A bilateral model system for the molecular investigation of diatom-bacteria interactions

by

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Doctor of Philosophy in Marine Microbiology

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Summary

Diatom blooms are frequently terminated by aggregation of algae and subsequent sedimentation in form of marine snow, and contribute significantly to carbon flux thereby playing an important part in the global carbon cycle. Transparent exopolymer particles (TEP) are produced by phytoplankton or bacteria and play an important role in aggregate formation. This study was aimed at analyzing the interactions between bacteria and phytoplankton and their influences on TEP formation, aggregation dynamics, and sedimentation of phytoplankton.

For this, an *in vitro* model system consisting of the diatom *Thalassiosira weissflogii* and the marine *Gammaproteobacterium Marinobacter adhaerens* HP15 was established in the course of this thesis and was analyzed in rolling tank experiments. From the results it could be concluded, that interaction of *M. adhaerens* HP15 with *T. weissflogii* increased aggregate formation and particle sinking, and thus enhanced the efficiency of the biological pump. Detailed investigation of this interaction revealed differences in the response of *T. weissflogii*, towards bacteria, namely diatom growth and exopolymer production, which greatly depended on nutrient availability. A careful interpretation of these results suggested a close and specific interaction of *M. adhaerens* HP15 with the diatom in a mutualistic relationship. The type of interaction shifted to commensalism during nutrient stress conditions. Depending on the nature of *M. adhaerens* HP15 - *T. weissflogii* interactions the extent and quality of algal exudation in form of TEP dramatically changed and consequently influenced aggregate formation.

Another part of this thesis focused on the chemotaxonomic and genomic characterization of *M. adhaerens* HP15 as the bacterial counterpart of the established model system. In this context the bacterial genome sequence was determined and a genetic system for molecular work has been established. A site-directed deletion mutation of the flagellin-encoding gene *fliC* of HP15 was generated and confirmed the suitability of *M. adhaerens* HP15 as a genetically accessible model organism.

With this bilateral model system being generated, a species-specific interaction can now be intensively and mechanistically studied in depth.

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List of Abbreviations

ALA	5-aminolevulinic acid
BSA	Bovin serum albumin
CCMP	Center for Culture of Marine Phytoplankton
Con-A	Concavalin-A
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
Cm	Chloramphenicol
DAPI	4´,6-diamino-2-phenylindole
DFAA	Dissolved free amino acids
DCAA	Dissolved combined amino acids
DFCHO	Dissolved free carbohydrates
DCCHO	Dissolved combined carbohydrates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DNA	Deoxyribonucelic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
ESD	Equivalent spherical diameter
EPS	Exopolysaccharides
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
IVET	*In vivo* expression technology
LB	Luria-Bertani
MB	Marine Broth
MIC	Minimal inhibitory concentrations
OPA	Ortho-phthal-dialdehyde
PAM	Pulse-amplitude-modulated
PCR	Polymerase chain reaction
POC	Particulate organic carbon
POM	Particulate organic matter
PTP	Picotiterplate
SEM	Scanning electron microscopy
SOC	Super Optimal Broth with Catabolite repression medium
TEM	Transmission electron microscopy
TEP	Transparent exopolymer particles

Contents

1 Introduction

1.1 Biological pump

Oceans are the largest active reservoir in the global carbon cycle, and therefore may play a major role in alleviating the effects of global warming and increased anthropogenic emissions of carbon dioxide (CO_2) (Siegenthaler and Sarmiento 1993). When CO_2 is fixed and turned into organic material during photosynthesis by phytoplankton, the carbon is converted from dissolved inorganic to particulate organic carbon (POC). This transformation enables downward flux of carbon, since particles sink whereas solutes do not (Kiørboe 2001). The amount of organic carbon reaching the deep sea is determined by settling speed and degradation of sinking particles (Alldredge and Gotschalk 1989; Fowler and Knauer 1986). Therefore, a rapid transit time from the ocean surface to the sea floor shortens the time available for decomposition of organic particles and consequently enhances the organic carbon export to the depth. If organic matter were to settle as individual phytoplankton cells they would need ~10 years to reach the ocean's sea floor (Smayda 1971). Within that time the organic matter would be grazed or remineralized by herbiviores and microbes. Therefore, the actual deep sea carbon flux is mainly mediated by fast sinking, large-size aggregates such as marine snow particles, whose sinking speed is greater than 100 m d^{-1} (Alldredge and Gotschalk 1989; Turner 2002).

Hence, aggregation of algae forming large aggregates predominantly in form of marine snow is a key mechanism for transporting carbon to depth. This sink of organic carbon is important for marine biogeochemical cycles and the efficiency of the so-called 'Biological pump' (Fig. 1) (De La Rocha et al. 2007; Fowler and Knauer 1986; Jahnke 1996). The Biological carbon pump can be divided into the production, the settling, and the decomposition of POC. When carbon is transported to the deep ocean via the Biological pump, CO_2 is removed from the surface waters and enables more CO_2 absorption from the atmosphere (Sarmiento and Bender 1994). Once the fixed carbon reaches the deep ocean's sediment most of it is sequestered for millions of years (Raven and Falkowski 1999).

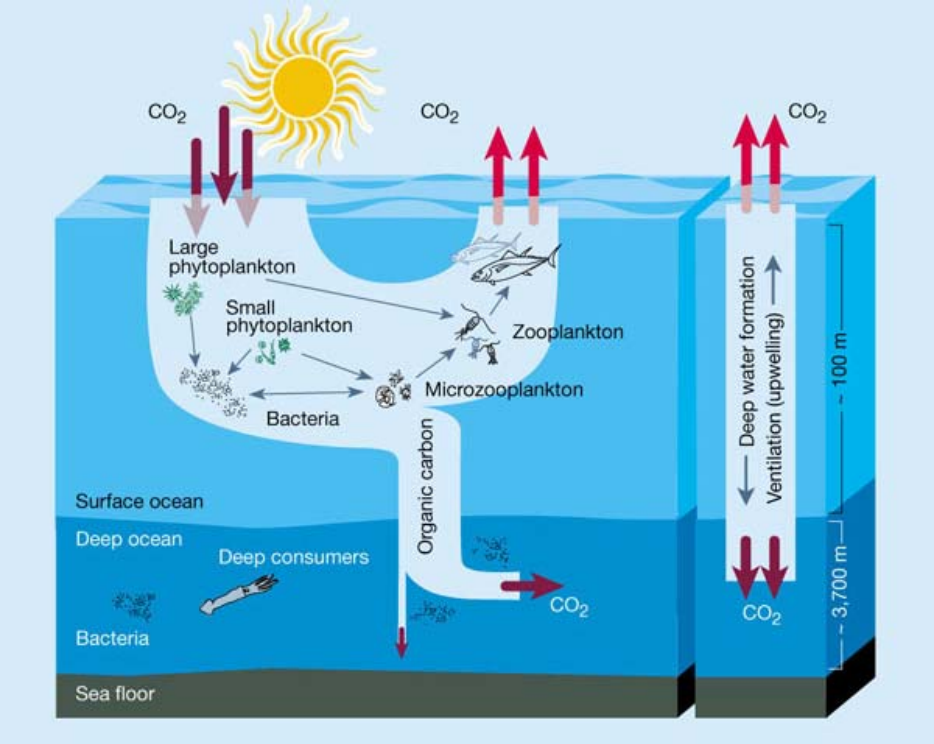

Figure 1: The Biological pump showing the processes that contribute to the removal of carbon from the surface water by sinking biological particles (Chisholm 2000).

1.2 Marine aggregates

Marine snow is defined as a diverse group of large aggregates with diameters greater than 0.5 mm and composed of phytoplankton aggregates, zooplankton fecal material, inorganic particles, or large organic structures, including mucous feeding nets from foraminiferas and pteropods (Alldredge and Silver 1988; Kiørboe et al. 2003). Marine snow can consist of up to 63% of total particulate organic carbon (Alldredge and Silver 1988). Large fluxes are mostly coupled to primary productivity and are typically associated with the termination of phytoplankton blooms (Alldredge and Gotschalk 1989; Smetacek 1985).

In this context, various studies recently reported on a) formation and properties of marine aggregates (Alldredge and Silver 1988; Jackson 2001; Passow 2002b; Simon et al.

2002; Verdugo et al. 2003); b) the flux of POC (Fowler and Knauer 1986; Jackson 2001; Jahnke 1996); c) the suspension, sinking and degradation of particles (Grossart et al. 2003; Ploug et al. 1999; Ziervogel et al. 2007); and d) the role of zooplankton in particle flux (Steinberg et al. 1994; Turner 2002). The formation of phytoplankton aggregates depends on the abundance and size (Jackson 2001) of particles as well as their physical environment and stickiness (Kiørboe et al. 1990; Kiørboe and Hansen 1993), which in turn depend on phytoplankton growth stage and physiological conditions (Jackson 2001; Passow 2002a).

The formation of larger particles from the collision and joining of smaller particles is termed coagulation (Jackson 1990) and has emerged as a significant process in controlling particle size, thereby determining particle fate. Particle dynamics describes the fate of particles as they collide to form new particles and ultimately settle out of their system. Classical coagulation models emphasize three mechanisms for particle-to-particle contact: Brownian diffusion, shear (laminar and turbulent), and differential sedimentation (Jackson 2001).

The sticking efficiency of algal particles is believed to be a direct result of cellular exudation (Passow and Alldredge 1995a), cell morphology (Kiørboe et al. 1990), and surface properties (Waite et al. 1995). Therefore, cell-to-cell interactions depend on the sticking efficiency, which are species-specific and depend on cell size and shape (Logan et al. 1994). Bacteria can inhibit as well as enhance the aggregation state of the system by reducing the stickiness by enzymatic activities such as hydrolases (Hoppe et al. 1988; Smith et al. 1992) or by increasing it by mucus production (Bhaskar and Bhosle 2005; Cole 1982).

1.3 Transparent exopolymer particles (TEP)

Transparent exopolymer particles (TEP) are produced by phytoplankton or bacteria and play an important role in aggregate formation (Passow 2002a).

Marine algae produce different types of carbohydrates with different functions. Aside from the production of internal storage polysaccharides, many phytoplankton species release large amounts of carbohydrates to the surrounding water (Myklestad and Haug 1972) and

contribute quantitatively and – in terms of reactivity – significantly to the pool of dissolved organic matter (DOM) in the ocean (Hernes and Benner 2002; Zhou et al. 1998). A significant proportion of carbon fixed by phytoplankton can be released as assembled gel-forming polysaccharides and macro-gels. Thereby formed TEP contain mostly acidic polysaccharides and are defined as Alcian-Blue-stainable particles retained on filters (Fig. 2) (Alldredge et al. 1993). TEP are particularly important for sedimentation processes because they affect aggregation of particulate organic matter (POM) and the formation of marine snow by generating the matrix or 'glue' of micro- and macro-aggregates (Alldredge et al. 1993; Logan et al. 1995; Passow 2002a) leading to the sticking of colliding particles (Kiørboe and Hansen 1993). Formation of TEP is the result of both, abiotic and biotic processes. These processes include the formation from dissolved precursor material released by microorganism (Bhaskar et al. 2005; Passow and Alldredge 1994a) and the formation via self coagulation of high molecular weight DOM (Chin et al. 1998; Verdugo et al. 2004).

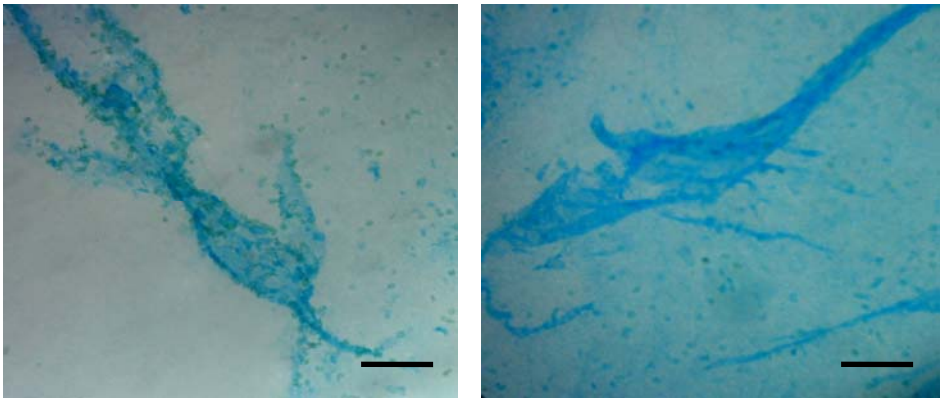

Figure 2: Microscopic view of transparent exopolymer particles (TEP) stained with the polysaccharide-specific dye Alcian Blue. Scale bar: 100 µm (this study).

TEP sizes range *in situ* from 2 to 500 µm in diameter and concentrations vary from 1 to 10^4 particles ml^{-1} (Mari and Burd 1998; Passow and Alldredge 1994b). Although TEP are the most thoroughly investigated gel particles, their relative composition is still poorly

understood. The dominant single sugars of TEP seem to be fucose, rhamnose, and arabinose (Passow 2002b). The observed variability in TEP composition may depend on phytoplankton species and the nutrient composition of the medium, in which the phytoplankton cells grow (Corzo et al. 2000; Engel and Passow 2001; Myklestad 1974; Myklestad and Haug 1972). Since TEP are mainly composed of acidic polysaccharides, TEP production increases when cell growth is limited by nitrogen deficiency (Beauvais et al. 2006; Corzo et al. 2000; Staats et al. 2000). The net rate of TEP production was shown to be affected by a variety of stochiometric N:P ratios (Obernosterer and Herndl 1995). Mari and Burd (1998) provided *in situ* evidence that TEP abundance increased when nitrate and the inorganic N:P ratio were low in the surface mixed layer. Under nitrogen deficiency and when protein synthesis is reduced, a larger proportion of photosynthetically fixed carbon seems to be exudated in form of TEP (Myklestad and Haug 1972). Stickiness of TEP is also influenced by other phytoplankton growth conditions and may be related to the molecular weight distribution of the secreted polymers. High molecular weight mucopolymers are preferentially produced during phytoplankton blooms and shift to even higher values as a result of bacteria-algae interactions. An important feature of TEP is its ability to scavenge nitrogen-rich substances from the water column including low molecular weight amino acids (Schuster and Herndl 1995) as well as to bind diverse trace elements and heavy metals (Engel 2004).

Although phytoplankton are typically considered the major producer of TEP, both, laboratory and field studies, have shown that bacteria also produce TEP (Passow 2002a). Stroderegger and Herndl (1999) estimated that in the North Sea 4-5 % of total mass abundances of exopolymeric particles were derived from bacteria. Various additional *in situ* and *in vitro* studies revealed that bacteria produce significant amounts of exopolymeric substances and thereby increase the amount of TEP (Bhaskar and Bhosle 2005; Bhaskar et al. 2005; Decho 1990a; Passow 2002a; Sastry and Rao 1994). Thus, bacteria contribute to phytoplankton aggregation by producing precursor material for the formation of TEP and may stimulate algal exudation (Azam et al. 1994; Decho 1990a; Stoderegger and Herndl 1999). Microbial activities associated with TEP formation influence aggregate formation in dependence of a) the nutritional environment (Myklestad 1977); b) the distribution of heterotrophic bacteria between particulate and dissolved fractions (Mari and Kiørboe 1996;

Mari et al. 2004) c) grazing pressure (Dutz et al. 2005; Prieto et al. 2001); and d) the production of enzymes as well as extra-cellular products (Alderkamp et al. 2007). Additionally, TEP can also be colonized and modified by bacteria (Mari and Kiørboe 1996; Passow and Alldredge 1994b; Verdugo et al. 2004), presumably serving as bacterial niches and substrate sources.

1.4 Microbial extra-cellular polymeric substances in marine environments

Microbial extra-cellular polymeric substances (EPS) are widely distributed in marine environments and are found in dissolved form, colloids, distinct particles such as TEP, and/or associated with other particulate matter including cell aggregates, detritus, biofilms, and microbial mats. Microbial EPS perform various functions and are involved in diverse marine processes (Fig. 3). The unique gelling properties of microbial EPS are considered important in the transport and transformation of organic matter, in complexing dissolved metals, and in biogeochemical cycling of other elements (Bhaskar and Bhosle 2005). Bacteria produce copious amounts of EPS involved in phytoplankton aggregate formation (Grossart et al. 2006b; Passow 2002a; Passow and Alldredge 1994b). The release of EPS by microorganisms depends on the individual species, the physiological state, and environmental growth conditions (Myklestad 1995).

Bacteria influence the structure and physical properties of the predominately algae-derived matrices of aggregates in two major ways. On the one hand, macromolecules within fibrils may be cleaved due to bacterial exo-enzyme activities with the resulting mono- and oligomers being taken up by bacteria. Modifications of algae-borne polymers by bacterial activities result in more sticky polymers, which can easily collide (Karner and Herndl 1992; Smith et al. 1995; Stoderegger and Herndl 1998). On the other hand, it was shown that the presence of surfaces allowing attachment stimulated the production of bacterial EPS. Thus, microbial EPS may contribute to the pool of DOC (Costerton et al. 1994).

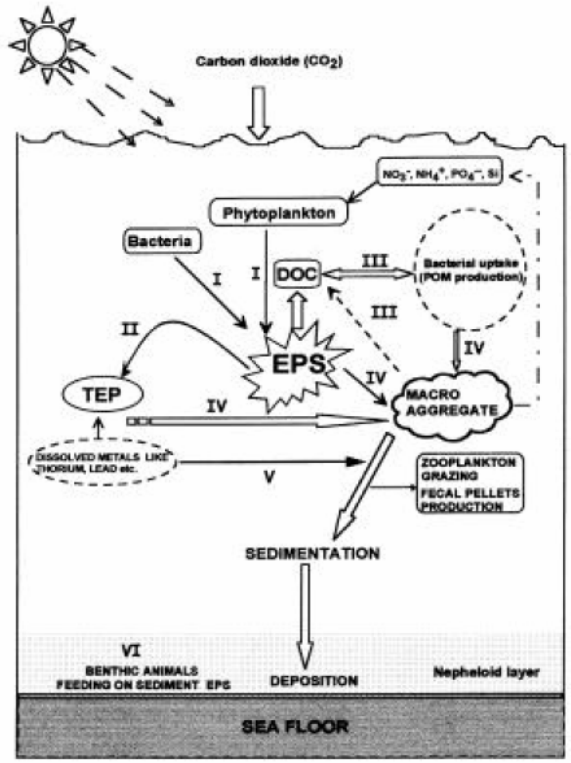

Figure 3: Schematic representation of the various roles of EPS in the marine environment. Numbering indicates the different processes involving EPS. I, Production of EPS by bacteria and phytoplankton; II, Production of TEP from EPS; III, The microbial loop; IV, Formation of particles; V, Chelation of dissolved metals and VI, EPS as a carbon source for the benthic community (Bhaskar and Bhosle 2005).

Bacterial multiplication and enzymatic activities were substantially enhanced in presence of a surface, to which these bacteria could attach. Zobell (1943) observed that the number of surface-attached bacteria was dramatically higher than cell numbers in the surrounding seawater. It is now widely accepted that aquatic microorganisms dominantly live in a highly structured environment that provides physical surfaces, such as aggregates or phytoplankton cells, and is characterized by diverse chemical gradients (Smith et al. 1992). The production of EPS by bacteria has been described as an adaptive strategy for growth in natural systems (Costerton 1999a).

In the ever fluctuating environment of the pelagic ocean, EPS and biofilms form protective micro-environments and may structure a range of microbial processes (Decho 2000). Bacterial EPS matrices form sticky coatings on individual particles and living surfaces. Thus, they act as buffering stabilizers for extra-cellular processes during frequently changing physical stresses (e.g. salinity, temperature, UV radiation). Likewise, they may decrease susceptibility of bacterial cells towards host defense mechanisms such as the production of antimicrobial substances (Costerton 1999b). Concurrently, EPS

appear to concentrate extra-cellular enzyme activities of bacteria and hence contribute to the efficient bio-mineralization of DOM and POM, namely phytoplankton-borne secreted polymers or substances (Decho 1990a; Ding et al. 2009; Hoffman and Decho 2000). Capsular polysaccharides may also provide planktonic bacterial cells with a protective barrier against toxic substances in the water column or may prevent grazing by other marine organisms, such as dinoflagellates (Flemming 2000; Loaec et al. 1998; Matz et al. 2004; Wuertz et al. 2000). In marine water columns, nutrients required to support maximal microbial growth are rarely present in sufficient quantities (Azam et al. 1994). Microbial attachment to fixed surfaces however is likely to enhance nutrient uptake. A biofilm polymer may be understood as a sorptive 'sponge', which binds and concentrates organic molecules and ions (Nichols et al. 2005).

TEP are produced by both, diatoms and bacteria, however, recent studies have shown that TEP can be generated abiotically from bacterial EPS (Alldredge et al. 1993; Bhaskar et al. 2005). Furthermore, high bacterial concentrations on phytoplankton cell surfaces induce increased production of polymers by the phytoplankton cells (Azam et al. 1991).

1.5 Bacteria-phytoplankton interactions

Analyzing the significance of algae-associated bacteria in marine aggregate formation is of major interest to better understand the mechanisms of regulation of downward carbon flux in the ocean and the detailed processes of the biological pump. Presence of particular bacterial strains can be of significance for phytoplankton aggregation (Grossart et al. 2006a), hence studies on bacteria-phytoplankton interactions are of particular importance.

As an analog of the 'rhizosphere' of higher terrestrial plants, phytoplankton cells and bacteria closely interact in the 'phycosphere', which is the micro-zone surrounding algal cells and acts as an important habitat for marine microorganisms (Bell and Mitchell 1972b) (Fig. 4).

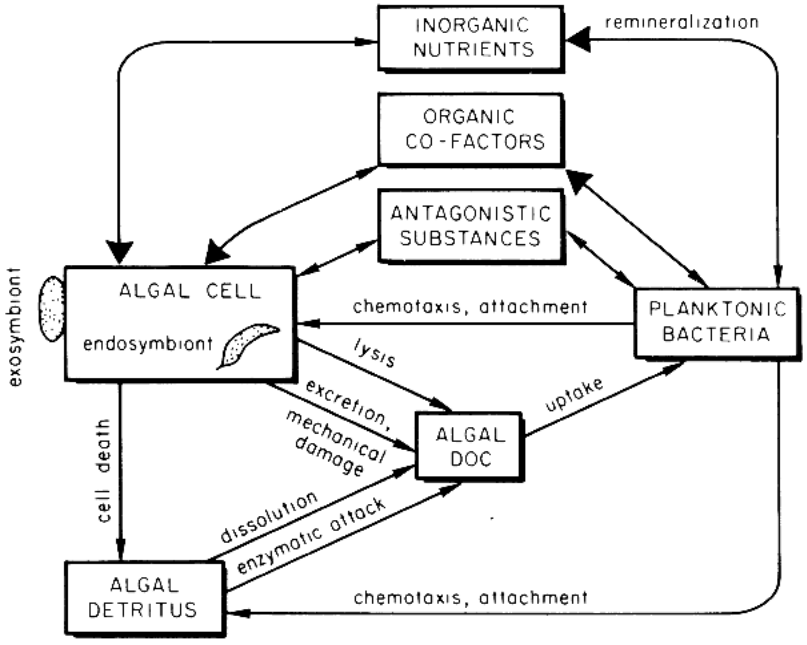

Figure 4: Simplified diagram of bacteria-phytoplankton interactions that occur in the phycosphere. The size of arrow heads denote the predominate direction of the process (Cole 1982).

In the phycosphere, bacteria may be free-living (Blackburn *et al*. 1998), may be attached to the algal surface (Kogure *et al.* 1981), or may occur as intracellular algal symbionts (Lewis *et al.* 2001). Often, bacteria profit from carbon sources provided by phytoplankton (Wang and Priscu 1994), whereas the algae utilizes remineralized organic matter released as nitrate, ammonium, or vitamins (Croft et al. 2005). These types of interaction were first described by the so-called 'cluster hypothesis' (Azam and Ammerman 1984). Bacteria may also be parasites of phytoplankton cells thus penetrating them or residing in intercellular spaces of multi-cellular host organisms, where they might cause cell lysis and release of nutrients (Bidle and Azam 1999; Cole 1982; Lovejoy et al. 1998). To prevent bacterial parasitism many algal species produce antibiotic substances (Sastry and Rao 1994). Diverse algae have evolved defense mechanisms, such as extensive production of surface mucus (polysaccharides and proteoglycans), which influence the

settlement, growth, and survival of microorganisms (Azam and Smith 1991). Commensalic bacteria benefit from phytoplankton without having any negative impact on algal growth (Bell and Mitchell 1972b). However, the distinction between commensalism and parasitism is transient and slight changes (e.g. during abiotic stress) might impact the actual outcome of the interaction. When loosely associated with algae, bacteria may compete for nutrients such as phosphate, iron, or vitamins (Rothhaupt and Güde 1992a). Hence, in natural environments phytoplankton-bacteria interactions are multifaceted with high variations in time and space depending on the actual environmental conditions (Grossart 1999).

Bacterial cells cluster around phytoplankton cells to create considerably higher cell concentrations than the average ~10^6 CFU ml^{-1} in bulk seawater. They exhibit an arsenal of hydrolytic enzymes, which allows them to efficiently utilize polymeric substances such as phytoplankton exudates (Grossart 1999; Hoffman and Decho 2000; Schäfer et al. 2002; Skerratt et al. 2002), thus controlling algal stickiness and aggregation (Smith et al. 1995).

Molecular community studies on diatom-associated bacteria were performed in different habitats and revealed that most of such interacting bacteria belonged to the *Cytophaga-Flavobacteria-Bacteriodites* group (Bruckner et al. 2008; Grossart et al. 2005; Riemann et al. 2000). Likewise, dinoflagellate-associated bacteria were assigned to the *Roseobacter* clade (Brinkmeyer et al. 2000; Jasti et al. 2005; Sapp et al. 2007). Very little is known about whether or not specific algal species attract distinct bacteria or bacterial communities (Schäfer et al. 2002). Several researchers have reported species-specific interactions between bacteria and phytoplankton, with species-specific responses to certain carbohydrates and amino acids (Butler and Camilli 2005; Parales and Harwood 2002). This led them to propose that bacteria may play a major role in controlling phytoplankton dynamics (Grossart et al. 2006b; Grossart et al. 2005; Lovejoy et al. 1998; Skerratt et al. 2002).

Although heterotrophic bacteria were shown to cluster near or attach onto phytoplankton, and thus control the development of marine phytoplankton aggregates (Gärdes et al. submitted-a; Grossart et al. 2006a; Grossart et al. 2005), specific functions of individual bacterial species on diatom aggregation have not been explored in detail. Bacteria contribute to phytoplankton aggregation by producing, inducing, or modifying TEP (Azam et al. 1994; Bhaskar et al. 2005; Decho and Herndl 1995; Passow 2002a;

Stoderegger and Herndl 1999). Hence, bacteria loosely or tightly associated with phytoplankton may efficiently increase sedimentation and thus the removal of organic matter from the upper water column (Azam and Long 2001).

1.5.1 Bacterial enzyme activities

Pelagic heterotrophic bacteria possess a diversity of hydrolytic enzymes, which allow them to efficiently utilize an array of polymeric substances such as phytoplankton exudates (Arnosti et al. 2009; Grossart 1999; Hoffman and Decho 2000; Skerratt et al. 2002). Measurements of enzymatic activities demonstrated that microbes produce extra-cellular enzymes for the degradation of i.e. laminarin (Alderkamp et al. 2007), fucoidin, and chondroitin sulfate, as well as other phytoplankton-borne exopolysaccharides (Passow and Arnosti; unpublished). Alternatively, bacteria may directly hydrolyze the mucus deposited on diatom surfaces (Smith et al. 1995). TEP represent hot spots of high substrate concentrations and thus may serve as nutrient and/or attachment surfaces for bacteria. Respective nutrients become accessible to bacteria through polymer hydrolysis by proteases, glycosidases, lipases, and nucleases (Martinez et al. 1996). The outcome of the phytoplankton-bacteria interaction will thus depend not only on the substrate concentrations found in the gel matrix but also on the polymer composition and the type and level of bacterial exo-enzymes being expressed. Simultaneous expression of monomer permeases and exo-enzymes could enable a tight coupling of hydrolysis and substrate uptake (Hoppe et al. 1988). Most bacterial hydrolyses are cell surface-bound (therefore also termed ‘ecto-enzymes’) (Ziervogel et al. 2007). Consequently, their enzymatic action on algae surfaces or TEP is spatially explicit. However, a small but variable fraction of hydrolase activities is often found not to be cell-associated but still may be derived from bacteria. Dissolved hydrolytic activities might also be a significant variable in TEP dynamics (Murray et al. 2007; Smith et al. 1992). Currently, there are no published studies addressing the relative or detailed impacts of the sources or the stabilities of released microbial exo-enzymes in seawater.

The enzymatic activities of bacterial cell surface-associated proteases and glucosidases on diatom surfaces might reduce or enhance diatom cell ‘stickiness’ and their

aggregation potential (Azam et al. 1991). This enzymatic 'pruning' of the diatom mucus would require micro-scale interactions between diatoms and bacteria. High concentrations of bacteria in the phycosphere would probably increase the rate of this process.

1.5.2 Microscale interactions – Bacterial motility

Cross-feeding or chemical interactions between bacterioplankton and diatoms can happen only in close distance, and various adaptive mechanisms allow bacteria to attach to phytoplankton cells (Baker and Herson 1978; Bell and Mitchell 1972b; Cole 1982; Decho 1990a; Grossart and Simon 1998; Kiørboe et al. 2002). The close proximity (0-1 mm) of individual cells suggests that there is potential for many cell-to-cell interactions. Motility and sensing enable bacteria to adapt to environments that contain substrate gradients. By this, bacteria may achieve spatial coupling with living or dying algae, or with protists (Barbara 2003; Blackburn et al. 1998). Chemotaxis-driven movement towards and attachment to the algal cell appear to be essential first steps for any interaction (Grossart et al. 2001).

Primary adhesion of bacteria to abiotic surfaces is often mediated by non-specific, e.g. hydrophobic interactions, whereas adhesion to living cell surfaces is accomplished through specific molecular docking mechanisms utilizing i.e. lectins, ligands, flagella, or adhesive proteins (Carpentier and Cerf 1993). The initial attachment of bacteria to biotic surfaces has been demonstrated to be flagella-mediated. In this context, molecular studies with *Pseudomonas aeruginosa* showed that flagella-defective mutants indeed do not adhere efficiently to surfaces (O' Toole and Kolter 1998). Bacterial flagella are composed of three structural elements: i) a basal body, which is embedded in the cell membrane and contains the proteins required for ATP hydrolysis, proton-motive force-mediated movement, and signal coupling to the chemotaxis apparatus; ii) an external helically shaped filament that acts as 'propeller' when rotated at its base; and iii) a hook that serves as a joint between the basal body and the flagella filament (Fig. 5). The flagellar filament of *P. aeruginosa* and other related organisms is a copolymer of flagellin subunits encoded by the *fliC* gene (Asakura 1970; Jarrell 2009; Totten and Lory 1990). Components of the axial structure of the filament are exported from the cytoplasm to the periplasm by the general secretory (Type II) pathway before these proteins are secreted from the periplasm to reach

their final assembly destination via a flagellum-specific pathway (Blocker et al. 2003). Thus, first the flagellar hook and then the filament grow by the incorporation of new flagellin subunits at the distal end of the filamentous structure.

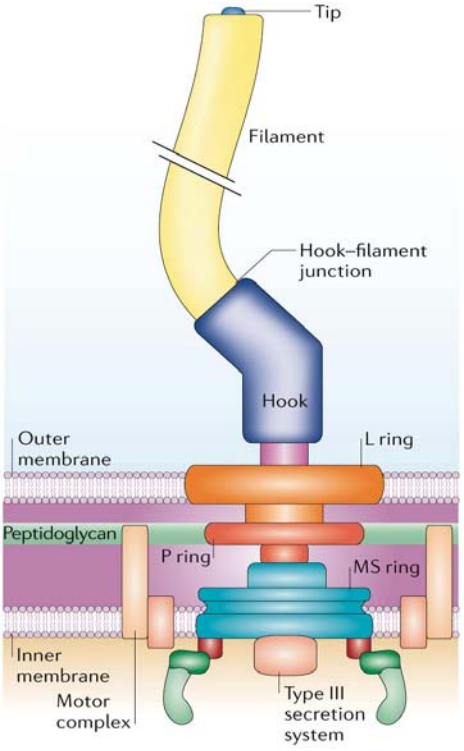

Figure 5: The bacterial flagellum consist of a basal body and two axial structures, the hook and the filament, which are joined with the hook-filament junction (Pallen and Matzke 2006).

