Identification of bacterial genes required for diatom–bacteria interactions

by

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SUMMARY

Aggregate formation in form of marine snow is an essential mechanism in the oceans that mediates the sinking of organic carbon to depth. Interactions between bacteria and diatom play an important role during this process by inducing secretion of different polymers, which increase the size of marine aggregates. Not much is known about the molecular mechanisms responsible for the diatom-bacteria interaction. To address this issue, a bilateral model system consisting of *Marinobacter adhaerens* HP15 and the diatom *Thalassiosira weissflogii* has been established. This bacterium specifically attaches to *T. weissflogii* cells thereby increasing its aggregation and inducing an increased formation of transparent exopolymeric particles. A genetic tool system was developed for *M. adhaerens* HP15, in which successful expression of reporter genes revealed useful tools for gene expression analyses. In addition, several bacterial genes potentially important during the interaction have been investigated. However, genes specifically expressed *in vivo* are still unknown.

In this work we identified bacterial genes that are induced during the interaction with *T. weissflogii* by two different approaches. First, an *In vivo* expression technology (IVET) screening was conducted, constructing a promoter-trap vector containing a fusion between a promoterless selection marker gene and a reporter gene. Generation of a library of plasmids carrying genomic fragments upstream of the fusion and its subsequent transformation into a selection marker mutant allowed the selection of bacterial promoters specifically expressed during the interactions with *T. weissflogii*. Second, bacterial proteins expressed in response to the presence of *T. weissflogii* were identified by comparison of protein profiles of bacterial cultures incubated with or without diatom cells and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). By these approaches several genes expressed during the co-cultivation with diatoms were identified. Sequence analyses of these genes showed that they are required for central intracellular metabolism, cell envelope structure, nutrient scavenging, regulation, chemotaxis, secretion, stress response and DNA

transfer. These genes may play an important role during the interaction between *M. adhaerens* HP15 and *T. weissflogii*. The results obtained in this study contribute to a better understanding of the molecular and biochemical mechanisms responsible for diatom-bacteria interactions.

Additionally, the tight adherence (*tad*) gene locus present on the 187-kb HP15 plasmid and consisting of 9 genes (*flp*, *rcpCA*, *tadZABCDG*), was analyzed. This locus is found in several Gram-negative bacteria encoding for a type IVb fimbrial low-molecular-weight (Flp) pilus, which plays a role in adherence and biofilm formation. The identification of a constitutively active promoter upstream the *flp* gene of *M. adhaerens* HP15 suggested the *tad* locus is transcribed. A *flp-rcpCA* deletion mutant was generated and analyzed in terms of its motility and attachment to abiotic and biotic surfaces. Under the experimental conditions tested, the mutant did neither show a phenotype in terms of surface attachment nor motility. However, the preliminary results of the current study encourage an in-depth analysis of the role of the *tad* locus in *M. adhaerens* HP15.

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LIST OF ABBREVIATIONS

2-DE	Two-dimensional protein electrophoresis
ABC	ATP-binding cassette
ACN	Acetonitrile
ALA	5-aminolevulinic acid
Amp	Ampicillin
ATP	Adenosine-5'-triphosphate
CE4	Carbohydrate Esterase family 4
CFU	Colony Forming Unit
Cm	Chloramphenicol
Cm^R	Chloramphenicol resistance
CO_2	Carbon dioxide
DHDPS	Dihydropicolinate synthase
DIC	Dissolved Inorganic Carbon
DNA	Deoxyribonucleic acid
DOC	Particulate Organic Matter
DOM	Dissolved Organic Matter
EDTA	Ethylenediamine Tetra-acetic acid
EPA	Eicosapentaenoic acid
EPS	Extracellular Polymeric Substances
ETF	Electron Transfer Flavoprotein
f/2 GLUT	f/2 medium supplemented with 5 g l^{-1} glutamate
Flp	Fimbrial low-molecular-weight
HCCA	α-cyano-4-hydroxycinnamic acid
HTA	Hexadecatrienoic acid
IVET	*in vivo* Expression Technology
Km	Kanamycin
LB	Luria-Bertani

MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MB	Marine broth
MIC	Minimal inhibitory concentrations
MMSDH	Methylmalonate-semialdehyde dehydrogenase
MS	Mass Spectrometry
MSHA	Mannose-sensitive haemagglutinin
NCBI	National Center of Biotechnology Information
NGS	Next-Generation Sequencing
OD	Optical density
ON	Over night
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
POM	Particulate Organic Matter
PPP	Prokaryotic Promoter Prediction
PTS	Phosphoenolpyruvate:carbohydrate phosphotransferase system
PUA	Polyunsaturated aldehydes
RDOM	Recalcitrant Dissolved Organic Matter
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RT	Room temperature
RT-qPCR	Reverse-transcription quantitative real-time PCR
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulfate
T2SS	Type II secretion system
TEP	Transparent Exopolymeric Particles
Tet	Tetracycline
TFA	Trifluoracetic acid
TRAP	Tripartite ATP-independent periplasmic
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. INTRODUCTION

1.1. The biological Pump

Through the process of the biological pump, which describes the fate of carbon in the ocean, atmospheric CO_2 concentration is lowered by the sinking of organic carbon from the surface water to the deep ocean (De la Rocha 2003), which then is important for climate changes (Jahnke 1996, Raven & Falkowski 1999, Riebesell et al. 2007). Photosynthesis decreases the concentration of dissolved CO_2 in surface waters and enables more CO_2 to be absorbed from the atmosphere (De la Rocha 2003). In addition, the biological pump impacts the cycling of other elements such as nitrogen, phosphorous, or silicon as well as trace elements (De la Rocha 2003, Fowler & Knauer 1986). The biological pump is however not fully efficient. Not all nutrients present at the surface are used during photosynthesis, due to the limitation of primary production by imbalance in trace element concentrations (Sarmiento & Orr 1991, De la Rocha 2003). For example, in high-nitrate, low-chlorophyll waters, iron-limitation affects the growth of phytoplankton (Martin 1990, Martin & Fitzwater 1988, Boyd et al. 2000, Abraham et al. 2000, Takeda 2011). In addition, nutrient imbalance also plays a role; for example, low concentrations of silica can limit growth of diatoms (Pichevin et al. 2009, Brzezinski & Nelson 1996, Brzezinski et al. 2011).

