification of zinc

Zinc concentrations were determined for both free-living and attached fractions of all assays. Zinc concentrations in the attached fractions were related to the TEP concentrations to show the actual concentration in the particles (Fig. 4).

In the assays with $ZnSO_4$ addition, there were only small differences detectable between the free-living fractions of any of the co-cultures and controls. Measured zinc concentrations ranged from 156 to 201 µM.

In the attached fractions, there was no significant difference in zinc concentrations ($p > 0.05$, Table S4) observed between the co-cultures with *M. adhaerens* HP15 wild-type or those with mutant ΔczcCBA.1/2 (Fig. 4), indicating that both bacterial organisms were exposed to similar conditions inside the TEP. The measured zinc concentrations were below the MIC for $ZnSO_4$ for bacterial incubation at 18°C, enabling the bacteria to survive the $ZnSO_4$ treatment throughout the experiment. Although the zinc concentration inside the TEP was similar in both the co-culture and the bacterial control, there was more zinc present in the attached fractions of the co-culture. Looking at the raw data of the zinc concentration in mM in the attached fraction before relating them to the TEP amount in the assays, we observed that there was significantly more ($p < 0.05$, Table S5) zinc present in the attached fractions of the co-cultures as compared to their bacterial controls (Table S5). As there was also more TEP present in the co-culture assays, these results suggest an accumulation of zinc in TEP.

In both, the f/2 medium control and the *T. weissflogii* control, the zinc concentrations were higher than in the assays with bacteria (Fig. 4). However, only the differences between the co-culture with *M. adhaerens* HP15 wild-type and both axenic controls were significant ($p < 0.05$, Table S4).

The naturally occurring zinc concentrations in f/2 medium, as measured in the assays without $ZnSO_4$ addition, did not significantly contribute to the above-mentioned values. In both the free-living and the attached fractions, the zinc concentrations were 2 orders of magnitude lower than in the assays with $ZnSO_4$ addition (Fig. S6).

4. DISCUSSION

4.1. TEP dynamics

We observed clear differences in TEP production between the treatments with and without $ZnSO_4$ addition (Fig. 2). In treatments without $ZnSO_4$ addition, TEP concentrations were in a similar range as observed in previous studies on the diatom–bacteria interaction model system (Gärdes et al. 2011, Seebah et al. 2014). Interestingly, in the present study we observed higher TEP concentrations in the axenic

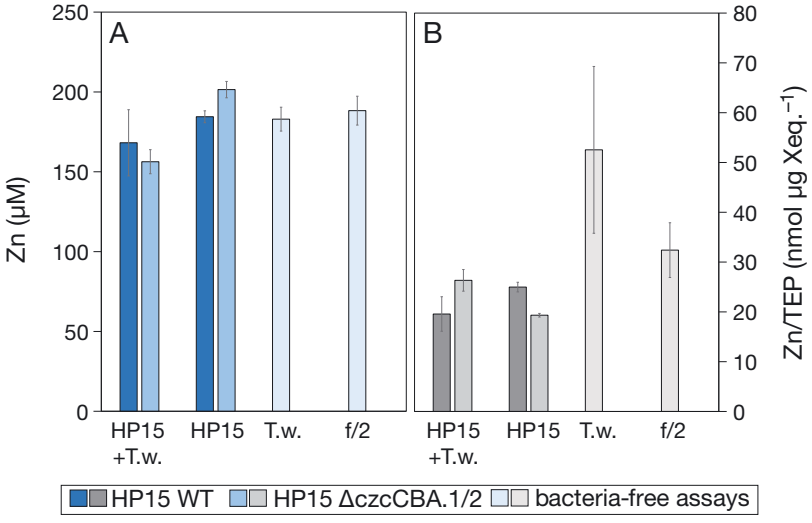

Fig. 4. Zinc concentrations in the (A) free-living (blue) and (B) attached (grey) fractions of the attachment assays with supplementation of 0.3 mM $ZnSO_4$ shown relative to the TEP concentration. Concentration of zinc in the samples was quantified using a photometric ligand-binding assay. HP15 + T.w.: co-cultures of *Marinobacter adhaerens* HP15 and *Thalassiosira weissflogii*; HP15: *M. adhaerens* HP15 control; T.w.: *T. weissflogii* control; f/2: f/2 medium control. Results shown as mean ± SD, n = 3. WT: wild-type

Thalassiosira weissflogii control than in the co-culture, contradicting the previous findings that *Marinobacter adhaerens* HP15 is needed for aggregate formation and that bacterial co-cultures lead to higher TEP production (Gärdes et al. 2011, 2012). It is noteworthy that the previous experiments were conducted over a period of 7 d. When results of the current assays without zinc treatment are compared with our previous data (Gärdes et al. 2011, 2012), it has to be assumed that the presence of bacteria may lead to more TEP release by the diatom only after 24 h of co-cultivation.