1.6 Model system to study phytoplankton-bacteria interactions

In order to understand the molecular interaction between phytoplankton and bacterial cells, an *in vitro* model system consisting of the diatom *Thalassiosira weissflogii*, and the marine *Gammaproteobacterium Marinobacter adhaerens* HP15, will be established in the course of this thesis (Fig. 6). With this bilateral model system being generated, a species-specific interaction can be intensively and mechanistically studied in terms of the attachment processes, EPS and TEP formation, as well as the characterization of diatom aggregation features.

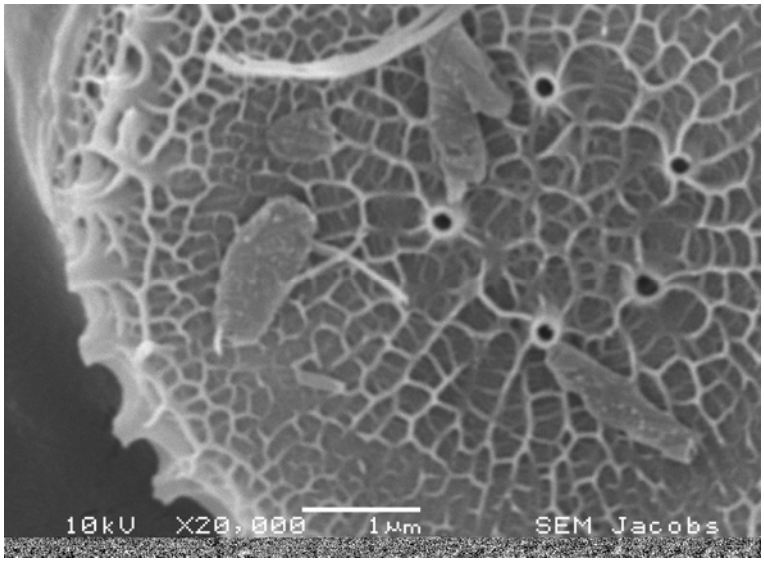

Figure 6: Scanning electron microscopic picture showing *M. adhaerens* sp. nov HP15 and *T. weissflogii* in close contact (this study).

1.6.1 Thalassiosira weissflogii

Diatoms are the most abundant group of phytoplankton, are ubiquitously distributed in the ocean, contribute up to 50 % of marine primary production (Nelson et al. 1995), and their aggregates are thought to be responsible for the transport of CO_2 from the surface to the deep (Morel et al. 2002). *T. weissflogii* has for a long time been considered as a marine diatom model organism and possesses suitable attributes: it is easy to cultivate, has a short replication time, and considerable genetic background information for this organism is available making it suitable for genetic manipulations (Armbrust 1999). *T. weissflogii* is a centric, radially symmetrical marine diatom assigned to the following phylogenetic classification:

Phylum:	*Bacillariophyta*
Class:	*Coscinodiscophyceae*
Order:	*Thalassiosirales*
Family:	*Thalassiosiraceae*
Genus:	*Thalassiosira*

Typical for diatoms, *T. weissflogii* has a characteristic ornamented siliceous cell wall and a frustule composed of two overlapping thecae: an epitheca and a hypotheca (Fig. 7). Each theca consists of a silica valve and one or more girdle bands, which possess a distinct micro-architecture of pores and slits displayed in a highly organized arrangement. These structures facilitate cell expansion and growth and provide routes for nutrient and gas exchange and for the secretion of exopolysaccharides (Molino and Wetherbee 2008). The cells are equipped with long spines made of chitin, which are presumed to be floating appendages (Smetacek 1985).

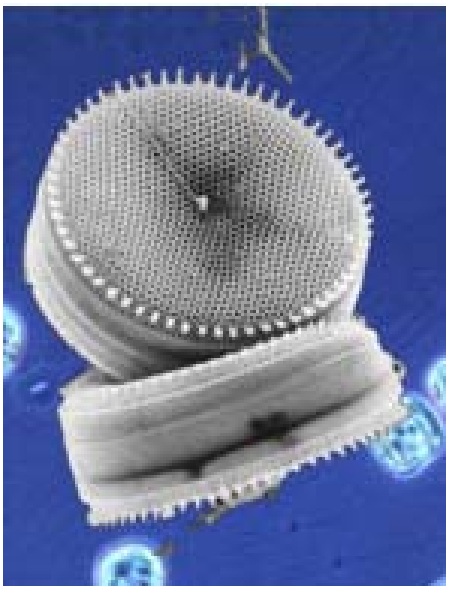

Figure 7: False-colored scanning electron micrograph of the marine diatom *Thalassiosira weissflogii* (Lane and Morel 2000).

Cell diameters of *T. weissflogii* range between 12-22 µm, and the cells grow with a doubling time of ~1.1 days until stationary phase is reached at approximately 120,000 cells per ml (Ribalet et al. 2007; Vrieling et al. 1999). *T. weissflogii* is a ubiquitously distributed species and has been isolated from the waters of Southeast Asia, Europe, Australia, and other regions (Dassow et al. 2006). *T. weissflogii* belongs to a genus with approximately 67 species, which exhibit a broad distribution spectrum covering marine environments ranging from tropic waters to arctic zones, i.e. *T. bipartita* (Hallegraeff and Bolch 1992) and *T. antarctica* (Doucette and Fryxell 1985). So far, the genome sequence of *T. pseudonana* isolated from Moriches Bay (Long Island, New York) has been determined as this species was considered a model for diatom physiology studies (Armbrust et al. 2004).

1.6.2 Marinobacter adhaerens sp. nov. HP15

The bacterial strain sp. nov. HP15 was isolated from particles sampled in surface waters of the German Wadden Sea (Grossart et al. 2004a). In the current study, it was identified among 82 diverse but particle-associated isolates due to its high potential to induce production of TEP and aggregate formation while interacting with the diatom *T. weissflogii*. According to 16S rRNA gene sequence analysis in the Ribosomal Database Project (Cole et al. 2005) the closest relative of *M. adhaerens* sp. nov. HP15 (GenBank accession no. AY241552) is *M. flavimaris* strain SW-145 (GenBank accession no. AY517632) isolated from the Yellow Sea offshore of Korea (Yoon et al. 2004a) with a similarity score of 0.99. The whole-genome relatedness of strain HP15 to *M. flavimaris* was determined as 66.2 % by DNA-DNA hybridization (This study). On the basis of phenotypic and chemotaxonomic properties and phylogenetic analysis, the strain HP15 (=DSM XXX^T = CIP XXX^T; pending) was proposed to represent a novel species, which was named *Marinobacter adhaerens* sp. nov..

M. adhaerens sp. nov. HP15 is a Gram-negative heterotrophic marine bacteria belonging to the following phylogenetic classification:

Phylum:	*Proteobacteria*
Class:	*Gammaproteobacteria*
Order:	*Alteromonadales*
Family:	*Alteromonadaceae*
Genus:	*Marinobacter*

The bacterium forms brownish, mucoid colonies when grown on Marine Broth (MB) agar plates and appears as a rod-shaped (0.6-0.8 x 1.7-2.4 µm), non-spore-forming cell with a single polar flagellum when observed by transmission electron microscopy (Fig. 8). HP15 grows optimally at temperatures ranging from 34°C to 38°C with a doubling time of ~3.1 hours (Kaeppel et al. submitted).

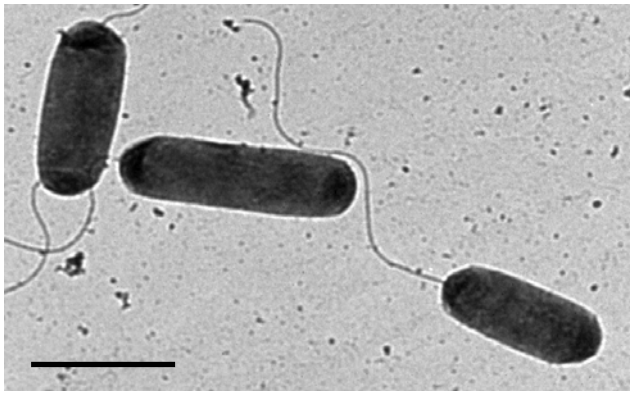

Figure 8: Transmission electron microscopic photograph of *M. adhaerens* sp. nov HP15. Scale bar: 1 µm (this study).

So far, more than 26 species belonging to the genus *Marinobacter* have been identified. The genus was first described by Gauthier (1992a) and has become of increasing interest because together with several other bacterial genera – namely *Alcanivorax*, *Thallassolituus*, *Cycloclasticus*, *Oleispira* and a few others - *Marinobacter* forms the obligate hydrocarbonoclastic group of bacteria recognized to play a significant role in the biological removal of petroleum hydrocarbons from polluted marine waters (Boyd et al. 2007). Further, different *Marinobacter* species are ubiquitously distributed and have been isolated from a variety of marine environments ranging from oil-contaminated environments (*M. aquaeolei*) (Huu et al. 1999b; Marquez and Ventosa 2005) to surface waters (*M. litoralis*) (Zhuang et al. 2009) and antarctic environments (*M. guineae*) (Montes et al. 2008a). Two *Marinobacter* species were identified due to their interactions with other organisms - *M. algicola* in dinoflagellate cultures and *M. bryozoorum* from *Bryozoa* (Green et al. 2006; Romanenko et al. 2005). Affiliation of the strain HP15^T to the genus *Marinobacter* was confirmed by BLASTn analysis of the 16S rRNA gene sequence. HP15 was most closely related to the type strains of *M. flavimaris* (99 %), *M. salsuginis* (98 %) and *M. algicola* (96 %). These four species form a discrete cluster in the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (Fig. 9).

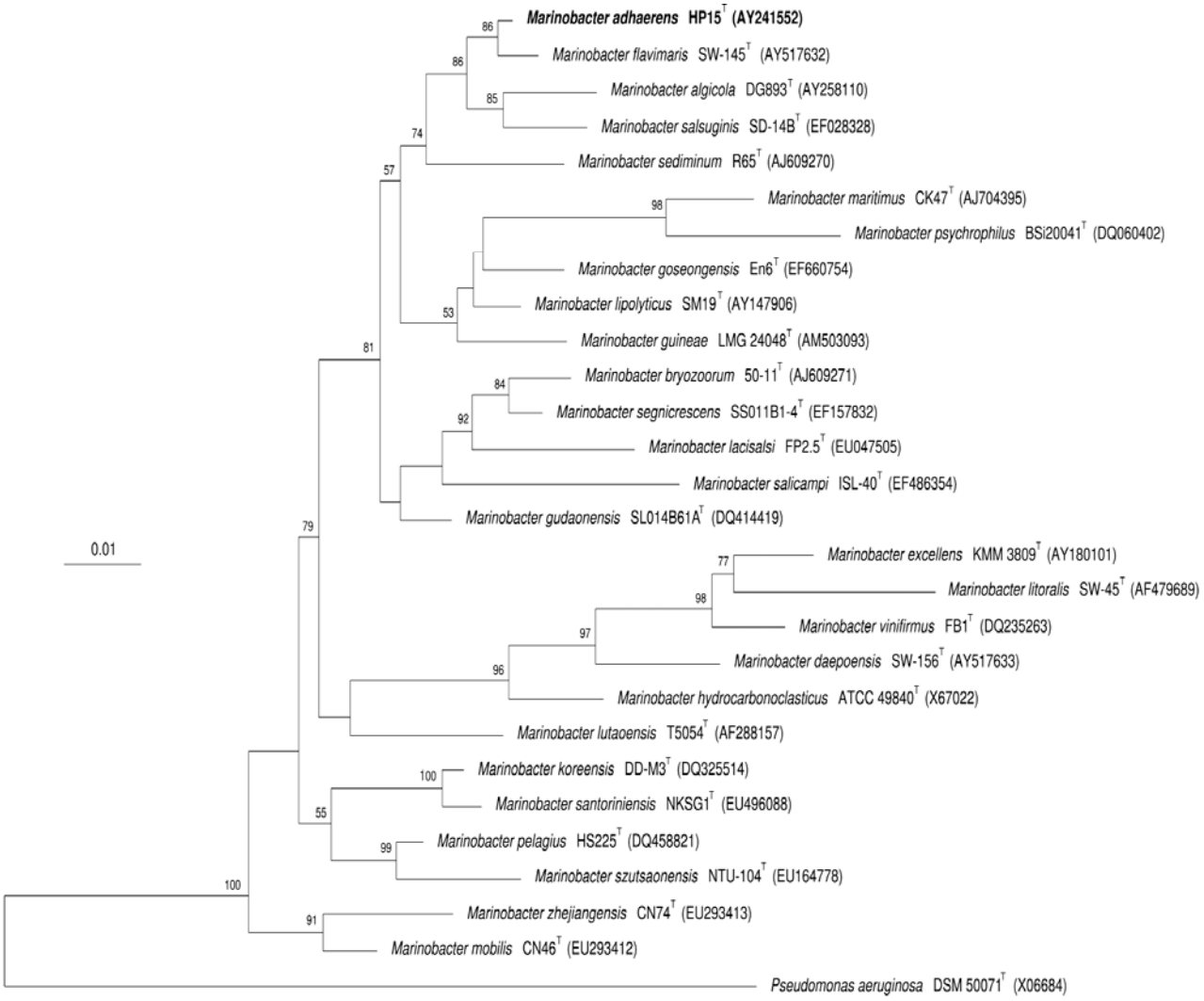

Figure 9: Phylogenetic relationship of *Marinobacter adhaerens* sp. nov. HP15 to other *Marinobacter* type strains. *Pseudomonas aeruginosa* PAO1 was used as an outgroup. The tree was generated by the neighbor-joining tree building algorithm. Bootstrap values are shown as branch points. Bar: 0.01 nucleotide substitutions per site.

2 Aims of the work

The main goal of the work is to answer some of the major questions regarding the role of marine heterotrophic bacteria interacting with algae and their impact on polysaccharide production, transparent exopolymer particle formation, and aggregation of dissolved and particulate organic matter. This is to better understand the cellular basis of vertical carbon flux in the ocean and the particular contribution of diatom-associated bacteria in this process.

For this, a genetically accessible bacterial model system was generated and investigated, which allows intensive and mechanistic studies on bacteria-algae interactions in terms of EPS and TEP formation, as well as the characterization and revealing the function(s) in phytoplankton aggregation. The aims of the current work can be divided into five major topics:

I Investigation of the role of diatom-associated bacteria in formation and dynamics of marine aggregates.

II Characterization of the concurrent diatom-bacteria interaction under fluctuating nutrient conditions and its effects on released diatom exudates.

III Establishment of a genetically accessible model system for molecular in-depth studies of diatom-bacteria interactions.

IV Chemotaxonomic and genomic characterization of *Marinobacter adhaerens sp. nov*. as the bacterial counterpart of the bilateral model system.

V Identification and mutational analysis of genes required for diatom-bacteria interactions, such as adhesion factors, in *Marinobacter adhaerens sp. nov*.

3 Results

Results of the current thesis are represented by the following manuscripts originated during the PhD thesis work:

Diatom-associated bacteria are required for *Thalassiosira weissflogii* aggregation.
Astrid Gärdes, Morten H. Iversen, Hans-Peter Grossart, Uta Passow and Matthias S. Ullrich
(submitted to ISME Journal)

Effects of *Marinobacter adhaerens* HP15 on polymer exudation by *Thalassiosira weissflogii* grown at different nitrogen-to-phosphorus ratios.
Astrid Gärdes, Yannic Ramaye, Hans-Peter Grossart, Uta Passow and Matthias S. Ullrich
(to be submitted)

Marinobacter adhaerens* sp. nov., prominent in aggregate formation with the diatom *Thalassiosira weissflogii.
Eva C. Kaeppel, Astrid Gärdes, Shalin Seebah, Hans-Peter Grossart and Matthias S. Ullrich
(submitted to International Journal of Systematic and Evolutionary Microbiology)

Complete genome sequence of *Marinobacter adhaerens* sp. nov. HP15, a diatom-interacting microorganism.
Astrid Gärdes, Eva C. Kaeppel, Aamir Shezad, Shalin Seebah, Hanno Teeling, Pablo Yarza, Hans-Peter Grossart, Frank Oliver Glöckner and Matthias S. Ullrich
(submitted to Standards in Genomic Science)

Analysis of genetic accessibility of *Marinobacter adhaerens* sp. nov. HP15, a marine bacterium interacting with diatoms.
Astrid Gärdes*, Eva C. Kaeppel*, Shalin Seebah and Matthias S. Ullrich
* these authors contributed equally
(to be submitted)

3.1 Diatom-associated bacteria are required for *Thalassiosira weissflogii* aggregation

Diatom-associated bacteria are required for aggregation of *Thalassiosira weissflogii*

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Abstract

Aggregation of algae, mainly diatoms, is an important process in marine systems leading to the settling of particulate organic carbon predominantly in form of marine snow. Exudation products of phytoplankton form transparent exopolymer particles (TEP), which are important for particle aggregation. Heterotrophic bacteria interacting with phytoplankton may control TEP formation and phytoplankton aggregation. This bacterial impact has not been explored in detail. It is hypothesized that bacteria attaching to *Thalassiosira weissflogii* might interact in a yet-to-be determined manner, which could be instrumental for TEP and aggregation dynamics. Herein, the role of defined *T. weissflogii*-attaching and free-living bacterial isolates for TEP production and diatom aggregation was investigated *in vitro*. *T. weissflogii* did not aggregate in axenic culture, and striking differences in aggregation dynamics and TEP abundance were observed when diatom cultures were treated with either diatom-attaching or free-living bacteria. Free-living bacteria did not influence aggregation whereas bacteria attaching to diatom cells significantly increased aggregate formation. Interestingly, metabolically inactivated *T. weissflogii* cells did not aggregate regardless of presence of bacteria. Comparison of aggregate formation, TEP production, aggregate sinking velocity, and solid hydrated density revealed remarkable differences. Both, metabolically active *T. weissflogii* and specific diatom-attaching bacteria were required for aggregation. It was concluded that interactions between heterotrophic bacteria and diatoms increased aggregate formation and particle sinking and thus may enhance the efficiency of the biological pump.

Running head: Bacterial-diatom interaction for aggregate formation

Key words: bacteria-phytoplankton interactions/ marine aggregates/ sinking flux/ TEP/ attachment assay

Introduction

Diatom blooms are frequently terminated by aggregation and subsequent sedimentation (Alldredge and Gotschalk 1989; Jackson 1990). The formation of large, fast sinking aggregates was proposed to be an integral part of the life cycle of many diatom species (Smetacek 1985) but also contributes significantly to carbon flux thus being important for the global carbon cycle (Fowler and Knauer 1986). Aggregation rates depend on the abundance and size of particles as well as on their environment, their relative sinking velocities, and their stickiness (Jackson 2001; Kiørboe and Hansen 1993). The sticking

efficiency of particles is thought to be a result of cellular exudation (Passow and Alldredge 1995a), cell morphology (Kiørboe et al. 1990), and surface properties (Waite et al. 1995). A major class of phytoplankton exudates are transparent exopolymer particles, TEP, which form the matrix of diatom aggregates (Alldredge et al. 1993) and promote aggregation of particles (Engel 2000; Jackson 1995; Logan et al. 1995). TEP, which form abiotically from precursor material released by diatoms (Passow 2002b), accumulate during bloom development (Jackson 2005; Kiørboe et al. 1994). Release of exudates by diatoms is species-specific and depends on environmental conditions (Kahl et al. 2008; Myklestad 1995). In laboratory experiments, three different types of aggregation behavior were distinguishable: (i) none-aggregating species that produce very little TEP (e.g. *Thalassiosira weissflogii*), (ii) aggregating species that generate a cell coating of sticky TEP-like material but produce no free TEP (e.g. *Nitschia angularis*) and (iii) species that generate copious amounts of TEP and form TEP-rich aggregates (*Chaetoceros spp*.) (Crocker and Passow 1995; Logan et al. 1994). *In situ* TEP concentration is coupled tightly with diatom concentrations suggesting that diatoms are the major producers of TEP (Passow 2002a). Bacteria also produce TEP (Azam et al. 1994; Decho 1990a; Grossart et al. 2006a; Stoderegger and Herndl 1999) and play a crucial role in determining the sticking efficiency of algal cells (Smith et al. 1995). Bacteria degrade or transform, and thus utilize TEP as an energy source (Bhaskar et al. 2005; Grossart and Simon 2007). Microbial activities influence TEP formation in dependence of a) the nutritional environment (Myklestad 1977); b) heterotrophic bacteria acting on particulate or dissolved fractions (Mari and Kiørboe 1996; Mari et al. 2004); c) grazing pressure (Dutz et al. 2005; Prieto et al. 2001); and d) the production of enzymes and extra-cellular products (Alderkamp et al. 2007). Grossart et al. (2006b) showed that heterotrophic bacteria control the development and aggregation of marine diatoms such as *T. rotula* and *Skeletonema costatum*. Although plant-microbe interactions have been extensively studied in terrestrial systems, e.g. in the rhizosphere (Kameneva and Muronets 1999), very little is known about the relationship between marine bacteria and diatoms. Both organism groups are thought to closely interact in the 'phycosphere', which is the micro-zone surrounding algal cells (Bell and Mitchell 1972b). Therein, bacteria may be free-living (Blackburn et al. 1998), may be attached to the algal surface (Kogure et al. 1981), or may occur as intracellular algal symbionts (Lewis et al. 2001). The attachment of bacteria to algal cells may represent a tight interaction (Cole 1982; Grossart et al. 2005).

Aim of the present study was to analyze diatom-bacteria interactions, which induce TEP and aggregate formation *in vitro*. *T. weissflogii* was chosen as test organism in order to avoid massive self-aggregation (Grossart et al. 2006b) and to focus on the bacterial influence on aggregate formation. The individual impact of attaching versus free-living bacteria as well as the metabolic state of diatom cells on various characteristics of aggregates was studied in rolling tank experiments. It was hypothesized that formation rates, size-dependent densities, and settling velocities of diatom aggregates differ in dependence of the physiological state of *T. weissflogii* and are influenced by presence of specific bacterial strains.

Material and Methods

Bacterial isolates

Five bacterial strains designated H14, Ex5, HP15, HP10, and HP2 were pre-selected from a total of 85 marine bacterial isolates (**Supplementary Tables 1, 2 and 3**). Selection was based on results of the *T. weissflogii* attachment assay described below. Strains HP15, HP10, and HP2 were isolates from marine particulate samples collected in June and October 2000 from surface waters of the German Wadden Sea (53°43′20″N, 07°43′20″E) using a 100-µm pore size plankton net (Grossart et al. 2004a). Bacterial strains H14 and Ex5 were isolated from the sponge, *Halichondria panacea*, collected near the island of Helgoland, German Bight, in October 1997 and January 1999, respectively (Wichels et al. 2006). Based on results of the *T. weissflogii* attachment assay, strains H14, Ex5, HP15, and HP10 were diatom-attaching while strain HP2 served as a free-living, non-attaching control strain. Bacterial isolates were grown on marine broth (MB) agar plates (Zobell 1941) at 28°C. Bacterial cells were washed twice with and resuspended in sterile f/2 medium prepared with >6 months-old filtrated seawater (Guillard 1975) to minimize input of bacterial growth-derived matter into diatom cultures as described earlier (Grossart 1999).

Axenic diatom cultures

Axenic cultures of *T. weissflogii* (CCMP 1336) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton

(Maine, USA). Diatom cultures were grown at 16°C in f/2 medium prepared as described above in 150-ml cell culture flask using a 12-h light period at 115 µmol photons m^{-2} s^{-1}. After 9 days of growth, diatoms were transferred to fresh f/2 medium. Cultures were checked regularly for bacterial contamination by microscopic observations and agar plating.

T. weissflogii attachment assay

The attachment assay was conducted to select four bacterial isolates, which attached to *T. weissflogii*, and one bacterial isolate that remained non-attached. A total of 85 marine bacterial isolates (**Supplementary Table 1, 2 and 3**) was tested for attachment. Three pools of bacterial isolates were generated according to the growth rates of the individual strains, which were determined by spectrometric analysis of the optical density (OD=600). Cells of each pool (27-30 bacterial isolates per pool) were collected, washed, and incubated with 200 ml cultures of axenic *T. weissflogii* for 24 h. Control treatments without diatom cells were conducted in parallel (bacteria only). Subsequently, cultures were carefully passed through a 10-µm plankton sieve by gravity flow to separate free-living bacterial cells from those attached to diatoms. Numbers of attached and free-living bacteria were determined by dilution plating on MB agar and counts of colony forming units (CFU ml^{-1}), which allowed determination of viable bacterial cells in both, the >10 µm fraction (attached bacteria) and the fraction of free-living bacteria. Colonies from the >10 µm fraction were picked after 3 days of incubation at 22°C and identified by 16S rRNA gene amplification and sequencing as follows: Universal 16S rRNA primers, GM3F and GM4R, specific for the domain *Eubacteria* (Muyzer and Ramsing 1995) were used to amplify 16S rRNA PCR fragments (5 fmol $µl^{-1}$) for nucleotide sequencing using BIG dye terminator Sequencing kit (Applied Biosystems Inc, USA**)**. Scanning electron microscopy (SEM) of the > 10 µm fraction was used to visualize and verify bacterial attachment to diatom cells following the procedure described by (Martin-Cereceda et al. 2007).

Rolling tank experiments

Rolling tanks mimic continuous sinking of aggregates and promote the formation of natural aggregates (Ploug and Passow 2007; Shanks and Edmondson 1989). Forty liters of diatom pre-cultures were grown in two 20-L cylinder flasks for 9 days until they reached late logarithmic growth phase. Sterile air with a CO_2 partial pressure of 15 Pa was continuously pumped through the cultures to avoid inorganic carbon depletion. *T. weissflogii* cell abundance was monitored by counting in a Sedgwick-Rafter Cell S50 (SPI Supplies, West Chester, USA) using an inverted Axiovert 200 microscope (Zeiss, Jena, Germany). The photosynthetic activity of diatoms was assessed using a pulse-amplitude-modulated (PAM) fluorometer as described below.

Different combinations of *T. weissflogii* cells, bacterial cells, or glass beads were incubated in 1.15-L plexi glass cylinders with diameters of 14 cm and depths of 7.47 cm. Five treatments were set up in duplicate (10 roller tanks) with metabolically active *T. weissflogii* plus either HP15, HP10, Ex5, H14 or the free-living control strain HP2. A second series of 5 rolling tank treatments was consisting of metabolically inactive *T. weissflogii* and the same bacterial strains as above. Additionally, the following controls were run in parallel: (1) axenic *T. weissflogii* cultures; (2) each of the five individual bacteria strains without diatoms; and (3) each of the five bacteria strains with glass beads (0.25 – 0.5 mm bead diameter) (Roth, Karlsruhe, Germany). Individual co-incubations were started by addition of 1 ml of washed cells of a given bacterial strain to 1.0 L of the axenic diatom culture, which was then diluted with 600 ml of fresh f/2 medium. In the absence of diatoms, sterile f/2 media was added instead of cell suspensions. Initial diatom concentrations were 3,000 cells ml^{-1} and initial bacterial concentrations were 3 x 10^4 cells ml^{-1}. Glass beads were added at a final concentration of 3,000 beads ml^{-1}.

All 27 rolling tanks were rotated on a rolling table at three rotations per minute at 18 °C for 7 days. Experiments were conducted in darkness, except for a 2-3 h light period per day, during which aggregates were counted or samples taken. Kinetics of dark-arrested *T. weissflogii* showed that these cells were not able to divide and withstood darkness for more than 2 weeks (Vaulot et al. 1986). Bacteria and TEP concentrations were determined after 24, 96, and 168 h of incubation. Samples of 5 ml of aggregate-free water from rolling tanks were collected at each sampling time. From these, 200 µl were used for the determination of bacterial cell numbers and 4 ml were used for TEP measurements. Samples were collected under sterile conditions using syringes and were immediately processed. Furthermore, aggregate abundance and size-distribution in each roller tank was estimated after 24, 48, 96, and 168 h of incubation.

Inactivation of T. weissflogii

Diatom cell suspensions were transferred to sterile 1-L bottles, placed in a microwave set to 180 W for 90 s, and subsequently incubated for 1 h at 18 °C as described earlier (Judy et al. 1989). Cells were observed microscopically and photosynthetic activities were determined to ensure inactivation without physical destruction or cell lysis. Prior to and directly following the microwave treatment and again post incubation, photosynthetic activities were measured using a PAM fluorometer as described below.

Determination of photosynthetic activity

Photosynthetic activities of *T. weissflogii* cells were measured by the 'saturation pulse method' using a PAM fluorometer according to Schreiber (1986) aliquots of cell suspensions were removed, placed into the PAM fluorometer, and dark-adapted for 15 min before measuring the photosynthetic activity according to established protocols (Genty et al. 1989). The quantum yield of charge separation in PSII, φp, was determined according to equation (1).

$$\varphi p = (Fm - F0)/Fm \quad (1)$$

where *F*m is the maximum fluorescence and *F*0 represent the minimal fluorescence. Comparison of φp values verified the inactivation of microwaved *T. weissflogii* cells.

Determination of bacterial cell numbers

Two hundred µl of samples, collected from aggregate-free water in rolling tanks, were diluted 1:2 and 1:10 in sterile f/2 medium, fixed with 0.2-µm pore size pre-filtered, borate-buffered 2% formalin, and stained with 4´,6-diamino-2-phenylindole (DAPI; 1 µg ml-1) for 2 min. Stained samples were filtered onto 0.2-µm pore-sized polycarbonate Nucleopore filters (Whatman, Maidstone, England) and stored at -20°C. Bacterial cell numbers were counted using an epifluorescence Axioplan microscope (Zeiss, Jena, Germany) at 1,000x magnification according to an established method (Porter and Feig 1980). Samples were counted within two weeks after sampling.

Transparent exopolymeric particles (TEP) measurements

Free TEP concentrations were quantified using the dye-binding assay for spectrophotometric measurements (Passow and Alldredge 1995b). Four ml samples were taken from aggregate-free water of rolling tanks. Prior to staining, 1 ml aliquots were filtered in triplicates at low constant vacuum (max 150 mm of Hg) onto 0.4-µm pore sized Nucleopore polycarbonate filters. TEP were stained on the filter for 30 seconds with 500 µl pre-calibrated 0.02% aqueous solution of alcian blue (8GX) in 0.06% acetic acid (pH 2.5). Filters were then rinsed once with distilled water to remove excess dye, the dye was dissolved in sulfuric acid and measured in a spectrophotometer at 787 nm.

Determination of aggregate sinking velocity

Size-specific sinking velocity of aggregates was determined in a vertical flow system (Ploug and Jørgensen 1999). Individual aggregates were gently transferred from the rolling tanks to an open flow-through chamber using a wide-tipped pipette. The open flow-through chamber consisted of a 10-cm high Plexi glass tube (5 cm diameter) with a net located in the middle. The net created a relatively uniform flow field across the upper chamber when a flow was applied from below (Ploug and Jørgensen 1999). The sinking velocity of an aggregate was calculated by dividing the flow rate by the cross-sectional area of the flow chamber. Triplicate measurements of sinking velocity were made for each aggregate. The lengths of all three aggregate axes (x, y, and z direction) were measured in the flow system using a horizontal dissection microscope with a calibrated ocular. The aggregate volumes were calculated by assuming ellipsoid aggregate shapes. For comparison with other aggregates, the equivalent spherical diameter (ESD) was calculated (diameter of a sphere with equivalent volume).