The biological pump (**Figure 1**) can be divided in three steps (De la Rocha 2003): firstly, organic matter and biominerals production; secondly, transport of organic particles into the deep ocean; and thirdly, decomposition of sinking and settled particles. In the first step, dissolved inorganic carbon (DIC) and other dissolved inorganic materials (NO_3^-, PO_4^{3-}, $Si(OH)_4$) in euphotic zones are fixed by phytoplankton during photosynthesis into particulate organic matter (POM). The main components of the produced organic matter are carbon, nitrogen and phosphorus; it also contains silica and

trace elements such as magnesium, cadmium, iron, calcium and barium (Fabiano et al. 1996, De la Rocha 2003).

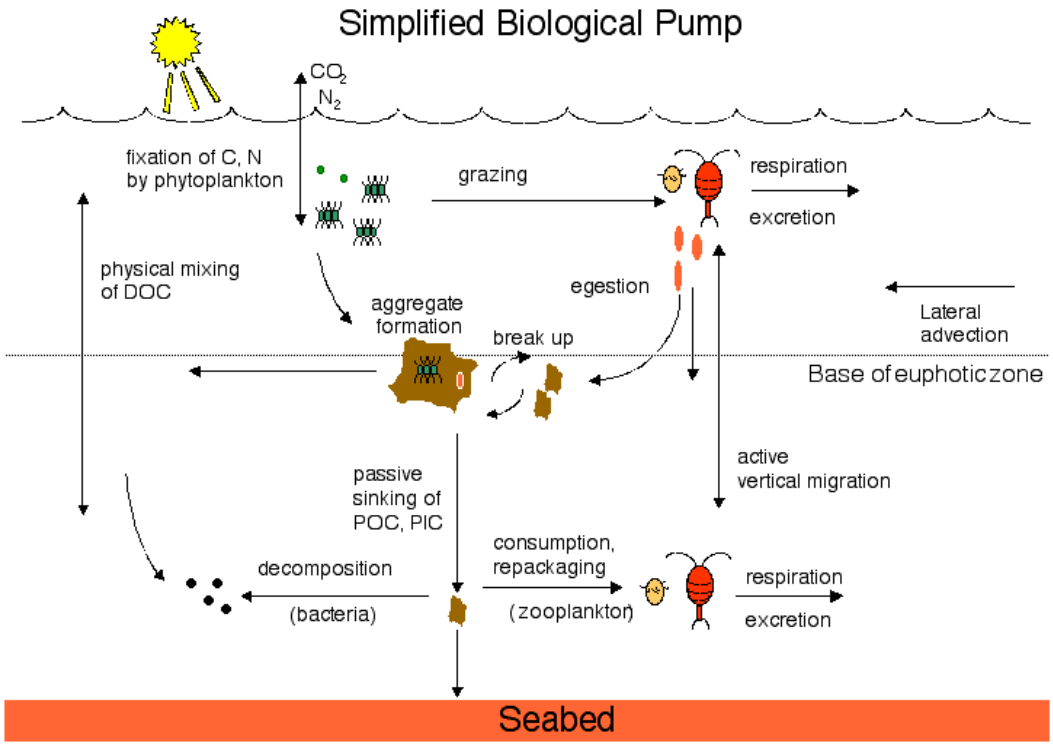

Figure 1. Principle components of the biological pump (Ducklow et al. 2001).

The organic matter produced in the upper waters can be immediately decomposed thereby recycling the fixed CO_2 and generating DIC and dissolved organic matter (DOM). Nevertheless, some of the organic matter is transported to deeper waters in the form of aggregates, known as marine snow (Alldredge & Silver 1988, Alldredge & Gotschalk 1989). The sinking rates depend on the density, shape and size of the aggregates, ranging from <1 to >1,000 m day^{-1} (Fowler & Knauer 1986). In the upper waters and while sinking, marine snow experience processes like bacterial degradation mediated by production of different enzymes (Smith et al. 1992, Bidle & Azam 1999, Azam & Long 2001). In addition, zooplankton consumption reduces the sinking flux of aggregates during grazing by decreasing the amount of POM and transforming large aggregates into smaller particles (Kiørboe 2011, Dilling & Alldredge 2000). Eventually, a relative small amount of fixed carbon reaches the sediments, less than 0.1 to 1.0%,

being sequestered over geologically long time spans (Lalonde et al. 2012, Ducklow et al. 2001, Hedges & Keil 1995, Raven & Falkowski 1999).

As part of the biological pump the microbial carbon pump, described originally by Jiao et al. (2010) (**Figure 2**), plays a particular role in the fate of the carbon in the oceans. During the export of carbon to deep ocean, bacteria and archaea mineralize DOM and POM, producing DIC and recalcitrant dissolved organic matter (RDOM). RDOM is resistant to biological decomposition thus persisting at any depth in the water column, being important carbon storage in the ocean (Jiao & Azam 2011). The precise ways of how RDOM is generated are still unknown, but RDOM contains wall-bound macromolecules such as lipopolysaccharides and muramic acid suggesting a direct exudation during bacterial growth, microbial viral lysis or by the degradation and transformation of POM and DOM (Gruber et al. 2006, Weinbauer et al. 2011, Benner & Kaiser 2003, Suttle 2007). The microbial carbon pump transforms organic carbon from low concentrated reactive carbon to high concentrated RDOM thus changing the chemical composition of the organic matter. RDOM has C:N:P ratios of ~3,511:202:1, suggesting that the microbial carbon pump converts and keep more carbon than nitrogen and phosphorus in the RDOM, therefore releasing inorganic nitrogen and phosphorus, providing essential nutrients for primary production. Jiao et al. (2010) stated that the connection between the biological pump and the microbial carbon pump is the microbial loop, where heterotrophic bacteria use DOM and then exporting the energy to the grazing food web.

The microbial loop (**Figure 3**) channels energy and carbon via bacteria to higher trophic levels (Pomeroy et al. 2007) thereby accelerating mineralisation and recycling of nutrients (Fenchel 2008). Bacteria use DOM produced by phytoplankton as an energy source and multiply. Bacterial populations are then controlled by i.e., flagellates which in turn are grazed by microzooplankton. Consequently, the energy stored in DOM is transferred to the main food chain (Azam et al. 1983). The functional groups involved apart from the previously mentioned include photosynthetic bacteria producing DOM (Cambell & Vaulot 1993) and viruses which lyse bacterial and eukaryotic algae thereby recycling DOM (Suttle 2007). In addition, due to the viral host-specificity, viruses control the successional changes of the bacterial community in the microbial loop (Fenchel 2008). In this way, nutrients become available for the production of new POM through photosynthesis or bacterial production (Verdugo et al. 2004).