When $ZnSO_4$ was added to the co-cultures, the measured TEP concentration was one order of magnitude higher than in assays without $ZnSO_4$ (Fig. 2). It has been previously suggested that the formation of TEP can be impacted by high heavy metal concentrations in the water as the metal cations bind to dissolved organic matter and affect the stickiness of TEP-precursors (Mari et al. 2007). This would explain why axenic diatom cultures also produced more TEP when zinc was added.

Another possible reason for the enhanced TEP production is the release of yet-to-be-determined exopolymeric substances (EPS) by *M. adhaerens* HP15. Bacteria are known to release EPS that can bind and chelate heavy metals to protect themselves (Kazy et al. 2002, González et al. 2010, Nocelli et al. 2016). The potential of binding heavy metals and protecting bacteria has also been reported for EPS released from marine bacteria from hydrothermal vents or associated with micro-algae and for *Marinobacter* sp. (Loaëc et al. 1998, Bhaskar & Bhosle 2006, Gutierrez et al. 2012). Bacterial EPS are chemically similar to diatom-derived TEP with acidic polysaccharides as a main component and might therefore also be quantified with the used Alcian Blue assay (Thornton et al. 2007). It was determined that the amount of bacterial EPS can reach a concentration that is up to one order of magnitude higher than that of phytoplankton-derived TEP (Sohm et al. 2011). It is thus likely that bacterial EPS contributed to the high TEP concentrations in the assays with $ZnSO_4$. This is supported by our finding that high amounts of TEP were measured in the bacterial controls without diatom cells (Fig. 2).

Although chemically similar, bacterial and diatom-derived polysaccharides often contain distinctive monosaccharides (Mühlenbruch et al. 2018). A possible way to analyze the different composition of aggregates in co-cultures and in diatom-free bacterial cultures and to elucidate the true contribution of bacterial EPS to the TEP pool is the use of techniques based on lectin staining (Uthicke et al. 2009). Lectin staining of aggregates has shown differences in the polysaccharide composition of aggregates with varying bacterial colonization (Grossart 1999, Gärdes et al. 2012, Bennke et al. 2013), suggesting that bacteria contribute to the exopolymer composition of aggregates in a different way as compared to phytoplankton.

Interestingly, in all assays with the mutant ΔczcCBA.1/2, TEP concentrations were higher as compared to assays with the wild-type, particularly when $ZnSO_4$ was supplemented (Fig. 2). It is known that bacteria release increased amounts of EPS under stress conditions (Jensen 1984). Since mutant ΔczcCBA.1/2 is more sensitive towards $ZnSO_4$ than the wild-type (Stahl et al. 2015), it is plausible that the lack of heavy metal resistance leads to more EPS release by the mutant as compared to its wild-type for protection from heavy metal stress.

4.2. Aggregates and diatoms as heavy metal carriers

In assays with $ZnSO_4$, more zinc was found in the attached fractions when diatoms and TEP were present (Fig. 4). Heavy metal cations can bind to both surface compounds in diatom cell walls (González-Dávila 1995, González-Dávila et al. 2000, Gélabert et al. 2006) and the negative charges of the acidic residues of the exopolymer substances (Kim et al. 1996) and thus be concentrated in aggregates (Puig et al. 1999). As marine aggregates also consist of bacterial EPS, both exopolymer substances might contribute to the binding of heavy metals. By this process, marine aggregates play an important role in the cycling of heavy metals in the ocean, for example by removing metals from surface water (Decho 1990), as a carrier for metals in the food chain (Bhaskar & Bhosle 2006) or by making metals available to phytoplankton (Gutierrez et al. 2012).

4.3. TEP colonizing behavior of HP15

M. adhaerens HP15 was chosen as the bacterial partner of the diatom–bacteria interaction model system because it had been isolated from marine aggregates and been found to be attaching to diatom cells and their TEP (Grossart et al. 2004, Gärdes et al. 2011). When determining bacterial CFU in the attached and free-living fractions of attachment assays, previous studies did not differentiate between bacteria attaching to diatoms directly or to TEP. By

using fluorescently labeled *M. adhaerens* HP15 strains and by simultaneously staining TEP with Alcian Blue, we could herein show that attached *M. adhaerens* HP15 cells are mainly found inside TEP and that only a minor fraction is actually attached to the diatom surface (Fig. 1). This result demonstrated that TEP may be a preferred habitat for *M. adhaerens* HP15, likely due to the presence of nutrients in TEP (Ploug et al. 1999). Such nutrients could for instance be particular amino acids for heterotrophic bacteria such as *M. adhaerens* HP15 (Stahl & Ullrich 2016).