Measurement of the solid hydrated density of aggregate constitutes

The solid hydrated density (ρ_s, g cm^{-3}) of the particles inside the aggregates was determined in a density gradient using a modified version of previously reported methods (Feinberg and Dam 1998; Schwinghamer 1991). Seven dilutions were made using Ludox TM colloidal silica, sucrose, and distilled water ranging in density from 1.05 to 1.43 g cm^{-3}. Dilutions were buffered to pH 8.1 with 0.0125 M Tris plus 0.0125 M Tris-HCl (final concentration). Thus, the produced gradient was iso-osmotic with seawater of a salinity of ~ 32 ‰ (Handbook Chem. Phys. 1968). Two ml of each dilution were gently transferred to a 20-ml centrifuge tube, beginning with the densest and finishing with the least dense dilution. The density gradients were stored at 5 °C overnight and allowed to warm to room temperature before use. One ml of sterile seawater (32 ‰) was gently applied on top of the density gradient.

Table 1. *T. weissflogii* attachment assay. Three bacterial pools were separately incubated with *T. weissflogii* cell cultures. Bacterial abundances in CFU ml^{-1} for attached bacteria and free-living bacteria were determined. Bacteria incubated without diatoms served as control.

	Bacterial pool 1 *T. weissflogii*		**Bacterial pool 2** *T. weissflogii*		**Bacterial pool 3** *T. weissflogii*	
Bacterial abundance ($x10^6$) CFU ml^{-1}	with	without	with	without	with	without
t=0, total	1.04	1.24	1.19	1.12	1.80	2.02
t=1d, total	0.88	0.58	0.33	0.78	2.40	1.80
t=1d, attached bacteria	0.19	0.0024	0.24	0.0018	0.88	0.0075
t=1d, free-living bacteria	0.40	0.38	0.19	0.60	1.30	1.50

Single aggregates were transferred to individual centrifugation tubes using a wide-tipped pipette letting the aggregates settle into the seawater layer without breaking. Next, samples were centrifuged at 3,000 rpm for 30 minutes and 1 ml from the density layers containing the aggregate were removed from the tube using a peristaltic pump and weighed using a Mettler Toledo fine-balance. Average densities were calculated. In case aggregates broke during settling, several 1-ml samples of the density gradient were screened for particles using a dissection microscope and subjected to weighing.

Statistical analyses

A simple One-way ANOVA analysis was carried out to assess differences in TEP, bacterial cell numbers, and aggregate abundance between axenic and bacteria-containing treatments. The same type of analysis was applied for cell numbers of the four bacterial strains attaching to diatoms (HP15, H14, HP10 and Ex5) and the free-living strain (HP2) when incubated in rolling tanks.

Results

Attachment assay

When pools of bacterial strains were incubated with *T. weissflogii* cell suspensions, variable numbers of bacterial cells were found to be attached to the diatoms but all three pools showed a similar tendency (**Table 1**). Numbers of diatom-attached bacteria were highest in co-incubations with pool 3 with 8.8 x 10^5 CFU ml^{-1} (attached fraction) thereby accounting for almost half of the initially inoculated cell number (1.8 x 10^6 CFU ml^{-1}). Data for the respective control treatment showed that a very small number of bacterial cells (7.5 x 10^3 CFU ml^{-1}) was retained on filters (attached fraction) when no diatom cells were present. This control demonstrated that there was specific attachment to *T. weissflogii* cells and that there was very limited unspecific binding of bacterial cells to the filters or aggregation of bacteria cells. and 16 S rRNA sequencing. Data were compared with those of the initial bacterial isolates (**Supplementary Tables 1, 2, and 3**).

Bacterial cells from the attached fractions of all three pools were subjected to colony typing Isolates HP15, HP10, Ex5 and H14 were found to be best represented in the attached fractions. Isolate HP2 was identified as a typical free-living bacterial strain isolated from the flow-through.

Diatom-attaching bacterial strains belonged to different phylogenetic groups: Strains HP15 was originally isolated from marine particles and assigned to the gammaproteobacterial genus *Marinobacter.* Strain HP10 was also isolated from marine particles and represented a Gram-positive species of the *Firmicutes*. The 16 S rRNA of strain Ex5 had 98% similarities to *Psychroserpens burtonensis* belonging to Flavobacteria. Strain H14 was another member of the *Gammaproteobacteria*. The free-living strain HP2 was found to be a member of the *Flexibacter* genus belonging to Bacteroidetes.

In order to verify the diatom-attaching phenotypes, all five strains were again individually subjected to the attachment assay. As expected, strains HP15, HP10, Ex5, and H14 attached to diatoms while strain HP2 did not (Data not shown). Moreover, samples from the coincubation of strain HP15 with *T. weissflogii* were subjected to scanning electron microscopy (**Fig. 1**) showing a clear association of both organisms towards each other.

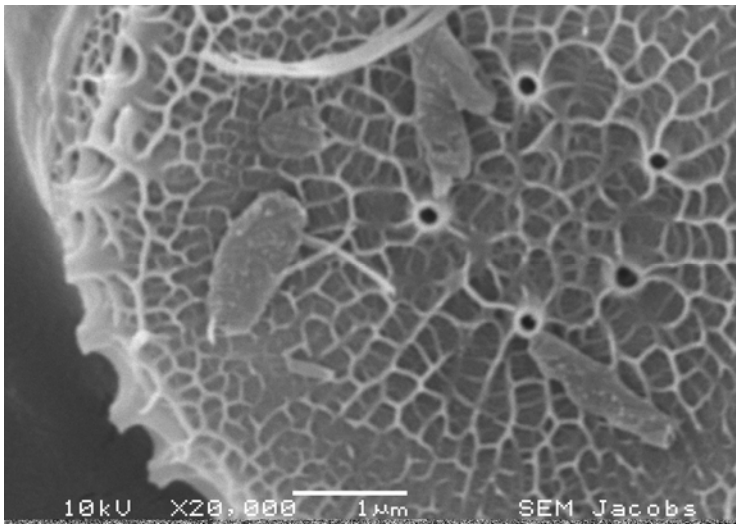

Figure 1. Scanning electron microscopy of a *T. weissflogii* cell with four cells of the attaching bacterial strain, HP15. Bacteria attached to *T. weissflogii* within 24 h of incubation. Scale bar 1 µm.

Aggregate formation in rolling tanks

Aggregate formation of metabolically active diatoms in rolling tanks differed significantly between axenic and bacteria-containing treatments ($p \leq 0.03$), and between treatments containing the attaching bacterial strains HP15, HP10, Ex5, and H14 or the free-living control strain HP2 ($p < 0.05$) (**Fig. 2**). No aggregates formed in rolling tanks containing axenic, metabolically active *T. weissflogii* cultures throughout the incubation period suggesting that activity of the diatom-attaching strains was vital for aggregate formation of this diatom. When *T. weissflogii* cells were co-incubated with any of the four attaching bacterial strains (HP15, HP10, Ex5, and H14), the total aggregated volumes increased to an average of ~14 cm^3 L^{-1} after 168 h of incubation. There was no significant difference in final aggregate abundance (30 ± 7.5 aggregates per tank with aggregate diameters of 3-5 mm) or total aggregated volume between incubations of the attaching-bacterial strains ($p < 0.262$). These results indicated that any of these bacterial species were capable to induce diatom aggregation. However, diatom aggregation dynamics differed for individual bacterial strains. Total aggregated volume increased steadily in rolling tanks containing bacterial strains H14 and HP15 while aggregation was delayed at 48 h and peaked at 96 h in treatments with strains HP10 and Ex5. In contrast to treatments with the attaching bacterial species the total aggregated volume in the treatment with the non-attaching bacterial strain, HP2, was roughly threefold lower after 168 h (4 cm^3 L^{-1}), and no aggregation occurred within the first 48 h (**Fig. 2**). When incubated with glass beads, none of the five bacterial isolates led to aggregate formation (Data not shown) indicating that none of the bacteria were able to induce aggregation of inorganic particles.

The impact of *T. weissflogii* metabolism on aggregate formation was investigated by co-incubations of bacteria with metabolically inactive diatom cells (**Fig. 2**). Analysis of the photosynthetic activities using PAM fluorometer determined active diatoms with $\varphi p = 0.57$ and inactive diatoms with $\varphi p = 0.21$. Microscopic observations confirmed that both, metabolically active and inactive diatoms, were colonized by *T. weissflogii*-attaching strains (Data not shown). However, metabolically inactive *T. weissflogii* cultures showed 9- and 12-fold lower aggregation rates when incubated with strains HP15 and HP2, respectively, as compared to metabolically active diatom cultures. Similar aggregate patterns were observed for metabolically inactive *T. weissflogii* cultures incubated with the bacterial isolates HP10, Ex5, and H14 (Data not shown). These results suggested that both, attaching bacteria and metabolically active *T. weissflogii* cells, were required for aggregate formation.

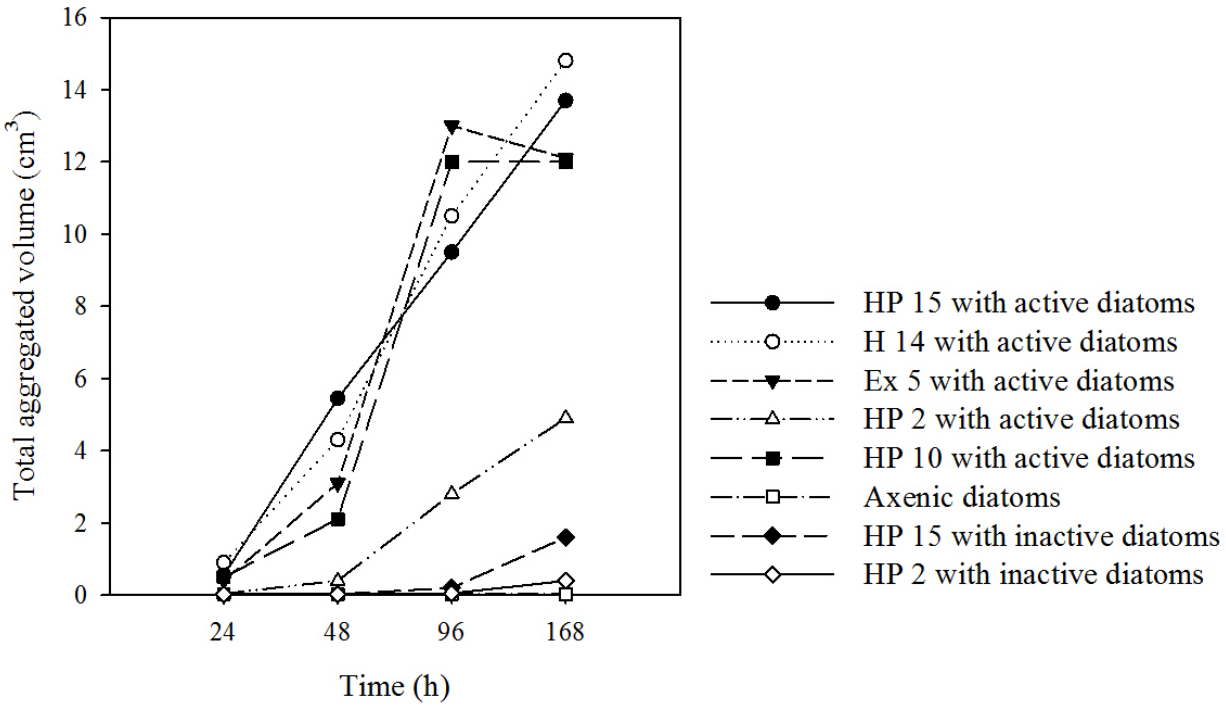

Figure 2. Total aggregated volumes (cm^{-3}) per rolling tank during 168 h of incubation. Metabolically active *T. weissflogii* cultures were separately incubated with the attaching bacteria strains, HP15, H14, HP10, and Ex5, or the non-attaching bacterial strain, HP2. Axenic *T. weissflogii* culture used as control. Metabolically inactive *T. weissflogii* cultures incubated with attaching strain, HP15, and non-attaching strain, HP2, as controls.

Aggregate characteristics

Sinking velocities and solid hydrated densities were determined for aggregates formed in rolling tank experiments from all five treatments with metabolically active diatoms and all five treatments with metabolically inactive diatoms as well as those formed with inactive axenic diatoms. Data were determined in replicates. Sinking velocities of aggregates generally increased with increasing aggregate sizes reaching 50 m d^{-1} for aggregates with an ESD of ~1 mm and 300 m d^{-1} for aggregates with an ESD of ~4 mm (**Fig. 3A**). Sinking velocity of aggregates formed in the presence of bacterial cells did not differ from those formed without bacteria. Using a power law, the correlation coefficient when plotting sinking velocities vs. the equivalent spherical diameters was high (R^2 = 0.81) (**Fig. 3B**).

Likewise, no differences were observed for the solid hydrated densities of aggregates between *T. weissflogii* cultures incubated with or without bacterial strains and ranged between 1.15 and 1.21 g cm^{-3} with an average value of 1.17 ± 0,021 g cm^{-3}. These data indicated that the measured aggregate characteristics were not influenced by the presence of attaching or free-living bacterial strains.

Dynamics of bacterial cell numbers and TEP formation

Bacterial cell numbers and TEP concentrations in aggregate-free rolling tank medium samples were quantified for treatments with metabolically active and inactive *T. weissflogii* cells (**Fig. 4**). When metabolically active *T. weissflogii* cell were incubated with the attaching strains, HP15, HP10, Ex5, and H14, bacterial cell numbers increased rapidly within the first 96 h and reached values ranging from 2 x 10^5 to 1 x 10^6 cells ml^{-1} (average= 8.9 x 10^5 ± 1.0 x 10^5 cells ml^{-1}) independent of the individual strains (**Fig. 4A**). These results suggested that bacterial cells potentially benefited from photosynthetic products released by *T. weissflogii*. After 96 until 168 h, bacterial cell numbers remained relatively stable. However, intermediate and maximum bacterial cell numbers of the non-attaching isolate, HP2, were significantly lower (on average 5.1 x 10^5 cells ml^{-1} ± 0.6 x10^5 cells ml^{-1}) as compared to those of attaching bacterial isolates. This indicated that the

non-attaching bacterial isolate could possibly not use diatom-derived substrates as efficient as the attaching bacterial strains.

TEP concentrations in treatments with metabolically active *T. weissflogii* and the attaching bacterial strains, HP15, HP10, H14 and Ex5, increased moderately within the first 96 h but more rapidly thereafter (**Fig. 4A**). At 168 h of incubation maximum values of with an average of 431 ± 27.2 µg Xequv L^{-1} were reached. Consequently, TEP concentrations of these treatments were higher than those of axenic *T. weissflogii* cultures. Interestingly, TEP formation was negligible when *T. weissflogii* cells were co-incubated with the non-attaching bacterial strain, HP2 (**Fig. 4A**) suggesting that this strain might not foster TEP formation by the diatom cells.

During the first 96 h bacterial abundances in treatments with metabolically inactive *T. weissflogii* (**Fig. 4B**) showed similar tendencies as those observed for metabolically intact diatoms. However, bacterial cell numbers decreased thereafter reaching maximum values of ~5 x 10^5 cells ml^{-1} (average 4.58 x 10^5 ± 1.2 x 10^5 cells ml^{-1}) (**Fig. 4B**). This decrease was even more pronounced for the incubation with the non-attaching strain, HP2. These results indicated that metabolically active diatom cells are required for optimal bacterial growth and that inactive *T. weissflogii* provide only a basic nutritional resource for bacteria. In controls with bacterial cells only or with bacteria and glass beads, the bacterial abundance decreased gradually from 4.7 x 10^4 ± 1.73 x 10^3 cells ml^{-1} at the beginning of incubation to 2.3 x 10^4 ± 7.81 x 10^3 cells ml^{-1} at 168 h (Data not shown).

When metabolically inactive *T. weissflogii* cultures were incubated with bacterial isolates, TEP concentrations decreased during the first 96 h but increased to an average of ~222 ± 41.8 µg Xequv L^{-1} at 168 h (**Fig. 4B**). Similar TEP concentrations were found for the axenic diatom controls (250 µg Xeuqv L^{-1}) after 168 h of incubation.

In summary, TEP concentrations correlated well with total aggregate volumes giving a linear regression R^2 = 0.89 (**Fig. 5**). With increasing total aggregate volumes TEP concentrations increased.

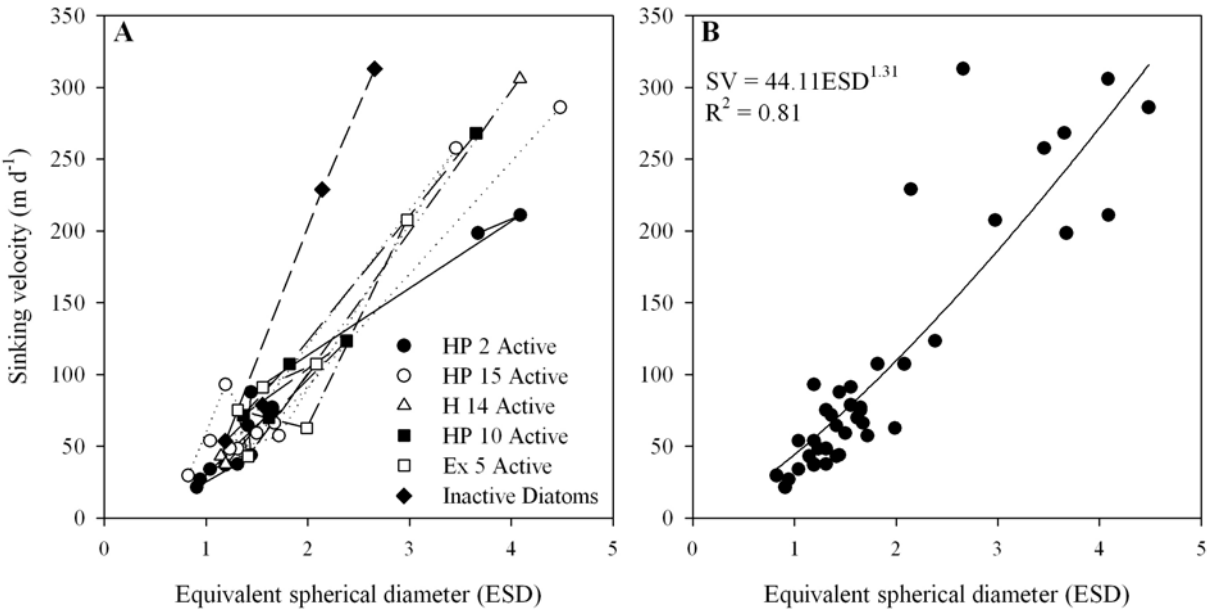

Figure 3. Sinking velocities of aggregates collected after 168 h of incubation as a function of aggregate diameter (Equivalent spherical diameter ESD in mm). **A**) Aggregates formed by *T. weissflogii* and the attaching bacterial strains, HP15, H14, HP10, and Ex5, as well as the non-attaching bacterial strain, HP2. **B**) Pooled sinking velocities (SV) for all aggregate types measured as a function of aggregate diameter. A power equation was fitted to the measurements: SV = 44.11 $ESD^{1.31}$; R^2 = 0.81.

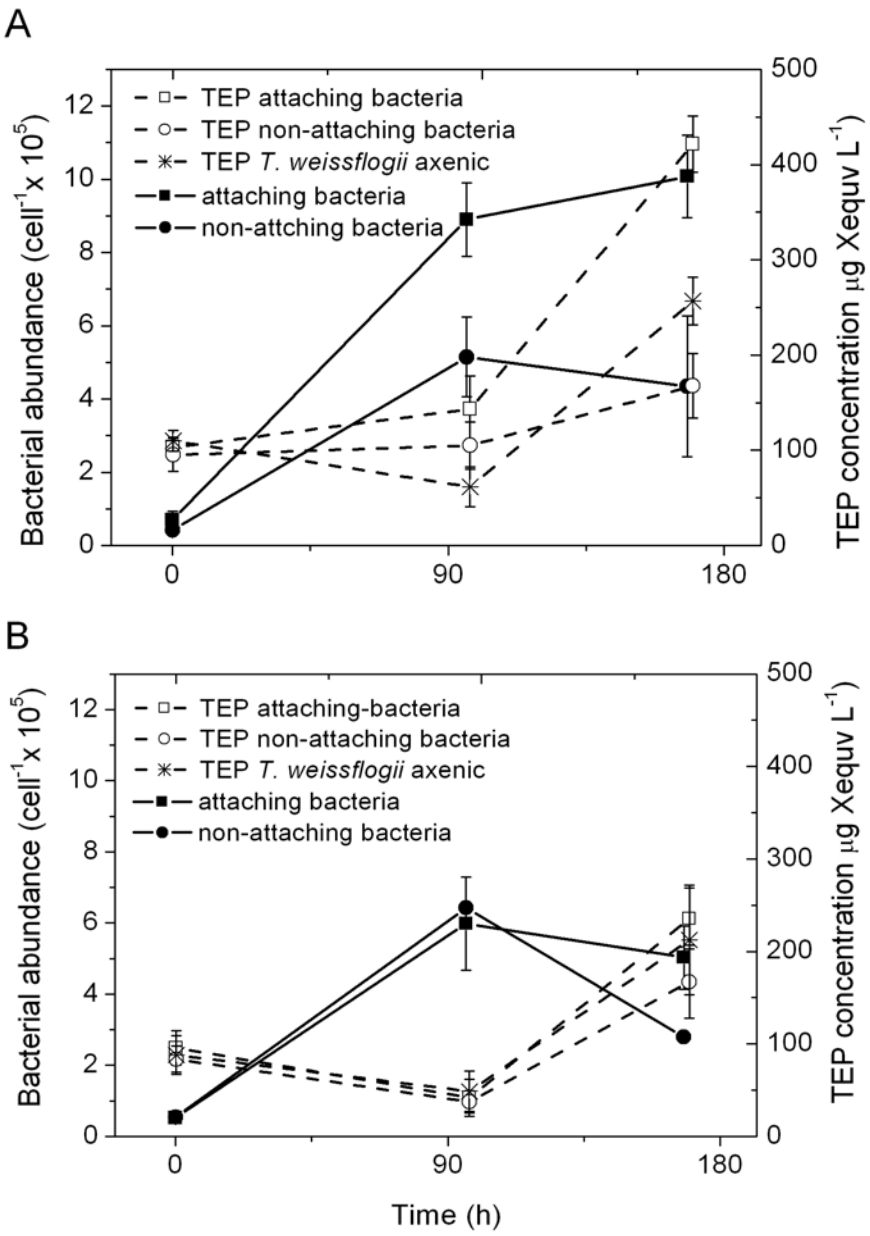

Figure 4. Average abundance of transparent exopolymer particles (TEP) and bacterial cell numbers from rolling tank experiments. **A**) Metabolically active *T. weissflogii* cultures and the attaching bacterial strains, HP15, H14, HP10, and Ex5. Average abundance of TEP from tanks with non-attaching bacterial strain, HP2, and the axenic *T. weissflogii* cultures were used as controls. **B**) Metabolically inactive *T. weissflogii* cultures and the attaching bacterial strains, HP15, H14, HP10, and Ex5. Average abundance of TEP from tanks of the non-attaching bacterial strain, HP2, and the inactivated axenic *T. weissflogii* cultures were used as controls.

Discussion

For the first time, this study demonstrated that under defined *in vitro* conditions specific bacterial strains attaching to *T. weissflogii* as well as metabolically active *T. weissflogii* cells are both required for TEP production and aggregate formation. Herein, it could be clearly demonstrated that aggregate abundance correlated with TEP concentration. This observed relationship had frequently been postulated but was not shown experimentally before (Azetsu-Scott and Passow 2004; Kiørboe and Hansen 1993; Passow and Alldredge 1995a).

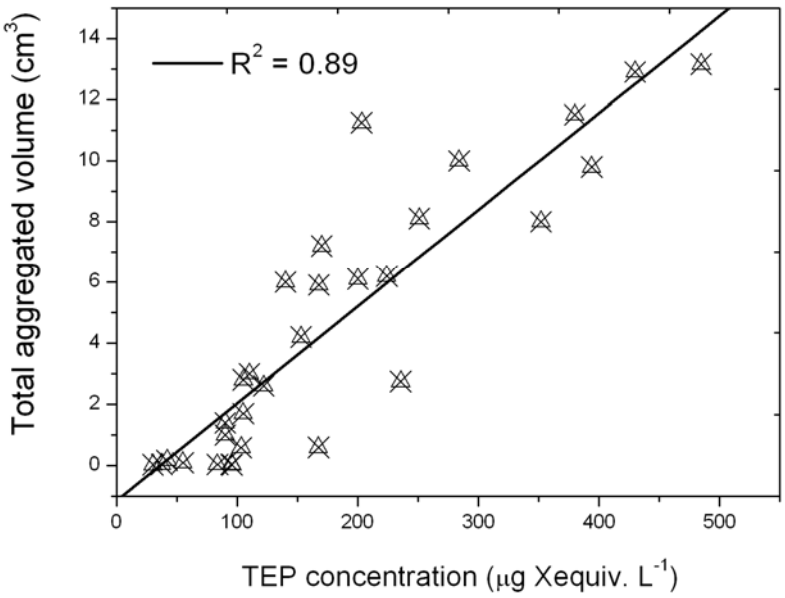

Figure 5. Correlation of total aggregated volumes and TEP concentrations in rolling tank experiments. Linear regression was fitted to measurements (R^2 = 0.89).

In an earlier study *T. weissflogii* was shown to formed little TEP and to not form aggregates (Crocker and Passow 1995). However, later it was observed that this diatom species generated copious amounts of TEP (Passow 2002a). Presumably the used diatom cultures of both studies differed in terms of bacterial contaminations, which according to results of the current study, might induce TEP formation and aggregation.

In contrast to previous studies (Grossart et al. 2006a; Grossart and Simon 2007), herein attaching and non-attaching bacterial isolates were comparatively analyzed with respect to their interactions with metabolically active and inactive diatom cells. It was demonstrated that not any type of bacterial isolate was able to elicit aggregation of *T. weissflogii* and TEP formation but that only attaching species did so. Moreover, photosynthesis by *T. weissflogii* was required to produce large amounts of TEP precursor material, which had previously been suggested to be required for aggregate formation through coagulation (Logan et al. 1995; Passow and Alldredge 1995a). Noteworthy, diatom exudates such as surface-active sugars might be affected by the herein applied inactivation procedure. In turn, this would change the sticking efficiency of diatom cells as described by others (Staats et al. 2000; Tien et al. 2005; Waite et al. 1995); which is an important parameter for aggregation (Jackson 1990).

Deducing from the current results, under natural conditions marine bacteria may benefit from diatom photosynthesis in form of either i) actively interacting with the algal thereby influencing TEP formation (and aggregation) or ii) in a non-interacting, free-living manner by utilizing dissolved organic matter released during the hydrolysis of TEP or other diatom exudates conducted by attaching bacterial species. Alternatively, bacteria itself may actively contribute to the TEP pool (Bhaskar et al. 2005; Passow 2002a; Stoderegger and Herndl 1999).

The observed aggregate characteristics did not differ between treatments with different bacterial strains attaching to *T. weissflogii*. A general tendency for increasing sinking velocity with increasing aggregate sizes was found thus confirming previous *in situ* results for natural aggregates (Iversen et al. 2010) and from laboratory experiments (Ploug et al. 2008). The average values for solid hydrated densities obtained herein were comparable with previously estimated values for *in situ* diatom aggregates (Alldredge and Gotschalk 1989). Results of the current study suggested a rather minor impact of bacteria on the estimated aggregate characteristics but showed their importance for *T. weissflogii* aggregation. Thus, this study provided

experimental evidence supporting a contribution of bacterial activities to the biological pump (Fowler and Knauer 1986).

A yet-to-be dissected cellular interaction of diatoms with bacteria may be instrumental for TEP and aggregation dynamics. This interaction might be as important for aggregation as are environmental conditions such as concentrations of nutrients, vitamins, and trace metals (Beauvais et al. 2006; Grossart 1999; Grossart and Simon 2007). The interaction might vary significantly *in situ* depending on changes in bacterial community composition, which possibly may explain the high variability of diatom aggregation patterns observed in previous studies (Azam and Malfatti 2007b; Kiørboe and Hansen 1993; Passow and Alldredge 1995a). At the moment, it cannot be ruled out that aggregation occurs as a result of nutrient stress of *T. weissflogii* (Grossart 1999; Grossart and Simon 2007) since the observed differential effects of non-attaching and attaching bacteria on aggregate formation may be the consequence of nutrient competition between algae and attaching bacteria.

The current results offer an experimental basis for establishing an *in vitro* model system, in which bilateral cellular interactions can be studied in-depth. This potentially will allow further studies on ecological consequences of bacteria-phytoplankton interactions not only in defined experimental set-ups but also in natural habitats.

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Supplementary Table 1. Phylogenetic affiliation of marine particle-associated bacterial isolates (This study).

Strain	Best match; similarity (%)
21-20-Bp	Uncultured *Acinetobacter sp.;94*
21-20-Bt	*Pseudomonas cf. stutzeri* V4.MO.16; 98
21-20-Bm	*Pseudoalteromonas elyakovii, 96*
21-20-Bj	*Arctic seawater bacterium* Bsw20400; 98
21-20-Bs	*Pseudoalteromonas sp.* G23; 97
21-20-Bv	*Pseudoalteromonas tetraodonis; 98*
21-20-Bl	*Pseudoalteromonas sp. 16; 97*
21-20-Bh	Marine bacterium 13; 94
21-20-Be	Marine bacterium 14; 96
21-20-Bc	Uncultured *Acinetobacter sp.; 96*
21-20-Bw	*Pseudoalteromonas sp.* 15; 96
21-20-Bn-2	*Pseudoalteromonas sp.* MMM18; 97
21-20-Bb	*Pseudoalteromonas sp*. CL19; 98
21-20-Bu	*Arctic seawater bacterium* Bsw20410; 98
22-20-Ab	*Pseudoalteromonas* BSi20460; 96
22-20-Ba	*Pseudoalteromonas sp.* CL19; 97
23-20-Aa	*Acinetobacter sp.* V4.ME.25; 96
23-20-Ab	Marine bacterium 13; 94
23-20-Ac	*Pseudomonas sp.* 168; 98
23-20-Ad	Uncultured *Acinetobacter sp.; 98*
24-0-B2	*Pseudoalteromonas sp.* BSi20548; 97
24-0-Ba	*Pseudoalteromonas sp.* CL1105; 97
25-20-Ba	*Psychrobacter glacincola, 97*
25-20-Bc	*Pseudoalteromonas tetraodonis, 99*
26-20-Ab	*Pseudoalteromonas sp.* BSi20325, 97
26-20-Ac	Marine bacterium Tw-4; 97
26-20-Ae	*Pseudoalteromonas sp.* RE10F/2; 98
26-20-Aa	*Pseudoalteromonas sp.* LOB-15; 98
26-20-Aa 2	*Pseudomonas sp.* 169; 90
28-20-Bb	*Pseudoalteromonas sp.; 96*
28-20-Ba	*Pseudoalteromonas sp.* D20; 94
29-20-Ab	*Pseudomonas sp.* V16; 99
29-20-Aa	*Acinetobacter sp.* WAB1931; 96

Supplementary Table 2. Phylogenetic affiliation of bacterial strains isolated from *Halichondria panacea* (Wichels et. al. 2006)

Strain	Best match; similarity (%)
Ex1	*Pseudoalteromonas nigrifaciens; 94*
Ex11	*Rugeria atlantika; 96*
Ex5	*Phsychroserpens burtonensis; 98*
Ex7	*Roseobacter literalis; 98*
Ex8	*Leifsonia aquatica; 89*
Ex9	*Phsychroserpens sp.; 97*
H11	*Pseudoalteromonas tetraodinis; 95*
H14	*Alteromonas sp.; 98*
H22	*Pseudoalteromonas sp.; 98*
H23	*Glaciecola sp.; 97*
H24	*Sulfitobacter sp.; 96*
H92	*Pseudoalteromonas carragenorra; 97*
H94	*Glaciecola sp.; 98*
H3	*Microscilla arenaria; 94*
Noc A1	*Marinobacter testaceum; 97*
NocA2	*Vibrio pecenticida; 98*
NocA3	*Micrococcus luteus; 99*
NocA4	*Vibrio pecenticida; 97*
NocA5	*Phsychrobacter immobilis; 90*
NocA6	*Pseudoalteromonas sp. ; 98*
NocA7	*Pseudoalteromonas sp.; 98*
NocA8	*Pseudolateromonas sp.;97*
NocA9	*Pseudolateromonas sp.; 97*
NocA10	*Marinobacter sp.; 90*
NocA11	*Marinobacter sp.; 94*
NocA12	*Marinobacter sp.; 94*
Zo12	*Pseudoalteromonas nigrifaciens; 97*
Zo2	*Vibrio tapefis; 97*
Zo20	*Alteromonas macleodii; 98*
Zo24	*Micrococcus antarticus; 97*
Zo5	*Vibrio splenadius; 98*

Supplementary Table 3. Phylogenetic affiliation of particle-associated bacterial isolates (Grossart et al. 2004a).