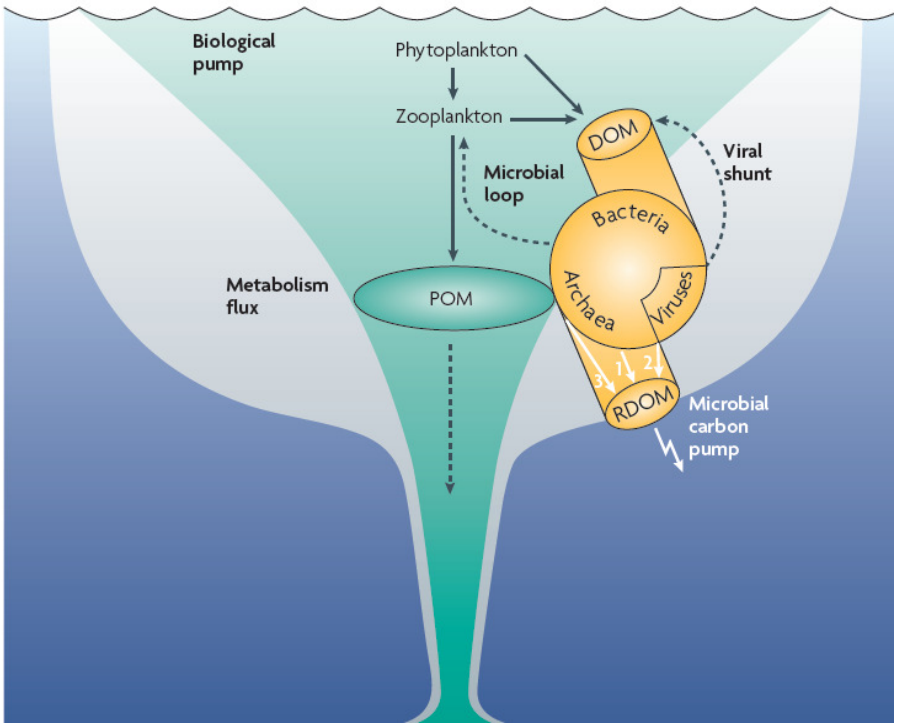

Figure 2. Main biological processes involved in carbon cycling in the ocean: The biological pump, the microbial loop, the viral shunt and the microbial carbon pump. POM: Particulate organic matter, DOC: Dissolved organic matter, RDOM: Recalcitrant dissolved organic matter (Jiao et al. 2010).

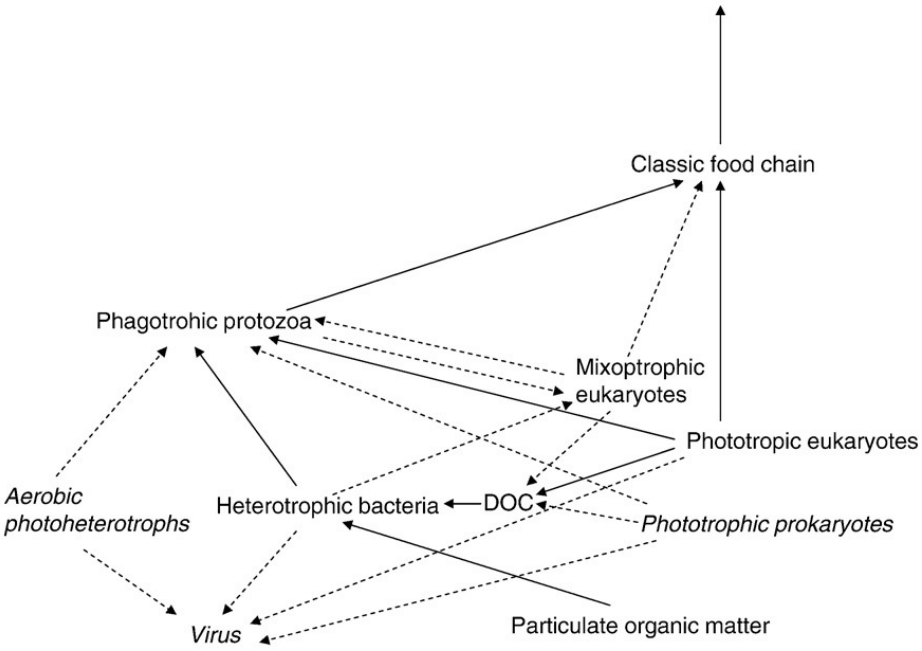

Figure 3. The microbial loop. DOC: Dissolved organic matter. (Fenchel 2008).

1.2. Marine snow

Marine snow (**Figure 4**) is defined as a diverse group of aggregates with diameters greater than 0.5 mm of different origins, morphologies, and characteristics (Alldredge & Silver 1988). The aggregates contain phytoplankton cells, bacteria, and diverse other microorganisms, fecal pellets, inorganic particles, mucus, feeding structures of foraminifera, appendicularians, and pteropods. These components of marine snow are glued together in three-dimensional fractal patterns (Alldredge & Silver 1988, Fowler & Knauer 1986, Kiørboe 2001). Marine snow can consist of about 20% carbon, is enriched in nutrients like nitrogen and trace elements, and is colonized by diverse cells communities at concentration higher than those found in the surrounding seawater. As such, marine snow forms microbial hotspots of metabolic activity, where biological processes like production, decomposition and recycling of nutrients take place (Alldredge & Silver 1988, Kiørboe 2001). Aggregates function as attachment surfaces for microorganisms, providing micro-niches and high concentration of nutrients, therefore playing a major role in the maintenance of microbial diversity (Long & Azam 2001, Verdugo et al. 2004). In addition, sinking of marine snow is the main mechanism for the transport of organic matter to the deep ocean, thus playing a significant role in the biological pump (Alldredge & Gotschalk 1990) and in determining the distribution of elements in the oceans (Fowler & Knauer 1986).