Comparing the number of attached bacteria in the assays with and without zinc stress, a significant difference could be found for both *M. adhaerens* HP15 wild-type and its mutant ΔczcCBA.1/2 (Fig. 3). Under heavy metal stress, bacteria may release EPS for protection (González et al. 2010), which in turn can be incorporated into TEP aggregates and which might then lead to a higher number of bacteria in the attached fraction. Percentages of attaching bacteria as high or even higher than the ones found in the current study when zinc was present have been reported in natural seawater during diatom bloom events (Alldredge et al. 1993) and in laboratory co-cultures of *Thalassiosira* diatoms and heterotrophic bacteria (Grossart et al. 2005, 2006).

Heavy metal resistance might help *M. adhaerens* HP15 colonizing TEP aggregates as shown by a higher cell density of HP15 wild-type inside TEP as compared to mutant ΔczcCBA.1/2 cells. An impact of heavy metal resistance during the interaction of *M. adhaerens* HP15 with diatoms was already suggested in one of our previous studies (Torres-Monroy & Ullrich 2018). During an IVET-based screening of interaction-induced genes, 2 genes (*merR1* and *czcC*) were identified as being controlled by promoters active during the interaction with *T. weissflogii*. Both genes are believed to be involved in the response and resistance against heavy metal stress. Moreover, Fontanez et al. (2015) found several bacterial heavy metal resistance genes, e.g. of the *czcCBA* type, enriched in particle-associated samples. These results imply an important role of heavy metal resistance for bacteria during their interaction with diatoms or during their association with TEP aggregates.

5. CONCLUSIONS

In the present study, we showed how the interaction between *Marinobacter adhaerens* HP15 and *Thalassiosira weissflogii* is affected by heavy metal stress (Fig. 5). When $ZnSO_4$ was added to the experiments, more Alcian Blue-stainable exopolymer substances were released by both *T. weissflogii* and *M. adhaerens* HP15. As these substances can protect bacteria from heavy metals, the amount of attached bacteria was higher under metal stress than without. However, the zinc-resistant *M. adhaerens* HP15 wild-type showed a higher cell density inside the TEP than the non-resistant mutant ΔczcCBA.1/2. This suggests that the heavy metal resistance helps *M. adhaerens* HP15 to withstand higher heavy metal concentrations inside aggregates. Thus, heavy metal resistance may be beneficial for bacteria during a TEP- or aggregate-associated lifestyle and may contribute to the bacterial colonization of nutrient-rich marine aggregates.

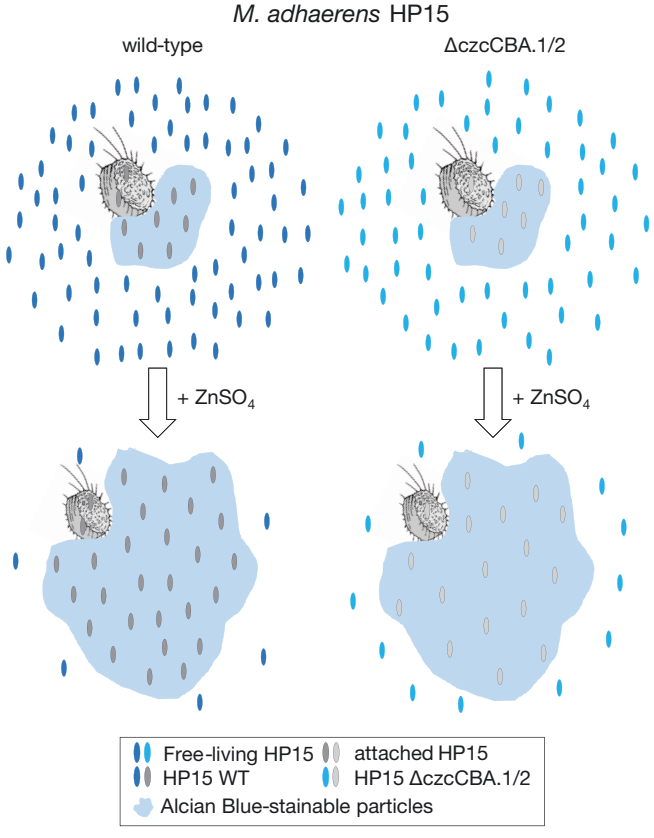

Fig. 5. Schematic model for the response of *Marinobacter adhaerens* HP15 and *Thalassiosira weissflogii* to zinc stress during their interaction. When $ZnSO_4$ is added to attachment assays, the production of Alcian Blue-stainable particles (blue clouds) is increased. Without $ZnSO_4$, most bacteria are free-living. When $ZnSO_4$ is added, more bacteria are found within aggregates. *M. adhaerens* HP15 wild-type (WT) shows a higher cell density inside aggregates than mutant ΔczcCBA.1/2 when $ZnSO_4$ is added. Blue bacteria: free-living *M. adhaerens* HP15; grey bacteria: attached *M. adhaerens* HP15. After addition of $ZnSO_4$, only 20% of free-living bacterial cells are shown

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