Strain	Accesion Number	Phylogenetic group
Ros7	AY841781	Alphaproteobacteria
HP2	AY241555	*Flexibacteriacae*
HP10	AY172664	*Firmicutes*
HP14	AY241558	*Flexibacteriacae*
HP15	AY241552	Gammaproteobacteria
HP25	AY241561	Flavobacteria
HP29w	AY239008	Alphaproteobacteria
HP30	AY239009	Alphaproteobacteria
HP31	AY241565	*Flexibacteriacae*
HP32	AY241566	*Flexibacteriacae*
HP34o	AY241567	*Flexibacteriacae*
HP37a	AY239011	Alphaproteobacteria
HP44w	AY841775	Alphaproteobacteria
HP45	DQ150527	*Firmicutes*
HP47a	AY841777	Alphaproteobacteria
HP49	DQ150528	*Flexibacteriacae*
HP52	DQ148416	Alphaproteobacteria
HP56	DQ148420	*Vibrio*
HP58	DQ148423	*Flexibacteriacae*
HP59	DQ148422	*Bacillus*
HP60(48)	DQ148424	Alphaproteobacteria

3.2 Effects of *Marinobacter adhaerens* HP15 on polymer exudation by *Thalassiosira weissflogii* grown at different N:P ratios

Effects of *Marinobacter adhaerens* HP15 on polymer exudation by *Thalassiosira weissflogii* grown at different N:P ratios

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Abstract

To better understand exopolymer accumulation, phytoplankton aggregation, and sinking fluxes of organic matter in the ocean, an in-depth investigation of the contribution of a diatom-interacting bacterial species and its effects on algal exopolymer production under differing environmental conditions was conducted. Physiological responses of the marine diatom, *Thalassiosira weissflogii*, to different nutrient limitations *in vitro* and the impact of the co-incubated bacterium, *Marinobacter adhaerens*, on algal exudation were simultaneously evaluated by analyzing the chemical composition and amount of released algal exudates. *T. weissflogii* cultures were grown at nutrient-balanced conditions (N:P=16) as well as at nitrogen- or phosphorus-deficient conditions, (N:P=1) and (N:P=95), respectively, with or without addition of *M. adhaerens*. Bacterial effects on algal growth, dissolved organic carbon release, transparent exopolymer particle (TEP) formation, and lectin-stainable exopolymeric substances depended on the N:P ratio used for the *T. weissflogii* cultures. At balanced N:P ratio, *M. adhaerens* had positive effects on *T. weissflogii* growth and stimulated TEP production. Under nutrient-limited conditions, however, polysaccharide secretion by *T. weissflogii* was enhanced regardless whether *M. adhaerens* was present or not. Differential lectin staining revealed that both, diatoms and bacteria, contributed to exopolymer formation. In conclusion, mechanistic understanding of bacteria-diatom interactions and their consequences on oceanic organic matter and nutrient cycling will require careful interpretation in an environmental context.

Key words: diatom-bacteria interactions, TEP, DOC, nutrient limitation, fluorophore-conjugated lectins, *Thalassiosira weissflogii*

Running head: Marinobacter adhaerens effects diatom polymer exudation

Introduction

Exudation products of marine phytoplankton can form transparent exopolymer particles (TEP), which are important for phytoplankton aggregation, oceanic organic matter cycling, and carbon sedimentation (Kiørboe et al. 1990; Passow and Alldredge 1995a). Heterotrophic bacteria interacting with phytoplankton may control TEP formation, cell aggregation, and sedimentation of phytoplankton as suggested earlier (Azam and Malfatti 2007a; Bhaskar et al. 2005; Grossart et al. 2006a; Smith et al. 1995).

This bacterial impact has not been explored in detail but may be important in the so called ”phycosphere”, which is the micro-zone surrounding algal cells (Bell and Mitchell 1972b). Bacteria attach to phytoplankton and cause hydrolysis of algal products (Bidle and Azam 1999; Grossart et al. 2007; Sapp et al. 2008b).

TEP can be colonized and modified by bacteria (Mari and Kiørboe 1996; Passow and Alldredge 1994b; Verdugo et al. 2004) thus serving as bacterial niches and substrate sources. Azam and Cho (1987) hypothesized that phytoplankton exudation may have evolved as a mechanism to establishing mutualism with bacteria and to attract bacteria as re-mineralizers.

Production of dissolved carbohydrates by marine phytoplankton is known to be highly variable depending on species, growth stage, and environmental conditions (Myklestad 1974). Phosphorus limitation, and for some species, nitrogen limitation can result in an increased extra-cellular release of photosynthesis products by cultured diatoms (Magaletti et al. 2004; Obernosterer and Herndl 1995).

Certain diatom species are known to release large amounts of carbohydrates and dissolved organic carbon (DOC) to the surrounding seawater (Biersmith and Benner 1998; Grossart et al. 2006a; Myklestad and Haug 1972). Hence, they contribute quantitatively and – in terms of reactivity –to the pool of dissolved organic matter in the ocean (Azam and Malfatti 2007a; Hernes and Benner 2002; Pakulski and Benner 1994). The most labile components of DOC are dissolved free amino acids and dissolved combined amino acids as well as dissolved free carbohydrates and dissolved combined carbohydrates. Total carbohydrates account for 20 to 30 % of the DOC pool in marine surface waters (Pakulski and Benner 1994) while amino acids exhibit a much smaller proportion of DOC (Kirchman et al. 2001).

A significant portion of organic carbon released by phytoplankton forms nano-gels (Chin et al. 1998) and TEP (Passow 2000; Passow and Alldredge 1994a). TEP contain acidic polysaccharides and may be retained as Alcian Blue-stainable particles on filters (Alldredge et al. 1993). TEP are particularly important for sedimentation processes because they promote aggregation of particulate organic matter and the formation of marine snow by providing the matrix or 'glue' of micro- and macro-aggregates (Alldredge et al. 1993; Logan et al. 1995; Passow 2002a).

Very little is known about the relative contribution of diatom-associated bacteria to the production of exopolymers by this type of phytoplankton. Likewise, there is lack of information about environmental triggers such as nutrient availability, which may control exopolymer accumulation and thus diatom aggregation and sinking flux of organic matter. Therefore, herein a series of *in vitro* experiments were conducted with the marine diatom *Thalassiosira weissflogii* and the bacterium, *Marinobacter adhaerens* HP15, which had been isolated from marine aggregates (Grossart et al. 2004a). HP15 was recently described as a *T. weissflogii* surface-attaching bacterium (Gärdes et al. submitted-a). To evaluate the physiological response of *T. weissflogii* to different nutrient conditions and the concurrent impact of *M. adhaerens* HP15 on algal exudation, the composition and amount of released polysaccharides, and DOC were determined. It was hypothesized that a specific bacterial strain affects quantity and quality of algal exudates and that exudate accumulation is influenced by inorganic nutrient availability in dependence of alga-associated bacteria.

Material and Methods

Diatom batch cultures, bacteria, and experimental setup

Axenic *T. weissflogii* (CCMP 1336) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, Maine, USA) and routinely grown in sterile f/2 medium (Guillard 1975) in 150-ml cell culture flasks. The cultures were checked regularly for bacterial contamination by microscopic observation and agar plating. Diatom cells were quantified by cell counting as described below.

Five liters of sterile diatom pre-cultures were grown at 16 °C in f/2 medium using a 12-h light period at 150 µmol photons m^{-2} s^{-1} for nine days. Next, *T. weissflogii* cells were harvested by centrifugation (5,000 rpm, 10 min) and subsequently washed with sterile artificial seawater (ASW) to minimize nutrient carry-over. Aliqoutes of approximately 5,000 *T. weissflogii* cells were then used to inoculate six of seven experimental treatments (600 ml medium in 1 l cell culture flasks). The control flask contained no diatoms. The experimental setup was outlined in **Fig. 1**.

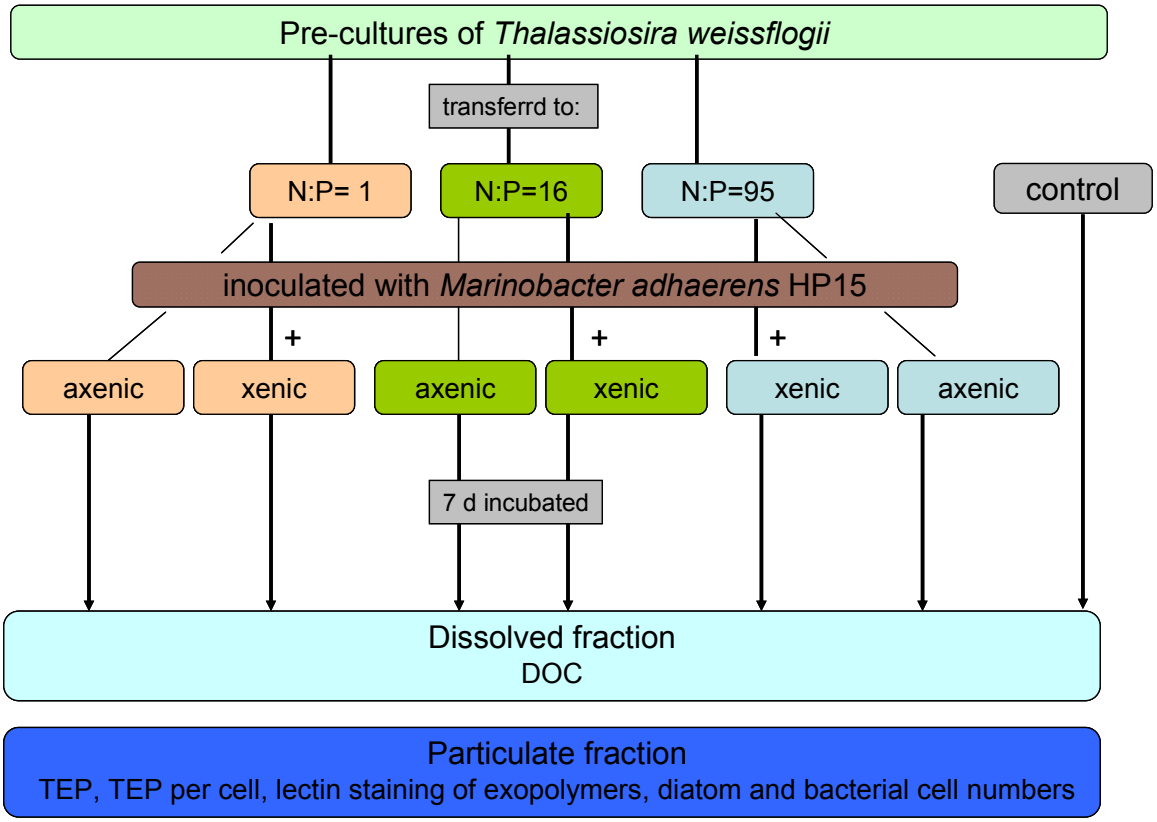

Figure 1: Outline of the experimental set-up used in this study. *T. weissflogii* cultures were incubated with or without *M. adhaerens* HP15. DOC: dissolved organic carbon; TEP: transparent exopolymer particles; Control: bacterial cells only.

Treatments 1 and 2 contained f/4 medium (N:P= 16), which was a twofold dilution of f/2 medium and was designated "nutrient-balanced". Medium for treatments 3 and 4 was reduced in nitrogen to an N:P ratio of 1 based on f/4 medium (N:P= 1) and was termed "nitrogen-deficient". Treatments 5 and 6 contained f/4 medium deficient in phosphate and designated "phosphorus-deficient" with an N:P ratio of 95 (N:P= 95). Treatments 1, 3, and 5 were kept axenic whereas bacterial strain *M. adhaerens* HP15 was added to treatments 2, 4, and 6 (xenic treatments). The control treatment 7 contained nutrient-balanced f/4 medium and the bacterial strain. *M. adhaerens* HP15 was isolated from marine aggregates in the German Wadden Sea (Grossart et al. 2004a). It was routinely grown in Marine Broth (MB) (Zobell 1941) in 100-ml Erlenmeyer flasks shaken at 150 rpm. To avoid carry-over of MB and bacterial metabolites, *M. adhaerens* HP15 cells were washed and incubated over night in nutrient-poor ASW (Grossart 1999). Thereafter, 1 ml of the bacterial suspension with a density of ~3 x 10^6 bacteria ml^{-1} was added to the respective treatments.

All cultures were incubated at 16 °C using a 12-h light period at 100 µmol photons m^{-2} s^{-1}. Twenty four hours after inoculation was considered as day 1 and the experiment lasted for 7 days. Samples were taken with sterile 20-ml syringes at days 1, 4, and 7 and subjected to a number of individual biochemical assays.

Determination of diatom and bacterial cell concentrations

Cell concentrations of diatoms were determined by cell counts in a Sedgwick-Rafter Cell S50 (SPI Supplies, West Chester, USA) using an inverted Axiovert 200 microscope (Zeiss, Jena, Germany). Bacterial cell numbers of the respective inoculum were determined by serial dilution platings, Bacterial cell numbers in treatments were determined as follows:

Two hundred µl of samples were diluted 1:2 and 1:10 in sterile f/4 medium, fixed with 0.2-µm pore size pre-filtered, borate-buffered 2% formalin,and stained with 4´,6-diamino-2-phenylindole (DAPI; 1 µg ml^{-1}) for 3 min (Porter and Feig 1980). Samples were filtered onto 0.2-µm pore-sized polycarbonate Nucleopore filters (Whatman, Maidstone, England) and stored at -20°C. Bacterial cell numbers were counted in replicates using an epifluorescence Axioplan microscope (Zeiss, Jena, Germany) at 1000x magnification.

Nutrient analysis

Concentrations of NO_3^- and PO_4^- were determined spectrophotometrically using the Auto Analyzer Evolution III apparatus (Alliance Instruments, Frepillon, France). 10-ml samples were pre-filtered (0.2 µm Nucelopore) and kept at -20°C until analysis, determination of nutrients were performed in duplicates according to an established method (Strickland and Parsons 1972). Nutritional parameters of this study are summarized in **Table 1**.

Staining of transparent exopolymer particles

For quantitative TEP analysis, replicate samples of 2-5 ml were filtered onto 0.2 µm pore size polycarbonate membranes, which were subsequently stained with Alcian blue (8GX, final conc. 0,05 % in Na-acetate buffer at pH5.8) (Alldredge et al. 1993). Filters were dried and placed on Cytoclear TM slides (Poretics, Livermore, USA). Twenty randomly selected grids were examined by light microscopy at 200x magnification. Image analysis was done using Analysis V 3.0 (Soft Imaging System, Münster, Germany) to enumerate TEP area and size distribution. A total of 300-700 TEP per slide were measured. The majority of TEP observed were small, ovate particles. TEP were grouped into geometric size classes based on maximal length. The maximal and minimum length axes were used to calculate the elliptical areas of TEP. The size spectrum from 20 grids was also used to calculate the total amount of TEP per slide.

Lectin staining

The following two lectins were used to test for their binding to diatom-derived extra-cellular polymers: Concanavalin A-fluorescein isothiocyanate (FITC-ConA) (Sigma, St. Louis, USA) specific for glucose and mannose residues and *Bandeiraea simplicifolia* lectin-tetramethyl rhodamine isothiocyanate (TRITC-Bandeiraea) (Sigma) specific for galactose and *N*-acetylgalactose residues.

Table 1. Concentrations of NO_3^- and PO_4^{3-} as determined for the different treatments conducted in this study

incubation	balanced nutrient conditions: N:P = 16				nitrogen deficient condition N:P = 1				phosphorus deficient condition N:P = 95				N:P = 16	
	axenic		xenic		axenic		mixed		axenic		mixed		control	
days	NO_3^- [µM]	PO_4^{3-} [µM]	NO_3^- [µM]	PO_4^{3-} [µM]	NO_3^- [µM]	PO_4^{3-} [µM]	NO_3^- [µM]	PO_4^{3-} [µM]	NO_3 [µM]	PO_4^{3-} [µM]	NO_3^- [µM]	PO_4^{3-}[µM]	NO_3^- [µM]	PO_4^{3-} [µM]
1	424± 26	24,1 ± 6	439± 41	26,1 ± 7,6	26,3± 1,8	29 ± 8,5	24,8 ± 2,2	27 ± 4,5	464 ± 22	4,8 ± 0,9	410,4 ± 10	5,57± 1	466 ± 37	21 ± 4,2
4	358 ± 11	8,35 ± 2,5	385± 9	7,5 ± 2	0,55± 0,06	23 ± 6	0,42 ± 0,09	25 ± 5,8	414,8 ± 27	0,51 ± 0,1	368 ± 25	2,25 ± 0,6	425 ± 21	15,5 ± 1,2
7	341± 16	4,35± 2,7	366± 37	3,9 ± 1,1	0,7± 0,1	23 ± 9,9	0,69 ± 0,1	24 ± 6,1	404,8 ± 15	0,18 ± 0,1	370 ± 31	1,98 ± 0,9	370 ± 26	10,9 ± 3,3

Staining with lectins according to (Liener et al. 1986) with modifications as published by (Wigglesworth-Cooksey and Cooksey 2005) was done in sterile eight-well cover slip chambers (Ibidi, Martinsried, Germany). Two hundred µl of samples were stained with lectin concentrations of 0.1 mg ml^{-1} in f/4 medium and incubated for 1-2 h in darkness. Unbound lectins were removed by adding 100 µl of bovin serum albumin (BSA) (1% in phosphate-buffered saline buffer). In all unstained controls, lectin solutions were replaced by BSA. FITC-ConA and TRITC-Bandeiraea binding activities on exocarbohydrates of axenic and xenic cultures were determined by confocal laser scanning microscopy (CLSM) using an Axiovert 400 (Zeiss) with one argon and one krypton laser (excitation wave lengths of 488 nm and 568 nm, respectively) and an emission wave length of 350 nm and 560 nm. Autofluorescence of chlorophyll *a* was detected at an emission wave length of 655 nm. Images were processed using the Zeiss LSM Image Browser.

Dissolved organic carbon

For analysis of DOC, replicates of 10-ml samples were collected in pre-combusted glass ampoules (Carl Roth, Karlsruhe, Germany) after filtration through pre-rinsed 0.2 µm polycarbonate membranes (Nuclepore). Samples were then acidified with 1% phosphoric acid, flame-sealed, and stored at -20°C (Grossart et al. 2006a). DOC analysis was performed using a high-temperature catalytic combustion TOC-5000 instrument (Shimadzu, Duisburg, Germany). Given DOC concentrations are averages of three injections from each sample per time point.

Statistical analyses

A simple regression analysis was carried out to assess the relationships between the measured parameters. One-way ANOVA was used to assess the differences in DOC, TEP, TEP size distribution, algal- and bacterial cell numbers. Results of all statistical analyses were summarized in **Table 2**.

Table 2. Statistical analyses using One-way ANOVA to test for significant differences between axenic and xenic *T. weissflogii* cultures with *M. adhaerens* in nutrient-balanced (N:P=16), phosphorus-deficient (N:P=95), and nitrogen-deficient (N:P=1) medium. DOC: dissolved organic carbon; TEP: transparent polymer particles abundance; NS: not significant

	significance value [p] between axenic and xenic cultures			significance value [p] between balanced and nutrient deficient conditions	
	N:P=16	N:P=95	N:P=1	axenic	xenic
DOC	0.024	NS	NS	NS	0.032
TEP	0.046	NS	NS	0.089	NS
TEP size frequency distribution	0.044	NS	NS	0.009	NS
Diatom cell numbers	0.01	NS	NS	NS	0.036
Bacterial cell numbers	/	/	/	/	0.048

Results

Phytoplankton and bacterial abundances

The experimental treatment scheme is depicted in **Fig. 1**. Cell numbers of *T. weissflogii* increased exponentially over 7 days of incubation in xenic nutrient-balanced medium (**Fig. 2A**). Increase in cell numbers was highest in the second half of the experiment from 1.4 x 10^4 ± 2.6 x 10^2 cells ml^{-1} to 4.7 x 10^4 ± 6.8 x 10^2 cells ml^{-1}. In nutrient-balanced but axenic conditions diatom cell numbers reached 2.4 x 10^4 ± 1.1 x 10^2 cells ml^{-1}. Thus, diatom growth was significantly ($p > 0.05$) higher in nutrient-balanced treatments if *M. adhaerens* HP15 cells were present as compared to axenic cultures. In xenic treatments, cell numbers of *M. adhaerens* HP15 increased rapidly within four days reaching 8.8 ± 1.1 x 10^6 cells ml^{-1} and remained nearly constant thereafter (**Fig. 3**).

As expected, in nitrogen- as well as in phosphorus-deficient media with ratios of N:P= 1 and N:P= 95, respectively, diatom growth was significantly weaker ($p < 0.05$) as compared to xenic nutrient-balanced medium incubation. Interestingly, diatom cell numbers were not affected by the presence of *M. adhaerens* HP15 under nutrient depreviation (**Fig. 2B and C**). Diatom cell numbers increased from 5 x 10^3 ± 0.42 x 10^3 cells ml^{-1} to only 2.1 x 10^4 ± 3.3 x 10^3 cells ml^{-1}. No significant differences between xenic and axenic treatments in nutrient-deficient treatments were observed. Consequently, final diatom cell numbers of xenic and axenic nutrient-deficient treatments were comparable to axenic nutrient-balanced conditions. Bacterial abundance in nitrogen-deficient medium reached a final concentration of 6.3 ± 0.9 x 10^6 cells ml^{-1} (**Fig. 3**). In phosphorus-deficient medium, bacterial cell numbers showed a similar pattern with the highest cell numbers at day 4 (5.9 ± 2.1 x 10^6 cells ml^{-1}) and being significantly lower as compared to nutrient-balanced conditions ($p < 0.05$).

In *T. weissflogii*-free but nutrient-balanced controls, bacterial abundances decreased over time to 3.8 ± 0.8 x 10^4 cells ml^{-1} (Data not shown) suggesting that bacteria lacked carbohydrate molecules possibly provided by the diatom.

Transparent exopolymer particle formation

TEP amounts differed significantly between axenic and xenic cultures in nutrient-balanced treatments ($p<0.05$). High TEP formation was observed in xenic cultures (8.0 ± 1.2 x 10^9 μm^2 ml^{-1}) while for axenic cultures TEP quantities decreased to 2.1 ± 0.2 x 10^9 μm^2 ml^{-1} (**Fig. 2A**).

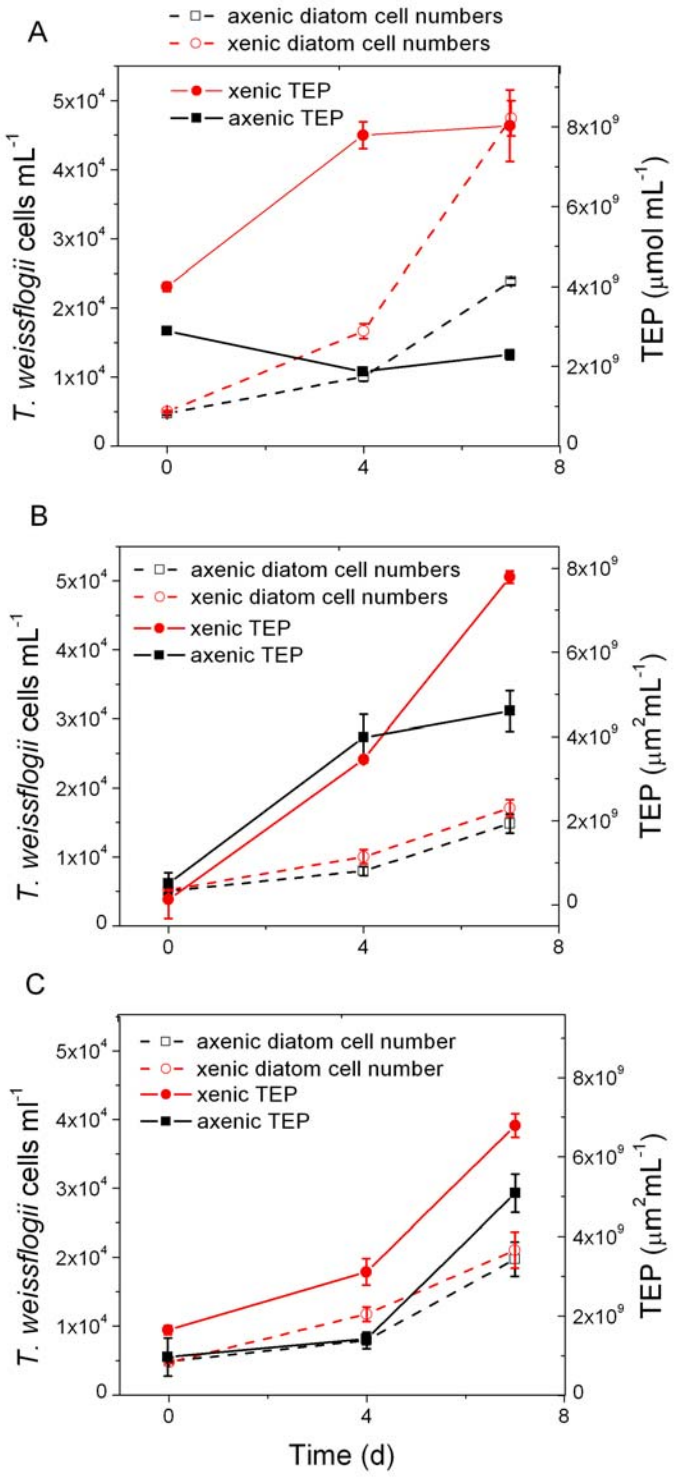

Figure 2: Total abundance of transparent exopolymer particles (TEP) and diatom cell numbers in **A**) nutrient-balanced medium (N:P=16), **B**) nitrogen-deficient medium (N:P=1), and **C**) phosphorus-deficient medium (N:P=95). *T. weissflogii* cultures were grown with (xenic) and without (axenic) *M. adhaerens* HP15.

In neither of the two types of nutrient-deficient treatment was there any significant difference in TEP formation rate between axenic and xenic treatments with average values of 7.5 ± 1.9 x 10^9 μm^2 ml^{-1} (**Fig. 2B and C**). However, final TEP values for axenic diatom cultures where generally higher in nutrient-deficient treatments as compared to the axenic nutrient-balanced condition ($p<0.05$).

Calculation of TEP production per diatom cell revealed highest TEP production rates in *M. adhaerens*-incubated and nitrogen-deficient treatments (**Table 3**). Size frequency distribution of TEP after 7 days of incubation differed between the three nutrient treatments (**Fig. 4**). Under nutrient-balanced conditions, a significantly larger number of TEP were >200 μm^2 in size in xenic treatments as opposed by the relative high number of smaller TEP (<200 μm^2) in axenic treatments. As expected, the proportion of TEP sizes >1,000 μm^2 was highest in the xenic nutrient-balanced treatment accounting for almost 8 % of total TEP numbers (**Fig. 4**). Similar to xenic nutrient-balanced treatments, axenic as well as xenic treatments under nutrient limitation revealed major proportions of TEP in the size class of >200 μm^2 ($p<0.05$). This effect was most pronounced during phosphate limitation.

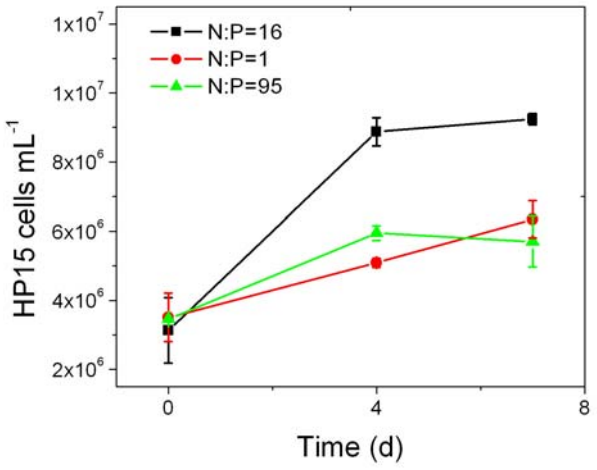

Figure 3: Cell numbers of *M. adhaerens* HP15 incubated with *T. weissflogii* under nutrient-balanced (N:P=16), nitrogen-deficient (N:P=1), and phosphorus-deficient (N:P=95) conditions.

Lectin stainable exopolymers

To gain first insights about the origin and nature of exopolymers formed during the diatom-bacteria interaction, fluorescence-assisted staining of samples from all treatments with lectins, FITC-ConaA and TRITC-Bandeiraea, was conducted and visualized by CLSM (**Figs. 5 I and II**). After the first day of incubation and in contrast to all other treatments, a thin ConA-stained layer on the diatom surface and some free exopolymers stained with the same lectin were observed in the axenic nitrogen-deficient treatment (**Fig. 5 IC**). Staining with TRITC-Bandeiraea did not yield signals in any of the treatments at day 1 of incubation.

After seven days of incubation, lectin staining revealed enhanced amounts of ConA-stained exopolymers for those nutrient-balanced and nitrogen-deficient treatments, in which the diatom surface had been colonized by *M. adhaerens* HP15 (**Fig. 5 IIA-D**). The exopolymer layer on the diatom surface in these xenic cultures was well developed and clearly stained with FITC-ConA (**Fig. 5 IIB and D**). In contrast, axenic nutrient-balanced and xenic phosphate-deficient *T. weissflogii* cultures did not reveal FITC-ConA-stained signals indicating the absence of polymeric material with glycosidic bounds (**Fig. 5 IIA and F**). Nitrogen-deficient treatments accumulated FITC-ConA-stained material mostly in free form regardless of bacterial presence (**Fig. 5 IIC and D**). In phosphate-deficient treatments, cell surface-bound FITC-ConA-stained material was only observed in axenic cultures (**Fig. 5 IIE and F**). Interestingly, lectin TRITC-Bandeiraea only gave a strong signal overlapping with the FITC-ConA signal in the xenic nutrient-balanced treatment (**Fig. 5 IIB**) suggesting that the FITC-ConA-stained exopolymer observed at day 1 had been somehow modified in the presence of bacteria.

Determination of dissolved organic carbon

In nutrient-balanced treatments, DOC concentrations differed significantly when axenic and xenic treatments were compared ($p<0.05$) (**Fig. 6A**).

Table 3. Relative production of transparent exopolymer particles (TEP) per diatom cell in axenic and xenic *T. weissflogii* cultures grown in nutrient-balanced (N:P=16), nitrogen-deficient (N:P=1), and phosphorus-deficient (N:P=95) media. As control only bacteria were incubated (HP15).