The production of marine snow is mediated by either biological production or physical aggregation, or a combination of both (Alldredge & Silver 1988). Marine snow is biologically produced by mucus-producing organisms such as from feeding structures of pteropods and larvaceans (Hansen et al. 1996, Kiørboe et al. 1996), transparent exopolymers (TEP) from diatoms (Alldredge et al. 1993, Passow 2002), or fecal pellets produced by zooplankton or small fishes (Kiørboe 2001, Fowler & Knauer 1986). Some of these precursors are already carrying bacteria and other microorganisms as in case of i.e. larvacean houses, since the later are use for filtrating water to collect food, while those and other precursors sink they may collect more particles (Alldredge & Silver 1988).

Coagulation and flocculation of particles lead to formation of marine snow during physical aggregation (Alldredge & Silver 1988). Coagulation is a two-step process. Firstly, nano- and micro-particles collide by physical process such as Brownian motion,

differences in sinking velocities, or fluid shear. Secondly, particles stick together after collision by electrostatic interactions or their chemical stickiness which ultimately increase attachment and collision probabilities (Kiørboe 1997, 2001 Alldredge & Silver 1988). The size of aggregates increase by the increase in coalescence and collision of particles (Jackson 2001). The sizes, concentrations and stickiness of particles, together with the intensity of colliding process, determine the aggregation rate of the particles (Verdugo et al. 2004). The sinking velocity of the aggregates also depends on the porosity, density, and size of particles (De la Rocha & Passow 2007)

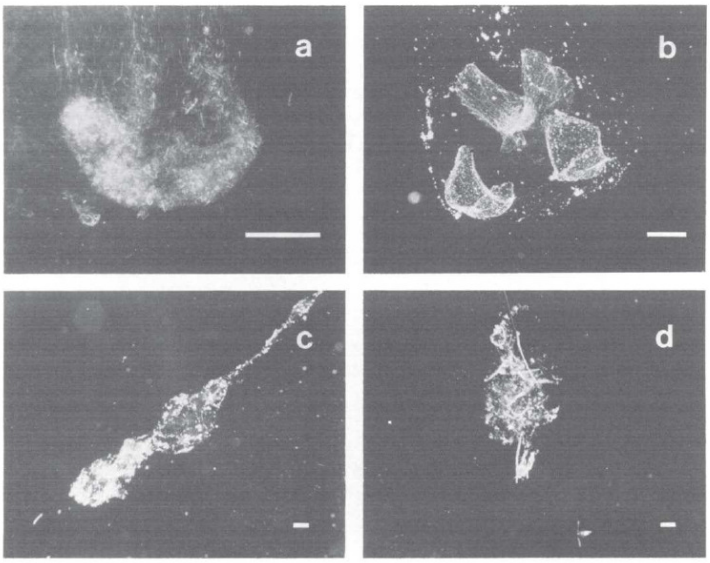

Figure 4. Different examples of marine snow. **A**. aggregate formed by the flocculation of living diatoms; **B**. abandoned filter net from an appendicularian; **C.** aggregate with a comet shape; **D.** aggregate containing macrocrustacean fecal pellets. Scale bar for A: 1 cm, for B–D: 1 mm. (Alldredge & Silver 1988).

Another important factor during the formation of marine snow is the presence of hydrogels in the oceans, which are three-dimensional networks of polymer imbedded in water (Chin et al. 1998, Verdugo et al. 2004). Polymeric DOM can reach high concentrations, which ultimately leads to the formation of nanogels, which in turn can anneal with each other to form microgels. Polysaccharides, proteins, and nucleic acids chains are the main components of microgels, which form non-covalently cross-linked physical polymer networks stabilized by calcium ion bonds (Verdugo et al. 2004, Wells & Goldberg 1994). Transparent exopolymeric particles (TEP) are larger macrogels, formed by the alignment of dissolved polysaccharide chains by cation linking (Verdugo et al. 2004, Alldredge et al. 1993, Logan et al. 1995, Passow 2002). TEP are defined as

Alcian Blue-stainable particles retained in filters (**Figure 5**), Alcian Blue stains negatively charged polysaccharides (Alldredge et al. 1993). TEP are important in sedimentation processes, during aggregation of diatoms blooms and marine snow formation, because they form the "glue" that sticks aggregates components together (Alldredge et al. 1993, Logan et al. 1995). In addition, TEP production leads to the transformation of DOC into POC thus representing an important way to transform DOC and sequestering it by sedimentation (Passow et al. 2001). Similarly, coomassie-stained particles (CSP) mainly formed by proteinaceous particles are also abundant in seawater (Long & Azam 1996).

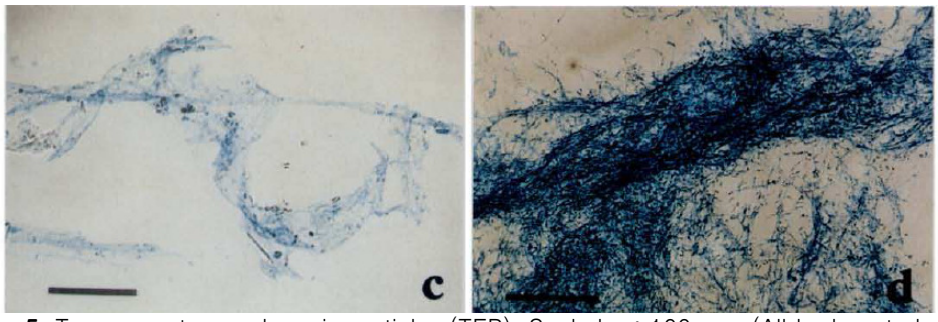

Figure 5. Transparent exopolymeric particles (TEP), Scale bar: 100 μm. (Alldredge et al. 1993)

There are two major biological processes by which marine snow aggregates change in size, composition, and characteristics: zooplankton or nekton grazing on them and microbial decomposition of marine snow particles (Alldredge & Silver 1988, Kiørboe 2001). Additionally, physical processes also play a role; lateral advection, particle settlement, fragmentation via turbulences and mixing (Alldredge & Silver 1988), or fragmentation due shear forces (Dilling et al. 2000, Graham et al. 2000). All these processes contribute to the rates of degradation, remineralizing, and recycling of aggregated material in the upper ocean, and thus leaving just a small proportion available for transport to the deep ocean (Kiørboe 2001).