	TEP production per diatom cell [μm^2 cell $^{-1}$]			TEP production per bacterial cell [μm^2 cells $^{-1}$]
	N:P=16	N:P= 95	N:P=1	control (HP15)
axenic	8,0 ± 0.14 x 10^4	2,6 ± 0.28 x 10^5	2,8 ± 0.09 x 10^5	3,0 ± 0.08 x 10^3
xenic	1,6 ± 0.34 x 10^5	3,8 ± 0.51 x 10^5	5,3 ± 0.68 x 10^5	/

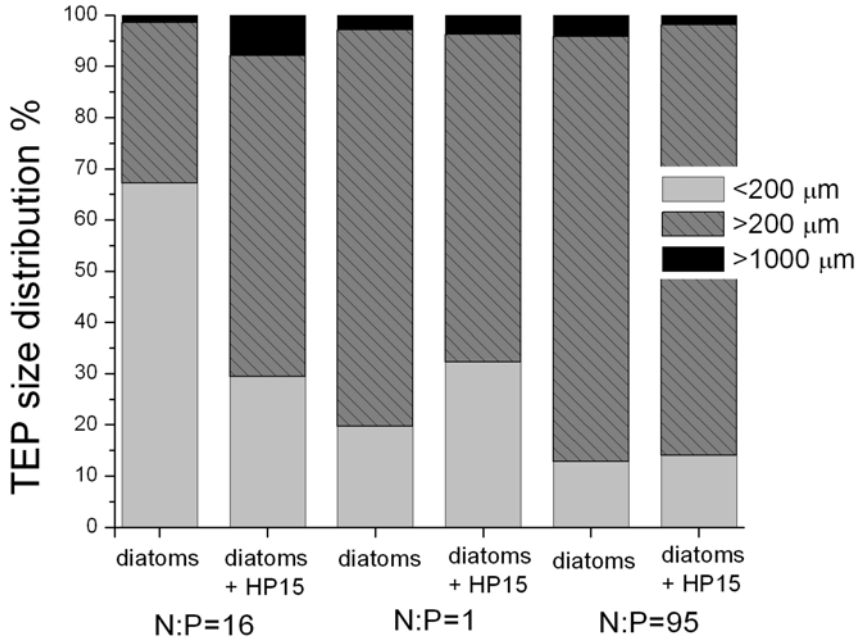

Figure 4. Size frequency of transparent exopolymer particles (TEP) formed in *T. weissflogii* cultures with (xenic) and without (axenic) *M. adhaerens* HP15 under nutrient-balanced (N:P=16), nitrogen-deficient (N:P=1), and phosphorus-deficient (N:P=95) conditions. TEP sizes were determined after 7 days of incubation and classified as <200 μm^2, >200 μm^2, and >1000 μm^2.

There were higher DOC concentrations found when diatom cells grew axenic. Initial DOC concentrations of axenic cultures increased from 150 ± 17.1 µM to 600 ± 43.2 µM at the fourth day of incubation and remained high thereafter. In xenic nutrient-balanced treatments, DOC concentrations increased in a similar manner as above but reached only 400 ± 12.8 µM (**Fig. 6A**). In nitrogen-deficient treatments, no differences in DOC concentrations for xenic and axenic cultures were observed.

The amount of DOC declined to 107 ± 20.9 µM (**Fig. 6 B**). In phosphorus-deficient treatments, high initial DOC concentrations were measured (average = 470.5 ± 31.2 µM) (**Fig. 6C**). These data suggested that a significant bacterial impact on DOC concentration could only be observed under nutrient-balanced conditions. Under nutrient-balanced conditions algal growth was enhanced by the presence of *M. adhaerens* HP15.

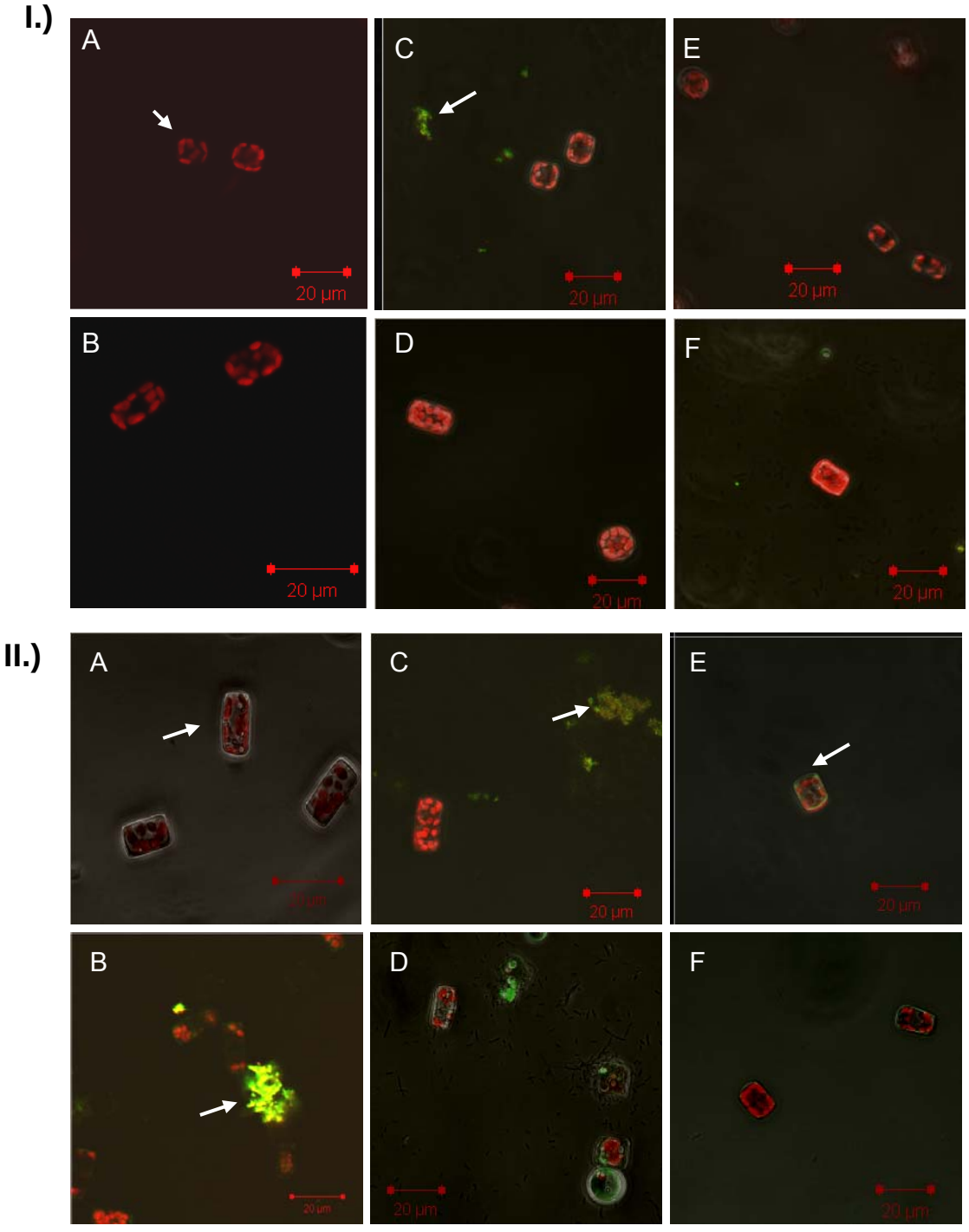

Figure 5: Confocal laser scanning microscopy of exopolymer staining with lectins, FITC-ConA (green signal) and TRITC-Bandeira (yellow signal), as well as diatom chloroplast autofluorescence (red signal) after **I.)** 1 day and **II.)** seven day of incubation. **A**) nutrient-balanced (N:P=16) axenic culture; **B**) nutrient-balanced (N:P=16) xenic culture; **C**) nitrogen-deficient (N:P=1) axenic culture; **D**) nitrogen-deficient (N:P=1) xenic culture; **E**) phosphorus-deficient (N:P=95) axenic culture; and **F**) phosphorus-deficient (N:P=95) xenic culture. White arrows indicate diatom cells and free exopolymers

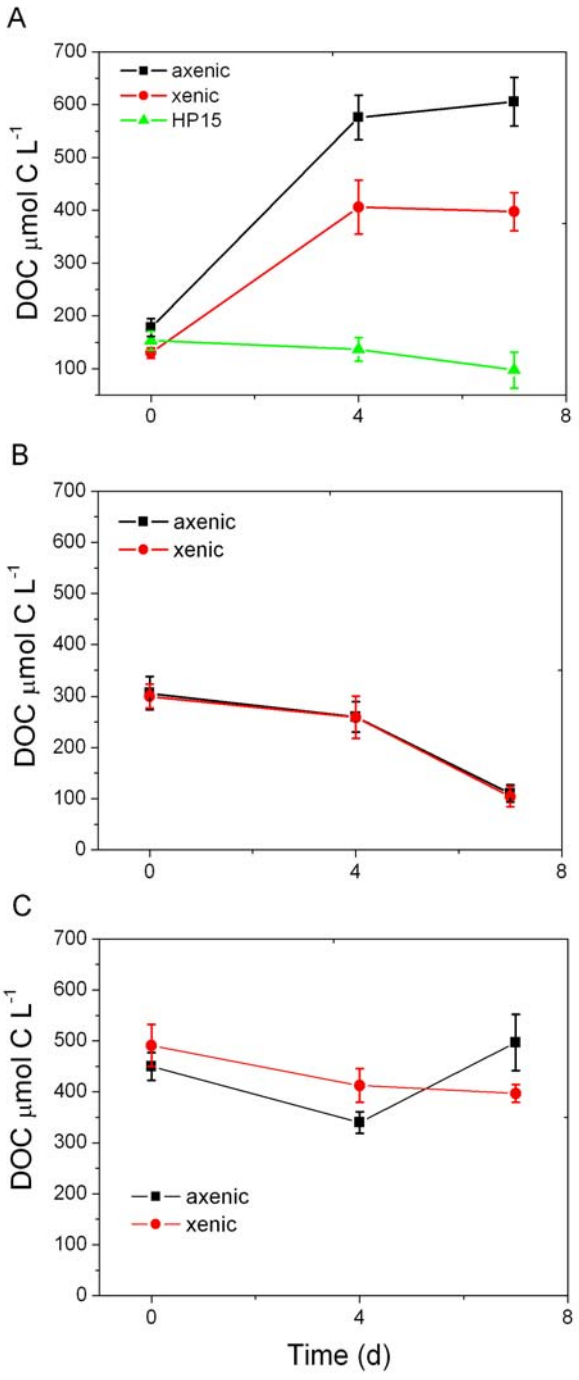

Figure 6: Concentrations of dissolved organic carbon (DOC) in *T. weissflogii* cultures grown in **A**) nutrient-balanced medium (N:P=16), **B**) nitrogen-deficient medium (N:P=1), and **C**) phosphorus-deficient medium (N:P=95). *T. weissflogii* cultures were grown with (xenic) and without (axenic) *M. adhaerens* HP15.

Discussion

Obtained under strictly defined *in vitro* conditions, results of this study demonstrated distinct effects of the diatom-associated bacterial strain, *M. adhaerens* HP15, on exopolymer formation by *T. weissflogii* during nutrient-balanced versus nutrient-deficient growth. The herein observed bacteria-diatom interactions strongly depended on a balanced availability of phosphate and nitrate, respectively, and were most pronounced when diatoms grew optimally. Therefore, one is tempted to conclude that there is indeed a yet-to-be dissected trophic interplay between these organisms, which is rather of biotrophic than necrotrophic nature.

Likewise, formation of TEP was induced. Photosynthesis, an active metabolism, and cell division of *T. weissflogii* were previously shown to be required for bacteria-induced aggregate formation (Gärdes et al., submitted). Herein, it was further shown by lectin staining of exopolymers that the bilateral interaction may result in either modification or *de novo* synthesis of exopolymers. These results are consistent with either those of (Grossart and Simon 2007), who found that presence of bacteria might favor TEP formation as suggested earlier (Bhaskar et al. 2005; Stoderegger and Herndl 1999) or alternatively might lead to degradation of TEP as concluded by other researchers (Alldredge et al. 1993; Passow et al. 1994). In this context, it should be note that despite the lack of any experimentally supplemented carbohydrates the bacterial organism benefited from optimal growth of the diatom thus suggesting a mutualistic relationship.

Under both tested nutrient-deficient conditions, diatom growth was low irrespective of bacterial cells being present or not. Likewise, *M. adhaerens* could not multiply under these conditions. The observed TEP formation rates determined under nutrient deficiency were not significantly influenced by the bacterial strain although they were still higher than the minor TEP formation by axenic algae. When the relative TEP amount produced per diatom cell was calculated, higher specific TEP formation rates in nutrient-deficient treatments became obvious regardless of presence of bacteria. These results are in accordance to previous findings of Mari and Burd (1998), who provided *in situ* evidence that TEP abundance increases when nitrate and the inorganic N:P ratio were low in the ocean's surface mixed layer.

Since TEP are mainly composed of acidic polysaccharides, it had been speculated that TEP production increases when algal growth is nitrogen-limited (Beauvais et al. 2006; Corzo et al. 2000; Staats et al. 2000). Corzo (2000) could demonstrate that the net rate of TEP production was affected by initial nitrogen and phosphorus concentrations. We hypothesize that under nitrogen- and phosphorus-deficient conditions when protein synthesis is reduced, a larger proportion of photosynthetically fixed carbon is exudated in form of TEP. Herein obtained findings are in contrast to those reported by Alcoverro (2000) and Guerrini (2000), who showed that phosphorus starvation led to higher release of polysaccharides by *Achnanthes brevipes* as compared to nitrogen starvation.

Possibly, the "cluster hypothesis" proposed by (Azam and Ammerman 1984) may help to explain some of our results. Bacterial re-mineralization of N- and P-rich components of algal exudates might supply algae with ammonium and phosphate whereas bacterial growth might be enhanced by nutrient-rich algae exudates. Consequently, bacteria might act as re-mineralizers when nutrient levels are high. At nutrient-deficient conditions, however, bacteria efficiently compete with phytoplankton for nutrients (Rhee 1972; Rothhaupt and Güde 1992b; Thingstad et al. 1993). Therefore, a remarkable shift from mutualism to commensalism between *M. adhaerens* and *T. weissflogii* as a result of nutrient stress is hypothesized.

Lectin staining revealed that diatom exopolymeric surface layers and free exopolymers were best developing in xenic nutrient-balanced cultures. Additionally, it seemed that a potentially novel type of exopolymer stainable with TRITC-Bandieraea accumulated as bacterial colonization of algal surfaces proceeded. Lack of lectin-stainable exopolymers in aged xenic phosphorus-limited treatments indicated that bacteria might have re-mineralized any formed precursor or exopolymer, which is in line with earlier findings (Grossart 1999). In contrast and still puzzling, nitrogen limitation led to an early release of FITC-ConA-stainable exopolymer under axenic conditions, which was not observed for the nutrient-balanced condition. In line with this, it had been shown previously that the mode of interaction between alga and bacteria can dramatically change by environmental condition such as nutrient availability (Grossart 1999; Grossart et al. 2006a).

In the current study, bacteria affected the dynamics of DOC only when nutrient-balanced conditions were provided and diatom growth was optimal. It may be assumed that the observed DOC patterns in xenic cultures resulted likewise from the active release or passive leakage of organic matter from diatoms as well as from bacterial degradation of algal exudates. Marine bacteria possess high hydrolytic enzyme activities leading to increased DOC utilization, which results in enhanced bacterial replication (Grossart et al. 2005; Grossart et al. 2007; Martinez et al. 1996). Diatoms grown in nutrient-deficient media showed similar DOC dynamics in axenic and xenic cultures, respectively, indicating that bacterial DOC utilization was minor under these conditions and thus supporting earlier findings (Stoderegger and Herndl 1998; Thingstad et al. 2008). However, bacteria may actively transform DOC into particulate organic carbon when concentrations of inorganic nutrients and trace metals are low.

Conclusions

Nutrient-dependent differences in exopolymer production by diatoms can be related to biotrophic interactions with distinct bacterial organisms. The interplay of *M. adhaerens* HP15 and *T. weissflogii* under nutrient-balanced conditions greatly affected the dynamics of TEP formation, cell growth, and DOC accumulation, as well as potentially the nature of produced exopolymers. Under nutrient-deficient conditions bacteria seem to be less important for organic matter re-mineralization. Phytoplankton exudation may have evolved as a mechanism to establish mutualism with bacteria, which may shift to comensalism if nutrients become limited. In the field, bacterial as well as algal populations are diverse and hence bacteria-algae interactions are likely to be very complex including a multitude of additional processes, which deserve further investigation.

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3.3 *Marinobacter adhaerens* sp. nov., prominent in aggregate formation with the diatom *Thalassiosira weissflogii*

Marinobacter adhaerens sp. nov., prominent in aggregate formation with the diatom *Thalassiosira weissflogii*

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Abstract

The Gram-negative, motile, and rod-shaped bacterial strain, HP15^T, was isolated from particles sampled in surface waters of the German Wadden Sea. It was identified among 82 other marine isolates due to its high potential to induce production of transparent exopolymeric particles (TEP) and aggregate formation while interacting with the diatom, *Thalassiosira weissflogii*. HP15^T grew optimally at a range of 34-38 °C, a pH of 7-8.5 and was able to withstand salt concentrations between 0.4-20 % (w/v) NaCl. HP15^T was chemotaxonomically characterized by possessing ubiquinone-9 as the major respiratory lipoquinone as well as $C_{16:0}$, $C_{18:1}\omega9c$, $C_{16:1}\omega7c$/iso, and $C_{15:0}$ 2-OH as the predominant fatty acids. The G + C content of its DNA was 56.9 mol%. The closest relative by means of 16S rRNA was *Marinobacter flavimaris* with a similarity level of 99 %. The whole-genome relatedness to *M. flavimaris* was determined to be 66 % by DNA-DNA hybridization. On the basis of phenotypic and chemotaxonomic properties as well as phylogenetic analyses, strain HP15^T (=DSM XXX^T = CIP XXX^T) is proposed to represent the novel species, *Marinobacter adhaerens* sp. nov.

Running title: Description of the novel species *Marinobacter adhaerens*

Subject category: New Taxa (*Proteobacteria*)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HP15^T is AY241552. The comparison of cellular fatty acid composition between strain HP15^T and type strains of seven other *Marinobacter* species is available as supplementary material in IJSEM Online.

Introduction

The genus *Marinobacter* was established with the species *Marinobacter hydrocarbonoclasticus* in 1992 (Gauthier et al. 1992b). A total of 26 further species have been described until today. These species are tolerant to various conditions as they were isolated from diverse locations - from the sediment (Gorshkova et al. 2003), the water column (Yoon et al. 2004b), from coastal (Roh et al. 2008) and deep sea waters (Takai et al. 2005), from the Antarctic (Montes et al. 2008b) and from the Red Sea (Antunes et al. 2007). Furthermore, representatives of this genus were isolated from oil-contaminated areas (Huu et al. 1999a), hot springs (Shieh et al. 2003), and salines (Martin et al. 2003). Two species were identified based on their interactions with other organisms - *M. algicola* isolated from dinoflagellate cultures (Green et al. 2006) and *M. bryozoorum* derived from Bryozoa (Romanenko et al. 2005).

The aggregation of phytoplankton cells is an important process in marine ecosystems leading to the sinking of particulate organic matter in form of marine snow. Heterotrophic bacteria were suggested to increase aggregation of microalgae

and other particles (Decho 1990b). To study the interaction of diatoms with bacteria and its role in aggregate formation, a bilateral model system was established (Gärdes et al. 2010a). Among 82 bacterial isolates from aggregates (0.1-1 mm in diameter) sampled in surface waters of the German Bight (Grossart et al. 2004b), strain HP15^T was shown to induce highest transparent exopolymeric particle (TEP) production and aggregate formation during its interaction with the diatom *Thalassiosira weissflogii*. Thus, strain HP15^T proofed to be a suitable model organism to study bacteria-microalgae interactions and its consequences for the organic matter sinking flux in the sea. The aim of the present study was to determine the taxonomic position of this species by analyzing its phenotypic properties and genotypic relatedness.

Methods

For phenotypic examination, HP15^T was grown aerobically on Marine Broth (MB) agar plates (5 g peptone, 1 g yeast extract, 0.1 g $FePO_4$, 6 g agar in 750 ml of North Sea water and 250 ml of distilled water, pH adjusted to 7.4) at 28 °C for 48 h. The reference strain *Marinobacter flavimaris* DSM 16070^T was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

The Gram staining reaction and cell morphology was examined by light microscopy and transmission electron microscopy (EM 900, Zeiss). Enzyme activities were analyzed by using the API 20E system (bioMérieux). Determination of carbon utilization patterns using the BIOLOG GN2 was carried out by DSMZ. The analysis of growth conditions, i.e. temperature (4-60 °C), pH (4-10), salinity (3-35 % (w/v) NaCl), cellular fatty acid composition, quinones, and spectroscopic DNA-DNA hybridization tests were performed by DSMZ. For the latter, HP15^T was compared with *M. flavimaris* in two replicates. Optimal growth temperature was determined by incubation of HP15^T in MB at 250 rpm and temperatures of 28, 30, 32, 34, 36, 38, 40, and 42 °C. The detailed salinity range was studied between 0 and 3 % (w/v) NaCl. For quinone analysis, cells were grown in MB at 28 °C and 250 rpm to an OD of 0.3 and harvested. For DNA-DNA hybridization, cells were grown using the same procedure, harvested, and stored in isopropanol-H_2O (1:1).

The 16S rRNA gene of HP15^T was completely sequenced (GenBank accession no. AY241552, 1531 bp). The phylogenetic position of *Marinobacter* sp. HP15^T based on its 16S rRNA sequence was analyzed using the ARB software package (Ludwig et al. 2004) and the reference alignment was provided by the Living Tree Project database (Yarza et al. 2008). The phylogenetic tree was based on the HP15^T sequence, all type strains of the genus *Marinobacter*, and the type strains of *Halospina denitrificans* HGD 1-3^T (DQ072719) and *Salicola marasensis* 7Sm5^T (DQ019934) as outgroups. The G + C content of the HP15^T genome was calculated using the complete genomic sequence (GenBank accession no. GRP 79971).

Results and Discussion

The cells of strain HP15^T were rod-shaped, stained as Gram-negative, and motile by one polar flagellum **(Fig. 1)**. On MB agar, colonies were small (1-2 mm) and roundish with smooth margins after 2 days of incubation. The colony appearance was translucent and slightly brownish and the color intensity increased with time of incubation. HP15^T grew between 4 and 45 °C with an optimum at 34-38 °C and at a pH ranging from 5.5 to 10 with an optimum of pH at 7 to 8.5. No growth was observed at a pH of 4 or lower. The strain grew within one day at NaCl concentrations from 0.4 to 10 % (w/v). It resisted up to 20 % (w/v) NaCl, but not 35 % (w/v) NaCl.

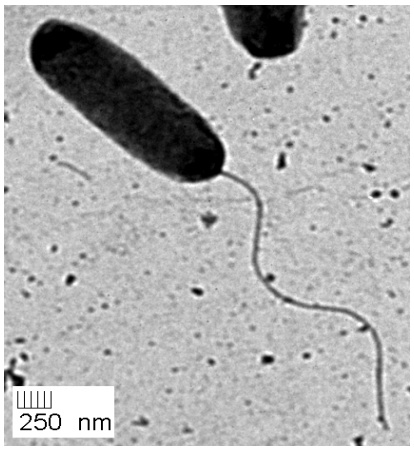

Fig.1. Transmission electron micrograph of strain HP15^T cultivated in marine broth for 24 hours.

The API 20 E test of HP15^T was negative for all reactions (β-galactosidase, arginine dihydrolase, tryptophan deaminase, lysine and ornithine decarboxylase, hydrogen sulfide production, urease, indole and acetoin production, gelatinase, utilization of citrate, D-glucose, D-mannitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose). The results of the BIOLOG GN2 plate were positive for the utilization

Table 1. Differential phenotypic characteristics between HP15^T and the closest related *Marinobacter* type strains. Strains: 1, HP15^T; 2, *M. flavimaris* SW-145^T; 3, *M. salsuginis* SD-14B^T; 4, *M. algicola* DG893^T. +, positive; -, negative; ND, not determined.

Characteristics	1	2	3	4
Temperature range (°C)	4-45	4-45	10-45	5-40
Optimal temperature (°C)	34-38	37	35-37	25-30
Urease activity	-	-	ND	+
Gelatinase activity	-	-	+	-
Utilization of:				
Glycerol	-	+	+	+
D-Fructose	-	+	-	+
D-Glucose	-	-	+	+
Maltose	-	-	-	+
D-Mannitol	-	-	-	+
Citric acid	-	-	-	+
DL-Lactic acid	+	-	-	+
D-Gluconate	-	+	+	+
L-Alanine	+	-	+	+
L-Phenylalanine	-	-	+	+
L-Glutamate	+	-	+	+
L-Arginine	-	-	-	+
G + C content (mol%)	56.9	58.0	55.9	57.0

of dextrin, Tween 40 and 80, pyruvic acid methyl ester, succinic acid mono-methyl-ester, cis-aconitic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-leucine, and L-proline.

As sole carbon source, HP15^T did not utilize α-cyclodextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, m-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, p-hydroxy phenylacetic acid, itaconic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-alanyl-glycine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ-amino butyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate, and D-glucose-6-phosphate. The fatty acid profile of HP15^T was composed of $C_{16:0}$ (21.7 %), $C_{18:1}\omega 9c$ (21.6 %), $C_{16:1}\omega 7c$/iso $C_{15:0}$ 2-OH (14.6 %), $C_{16:1}\omega 9c$ (9.0 %), $C_{12:0}$ 3-OH (7.9 %) and $C_{12:0}$ (6.0 %). Thus, it is similar to that of other *Marinobacter* type strains **(Supplementary Tab. S1 in IJSEM Online)**. The predominant ubiquinone was ubiquinone-9, which is consistent with that of other *Marinobacter* species except *M. lutaeoensis*, which contained ubiquinone-8 (Shieh *et al*., 2003).

Based on its 16 S rRNA sequence, strain HP15^T was affiliated to the *Marinobacter* genus of the *Gammaproteobacteria*. It is most closely related to the type strains of *Marinobacter flavimaris* (99 %), *Marinobacter salsuginis* (98 %)

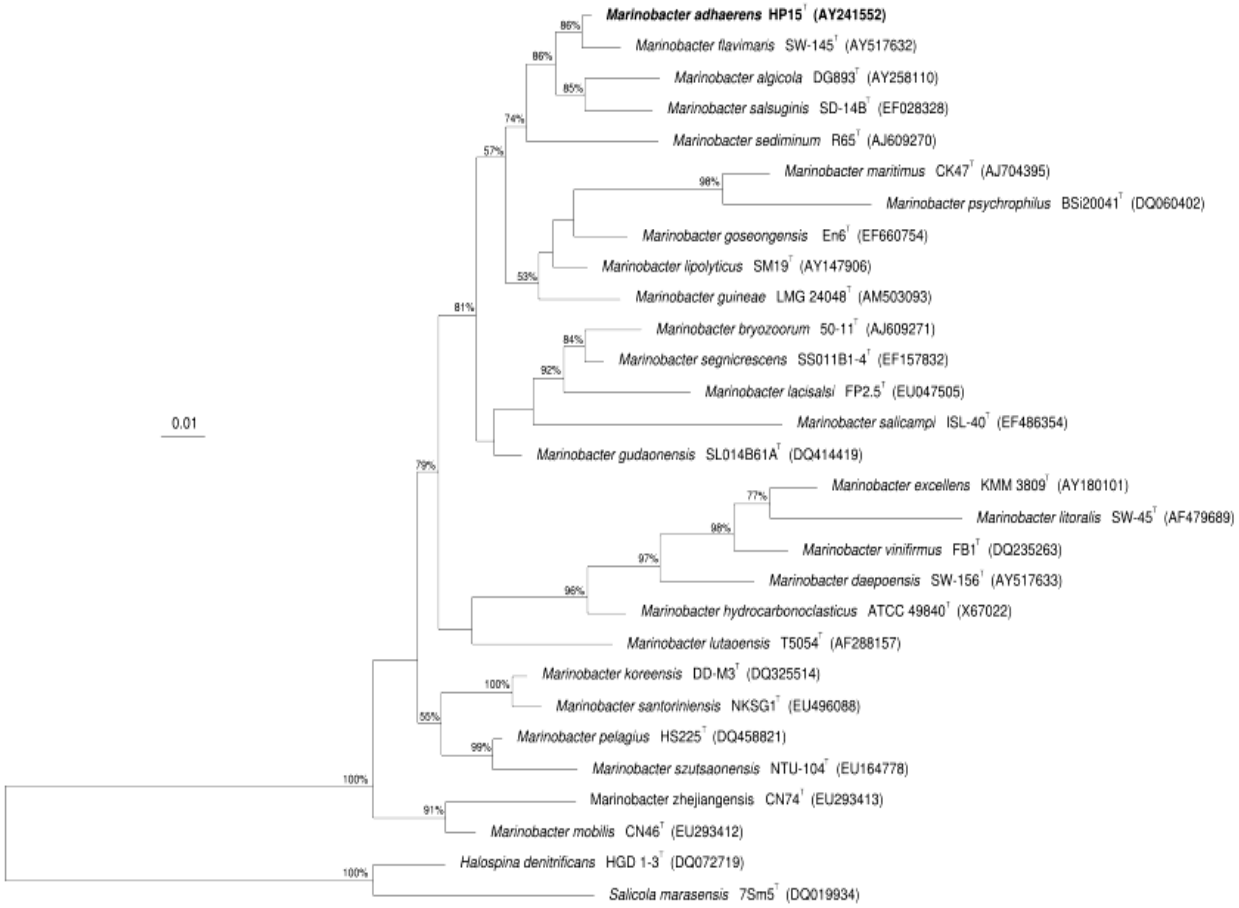

Fig. 2. Maximum likelihood phylogenetic tree based on 16 S rRNA sequences of HP15^T, all type strains of the genus *Marinobacter* and the type strains of *Halospina denitrificans* HGD 1-3^T (DQ072719) and *Salicola marasensis* 7Sm5^T (DQ019934) working as outgroups. The tree was inferred from 1,531 alignment positions using the RAxML algorithm (Stamatakis 2006b). Support values from 1,000 bootstrap replicates are displayed above branches if larger than 50 %. Bar, 0.01 nucleotide substitutions per site.

and *Marinobacter algicola* (96 %) (Antunes et al. 2007; Green et al. 2006; Yoon et al. 2004b). These four type strains form a discrete cluster in the phylogenetic tree **(Fig. 2)**. As determined by DNA-DNA hybridization, the genome of HP15^T showed a similarity of about 64 % to that of *Marinobacter flavimaris*, indicating that this similarity is below the generally accepted species differentation limit of 70 % (Wayne et al. 1987a). Furthermore, HP15^T differed from *M. flavimaris* and the other *Marinobacter* species in a number of chemotaxonomic properties **(Tab. 1)** such as utilization of glycerol, fructose, lactic acid, gluconate, alanine, and glutamate.

The fatty acid profile of HP15^T is similar to those of other *Marinobacter* species **(Supplementary Tab. S1)**. The same holds true for the G + C content of the HP15^T genome, which was 56.9 mol% **(Tab. 1)**. Based on the herein determined phenotypic and phylogenetic characteristics and the genomic differences towards other *Marinobacter* type strains, strain HP15^T should be placed in the genus *Marinobacter* and should be considered as a novel species. Due to its unique and characteristic attachment properties in the presence of marine particle surfaces, we propose for HP15^T the name *Marinobacter adhaerens* sp. nov.

Description of *Marinobacter adhaerens* sp. nov.

Marinobacter adhaerens [ad.hae´rens. part. adj. adhaerens: hanging on, sticking to]
The cells are motile by means of a single polar flagellum, Gram-negative, and non-spore-forming rods (0.6-0.8 x 1.7-2.4 µm). Colonies on MB agar are brownish translucent and have a circular shape (1-2 mm in diameter) with smooth edges after 2 days of incubation at 28 °C. Growth occurs between 4 and 45 °C with an optimum at 34-38 °C, within a pH range of 5.5-10 with an optimum at 7-8.5, and between 0.4-20 % (w/v) NaCl. The strain is negative for urease, gelatinase, β-galactosidase, arginine dihydrolase, tryptophan deaminase, lysine and ornithine decarboxylase activity, indole and acetoin production, and positive of the utilization of Tween 40 and 80, cis-aconitic acid, lactic acid, alanine, glutamate, leucine, and proline. The major fatty acids are $C_{16:0}$ (21.7 %), $C_{18:1}\omega 9c$ (21.6 %), and $C_{16:1}\omega 7c$/iso $C_{15:0}$ 2-OH (14.6 %). The quinone system consists of quinone-9. The type strain is HP15^T (=DSM XXX^T; =CIP XXX^T). The G + C content is 56.9 mol%. The strain was isolated from marine aggregates (0.1-1 mm) of surface waters of the German Bight.

Acknowledgements

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Supplementary Table S1. Cellular fatty acid composition (%) of *Marinobacter* type strains.

Strains: 1, HP15^T; 2, *M. flavimaris* SW-145^T; 3, *M. salsuginis* SD-14B^T; 4, *M. algicola* DG893^T; 5, *M. guineae* LMG 24048^T; 6, *M. lipolyticus* SM-19^T; 7, *M. sediminum* R65^T; 8, *M. aquaeolei* VT8^T; Additional data for reference strains were taken from Yoon *et al.* (2004b), Antunes *et al.* (2007), Green *et al.* (2006), Montes *et al.* (Montes et al. 2008b), Martin *et al.* (2003), Romanenko *et al.* (2005), and Huu *et al.* (1999a). Values represent the percentages of the total fatty acid content. ND, not determined.

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
$C_{10:0}$	0.2	0.5	<0.5	0.4	<1	1.5	ND	0.6
$C_{12:0}$	6.0	9.1	7.3	7.3	5.2	8.3	4.2	7.9
$C_{14:0}$	1.2	1.1	1.1	0.5	<1	0	0.9	2.6
$C_{15:0}$	0	0.7	<0.5	0	<1	1.0	0	2.2
$C_{16:0}$	21.7	26.7	22.9	25.5	16.9	28.5	21.8	22.6
$C_{17:0}$	1.1	3.7	0.5	1.7	2.5	3.6	1.3	3.8
$C_{18:0}$	3.4	3.3	2.9	2.2	2.2	2.7	2.2	1.4
$C_{16:1}\omega 9c$	9.0	10.2	10.5	4.0	7.1	10.5	13.3	11.6
$C_{17:1}\omega 8c$	2.0	3.8	3.8	2.6	4.4	2.9	2.8	4.5
$C_{18:1}\omega 7c$	3.7	1.2	ND	6.2	6.8	2.3	2.9	ND
$C_{18:1}\omega 9c$	21.6	17.4	17.2	10.4	14.0	13.9	16.1	19.8
$C_{16:1}\omega 7c/C_{15:0}$ iso 2-OH	14.6	6.8	<0.5	19.8	ND	ND	15.9	6.8
$C_{11:0}$ 3-OH	0.2	<0.4	0.5	0.4	ND	ND	ND	0.6
$C_{12:0}$ 3-OH	7.9	10.5	9.3	8.7	8.5	11.3	8.0	9.9
$C_{16:0}$ 10-methyl	0.6	0.2	<0.5	7.7	4.1	4.0	0	2.8

3.4 Complete genome sequence of *Marinobacter adhaerens* HP15

Complete genome sequence of *Marinobacter adhaerens* type strain (HP15), a diatom-interacting marine microorganism

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Abstract

Marinobacter adhaerens HP15 is the type strain of a newly identified marine species, which is phylogenetically related to *M. flavimaris*, *M. algicola*, and *M. aqueoli*. It is of special interest for research on marine aggregate formation because it showed specific attachment to diatom cells. *In vitro* it led to exopolymer formation and aggregation of these algal cells to form marine snow particles. *M. adhaerens* HP15 is a free-living, motile, rod-shaped, Gram-negative *Gammaproteobacterium*, which was originally isolated from marine particles sampled in the German Wadden Sea. *M. adhaerens* HP15 is growing heterotrophically on various media, is easy to access genetically, and serves as a model organism to investigate the cellular and molecular interactions with the diatom, *Thalassiosira weissflogii*. Here we describe the complete genome sequence and its annotation as well as some details on flagella-associated genes. *M. adhaerens* HP15 possesses three replicons; the chromosome comprises 4,422,725 bp and codes for 4,180 protein-coding genes, 46 tRNAs and three rRNA operons, while the two circular plasmids are ~187 kb and ~42 kb in size and contain 178 and 52 protein-coding genes, respectively.

Keywords: marine heterotrophic bacteria, diatoms, attachment, marine aggregate formation

Introduction

Strain HP15 (DSM and JCM assignments pending) is the type strain of the newly established species *Marinobacter adhaerens* sp. nov. and represents one of 26 species currently assigned to the genus *Marinobacter* [1]. Strain HP15 was first described by Grossart *et al.* in 2004 [2] as a marine particle-associated, Gram-negative, γ-proteobacterium isolated from the German Wadden Sea. The organism is of interest because of its *in vitro* capabilities to specifically attach to the surface of the diatom, *Thalassiosira weissflogii*, to induce exopolymer and aggregate formation, and to thus generate marine snow particles [3]. Marine snow formation is an important process of the biological pump, by which atmospheric carbon dioxide is taken up, recycled, and partly exported to the sediments. This sink of organic carbon plays a major role for marine biogeochemical cycles [4]. Several studies reported on the formation and properties of marine aggregates [5–8]. Although it was shown that heterotrophic bacteria control the development and aggregation of marine phytoplankton [2], specific functions of individual bacterial species on diatom aggregation have not been explored thus far. A better understanding of the molecular basis for bacteria-diatom interactions leading to marine snow formation is currently gained by establishing a bilateral model system, for which *M. adhaerens*

sp. nov. HP15 serves as the bacterial partner of the easy-to-culture diatom, *T. weissflogii* [3]. Herein, we present a set of features for *M. adhaerens* sp. nov. HP15 (**Table 1**) together with its annotated complete genomic sequence, and a detailed analysis of its flagella-associated genes.

Classification and features

M. adhaerens sp. nov. strain HP15 is a motile, Gram-negative, non-spore-forming rod (**Figure 1**). Based on its 16S rRNA sequence, strain HP15 was affiliated to the *Marinobacter* genus of γ-proteobacteria. Its 16S rRNA gene is most closely related to those of the type strains of *M. flavimaris* (99%), *M. salsuginis* (98%) and *M. algicola* (96%). These four type strains form a discrete cluster in the phylogenetic tree (**Figure 2**). In contrast, DNA-DNA hybridization experiments revealed that the genome of *M. adhaerens* sp. nov. HP15 showed a similarity of about 64% to that of *M. flavimaris* [1], which is below the generally accepted species differentiation limit of 70% [9].

Chemotaxonomy

The heterotrophic bacterium *M. adhaerens* sp. nov. HP15 was experimentally demonstrated to interact with the diatom *Thalassiosira weissflogii* [3]. Strain HP15 can grow in artificial sea water with a nitrogen-to-phosphorus ratio of 15:1 and supplemented with glucose as the sole carbon source. In presence of diatom cells but without glucose, HP15 utilizes diatom-produced carbohydrates as the sole source of carbon. Furthermore, *M. adhaerens* sp. nov. HP15 differed from *M. flavimaris* and other *Marinobacter* species in a number of chemotaxonomic properties such as utilization of glycerol, fructose, lactic acid, gluconate, alanine, and glutamate [1]. Additionally, strain HP15 showed a unique fatty acid composition in quantitative terms.

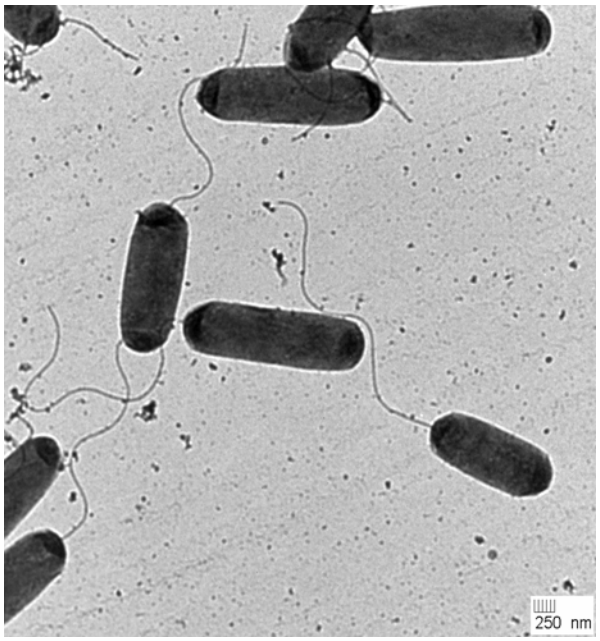

Figure 1. Transmission electron micrograph of *M. adhaerens* sp. nov. strain HP15.

Genome sequencing and annotation

Genome project history

This organism was selected for sequencing on the basis of its phylogenetic position, its particular feature as a diatom-interacting marine organism [3], and its feasible genetic accessibility to act as a model organism. The genome project is deposited in the Genome OnLine Database [13] and the complete genome sequence in GenBank. Sequencing and finishing were performed by AGOWA GmbH, Berlin, Germany. The annotation was performed in cooperation with the Max Planck Institute for Marine Microbiology, Bremen, Germany. The main project information is summarized in **Table 2**.

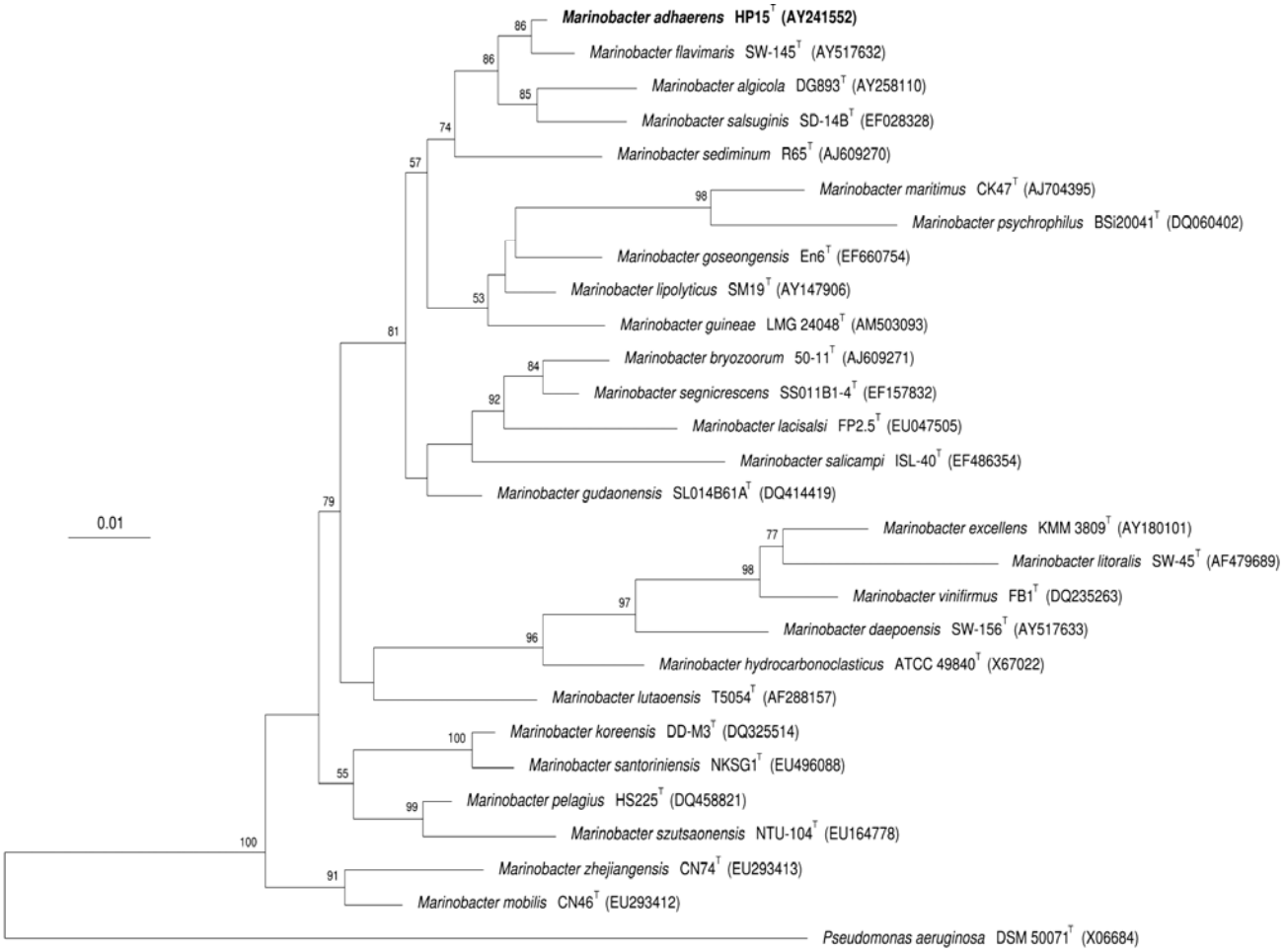

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Figure 2. Maximum likelihood phylogenetic tree based on 16S rRNA sequences of HP15, all type strains of the genus *Marinobacter* and the *Pseudomonas aeruginosa* type strain DSM50071 as an outgroup. The tree was inferred from 1,531 alignment positions using the RAxML algorithm (Stamatakis 2006a). The branches are scaled in terms of the expected number of substitutions per site. Support values from 1,000 bootstrap replicates are displayed below branches. Bar, 0.01 nucleotide substitutions per site.

Growth conditions and DNA isolation

M. adhaerens sp. nov. HP15 was grown in 100 ml Marine Broth medium [14] at 28°C. A total of 23 µg DNA was isolated from the cell paste using a Qiagen Genomic 500 DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Table 1. Classification and general features of *M. adhaerens* sp. nov. HP15 in accordance to the MIGS recommendations [11]

MIGS ID	Property	Term	Evidence code
		Domain *Bacteria*	
		Phylum *Proteobacteria*	
		Class *Gammaproteobacteria*	TAS [12]
	Current classification	Order *Alteromonadales*	TAS [12]
		Family *Alteromonadaceae*	TAS [12]
		Genus *Marinobacter*	TAS [12]
		Species *Marinobacter adhaerens*	TAS [1]
		Type strain HP15	TAS [1]
	Gram stain	negative	IDA
	Cell shape	rod-shaped	IDA
	Motility	motile, single polar flagellum	IDA
	Sporulation	non-sporulating	NAS
	Temperature range	mesophilic	IDA
	Optimum temperature	34-38°C	IDA
	Salinity	0.4-10 g NaCl/l (optimum/growth within 1 day)	IDA
MIGS-22	Oxygen requirement	strictly aerobic	IDA
	Carbon source	dextrin, Tween 40 and 80, pyruvic acid methyl ester, succinic acid mono-methyl-ester, cis-aconitic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-leucine and L-proline	IDA
	Energy source	chemoorganoheterotrophic	IDA
MIGS-6	Habitat	sea water	IDA
MIGS-15	Biotic relationship	free-living and particle-associated	TAS [2]
MIGS-14	Pathogenicity	none	NAS
	Biosafety level	1	NAS
	Isolation	Marine aggregates (0.1-1 mm)	TAS [2]
MIGS-4	Geographic location	German Wadden Sea	TAS [2]
MIGS-4.1	Latitude	53°43'20''N	TAS [2]
MIGS-4.2	Longitude	07°43'20''E	TAS [2]
MIGS-4.3	Depth	Surface waters	TAS [2]
MIGS-4.4	Altitude	Not reported	
MIGS-5	Sample collection time	15 June and 10 October 2000	TAS [2]

Evidence codes – IDA: inferred from Direct Assay (first time in publication); TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property of the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [13]. If evidence code is IDA, then the property was directly observed for a live isolate by one of the authors or an expert mentioned in the acknowledgements.

Table 2: Genome sequencing project information for *M. adhaerens* sp. nov. HP15

MIGS ID	Property	Term
MIGS-31	Finishing quality	Finished
MIGS-28	Library used	454 pyrosequencing standard library
MIGS-29	Sequencing platforms	454 FLX Ti
MIGS-31.2	Sequencing coverage	22.5x pyrosequencing
MIGS-30	Assemblers	Newbler version 2.0.00.22
MIGS-32	Gene calling method	GLIMMER v3.02, tRNAScan-SE
		CP001978 (chromosome)
	Genbank ID	CP001979 (pHP42)
		CP001980 (pHP187)
	Genbank Date of Release	September 18, 2010
	GOLD ID	
	NCBI project ID	46089
	Database: IMG	
	Project relevance	Marine diatom-bacteria interactions

Genome sequencing and assembly

The *Marinobacter adhaerens* sp. nov. HP15 genome was sequenced at AGOWA (AGOWA GmbH, Berlin, Germany) using the 454 FLX Ti platform of 454 Life Sciences (Branford, CT, USA). The sequencing library was prepared according to 454's instructions from genomic *M. adhaerens* sp. nov. HP15 DNA with a final concentration of 153 ng/µl. Sequencing was carried out on a physically quartered 454 picotiterplate, yielding 258.645 reads with an average length of 405 bp, totaling to almost 105 Mb. These reads were assembled using the Newbler assembler version 2.0.00.22 (Roche), resulting into 253.285 fully and 4.763 partially assembled reads, leaving 932 singletons, 226 repeats and 371 outliers. The assembly comprised 112 contigs, with 40 exceeding 500 bp. The latter comprised more than 4.6 Mb, with an average contig size of almost 116 kb and a longest contig of more than 1.2 Mb. Gaps between contigs were closed in a conventional PCR-based gap closure approach, resulting into a fully closed circular chromosome of 4.421.911 bp, and two plasmids of 187.465 bp and 42.349 bp, respectively. Together all sequences provided 22.5x coverage of the genome. The error rate of the completed genome sequence is about 3 in 1,000 (99.7%).

Genome annotation

Potential protein-coding genes were identified using GLIMMER v3.02 [15], transfer RNA genes were identified using tRNAScan-SE [16] and

ribosomal RNA genes were identified via BLAST searches [17] against public nucleotide databases. The annotation of the genome sequence was performed with the GenDB v2.2.1 system [18]. For each predicted gene, similarity searches were performed against public sequence databases (nr, SwissProt, KEGG) and protein family databases (Pfam, InterPro, COG). Signal peptides were predicted with SignalP v3.0 [19, 20] and transmembrane helices with TMHMM v2.0 [21]. Based on these observations, annotations were derived in an automated fashion using a fuzzy logic-based approach [22]. Finally, the predictions were manually checked with respect to missing genes in intergenic regions and putative sequencing errors, and the annotations were manually curated using the Artemis 11.3.2 program and refined for each putative gene [23].

Genome properties

The genome of strain HP15 comprises three circular replicons: the 4,422,725 bp chromosome and two plasmids of ~187 kb and ~42 kb, respectively (**Table 3A**). The genome possesses a 56.86% GC content (**Table 3B**). Of the 4,482 genes predicted, 4,422 were protein coding genes, and 55 RNAs; 391 pseudogenes were also identified. The majority of the protein-coding genes (67.5%) were assigned with a putative function, while those remaining were annotated as hypothetical proteins. The distribution of genes into COGs functional categories is presented in **Table 4**.

Table 3A. Genome composition for *M. adhaerens* HP15

Label	Size (Mb)	Topology	INSDC identifier	RefSeq ID
Chromosome[§]	4.423	circular	-	CP001978
Plasmid pHP-187[¶]	0.187	circular	-	CP001980
Plasmid pHP-42*	0.042	circular	-	CP001979

[§] Number of protein-coding genes: 4,180, [¶] Number of protein-coding genes: 178
***** Number of protein-coding genes: 52

Table 3B. Genome statistics for *M. adhaerens* HP15

Attribute	**Genome (total)**	
	Value	% of total[a]
Genome size (bp)	4,651,725	
DNA Coding region (bp)	4,178,502	89.80
DNA G+C content (bp)	2,644,970	56.86
Number of replicons	3	
Extrachromosomal elements	2	
Total genes[b]	4,410	
tRNA genes	46	1.13
5S rRNA genes	3	0.07
16S rRNA genes	3	0.07
23S rRNA genes	3	0.07
Protein-coding genes	4,355	98.66
Genes assigned to COGs	3,027	67.54
Genes with Pfam domains	2,918	65.1
1 Pfam domain	2,041	45.54
2 Pfam domains	598	13.34
3 Pfam domains	194	4.33
4 or more Pfam domains	85	1.9
Genes with signal peptides	765	17.07
Genes with transmembrane helices	1,043	23.27
1 transmembrane helix	341	7.61
2 transmembrane helices	154	3.44
3 transmembrane helices	72	1.61
4 or more transmembrane helices	476	10.62
Genes in paralogous clusters	570	12.72
Genes with 1 paralog	364	8.12
Genes with 2 paralogs	63	1.41
Genes with 3 paralogs	26	0.58
Genes with 4 or more paralogs	117	2.61
Pseudo/hypothetical genes	391	8.72
Conserved hypothetical genes	668	14.90
Genes for function prediction	3,363	75.03

a) The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.
b) Also includes 54 pseudogenes and 5 other genes.

Table 4. Number of genes associated with the 21 general COG functional categories

Code	Value	% of total[a]	Description
J	162	3.7	Translation
A	0	0	RNA processing and modification
K	161	3.6	Transcription
L	132	3	Replication, recombination and repair
B	0	0	Chromatin structure and dynamics
D	32	0.7	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	0	0	Defense mechanisms
T	199	4.5	Signal transduction mechanisms
M	151	3.4	Cell wall/membrane biogenesis
N	166	3.8	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	0	0	Intracellular trafficking and secretion
O	127	2.9	Posttranslational modification, protein turnover, chaperones
C	192	4.3	Energy production and conversion
G	82	1.9	Carbohydrate transport and metabolism
E	254	5.7	Amino acid transport and metabolism
F	51	1.1	Nucleotide transport and metabolism
H	97	2.2	Coenzyme transport and metabolism
I	141	3.2	Lipid transport and metabolism
P	138	3.1	Inorganic ion transport and metabolism
Q	76	1.7	Secondary metabolites biosynthesis, transport and catabolism
R	330	7.5	General function prediction only
S	251	5.7	Function unknown
multiple COGs	285	6.4	
-	3027	68.6	Total
-	1383	31.4	Not in COGs

a) The total is based on the total number of protein coding genes in the annotated genome

Flagella-associated gene clusters of *M. adhaerens* HP15

Because *M. adhaerens* HP15 was experimentally shown to adhere to diatom cells, gene clusters coding for secretion, assembly, and mechanistic function of the polar flagellum were analyzed in detail (**Figure 3**). Bacterial flagella and other cell appendages had previously been shown to be instrumental for chemotactic movement towards and adhesion to biotic surfaces [24, 25]. The amino acid sequences of proteins encoded by the three identified gene clusters showed significant similarities to orthologous gene products of *P. aeruginosa* PAO1 and various other bacterial species. Not surprisingly, hook and motor switch complex components were most conserved. However, gene products involved in flagellar filament formation encoded by Cluster II also showed 53 to 78% identity to the respective PAO1 proteins. Mutagenesis of flagella-associated genes of *M. adhaerens* HP15 will be carried out in the near future to study the role of flagella in bacteria-diatom interactions and to further our understanding of the cell-to-cell communication between those organisms.

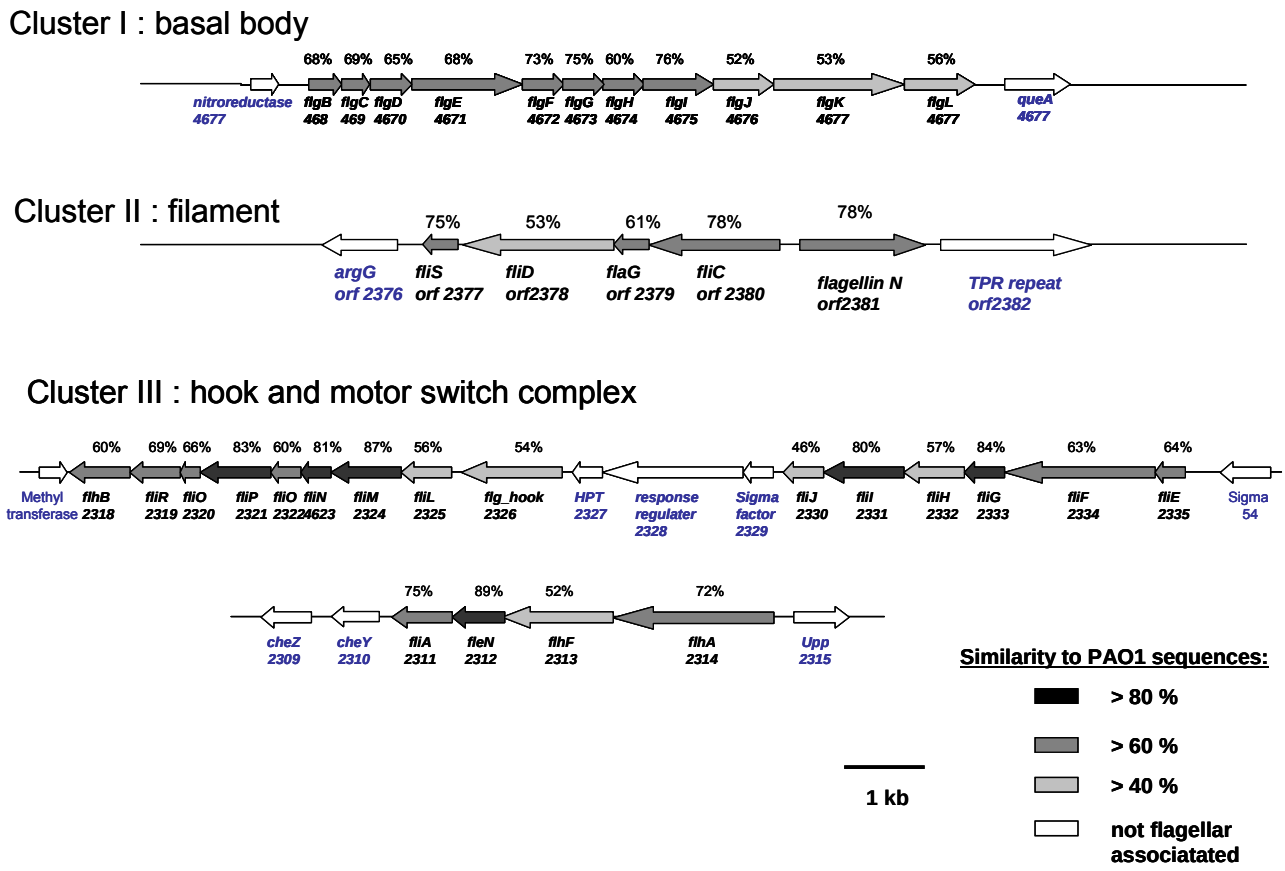

Figure 3. Schematic presentation of the three flagella-associated gene clusters of *M. adhaerens* HP15 coding for the basal body, the filament, and the hook and motor switch complex. Identities to the respective orthologs in the genome of *P. aeruginosa* PAO1 are indicated by gray-scale code. Numbers of CDS are shown below gene names.

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3.5 Analysis of genetic accessibility of *Marinobacter adhaerens* sp. nov. HP15, a marine bacterium interacting with diatoms

Analysis of genetic accessibility of *Marinobacter adhaerens* sp. nov. HP15, a marine bacterium interacting with diatoms

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Abstract

Various *Marinobacter* species are ubiquitously found in diverse marine environments. *Marinobacter adhaerens* HP15 was isolated from marine aggregates and shown to induce aggregate formation while interacting with the diatom, *Thalassiosira weissflogii*. Diatom aggregation is substantial for the flux of organic carbon from the photic zone to the sea bed. To analyze this effect at the molecular level, a genetic tool system was developed for *M. adhaerens* HP15. The antibiotics susceptibility spectrum of this organism was determined, and transformation protocols using both, electroporation and plasmid conjugation, were established. Among various plasmids of different incompatibility groups, only plasmids pBBR1MCS and pSUP106 were shown to replicate in *M. adhaerens* HP15. For conjugation 1.4×10^{-3} and for electroporation 5.1×10^{-5} transformants per number of recipients (1.1×10^{5} CFU) per µg of DNA were obtained for the broad-host-range vector pBBR1MCS. Additionally, transposon mutagenesis using a suicide plasmid and site-directed mutagenesis using homologous recombination were conducted for flagellum biosynthetic genes. The resulting mutant phenotypes were confirmed by soft agar assay and transmission electron microscopy. A genomic cosmid library was constructed to screen for genes and to functionally complement mutants. Expression of the reporter gene encoding for enhanced green fluorescent protein in *M. adhaerens* HP15 was successfully done revealing a useful tool for gene expression studies. This study is the first report for the genetic manipulation of a member of the *Marinobacter* genus.

Key words: Marinobacter, conjugation efficiency, electroporation, mutagenesis, *fliC*

Running title: Genetic accessibility of *Marinobacter adhaerens* HP15

Introduction

Heterotrophic bacteria interacting with micro-algae play an important role in the formation of marine snow particles and are thus important for the carbon cycling in pelagic systems (Grossart et al. 2006; Sapp et al. 2008a). Besides their role in degradation of organic carbon and remineralization of nutrients (Cole 1982), these bacteria promote aggregation of phytoplankton (Decho 1990b). The concept of the ”phycosphere“ describes the area surrounding micro-algae, in which microorganisms interact with algae via diverse cell-to-cell mechanisms (Bell and Mitchell 1972a). Bacteria interacting with algal cells could feed on them or their products, respectively, or support their growth by remineralization of nutrients. Since various scenarios could be imagined, it remained to be determined whether bacteria enhancing aggregate formation inhibit or promote the growth of algae. Most previous studies focused on bacterial communities associated with phytoplankton at the ecological level, and little is known about the specific role of single bacterial strains, their genes, and gene products for this interaction.

For in-depth molecular analysis of diatom-bacteria interactions, a bilateral model system consisting of the diatom, *Thalassiosira weissflogii*, and the bacterial strain, *Marinobacter adhaerens* HP15, was recently established (Gärdes et al. 2010a). Close interaction of the two organisms was demonstrated by attachment assays and determination of transparent exopolymer particle (TEP) production concluding that *M. adhaerens* HP15 plays an important role in *T. weissflogii* aggregation dynamics. Members of the genus *Marinobacter* were found in various marine habitats (Gorshkova et al. 2003) as well as in interactions with eukaryotic organisms such as *Bryozoa* or dinoflagellates (Green et al. 2006; Romanenko et al. 2005).

Herein, for the first time the genetic accessibility of a *Marinobacter* species was comprehensively analyzed. Suitability of *M. adhaerens* HP15 for molecular studies was demonstrated by transfer of plasmids via electroporation and conjugation and by two types of mutagenesis. As proof-of-principle, motility-deficient mutants were generated by transposon insertion as well as by site-directed mutagenesis using homologous recombination. A genomic cosmid library of *M. adhaerens* HP15 was constructed and can be used for gene screening, heterologous gene expression, and mutant complementation in the future. Reporter gene expression using enhanced green fluorescent protein (*egfp*) was successfully demonstrated in *M. adhaerens* HP15 and might be a potential tool for further studies on promoter activities and transcriptional regulation.

Material & Methods

Bacterial strains, plasmids, and culture media

The bacterial strains and plasmids used are listed in **Table 1**. *M. adhaerens* HP15 was isolated from marine particulate samples collected from surface waters of the German Bight (Grossart et al. 2004b). *Marinobacter* cells were cultivated in marine broth (MB) medium as described in Kaeppel *et al.* (2010). For electroporation, cells were cultivated on MB agar medium for 2 days. *E. coli* strains were maintained in Luria-Bertani (LB) agar medium. For conjugation, *M. adhaerens* cells were grown in 100 ml liquid culture shaking at 250 rpm over night. The donor strain *E. coli* ST18 was grown in liquid LB medium containing 5-aminolevulinic acid (ALA, final concentration: 50 µg/ml). The following antibiotics were added to media when needed (in µg/ml): chloramphenicol, 25; kanamycin, 500; and ampicillin, 50.

To identify antibiotics susceptibility as selection marker for transformation, *M. adhaerens* HP15 was grown in MB liquid medium to an OD of 1, and 20 µl were spotted on MB agar medium containing various concentrations of ampicillin, chloramphenicol, gentamycin, kanamycin, spectinomycin, or tetracycline. The minimal inhibitory concentrations (MICs) for these antibiotics in MB were determined by the micro-dilution assay as described by Burse *et al.* (2004).