Microorganisms carry out different processes during the decomposition of aggregates: respiration, solubilization of POM, biomass production, hydrolysis and uptake of substrate, or release of synthesized substrates (Simon et al. 2002). During microbial decomposition, successional changes in the species composition and in the physico-chemical microenvironment take place (Simon et al. 2002, Alldredge & Silver 1988). Firstly, bacteria colonize the aggregates and start enzymatic activities which solubilise and remineralise the organic matter thereby increasing bacterial biomass (Smith et al. 1992). Then, zooplankton such as copepods and microflagellates colonize

the aggregate to consume the bacterial cells and other particulate components of the aggregate (Kiørboe 2010, Caron 1987, Tiselius & Kiørboe 1998). The remaining aggregated fragment can then be populated by other microbial communities (Alldredge & Silver 1988). Finally, zooplankton, fish and other higher order organisms either feed on the constituents or on the aggregate itself (Steinberg et al. 1994, Kiørboe 2001, Larson & Shanks 1996). Only minor amounts of marine snow continue to sink towards the bottom of the seafloor.

Colonization of aggregates by bacteria can either be passive, where bacteria randomly collide with an aggregate, or active, where mechanisms of aggregate detection are used (Kiørboe 2001). Bacteria use chemotaxis to sense chemical gradients and then swim towards areas of higher concentrations of the nutrient molecules (Mitchell et al. 1995, Grossart et al. 2001). Once close or attached to the aggregate, bacteria start producing extracellular or cell wall-bound lytic enzymes to degrade organic matter into DOM (Smith et al. 1992, Ziervogel & Arnosti 2008). The production of DOM is faster than its recycling, therefore, it leaks out from marine snow particles forming a plume of concentrated nutrients (**Figure 6**), which then readily attract other microorganisms to the sinking aggregate (Azam & Long 2001).

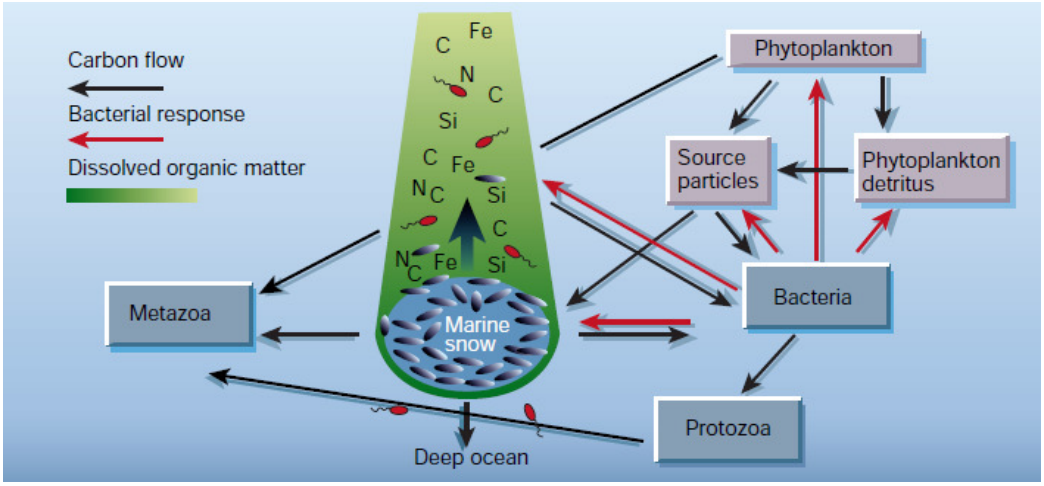

Figure 6. Carbon fluxes in marine snow during sinking, production of hydrolytic enzymes by colonizing bacteria release DOM containing carbon (C), nitrogen (N) silicon (Si) and iron (Fe). (Azam & Long 2001).

Bacteria associated with aggregates are usually bigger in size, more abundant (Herndl 1988, Simon et al. 2002), and phylogenetically different from the free-living bacteria in the surrounding water (Ploug et al. 1999, Simon et al. 2002, Delong et al.

1993). For example, the bacterial *Cytophaga-Flavobacterium* cluster was found to be usually more abundant on marine snow (Ploug et al. 1999, Delong et al. 1993, Moeseneder et al. 2001) possibly due to their capacity to degrade biopolymers including complex polysaccharides (Cottrell & Kirchman 2000, Simon et al. 2002, Verdugo et al. 2004). Another example is the ammonium-oxidizing *Nitrosomonas* cluster which was found to be associated to particles while the cluster *Nitrosospira* was reported as free-living bacteria (Phillips et al. 1999).

1.3. Diatom-bacteria interactions

Marine bacteria are ubiquitous and can reach up to 10^6 cells ml^{-1} (Fenchel 2008). Some of them are heterotrophic bacteria playing a role during the remineralization of organic matter (Cho & Azam 1988), hence influencing the biogeochemical cycles of nutrients. Phytoplankton occur in abundances ranging from 10^2 to 10^5 cells ml^{-1} (Cole 1982). Diatoms represent the most diverse group of phytoplankton and carry out approximately 20% of photosynthesis on earth (Nelson et al. 1995). Diatoms are unicellular, occur as solitary cells or in chains of cells, and are characterized by their siliceous cell wall know as frustule (Armbrust 2009, 1999). The frustule can protect diatoms from predation due to its strength (Hamm et al. 2003); furthermore it enables the diatoms to sink and thus plays an important role in the sinking of organic carbon to the deep ocean (Armbrust 2009).

The term phycosphere, in analogy to the rhizosphere, describes the zone extending outward from a colony or algal cell to an undefined distance, in which the algal extracellular products stimulate the growth of bacteria (Bell & Mitchell 1972). This region has a high concentration of microbial products important for nutrient fluxes (Amin et al. 2012). The phycosphere is the zone where interactions between bacteria and phytoplankton take place (**Figure 7**, Cole 1982). Bacteria can be free-living, be attached or live inside algal cells (Cole 1982, Martinez et al. 1983, Kodama et al. 1990). To be located in a close proximity to the phycosphere, bacteria can use chemotaxis and motility to sense the high nutrient concentrations (Barbara & Mitchell 2003, Mitchell et al. 1995, Stocker et al. 2008), or they can attach to the algal surface (Gärdes et al. 2011, Mayali et al. 2008, Biegala et al. 2002). The attachment can be mediated by the

production of TEP by diatoms and EPS by bacteria (Amin et al. 2012). It has been hypothesized that TEP produced by diatoms can be used as signal to attract bacteria, in a similar way as, i.e. the flavonoids secreted by plants stimulate the production of Nod factors in *Sinorhizobium meliloti*. (Gage 2004). Likewise, diatoms are observed to produce pheromones and bacteria produce autoinducers, suggesting that these compounds could be involved in signaling between these organisms and may play a role during the attachment (Amin et al. 2012).

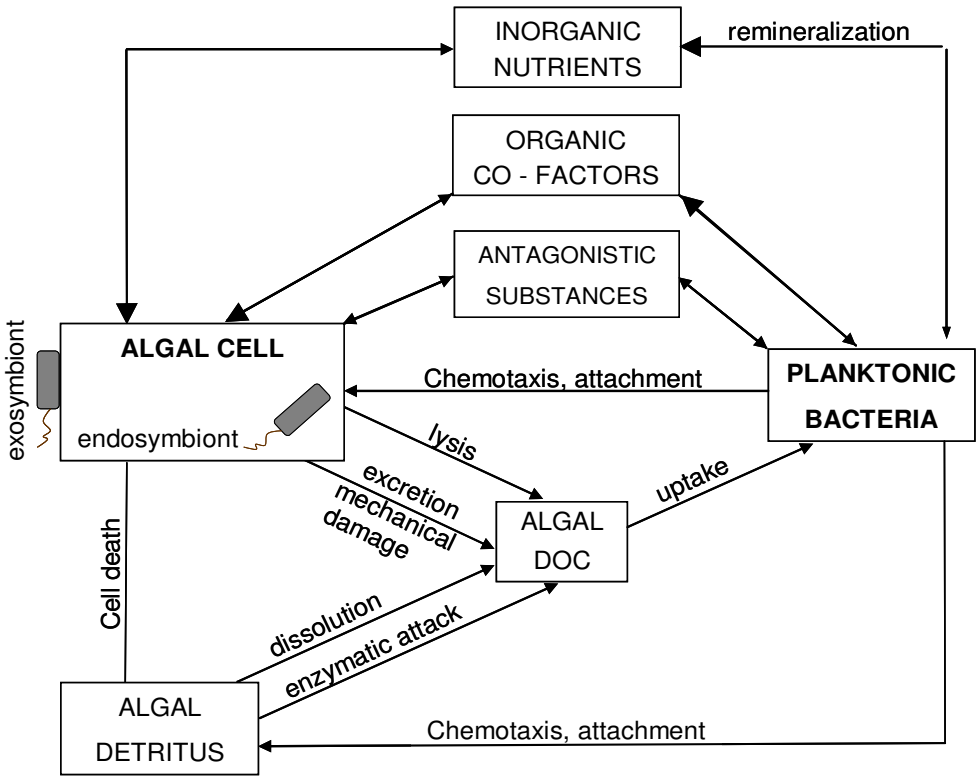

Figure 7. Bacteria–phytoplankton interactions that occur in the phycosphere. The size of arrow heads denote the predominate direction of the process (Cole 1982).

Many types of interaction between bacteria and diatoms have been described (**Figure 8**) including indirect associations due to the ability of bacteria to use algal exudates and remineralize organic matter produced by decaying diatoms, where the bacteria acts as a saprophyte (Cole 1982). Some other bacteria interact with diatoms through specific interactions (Grossart et al. 2005, Sapp et al. 2007). Several studies using diatom isolates or sampling during diatom blooms have identified the phyla *Bacteroidetes* and *Proteobacteria* as the most common phyla associated with diatoms, including the genera *Sulfitobacter*, *Roseobacter*, *Alteromonas* and *Flavobacterium* (Amin

et al. 2012). The diatom *Rhizosolenia styliformis* hosts the endophytic cyanobacterium, *Richelia intracellularis* (Alldredge & Silver 1982, Martinez et al. 1983). These studies indicate that there are specific relationships between bacteria and diatoms (Cole 1982).

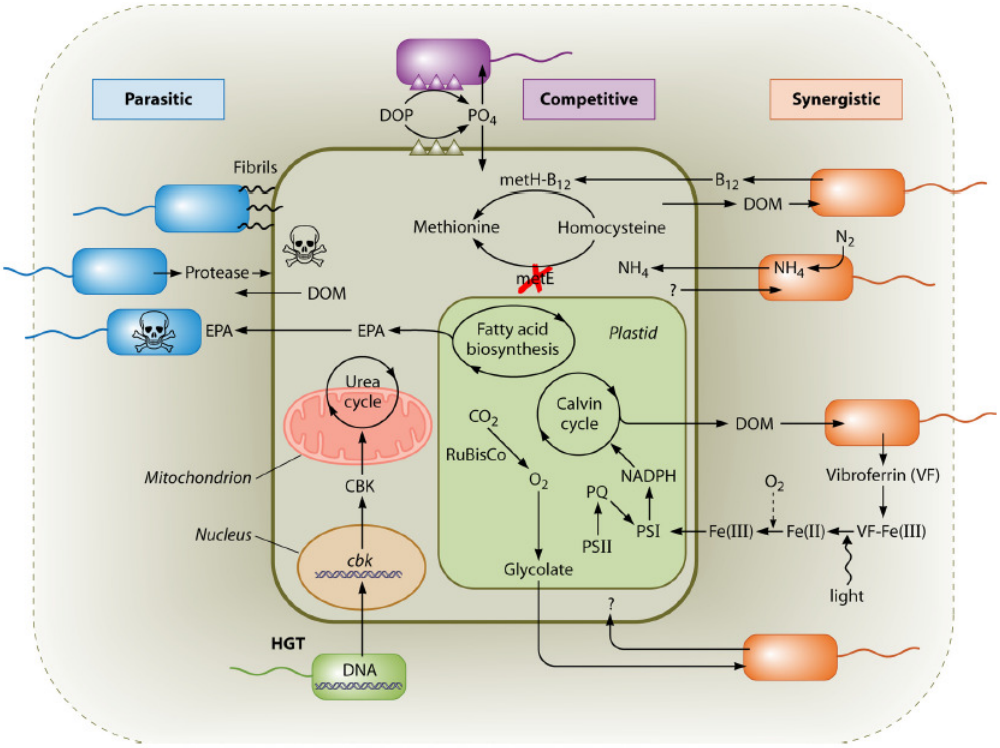

Figure 8. Schematic representation of bacterial interactions with diatoms, including competitive (purple), synergistic (orange), and parasitic (blue). An example of horizontal gene transfer (HGT) is also shown (green). The phycosphere is represented by background gradient and dashed line. DOP: dissolved organic phosphorus, DOM: dissolved organic matter, EPA: fatty acid eicosapentaenoic (Amin et al. 2012).