DNA procedures

Plasmids were isolated by NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany). Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturer (Fermentas, St. Leon-Rot, Germany). DNA fragments were resolved in 1% agarose gel and extracted with Nucleo Spin Extract kit (Macherey-Nagel, Düren, Germany). Preparation of gDNA was conducted with NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany).

Plasmid conjugation

Recombinant plasmids were introduced into the recipient *M. adhaerens* HP15 by biparental conjugation with *E. coli* ST18 as a donor. Additionally, triparental mating with the helper strain *E. coli* HB101, capable of plasmid mobilization, was performed. All strains were grown as described above over night and the OD was adjusted to 0.1 (~ $3x10^7$ cells/ml). 10^7-10^8 cells of donor and recipient were mixed in a ratio of 1:2. For triparental mating, recipient, donor, and helper strain were mixed in a ratio of 3:1:1. For both types of mating, cells were resuspended in 500 µl of LB medium supplemented with ALA (for biparental mating), spotted on LB or LB+ALA agar plates, respectively, and incubated for 24 h, 48 h, or 72 h at 28°C. After incubation, the cell mass was scraped off the agar plates and resuspended in MB medium for subsequent dilution plating. Transconjugants were selected on MB agar supplemented with chloramphenicol after incubation at 28°C for 2-5 days. As control, the non-replicating cosmid vector, pWEB™ (Epicentre Biotechnologies, Madison, USA) was used.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Bacterial strains		
Escherichia coli DH5α λpir	Φ80*dlacZ*ΔM15 Δ(*lacZYA- argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*	(Miller and Mekalanos 1988)
Escherichia coli ST18	S17 λ*pir* ΔhemA	(Thoma and Schobert 2009)
Escherichia coli HB101	carrying helper plasmid pRK2013	(Figurski and Helinski 1979)
Marinobacter adhaerens HP15	wild type	(Grossart et al. 2004b)
Δ*fliC*	*fliC* deletion mutant of HP15	This study
*fliG::*Tn*5*	transposon insertion mutant in *fliG* of HP15	This study
*fliR::*Tn*5*	transposon insertion mutant in *fliR* of HP15	This study
*chpA::*Tn*5*	transposon insertion mutant in *chpA* of HP15	This study
Plasmids		
pBBR1MCS	broad-host-range *mob* Cm^R	(Kovach et al. 1994)
pSUP106	IncQ *mob cos* Cm^R Tet^R	(Priefer et al. 1985)
pWeb-Cm	colE1 *cos* Cm^R; Cm resistance cassette from pFCM1 cloned into *Nhe*I site of pWEB™	This study
pGEM.Km	colE1 Km^R; Km resistance cassette from pMKm cloned into *Pst*I site of pGEM®-T Easy	This study
pEX18Tc	pMB1 oriT *sacB* Tet^R	(Hoang et al. 1998a)
pK18mob	pMB1 oriT Km^R	(Schäfer et al. 1994)
pLAFR3	IncP *cos* Tet^R	(Staskawicz et al. 1987)

pKNOCK-Cm	IncX oriT Cm^R	(Alexeyev 1999)
pPH1JI	IncP Cm^R Gm^R	(Hirsch and Beringer 1984)
pRK415	IncP oriT Tet^R	(Keen et al. 1988)
pSU18	pMB1 Cm^R	(Bartolome et al. 1991)
pBK-miniTn*7*-*gfp*1	pMB1 *mob* Amp^R, miniTn*7*-*gfp*1 with Km^R and Cm^R	(Koch et al. 2001)
pEP4351	IncX oriT Cm^R, Tn4351 with Em^R and Tet^R	(Cooper et al. 1997)
pRL27	IncX oriT, Tn5 with Km^R	(Larsen et al. 2002)
pBBR.egfp	Broad-host-range *mob egfp* Cm^R	(Weingart et al. 2004)
pGEM®-T Easy	colE1 *lacZ* Amp^R	Promega GmbH, Mannheim, Germany
pFCM1	Amp^R Cm^R	(Choi and Schweizer 2005b)
pK19mobsacB	pMB1 oriT Km^R	(Schäfer et al. 1994)
pEX18Ap	pMB1 oriT *sacB* Amp^R	(Hoang et al. 1998a)
pAS3	pGEM®-T Easy containing 1002 bp upstream *fliC* flanking region of HP15	This study
pAS4	pGEM®-T Easy containing 1236 bp downstream *fliC* flanking region of HP15	This study
pAS5	FRT sites and Cm resistance cassette (1135 bp) from pFCM1 cloned into *Kpn*I site of pAS3	This study
pAS6	FRT sites, Cm resistance and upstream region (2137 bp) from pAS4 cloned with *BamH*I/*Spe*I into pAS5	This study
pAS7	knock out fragment (3373 bp) from pAS6 cloned with *EcoR*I into pEX18Ap	This study
pAS8	knock out fragment (3373 bp) from pAS6 cloned with *EcoR*I into pk18mobsacB	This study

Table 2. Oligonucleotide primers used in this study.

Primer name	Sequence 5´ - ´3
TnF	TAACGGCTGACATGGGGG
TnR	GCATCTTCCCGACAACGC
FliCupF	ATCTCTGTTTGCAGCGCG
FliCupR	TAGGATCCCGGTACCCGCCGAACTCGTTGCTT
FliCdownF	ACGGATCCACCTTCGGGTTCCGGTTT
FliCdownR	TCGATAACGCCAGCGGAAA
FliCF	GATGCGCAGGCCGGAAGA
FliCR	GCCCGAGCCGGTGTTTGA
CmF	AGATCACTACCGGGCGTA
CmR	TGCCACTCATCGCAGTAC

Electroporation

Electro-competent *Marinobacter* cells were prepared directly before electroporation and kept on ice during all steps of the washing procedure. The cell mass of two fully covered MB agar plates was resuspended in 1 ml of pre-cooled 300 mM sucrose and washed two times with 1 ml of cold 300 mM sucrose with intermittent centrifugations at 13.000 rpm for 3 min at 4°C. The final pellet was resuspended in 200 µl of 300 mM sucrose to obtain a dense suspension (~10^{10} cells/ml). 50 µl of cell suspension was mixed with 0.3 to 1.5 µg of plasmid DNA for electroporation (cuvette width 0.1 cm, resistance 200 Ω, capacitance 25 µF, pulse: 2.5 kV for ~5 ms). Immediately after the pulse, 950 ml of SOC medium was added to the cuvette. The cell suspension was transferred to a 1.5-ml sterile tube and incubated shaking at 37°C for 15-20 hrs. 50 to 400 µl of suspensions were subsequently plated on MB agar medium supplemented with the appropriate antibiotics and incubated at 37°C. Electrotransformation of *M. adhaerens* HP15 was tested with the following plasmids: pBBR1MCS, pSUP106, pWeb-Cm, pGEM.Km, pEx18Tc, pK18mob, pLAFR3, pKnock-Cm, pPH1JI, pRK415, and pSU18 (**Table 1**).

Transposon mutagenesis

Plasmids pBK-miniTn7-gfp1, pEP4351, and pRL27 (**Table 1**) containing different transposons were tested for transposon mutangenesis efficiency using electroporation. Resulting mutant colonies were grown in MB medium supplemented with kanamycin in 96 well plates over night, resuspended in glycerol (final concentration: 15%), and stored at -80°C. For screening of flagella-deficient mutants, mutant cells were grown on MB medium containing kanamycin and stamped onto 10-fold diluted MB soft agar plates (0.3% agar). Swimming-deficient mutants were identified by lacking the typical motility pattern of the wild type. The genomic DNA of promising mutants was extracted, treated with restriction enzyme *Nco*I, religated with T7 DNA ligase, and introduced into *E. coli* DH5α by electroporation. Nucleotide sequencing of transposon-flanking DNA was conducted with primers TnF and TnR (**Table 2**). The obtained nucleotide sequences were compared to the GenBank protein sequence database using BlastX (Altschul et al. 1990).

Site-directed mutagenesis

For site directed mutagenesis, the flagellin-encoding gene *fliC* was selected by sequence analysis using the *M. adhaerens* HP15 genome sequence (GRP79971) (Gärdes et al. 2010b), GenDB 2.2 (Meyer et al. 2003) and BlastN analysis (Altschul et al. 1990). The upstream (1002 bp) and downstream (1236 bp) flanking regions of *fliC* were amplified using primer pairs FliCupF/FliCupR and FliCdownF/FliCdownR, respectively (**Table 2**). Both fragments were sub-cloned to vector pGEM®-T Easy (Promega, Mannheim, Germany) resulting in plasmids, pAS3 and pAS4 (**Table 1**). A chloramphenicol resistance cassette was excised from pFCM1 (**Table 1**) with a *Kpn*I restriction digest and inserted into *Kpn*I-treated pAS3 yielding plasmid pAS5. Plasmid pAS5 was treated with restriction enzymes, *BamH*I and *Spe*I, the insert fragment was purified, and ligated into *Bam*HI and *Spe*I-treated plasmid pAS4 resulting in plasmid pAS6, which contained the 6338-bp knock-out fragment consisting of the chloramphenicol resistance gene flanked by *fli*C upstream and downstream fragments. The knock-out fragment was excised with enzyme *Eco*RI and

ligated to *Eco*RI-treated suicide vectors pEX18Ap and pK19mobsacB, respectively, (**Table 1**) generating pAS7 and pAS8 knock-out constructs, both containing the *sacB* gene as a counter-selectable suicide marker. After biparental conjugation and subsequent homologous recombination, correct insertion of knock-out fragments into the *M. adhaerens* HP15 chromosome by double crossover was confirmed by PCR with primer pairs FliCF and FliCR as well as CmF and CmR (**Table 2**).

Determination of mutant phenotype by swimming assays and transmission electron microscopy (TEM)

Flagella-deficient mutants and the wild type were grown in MB liquid medium containing kanamycin or chloramphenicol, respectively, over night, stamped onto 10-fold diluted MB soft agar plates (0.3% agar), and incubated for 24 h. For transmission electron microscopy, cells were grown in MB liquid medium as described above. A 300-µm-mesh carbon coated copper grid (Plano GmbH, Wetzlar, Germany) was incubated for 30 s in 20 µl of cell suspension, excess liquid was removed, and adhering cells were stained with 1% uranyl acetate, washed with distilled water, and dried. The stained cells were visualized using an EM900 transmission electron microscope (Zeiss, Jena, Germany).

Generation of genomic cosmid library

A genomic cosmid library for *M. adhaerens* HP15 was constructed using the pWEB™ cosmid cloning kit (Epicentre Biotechnologies, Madison, USA). pWEB™ (GenBank accession number AF075573) is a cosmid vector derived from cosmid pWE15 (GenBank accession number X65279) (Wahl et al. 1987). Following the manufacturer's recommendations, genomic DNA of *M. adhaerens* HP15 was manually sheared and DNA fragments of ~40 kb were ligated via blunt-end cloning to the *SmaI* restriction site of pWEB™.

Subsequently, ligation products were packaged into bacteriophage λ heads and the packaging assembly products were allowed to infect *E. coli* host cells. A 10-fold dilution of the resulting cell suspension was spread on LB agar containing ampicillin and incubated for over night. Obtain colonies were individually picked, grown in LB liquid medium in 96-well microtiter plates, and stored at -80°C in 15% glycerol. In order to determine the number of cosmid clones required for full coverage of the genome, the following formula was applied: Genome coverage = [Number of clones x Size of insert] / Genome size.

Expression of enhanced green fluorescent protein in Marinobacter adhaerens HP15

M. adhaerens HP15 was labeled using enhanced green fluorescent protein (EGFP). The *egfp* gene-carrying broad-host-range plasmid pBBR.EGFP (**Table 1**) was introduced to *M. adhaerens* HP15 by electroporation. Expression of *egfp* in single cells of *M. adhaerens* HP15 was visualized by use of a LSM 510 meta confocal laser scanning microscope (Zeiss). The *M. adhaerens* HP15 wild type served as a negative control.

Results

Antibiotic susceptibility

Growth of *M. adhaerens* HP15 was inhibted by a number of commonly used antibiotics (**Table 3**). MICs were generally higher on agar than those observed in liquid medium. *M. adhaerens* HP15 showed lowest sensitivity to ampicillin on agar with an MIC > 1000 µg/ml, but highest in liquid medium with an MIC of 0.1 µg/ml. Highest susceptibility of *M. adhaerens* HP15 on agar medium with an MIC of 25 µg/ml was observed for chloramphenicol, which was later used as selection marker for transformation when appropriate.

Table 3. Minimal inhibitory concentration on 1.2% MB agar and in MB medium

Antibiotic	1.2% MB agar (µg/ml)	MB medium (µg/ml)
Ampicillin	> 1000	0.1
Chloramphenicol	25	2
Gentamycin	50	31.3
Kanamycin	100	62.5
Spectinomycin	100	15.6
Tetracycline	250	31.3

Transformation efficiency and expression of reporter gene

From various vectors tested, only plasmids pBBR1MCS and pSUP106 were found to stably replicate in *M. adhaerens* HP15. Other plasmids could not be transformed or did not replicate in *M. adhaerens* HP15 (Data not shown). Highest conjugation efficiencies were obtained via biparental mating at a donor-to-recipient ratio of 1:2 and after 24 hrs of mating time. For plasmid pBBR1MCS 1.4 x 10^{-3} transconjugants per number of recipients and for plasmid pSUP106 2.7 x 10^{-4} for were obtained (**Table 4**). Using electroporation, transformation efficiencies of 5.1 x 10^{-5} transformants per number of recipients for pBBR1MCS and 9.2 x 10^{-7} transformants per number of recipients for pSUP106 were observed. These values correspond to 1.1 x 10^{5} CFU/µg DNA for pBBR1MCS and 1.6 x 10^{3} CFU/µg DNA for pSUP106 as transformant numbers. When plasmid pBBR.*egfp* was introduced to *M. adhaerens* HP15, transformants exhibited fluorescence when excited with a wavelength of 488 nm, demonstrating that *egfp* was expressed (**Fig. 1**). In contrast, no fluorescence was observed for *M. adhaerens* HP15 wild type suggesting that *egfp* is a suitable reporter gene in this bacterium.

Transposon and site-directed mutagenesis of M. adhaerens HP15

The transposon-carrying plasmids pBK-miniTn7-gfp1, pEP4351, and pRL27 were assayed for mutagenesis of *M. adhaerens* HP15 via electroporation. Transformation with pBK-miniTn*7-gfp*1 and pEP4351 did not yield in transposon mutants. In contrast, transformation of strain HP15 with plasmid pRL27 carrying transposon Tn*5* resulted in an efficiency of 6.8 x 10^{2} CFU/µg DNA (1.8 x 10^{-7} mutants per number of recipients), which is roughly comparable to the efficiency of conjugation for plasmid pBBR1MCS. Testing a total of 768 transposon mutants in the swimming assay in soft agar revealed 16 swimming-deficient mutants. For those mutants, nucleotide sequencing of the transposon-flanking DNA regions revealed that the phenotype of three mutants could be correlated to transposon insertions in the motility-associated genes *fliG*, *fliR*, and *chpA*. A mutant with the transposon insertion in *fliG* termed *fliG*::Tn*5* was used for further phenotypic analysis.

Results of the nucleotide sequencing of all 16 transposon mutants proved the randomness of transposon insertion in the *M. adhaerens* HP15 genome, since every of the 16 determined genotypes were unique (Data not shown). Site-directed mutagenesis was conducted by introducing the suicide plasmids pAS7 and pAS8, respectively, harboring the *fliC* knock-out construct by biparental conjugation. In total, 371 chloramphenicol resistant transconjugants were selected after conjugation. Only 4% of those were found to represent a double crossover event in the *fliC* gene.

Table 4. Conjugation efficiencies for plasmids pBBR1MCS, pSUB106 and pWEB^{TM} in *M. adhaerens* HP15.

Plasmid	*E. coli* Donor	No. of transconjugants per recipient cell			Recipient to donor ratio	No. of replicates
		24h	48h	72h		
Triparental conjugation						
pBBR1MCS	DH5α	2.0 x 10^{-4}	5.8 x 10^{-5}	5.6 x 10^{-5}	1:3	4
pSUP106	DH5α	2.1 x 10^{-5}	6.1 x 10^{-6}	2.5 x 10^{-5}	1:3	3
Biparental conjugation						
pBBR1MCS	ST18	1.3 x 10^{-3}	2.2 x 10^{-4}	6.4 x 10^{-4}	1:2	2
pSUP106	ST18	2.6 x 10^{-4}	1.3 x 10^{-4}	1.2 x 10^{-5}	1:2	2
Control						
pWeb^{TM}	ST18	0	0	0	1:2	2

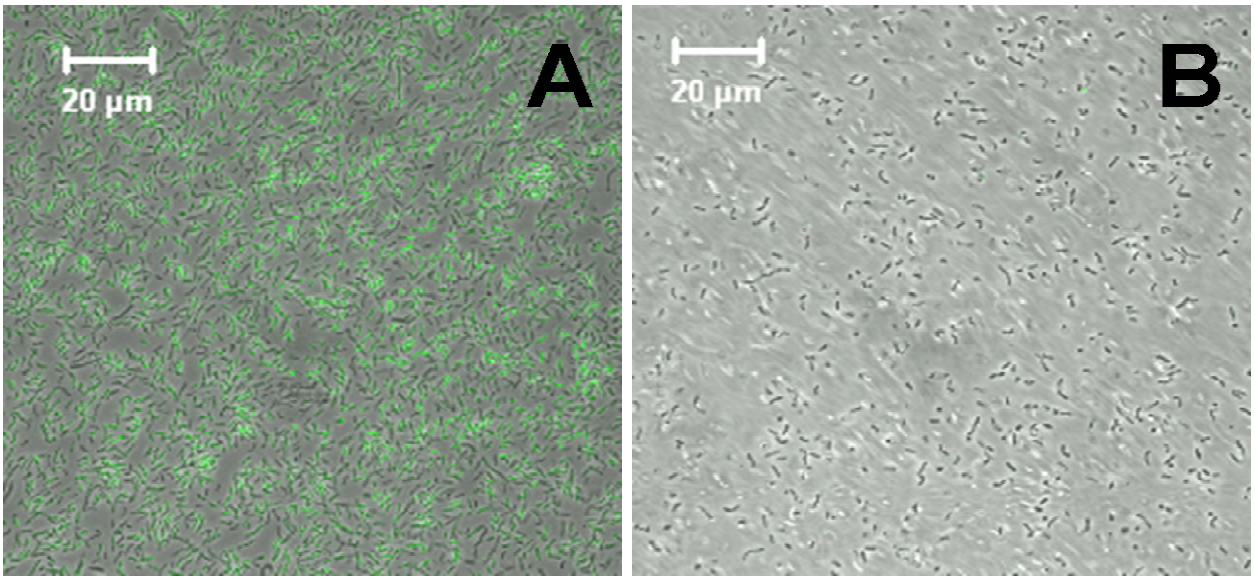

Figure 1. Fluorescence microscopy photographs of *Marinobacter adhaerens* HP15 harboring the reporter gene plasmid pBBR.egfp (A) and plasmid-free wild type control (B) excited at 488 nm.

Using PCR with primers FliCF and FliCR, the expected 1734-bp fragment was amplified for a selected mutant named Δ*fliC* (**Fig. 2**, lane 1). In contrast, PCR with the *M. adhaerens* HP15 wild type using the same primer set yielded an intact *fliC* amplificate of 2487 bp (**Fig. 2**, lanes 2 and 3). These results confirmed a successful site-directed mutagenesis using homologous recombination in *M. adhaerens* HP15.

Phenotypic characterization of M. adhaerens HP15 mutants

In contrast to the HP15 wild type, motility-deficient mutants Δ*fliC* and: *fliG::*Tn*5* were not motile in soft agar demonstrating that genes *fliC* and *fliG* of *M. adhaerens* HP15 were essential for flagellar movement (**Fig. 3**). Furthermore, TEM analysis revealed that HP15 wild type possessed one polar flagellum (**Fig. 4**) while the Δ*fliC* mutant did not produce a visible flagellum but retained the flagellar hook (**Fig. 4A**). However, transposon insertion in the hook-associated *fliG* gene led to a total loss of the flagellum as seen for mutant *fliG*::Tn5 (**Fig. 4B**).

Generation of the genomic cosmid library

Per bacteriophage λ infection round, a total number of ~8,000 cosmid clones could be obtained. With a genome size of *M. adhaerens* HP 15 of ~4.6 MB, this clone number corresponded to a >7-fold genome coverage indicating that the cosmid library may be considered comprehensive. The randomness of the inserts of the genomic cosmid library was assessed by isolating cosmid DNA from 15 randomly picked clones and digestion with restriction enzyme *Eco*RI (**Fig. 5**). The resulting DNA restriction patterns were random thereby confirming that insertion of genomic DNA fragments of strain HP15 was not biased during cosmid library construction. A fragment of ~8 kb found in every restriction treatment corresponded to linearized pWEB™ vector since a pair of *Eco*RI restriction sites flanks its multiple cloning site.

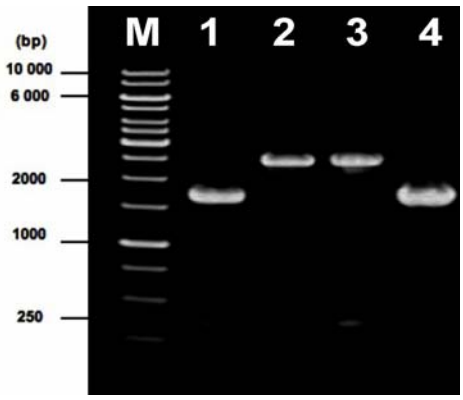

Figure 2. Confirmation of the genotype of *M. adhaerens* HP15 mutant Δ*fliC* using PCR with oligonucleotide primers FliCF and FliCR and the following templates: M, molecular weight marker; 1, colony of mutant Δ*fliC*; 2, genomic DNA of HP15 wild type; 3, colony of HP15 wild type; 4, plasmid pAS7.

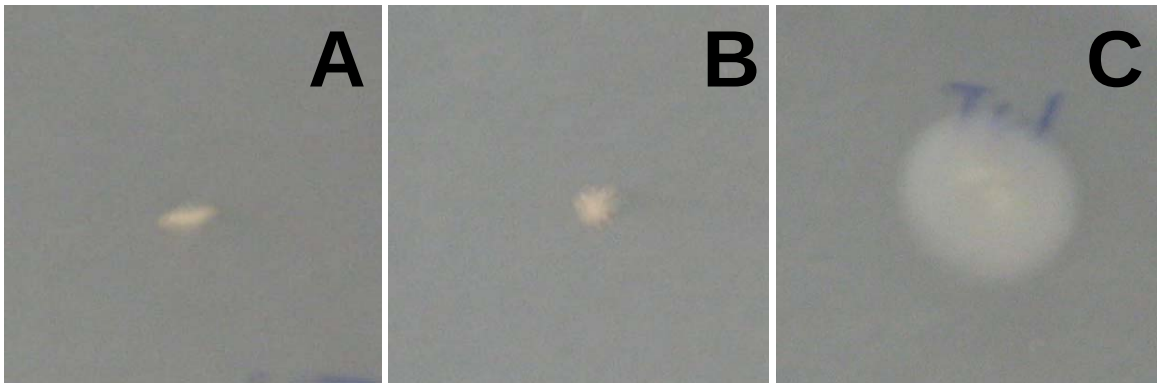

Figure 3. Phenotypic characterization of flagellum-deficient *M. adhaerens* HP15 mutants by 0.3% soft agar assay after 1 day of incubation: A, HP15 mutant Δ*fliC*; B, HP15 mutant *fliG::*Tn5; C, HP15 wild type.

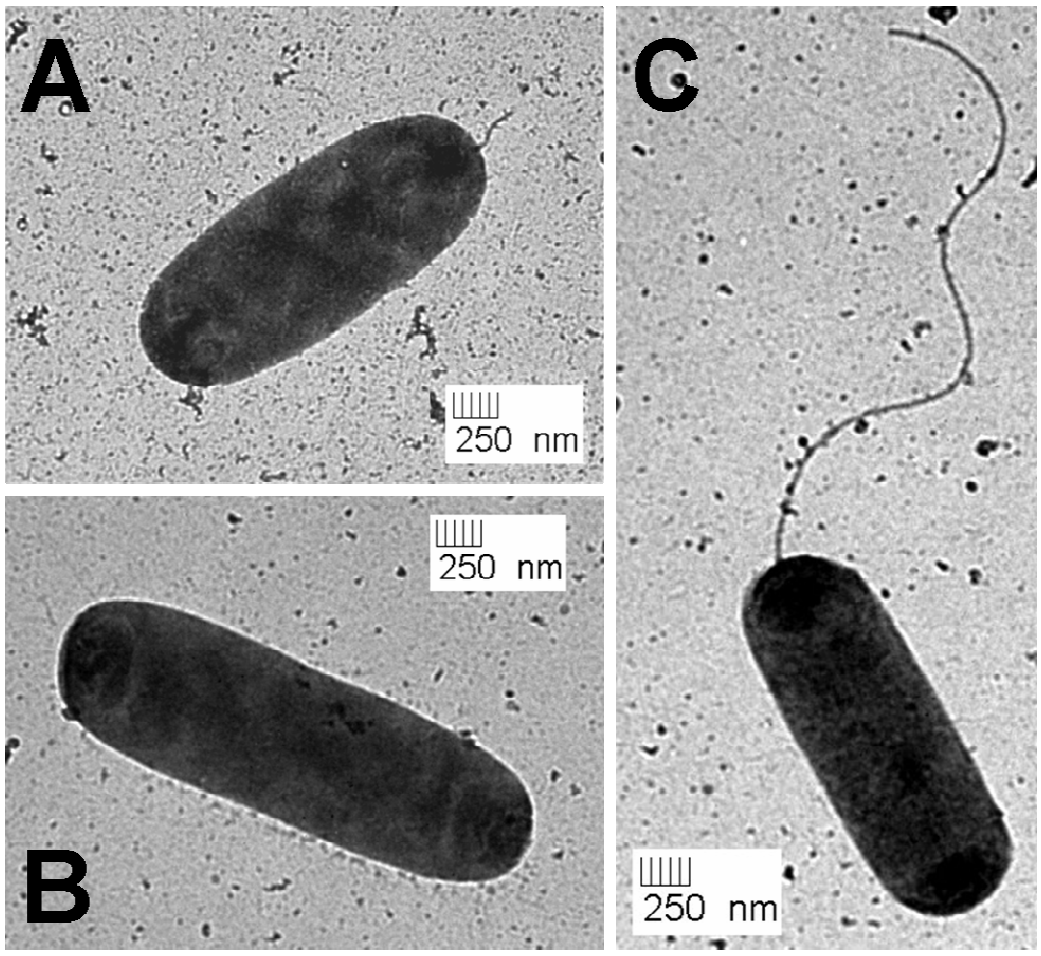

Figure 4. Phenotypic characterization of flagellum-deficient *M. adhaerens* HP15 mutants by transmission electron microscopy: A, HP15 mutant Δ*fliC*; B, HP15 mutant *fliG::*Tn5; C, HP15 wild type.

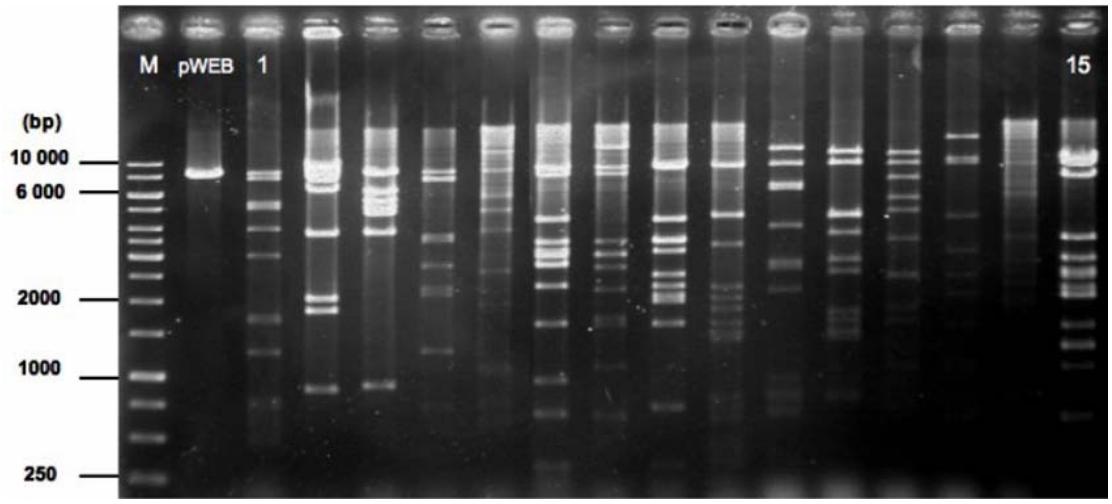

Figure 5. Confirmation of randomness of inserts of the *M. adhaerens* HP15 genomic cosmid library by agarose electrophoresis of restriction treatments: M, molecular weight marker; pWEB, pWEB™ cosmid vector DNA treated with *Eco*RI; Lanes 1-15, randomly picked cosmid clones treated with *Eco*RI.

Discussion

In contrast to well-established bacterial genetic model systems in medical, veterinary, or plant pathology as well as in microbial biotechnology, environmentally important microbes – particularly of marine origin - are often not readily accessible for molecular laboratory work. However, in order to understand the molecular basis of microbial activities in the oceans, genetically accessible model systems are needed. Essential methods to allow molecular analyses of a given bacterium are plasmid transformation techniques, different types of mutagenesis, and reporter gene expression. Herein, transformation of *M. adhaerens* HP15 by electroporation and conjugation, random and site-directed mutagenesis, as well as expression of a reporter gene were reported for the first time as a proof-of-principle. With the established techniques it is now possible to identify genes, gene products, and molecular signals important for the interaction of this bacterium with diatom cells.

The antibiotics susceptibility spectrum of *M. adhaerens* HP15 was determined to allow for selection of transformants or mutants by antibiotics resistance markers. In contrast to ampicillin, several other antibiotics were found suitable as markers for transformation or mutagenesis of *M. adhaerens* HP15. However, susceptibility of HP15 to some antibiotics was rather low, which might be due to the high salt concentration in the medium as concluded for other marine organisms previously (Piekarski et al. 2009). Antibiotics resistances were claimed to be usable taxonomic markers for marine bacteria (Gorshkova and Ivanova 2001). Herein obtained data are comparable to those for *M. aqueolei* (Huu et al. 1999a), but not to those of *M. vinifirmus* and *M. alkaliphilus* (Liebgott et al. 2006; Takai et al. 2005) and thus did not result in a clear genus-specific pattern.

Recombinant plasmids of different incompatibility groups were tested for replication in *M. adhaerens* HP15. Interestingly, transformation with plasmids of the incompatibility group IncQ was successful whereas plasmids of incompatibility groups IncP, IncX, colE1, or pMB1 did not replicate or could not be introduced to *M. adhaerens* HP15. It remains to be analyzed whether the two native plasmids of HP15 with molecular sizes of 42 and 187 kb, respectively, interfere with replication of the latter plasmid groups.

For transforming *M. adhaerens* HP15, preparation of competent cells, amount of plasmid DNA, and incubation period following electroporation were optimized. For successful electro-transformation of *M. adhaerens* HP15 a long recovery period post electroporation was needed. This might be due to a prolonged time needed for adequate expression of the antibiotic-resistance gene as described by others (Bakermans et al. 2009). The herein obtained electroporation efficiency was comparable to that of the marine *Gammaproteobacterium*

Pseudoalteromonas (Kurusu et al. 2001) but was lower than that described for *Alteromonas* (Kato et al. 1998). Upon optimization of the mating period, the plasmid conjugation efficiency for HP15 was found to be similar to those of other marine γ-proteobacteria (Dahlberg et al. 1998) or α-proteobacteria of the *Roseobacter* clade (Piekarski et al. 2009).