Positive interactions like mutualism take place where both organisms benefit from the excreted compounds of each other. For instance, diatoms require vitamins, especially B vitamins, to grow (Croft et al. 2005), as such they benefit from the vitamins produced by bacteria (Cole 1982). Accordingly, bacteria producing cobalamin (vitamin B12) were observed to improve diatom yield (Haines & Guillard 1974). Similarly, iron is limited in seawater; many marine bacteria produce siderophores which make iron available to diatoms (Amin et al. 2007). Conversely, the polysaccharides produced by diatoms can enhance the bioavailability of iron for bacteria (Hassler et al. 2011). In this way, bacteria which do not produce siderophores, can access iron (Amin et al. 2012). In addition, some other diatoms benefit from nitrogen-fixing bacteria (Fiore et al. 2010): the diatoms *Hemiaulus*, *Rhizosolenia* and *Chaetoceros* host the filamentous

heterocystous cyanobacteria *Richelia intracellularis* and *Calothrix rhizosoleniae* (Foster et al. 2011). Besides, bacteria can help diatoms in the detoxification of reactive oxygen species. The diatom *Amphiprora kufferathii* is associated to epiphytic bacteria from the genera *Sulfitobacter*, *Colwellia* and *Pibocella*, which consume hydrogen peroxide produced by the diatom (Hünken et al. 2008).

On the other hand, bacteria can also benefit from compounds produced by diatoms during photosynthesis. For example, DOC produced by diatoms has been shown to be used by heterotrophic bacteria (Bell et al. 1974, Grossart et al. 2006). Glycolate, a 2-carbon molecule, is released by diatoms; some bacteria carry the gene *glcD*, encoding the D-subunit of the enzyme glycolate oxidase, needed for glycolate utilization (Lau & Armbrust 2006, Lau et al. 2007). In addition, the mucus produce by phytoplankton, containing polysaccharides and proteoglycans, might provide protection to bacteria (Azam & Malfatti 2007).

Not all the interactions are mutually beneficial, however. Some bacteria produce algicidal compounds (Cole 1982, Imai et al. 1993, Paul & Pohnert 2011). The bacterium *Korida algicidal* for example, produces a protease which has activity against *Thalassiosira, Skeletonema* and *Phaeodactylum*. This protease is however not effective against *Chaetoceros* suggesting that algicidal activity can be species-specific (Paul & Pohnert 2011). The bacterium *Saprospira* sp. SS98-5 stimulates the aggregation of the diatom *Chaetoceros ceratosporum* and then produces extracellular microtubule-like structures which kill the diatoms by lysing the diatom cells (Furusawa et al. 2003).

Phytoplankton have on their side, evolved mechanisms against non-beneficial bacteria, through the production of antibacterial compounds such as fatty acids and esters (Sastry & Rao 1994, Lebeau & Robert 2003). The diatom *Phaeodactylum tricornutum* for example, produces the fatty acids: palmitoleic acid, hexadecatrienoic acid (HTA) and eicosapentaenoic acid (EPA), which inhibit bacterial growth (Desbois et al. 2009). Furthermore, polyunsaturated aldehydes (PUA) produced by diatoms as a defense against grazers, also have antibacterial effects (Ianora et al. 2004). Interestingly, the diatom-associated bacteria *Sulfibacter*, *Paracoccus* and *Erythrobacter* were found to be PUA-resistant (Ribalet et al. 2008).

Competition has also been observed between bacteria and diatoms. The growth of phytoplankton is limited by the availability of some nutrients such as nitrogen, iron, or phosphorous (Rothhaupt & Güde 1992). In habitats where these nutrients are limited,

competition between bacteria and algae can occur (Cole 1982, Rothhaupt & Giide 1992). For example, under phosphate limitation, the diatom *Cylindrotheca fusiformis* growing with bacteria showed slower growth compared with the growth under normally balanced phosphate conditions, suggesting that the bacteria were more efficiently taking up the available phosphate (Guerrini et al. 1998). It is important to point out that the types of interaction taking place between bacteria and diatoms are not constant and depend on the environmental conditions, a mutualistic relationship can switch to a parasitic relationship under stress conditions (Grossart 1999) or to a commensalism under nutrient-limiting conditions (Gärdes et al. 2012)

1.4. The *in vitro* model system

So far, the biochemistry of the diatom-bacteria interactions has been poorly understood (Azam & Malfatti 2007). Furthermore, the signaling processes between these organisms during the initiation of the interaction are still unknown (Amin et al. 2012). In addition, the molecular basis underlying diatom-bacteria interactions remains unclear. To further our understanding of the cell-to-cell interaction between diatoms and bacteria, a bilateral model system consisting of the diatom, *Thalassiosira weissflogii*, and the bacterial strain, *Marinobacter adhaerens* HP15, was therefore established (Gärdes et al. 2011, Sonnenschein et al. 2012).

1.4.1. *Thalassiosira weissflogii*

Thalassiosira is a genus belonging to the centric diatoms, which have a broad distribution in all open water masses (Armbrust 1999). This genus is taxonomically affiliated to the class of Coscinodiscophyceae and the phylum Bacillariophyta. Approximately 180 marine species belong to this genus; *T. weissflogii* and *T. pseudonana* are two well-studied examples (Round et al.1990). The genome of *T. pseudonana* is partially available making this genus important for understanding the evolution of diatoms (Armbrust et al. 2004).

T. weissflogii (**Figure 9**) has a broad distribution, being isolated from the waters of Europe, Southeast Asia, Australia, and other regions (Dassow et al. 2006). This diatom

has a frustule composed of two thecae with diverse long thread-like strutures composed of chitin (Durkin et al. 2009). *T. weissflogii* grows as solitary cells having diameters ranging from 12 to 22 μm (Provasoli Guillard National Center for Culture of Marine Phytoplankton (CCMP)). Diverse studies have used *T. weissflogii* as a marine diatom model system (Armbrust 1999, Gärdes et al. 2011, Vrieling et al. 1999, Grossart 1999, 2005, Durkin et al. 2009, Bilde & Azam 1999, Passow 2002).