Since random transposon insertion in bacterial genomes is a simple method to produce diverse mutant libraries, the transposon delivery plasmid pRL27 was used to generate random mutants of *M*. *adhaerens* HP15. The efficiency of mutagenesis was lower than that of the close relative *Pseudomonas stutzeri* (Larsen et al. 2002). However, it was sufficient to readily generate a library of ~2000 mutants. For homologous recombination, derivatives of the mobilizable vectors pEX18Ap and pK19mobsacB were used due to their inability to replicate in non-enterobacterial species (Hoang et al. 1998b; Schäfer et al. 1994). As expected, conjugation of these vectors into HP15 did not yield antibiotics-resistant transformants indicating that they could be used as suicide vectors for delivery of mutagenic DNA fragments to the HP15 chromosome.

To ensure randomness of transposon insertions and to demonstrate the ability to knock-out specific genes, motility of obtained mutants was screened. The flagella-deficient transposon mutants *fliG::*Tn*5*, *fliR::*Tn*5*, as well as the site-directed mutant Δ*fliC* were unable to swim in soft agar. As expected, in mutants *fliG::*Tn*5* and *fliR::*Tn*5* the flagellum was not formed at all since these genes are required for the hook formation as described earlier in *Salmonella enterica* by Thomas (2001). In contrast, the Δ*fliC* mutant exhibited the flagellar hook but was missing the flagellar filament confirming previous data obtained for *Heliobacter pylori* and other bacteria (Macnab 2003; Seong Kim et al. 1999). These results demonstrated that the *fliC* gene encodes for the flagellar filament of *M. adhaerens* HP15. Furthermore, the mutational analysis experimentally proofed that motility-associated genetic traits of *M. adhaerens* HP15 are comparable to those of e.g. *Pseudomonas aeruginosa* (Lillehoj et al. 2002; Stover et al. 2000) and thus confirmed our genome sequence data analysis (Gärdes et al. 2010b). The flagella-deficient mutants will next be tested during the interaction with diatoms to study the role of bacterial motility in chemotaxis and attachment.

The reporter gene *egfp* was introduced *in trans* to *M. adhaerens* HP15 and showed a clear phenotypic expression. Thus, *egfp* can be used for *in vivo* fluorescent labeling of *M. adhaerens* HP15 in future studies. The obtained genomic cosmid library of *M. adhaerens* HP15 was demonstrated to be comprehensive and adds as a powerful tool for genome-wide analysis.

In summary, this study established an easy-to-work-with and powerful genetic system for *M. adhaerens* HP15 making this bacterium a suitable model organism for molecular analysis of diatom-bacteria interactions.

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4 Summary and Concluding Remarks

Analyzing the role of algae-associated bacteria in marine aggregate formation is of major interest to better understand the regulatory mechanisms of downward carbon flux in the ocean as well as the detailed processes of the biological pump. As shown earlier, presence of particular bacterial strains can be of significance for phytoplankton aggregation (Grossart et al. 2006a). The main goal of this work was to establish and analyze a bilateral model system for bacteria-diatom interactions.

During this thesis work, a novel model system was developed, which enable us to investigate bacteria-algae interactions, bacterial adhesion, and the contribution of bacteria to TEP and aggregate formation. It is serving and will continue to serve for an in-depth molecular-ecological analysis. Screening of a total of 82 selected, particle-associated bacterial isolates and using a newly developed quantitative attachment assay revealed distinct *T. weissflogii*-attaching bacterial strains. Four attaching bacterial isolates including *M. adhaerens* sp. nov. HP15 were selected for further studies. The attachment of those bacterial strains to diatom cells was verified using scanning electron microscopy to further demonstrate that the attachment assay is a suitable tool to distinguish between attaching and non-attaching bacterial strains.

4.1 The role of *T. weissflogii*-attaching bacteria in aggregate formation

The significance of *M. adhaerens* sp. nov. HP15 and three other diatom-attaching bacterial strains for marine aggregate formation was determined in rolling tank experiments. With these, the formation of TEP, the synthesis of polymeric adhesive properties, and diatom aggregate dynamics were investigated in depth. Interestingly, under defined *in vitro* conditions, this study demonstrated for the first time that bacterial strains attaching to *T. weissflogii* as well as metabolically active *T. weissflogii* cells were both simultaneously required for marine aggregate formation and TEP production (Gärdes et al. submitted-a). In previous studies *T. weissflogii* was shown to be a low exudate species with low stickiness and absence of sticky TEP (Crocker and Passow 1995). Therefore, we

choose *T. weissflogii* as the test organism to avoid non-bacterial influenced aggregation (Grossart et al. 2006b).

Significant differences in aggregation were observed between axenic *T. weissflogii* cells and those incubated with bacteria, which points to the importance of attaching-bacteria for *T. weissflogii* aggregation. Presence of attaching bacteria increased TEP concentrations. Additionally both, bacterial and TEP abundance contributed to total abundance of particles. Thus, the frequency of colliding particles increased and aggregate formation was enhanced. It is known that the presence of TEP is one pre-requisite for the formation of aggregates through coagulation (Logan et al. 1995). Herein, it could be demonstrated that TEP concentration and aggregate abundance correlated significantly for *T. weissflogii,* and that both were depended on diatom metabolism and bacterial activities alike.

Use of another marine bacterium, which did not attach to *T. weissflogii* in attachment assays, resulted in no aggregate formation in rolling tank experiments and was thus comparable to axenic controls. These results showed clearly that there seems to be specificity in this interaction and that not any kind of marine bacterium may induce *T. weissflogii* aggregation.

In contrast to earlier studies (Grossart et al., 2006a; Grossart and Simon, 2007), we have comparatively analyzed attaching and non-attaching bacterial isolates with respect to their interactions with metabolically active and inactive diatom cells. Although metabolically inactive diatoms were colonized by bacteria, they did not form aggregates after elongated periods of incubation. Experiments with metabolically active diatoms as compared to metabolically inactivated, yet intact, *T. weissflogii* cells suggested that synthesis and the active release of polymers as well as a yet-to-be analyzed response towards the attaching-bacteria were important for *T. weissflogii* aggregation. In line with this, lower TEP concentrations were measured for metabolically inactive diatoms than for active diatoms. Both results suggested that inactive diatoms did not exude biopolymers, which act as precursors for TEP formation. Likewise, presence of bacteria could not enhance aggregation of metabolically inactive diatoms.

Others had previously shown that certain carbohydrates of the diatom surface alter its stickiness and that presence of such carbohydrates is dictated by the diatom cell's

physiology (Tien et al. 2005; Waite et al. 1995). We can assume that changes in diatom exudates, which remain attached to the surface as surface active sugars, are affected by the herein applied metabolism inactivation procedure, thus changing the sticking efficiency of diatom cells. Jackson (1990) described that the sticking efficiency of colliding particles is a significant parameter in aggregation.

Addition of abiotic particles such as glass beads or metabolically inactive diatoms did not increase aggregate abundance. These important controls highlighted that an appropriate binding between TEP, EPS, and the surface of colliding diatom particles seems essential for formation of larger aggregates. Since aggregate formation depends on the abundance and size of TEP as well as particle stickiness, cell morphology, and other surface properties, stimulation of TEP formation by attaching bacteria seemed crucial. We hypothesize that bacterial species, which attach to metabolically active *T. weissflogii* cells, might interact with those and that the potential yet-to-be determined interaction could be instrumental for TEP and aggregation dynamics. It is tempting to speculate that TEP are formed as a result of the cell-to-cell interaction between diatoms and bacteria.

It could be concluded that the interaction of *M. adhaerens* sp. nov. HP15 with *T. weissflogii* may be used as a model system to study micro-scale interactions, the impact of bacteria on diatom TEP formation, and aggregation of dissolved and particulate organic matter. This system will contribute to future aggregation models, which may involve different phytoplankton morphologies, TEP-particle interactions, and the dispersion of TEP. None of the current theories of TEP dynamics have sufficiently considered the species-specific interactions between bacteria and phytoplankton (Grossart et al. 2005; Passow 2000) and the thereof resulting effects on production and characteristics of TEP. A direct extrapolation to *in situ* conditions might still be difficult. *In situ* bacteria-diatom interactions might be much more complex as they also include reactions among different bacterial species (Long and Azam 2001) as well as temporal changes in composition and activities of bacterial and phytoplankton communities (Riemann et al. 2000).

For the first time, a bilateral model system relevant for marine snow formation can now be intensively and mechanistically studied. These future studies will be integrated into the overall understanding of the micro-scale scope of bacterial interactions with diatom

surfaces and should enhance our ability to predict the timing of diatom bloom termination and the regulation of carbon export in an ecosystem context.

4.2 Interactions of *M. adhaerens* sp. nov. HP15 and *T. weissflogii* depend on nutrient stoichiometry - Indications for the type of interaction

Even though phytoplankton and bacteria are key players in marine energy fluxes and nutrient cycles, our understanding of phytoplankton–bacteria interactions is still very limited. In natural environments phytoplankton-bacteria interactions can be multifaceted with high variations in time and space depending on environmental conditions (Grossart 1999). In order to evaluate the physiological response of *T. weissflogii* to different nutrient conditions and the concurrent interaction with *M. adhaerens* sp. nov HP15, another study of this thesis analyzed the quality and quantity of *T. weissflogii* exudates.

The *in vitro* study included *M. adhaerens* sp. nov. HP15 and *T. weissflogii* incubated under explicitly defined environmental conditions. Differences in exopolymer production may greatly depend on nutrient stoichiometry. Production of dissolved carbohydrates by marine phytoplankton is known to be highly variable depending on the species, its growth stage, and the environmental conditions (Myklestad 1974). Phosphorus limitation (Guerrini et al. 1998; Urbani et al. 2005) and – for some species – nitrogen limitation can result in an increase of photosynthesis-driven extracellular release of carbohydrates by cultured diatoms (Magaletti et al. 2004; Obernosterer and Herndl 1995). The amount, composition, and characteristics of released carbohydrates is of major interest since they might serve as precursor material for TEP formation (Passow 2000). These released carbohydrates effect the formation of particles via self-coagulation of organic matter (Chin et al. 1998) and phytoplankton aggregation (Passow 2000; Passow et al. 1994).

The herein conducted experiments showed that *T. weissflogii* produced the organic carbon source for growth of *M. adhaerens* sp. nov HP15, which in turn supported diatom growth. Apparently the bacteria released substances that enhanced *T. weissflogii* growth, or alternatively they consumed substances which might otherwise inhibit diatom growth (Gärdes et al. to be submitted-b). In order to distinguish between those two possibilities,

future experiments should be carried out with *T. weissflogii* growing in filter-sterilized culture medium, in which *M. adhaerens* sp. nov HP15 had grown before. This would possibly result in enhanced diatom growth by released bacterial substances. If not, bacterial cells might consume growth-inhibitory substances accumulated by diatom growth.

Under nutrient-deficient conditions diatom growth was low irrespective of the presence of bacteria. This result is in line with the so-called 'cluster hypothesis' (Azam and Ammerman 1984), which suggested that bacterial mineralization of N- and P-rich compounds of algal exudates supplies algae with ammonium and phosphorus and hence sustains algal growth. Bacteria become re-mineralizers at high nutrient levels but they efficiently compete with phytoplankton at low nutrient levels (Rothhaupt and Güde 1992a).

When grown at high nutrient levels, strong effects of *M. adhaerens* sp. nov HP15 on dynamics of DOC, amino acid production, and polymer formation were observed. With changing nutrient stoichiometry the amount of DOC and polymer formation was not influenced by *M. adhaerens* sp. nov HP15. It is speculated that the stimulation of release of organic matter by *T. weissflogii* under nutrient stress conditions might be to attract re-mineralizers. Simultaneously a stimulation of polymer formation and a subsequent utilization of those exudates by bacteria might take place. A careful interpretation of our results suggested a close interaction of *M. adhaerens* sp. nov HP15 with the diatom *T. weissflogii* in a mutualistic relationship, where both organisms benefit from each other when sufficient nutrients are available. But the type of interaction may be shifted to commensalism under nutrient stress, when bacteria may utilize algal exudates and benefit from algae without affecting algal growth (Fig. 10).

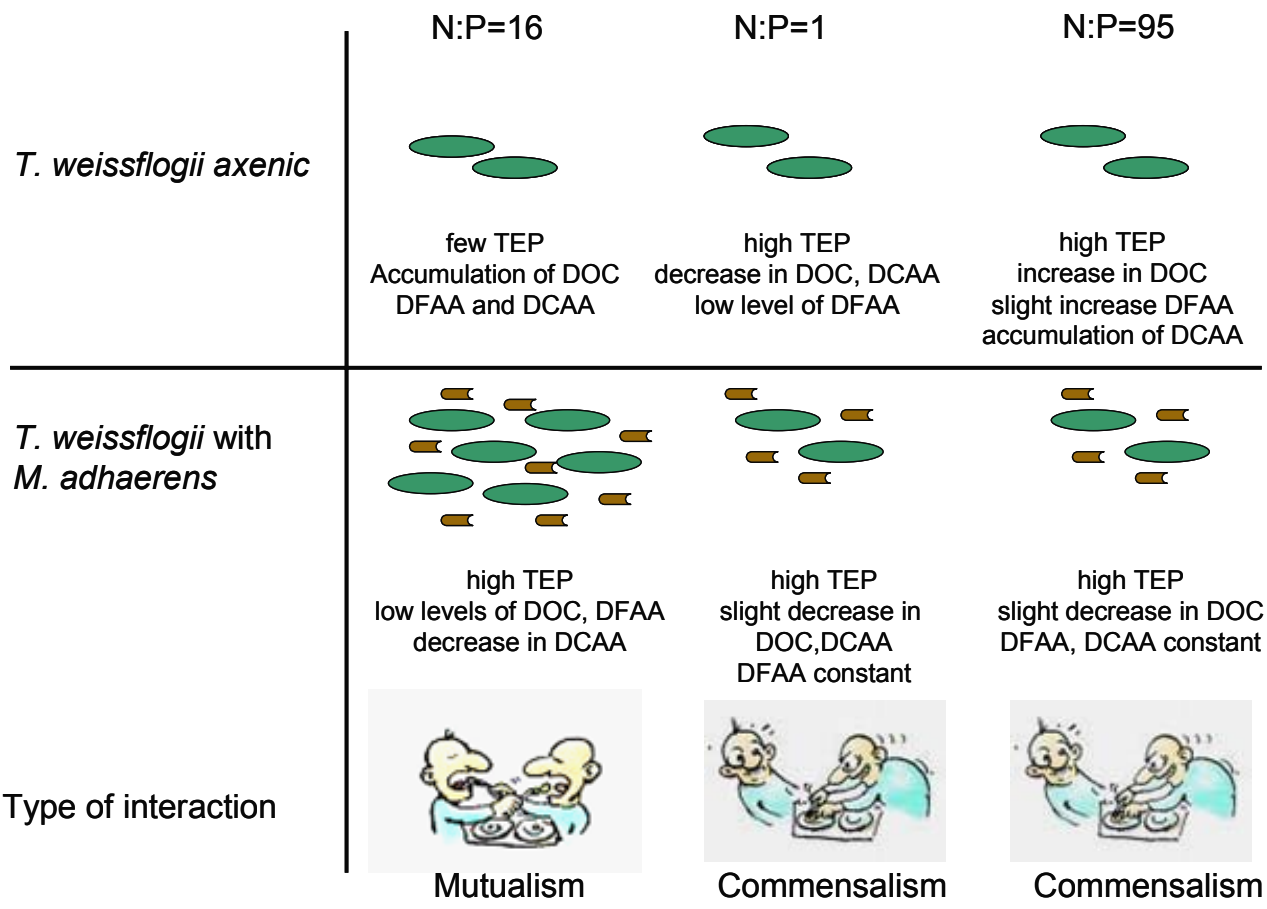

Figure 10: Schematic summary of major differences between the various N:P stochiometry conditions used. Axenic *T. weissflogii* cultures were inoculated with *M. adhaerens* HP15 grown at different nutrient stochiometry (N: P=16; N:P=1 and N: P=95). diatoms; bacteria.

The secreted polymers might change TEP quantity and quality when *M. adhaerens* sp. nov HP15 is present. The results furthermore confirmed our earlier findings of significantly higher TEP build-up and aggregate formation after five days of incubation of *T. weissflogii* with *M. adhaerens* sp. nov HP15 in rolling tank experiments. Thus, we speculated that the additional modified and/or secreted EPS play a major role in TEP formation, stickiness, and therefore aggregate formation. One future goal is to visualize and biochemically characterize the micro-scale architecture of the produced

polysaccharide matrix. Confocal laser scanning microscopy might be useful in this context since gels can be viewed in tree-dimensional space, where the physical relationship of bacteria and exopolymers may possibly be distinguished.

4.3 Chemotaxonomic and genomic characterization of *Marinobacter adhaerens sp. nov.* as the bacterial counterpart of the bilateral model system.

The chemotaxonomic and genomic characterization of *M. adhaerens* sp. nov HP15 as the bacterial counterpart of the established model system was a perquisite to study this organism in depth. We determined the taxonomic position by analyzing HP15's phenotypic properties and genotypic relatedness.

Chemotaxonomic properties and phylogenetic analysis suggested, that *M. adhaerens* strain HP15 (=DSM XXX^T = CIP XXX^T ; pending) represent a novel species. According to 16S rRNA gene sequence analysis in the Ribosomal Database Project (Cole et al. 2005) the closest relative of *M. adhaerens* sp. nov. HP15 (GenBank accession no. AY241552) is *M. flavimaris* strain SW-145 (GenBank accession no. AY517632) isolated from the Yellow Sea offshore of Korea (Yoon et al. 2004a) with a similarity score of 0.99. The phylogenetic position of *M. adhaerens* sp. nov HP15 based on its 16S rRNA sequence was analyzed using the ARB software package (Ludwig et al. 2004) and the reference alignment was provided by the Living Tree Project database (Yarza et al. 2008). The whole-genome relatedness of strain HP15 to *M. flavimaris* was determined as 64 % by DNA-DNA hybridization.

Stackebrandt & Goebel (1994) found that strains that are more than 3% divergent in 16S rRNA are almost always members of different species, whereas strains that are less than 3% divergent may or may not be members of the same species. A cut-off of 3% divergence was recommended as a conservative criterion for demarcating species. Therefore, a whole-genome relatedness of *M. adhaerens* sp. nov HP15 and *M. flavimaris* was conducted herein. Johnson (1973) determined that strains from the same species, as defined by phenotypic clustering, almost always shared 70% or more of their genomes and that strains from different species almost always shared less than 70%. A 70% cut-off level

of homology over the entire genome was thus adopted as a standard for determining whether two strains should be considered different species (Wayne et al. 1987b). HP15^T additionally differed from *M. flavimaris* and the other *Marinobacter* species in a number of chemotaxonomic properties such as utilization of glycerol, fructose, lactic acid, gluconate, alanine, and glutamate, thus confirming *M. adhaerens* sp. nov HP15 being a novel species.

The genus *Marinobacter* was established with the species *Marinobacter hydrocarbonoclasticus* in 1992 (Gauthier et al. 1992b). A total of 26 further species have been described until today. These species are tolerant to various conditions as they were isolated from diverse locations - from the sediment (Gorshkova et al. 2003), the water column (Yoon et al. 2004b), from coastal (Roh et al. 2008) and deep sea waters (Takai et al. 2005), from the Antarctic (Montes et al. 2008b) and from the Red Sea (Antunes et al. 2007). Representatives of this genus were isolated from oil-contaminated areas (Huu et al. 1999a), hot springs (Shieh et al. 2003), and salines (Martin et al. 2003). Interestingly, the bacterial flora of phytoplankton, such as *Gymnodinium catenatum,* was found to have high numbers of *Marinobacter* species (Green et al. 2004). This led to the hypothesis that *Marinobacter* species might have evolved selective mechanisms and adaptations to utilize algal extracellular products. Not surprisingly, the *in situ* abundance of *Marinobacter* species in seawater often correlated with phytoplankton blooms (Green et al. 2004). Two species were previously identified based on their interactions with other organisms - *M. algicola* isolated from dinoflagellate cultures (Green et al. 2006) and *M. bryozoorum* derived from Bryozoa (Romanenko et al. 2005).

In addition to the content of this thesis, preliminary metagenome studies using the ‘CAMERA’, ‘MGRast’, and Megx (marine ecological genomics) databases were conducted for the *Marinobacter* sp. HP15 16S rRNA sequence (Data not shown). Theses studies revealed several reliable hits along the JCVI Global Ocean Survey sampling sites and those of the Monterey Bay metagenome studies. A future analysis whether or not *Marinobacter* abundance correlates with phytoplankton blooms will give further hinds to the ecological relevance of this bacterial genus in phytoplankton dynamics.

4.4 Diatom-bacteria interactions at the micro-scale level

Relevant cross-feeding or chemical interactions between phytoplankton and diatoms can happen only in close distance and various adaptations allow bacteria to attach to phytoplankton (Baker and Herson 1978; Bell and Mitchell 1972b; Cole 1982; Decho 1990a; Grossart et al. 2006a; Grossart and Simon 1998; Kiorboe et al. 2002). Chemotaxis-driven movement towards and attachment to the algal cell appear to be essential first steps for any such interaction (Grossart et al. 2001). Adhesion to living cell surfaces is often accomplished through specific molecular docking mechanisms, which involve lectins, various types of ligands, or adhesive proteins (Donlan 2002). The initial attachment of bacteria to the surfaces has been demonstrated to be flagella-mediated (Carpentier and Cerf 1993). In this context, molecular studies with *Pseudomonas aeruginosa* demonstrated that flagella-defective mutants did not adhere well to surfaces (O' Toole and Kolter 1998) and were impaired in biofilm formation (Ramsey and Wozniak 2005). In order to get further insights in bacterial adaptations and strategies for diatom-bacteria interactions at the molecular level, especially the role of flagella in attachment to diatom cell surfaces, the identification and characterization of flagella biosynthetic genes in the herein used model organism, *M. adhaerens sp. nov*. HP15, was required.

Since *M. adhaerens* sp. nov HP15 had been determined as a suitable model organism for diatom-bacteria interaction studies in the course of this thesis, the genome sequence of this organism was determined and fully annotated (Gärdes et al. submitted-b). Genome analysis revealed that *M. adhaerens* sp. nov HP15 possesses three replicons; the chromosome comprises 4,422,725 bp and codes for 4,180 protein-coding genes, 46 tRNAs and three rRNA operons, while the two circular plasmids are ~187 kb and ~42 kb in size and contain 178 and 52 protein-coding genes, respectively.

Furthermore, extensive molecular investigations were done regarding the genetic accessibility of this strain (Gärdes et al. to be submitted-a). The bacterial organism was successfully tested in terms of transformation efficiencies with various plasmids using both, conjugative transfer and electroporation. Both, random transposon mutagenesis and site-directed mutagenesis were conducted to generate mutants deficient in flagellum synthesis.

This was mainly done to provide a proof-of-principle for successful genetic accessibility and to further test the potential involvement of bacterial motility in attachment to the diatom cell surface.

An *in silico* analysis of the *M. adhaerens* sp. nov HP15 genome and careful comparative blast searches revealed three major flagella gene clusters encoding for the basal body, the filament, the hook, and the motor switch complex (Fig. 11).

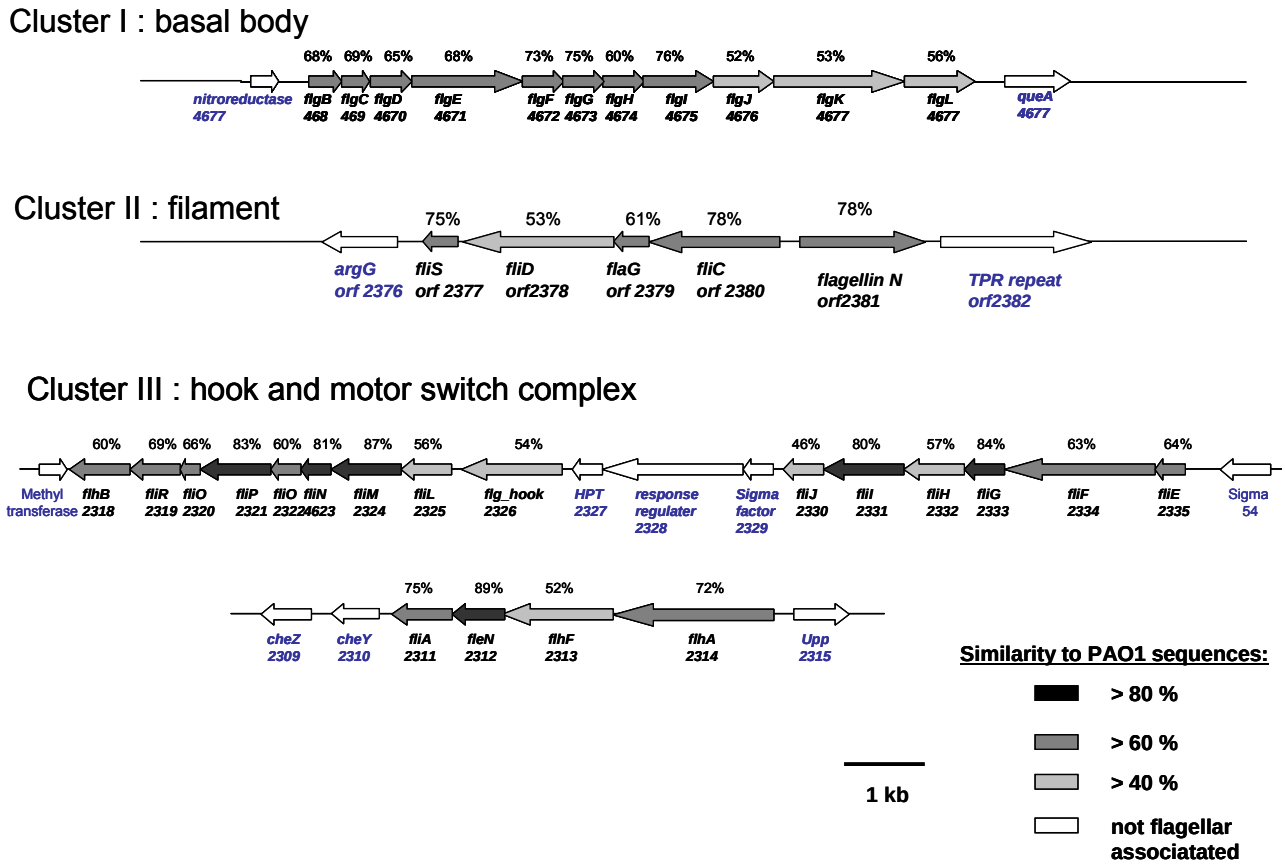

Figure 11: Flagella biosynthetic gene clusters identified in *the M. adhaerens* HP15 genome and protein sequence similarities to *Pseudomonas aeruginosa* flagella biosynthetic gene products.

Two major proteins form the flagellar filament and are encoded by the genes, *fliC* and *flagellinN*. FliC is the dominant subtype and FlagellinN the minor subtype, both have similar molecular mass (~50 kDa) and share considerable amino acid homology (68 %) in *M. adhaerens* sp. nov HP15 as well as in other bacterial species (Scharf et al. 2001; Schultheiss et al. 2004; Seong Kim et al. 1999; Stover et al. 2000). However, the respective genes are transcriptionally unlinked in *M. adhaerens* sp. nov HP15 and in other organisms (Jarrell 2009; Lillehoj et al. 2002; Scharf et al. 2001). Studies with *Heliobacter pylori* isogenic mutants of the respective genes, revealed that both genes are necessary for full motility and necessary for initial attachment and biofilm formation (Pratt 1998). Futhermore, in *P. aeruginosa* both flagellin genes (type a and type b, respectively) were shown to be responsible for its binding to Muc1 mucin on the epithelial cell surface (Lillehoj et al. 2002).

Consequently, a site-directed deletion mutation of *fliC* in HP15 was generated using homologous recombination resulting in a clearly flagellum-negative phenotype of the respective mutant as observed by transmission electron microscopy and swarming assays (Gärdes et al. to be submitted-a). As expected, a truncated flagellum was observed for the mutant with the flagellar hook and basal body being intact. This is in line with studies of Seong Kim et al. (1999), where mutants in *flaA* (the homolog of *M. adhaerens* sp. nov HP15 *fliC* gene) produced truncated flagellar. Thus, these mutants were impaired in motility and the ability to colonize the gastric mucosae of mice. In contrast, transposon insertion mutagenesis of the *M. adhaerens* sp. nov HP15 *fliG* gene, which is located in the hock and motor switch cluster, led to the total loss of the flagellum without the arrangement of the hook. A similar phenotype was previously described for *Salmonella enterica* by Thomas (2001) and thus confirmed the herein proposed gene cluster organization in *M. adhaerens* sp. nov HP15.

Our results confirmed the suitability of *M. adhaerens* sp. nov HP15 as a genetically accessible model organism, and proved the ability to create mutants, which will be used for gene characterization and functional studies of single genes. The obtained *fliC* mutant of HP15 will now be characterized in terms of attachment to the diatom cell surface using the previously developed attachment assay and rolling tank experiments. These future experiments will reveal any involvement and/or importance of flagella in the initial steps of

diatom-bacteria interactions. Gene *fliC* is the first gene identified and phenotypically characterized in *M. adhaerens* sp. nov HP15.

Finally, it should be noted that the flagellum of *M. adhaerens* sp. nov HP15 might be only one of several potential cellular factors involved in diatom-bacteria interactions. Other genetic loci responsible for e.g EPS production, biofilm formation, or adhesion such as pilus biosynthetic genes or chemotaxis as well as quorum sensing genes should be tested in future studies. Besides the generation of site-directed mutants for known genes, random transposon mutagenesis and the so-called *In vivo* expression technology (IVET) should be used in the future (Choi and Schweizer 2005a; Lee and Cooksey 2000; Silby et al. 2004). IVET is a strategy for selecting bacterial genes whose expression is specifically induced *in vivo*, i.e. in close proximity or during attachment to algal cells. The *in situ*-induced genes can be identified by the ability of their promoters to express a promoterless selection marker gene that is essential for survival. In order to distinguish this expression from constitutive gene transcription, a second non-essential promoterless reporter gene needs to be cloned directly downsteam of the essential reporter gene. Thus, constitutively active promoters will be excluded by counter selection for inactivity of this second reporter enzyme *in vitro*. Ultimately expression profiles of *M. adhaerens* sp. nov HP15 and *T. weissflogii* using whole genome micro-arrays or by the faster EST 454 sequencing method (Shendure and Ji 2008) as screening tools for model organisms such as *M. adhaerens* sp. nov HP15 and *T. weissflogii* may reveal important genes expressed during interaction.

5 Future Scope

The establishment and identification of a genetically accessible model system for molecular in-depth studies on diatom-bacteria interactions gave interesting new insights on how it is mediated, which type of interaction it may be, and how it impacts phytoplankton aggregation and thus carbon sequestration. Future studies and fine-tuned molecular analyses of *M. adhaerens* sp. nov HP15 genes encoding for polysaccharide synthesis and exo-enzymes degrading diatom-borne polysaccharides should be the next major steps to determine bacterial key enzymes important for the formation, stability, and stickiness of TEP and its potential role in phytoplankton aggregation and marine snow formation.

As TEP stickiness plays a major role in aggregation dynamics, the bacterial activities involved should be thoroughly studied. First, the identification and subsequent site-directed mutagenesis of EPS-encoding genes in *M. adhaerens* sp. nov HP15 should be conducted in order to reveal their contribution for the formation of TEP. Next, exo-enzyme activities of *M. adhaerens* sp. nov HP15 should be investigated to analyze potential modifications of polymeric substances released by *T. weissflogii* and the impact of those enzymes on TEP formation and TEP stickiness.

Additionally, the ecological significance of *Marinobacter* species should be addressed by determining the *in situ* abundance and natural relevance of *Marinobacter* species in marine ecosystems and processes relevant during diatom blooms. Such future experiments may verify the relevance of the herein described and analyzed model system in nature. Simultaneously, future studies with *M. adhaerens* sp. nov HP15 and *T. weissflogii* might test the hypothesis that *Marinobacter* species might have evolved selective mechanisms and adaptations to utilize algal extracellular products and therefore might specifically interact with diatoms as hosts. The combination of *in vitro* and *in situ* studies should be integrated into the overall understanding of the micro-scale scope of bacterial interactions with diatom surfaces. Ultimately, this should enhance our ability to predict the timing of diatom bloom termination and the regulation of carbon export in an ecosystem context.

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