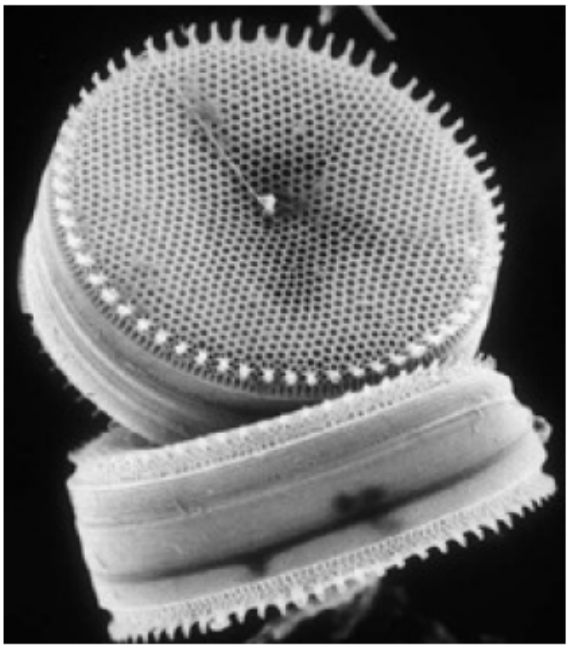

Figure 9. Scanning electron micrograph of the marine diatom *Thalassiosira weissflogii* (Courtesy of F. Hinz, Alfred-Wegener Institute, Bremerhaven, Germany).

1.4.2. *Marinobacter adhaerens* HP15

The ubiquitously distributed genus *Marinobacter* is taxonomically affiliated to the phylum Proteobacteria, class Gamma-proteobacteria and family Alteromonadales. More than 30 species isolated from a variety of environments are affiliated to this genus. For instance, *Marinobacter hydrocarbonoclasticus,* the first strain described, was isolated near a petroleum refinery in the French Mediterranean coast, and uses different hydrocarbons as sole carbon source (Gauthier et al. 1992). Other species interact with different organisms, such as *M. bryozoorum*, isolated from a Bryozoa specimen

(Romanenko et al. 2005), *M. algicola*, isolated from dinoflagellate cultures (Green et al. 2006), and *M. xestospongiae* from a marine sponge (Lee et al. 2012).

M. adhaerens HP15 (Käppel et al. 2012) was isolated from marine aggregates collected from surface waters of the German Bight (Grossart et al. 2004). *M. adhaerens* HP15 is a heterotrophic, aerobic, Gram-negative bacterium. Cells are rod-shaped (0.6-0.8 x 1.7-2.4 µm) and motile by means of a polar flagellum (**Figure 10A**). Its optimal growth temperature ranges from 28 to 37°C, and pH 7.0-9.0. On Marine-Broth (MB) agar plates *M. adhaerens* HP15 grows in form of brownish mucoid colonies (**Figure 10B**) (Käppel et al. 2012).

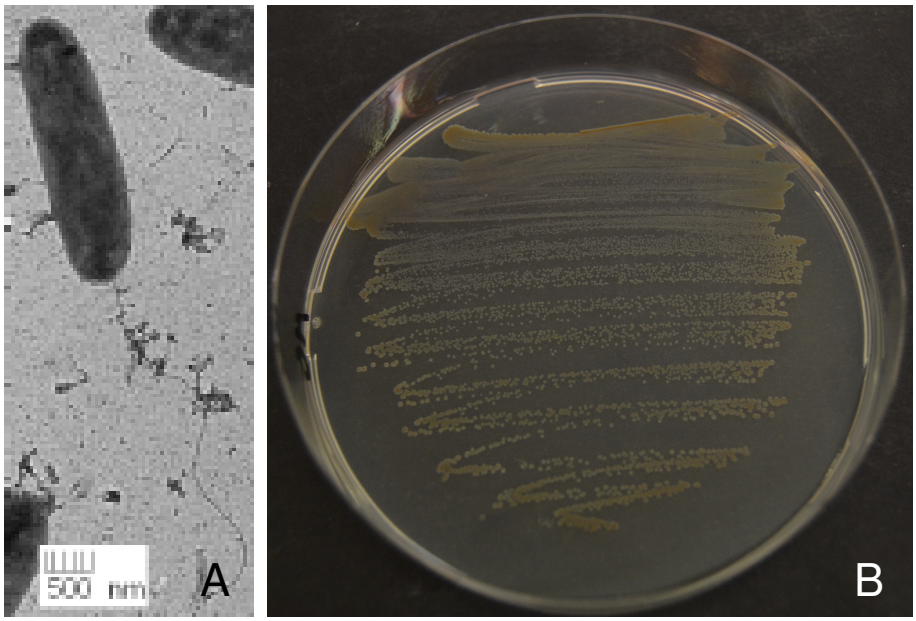

Figure 10. *M. adhaerens* HP 15. A, colonies plated on MB medium. B, Transmission electron microscopy picture (Sonnenschein et al. 2011).

M. adhaerens HP15 specifically attach to *T. weissflogii* inducing TEP production and aggregate formation (**Figure 11**, Gärdes et al. 2011), suggesting that this bacterium plays an important role during *T. weissflogii* aggregation dynamics. For this reason, *M. adhaerens* HP15 was selected for establishing an *in vitro* model system together with *T. weissflogii*, to study aggregate formation during diatom-bacteria interactions at the cellular and molecular level. In addition, its genome was sequenced and completely annotated (Gärdes et al. 2010). The genome comprises three replicons; a chromosome of approximately 4.5 Mb, and two plasmids of ~187 and ~42 Kb, respectively. The

genome sequence revealed some interesting features, supporting a role of this bacterium during interactions with eukaryotic cells. For instance, the presence of several pili and flagella-associated gene clusters, methyl-accepting chemotaxis proteins, chemotaxis gene clusters, two-component regulatory systems, multi-drug efflux systems, a siderophore receptor-encoding gene, exopolymer syntheses, and hydrolytic enzymes-encoding genes, among others. Furthermore, the genetic accessibility of *M. adhaerens* HP15 was comprehensively analyzed in the laboratory (Sonnenschein et al. 2011). Transformation of *M. adhaerens* HP15 by electroporation and conjugation, random and site-mutagenesis, as well as expression of reporter genes such as enhanced green fluorescent protein (*egfp*) and ß-galactosidase (*lacZ*) were successfully conducted.

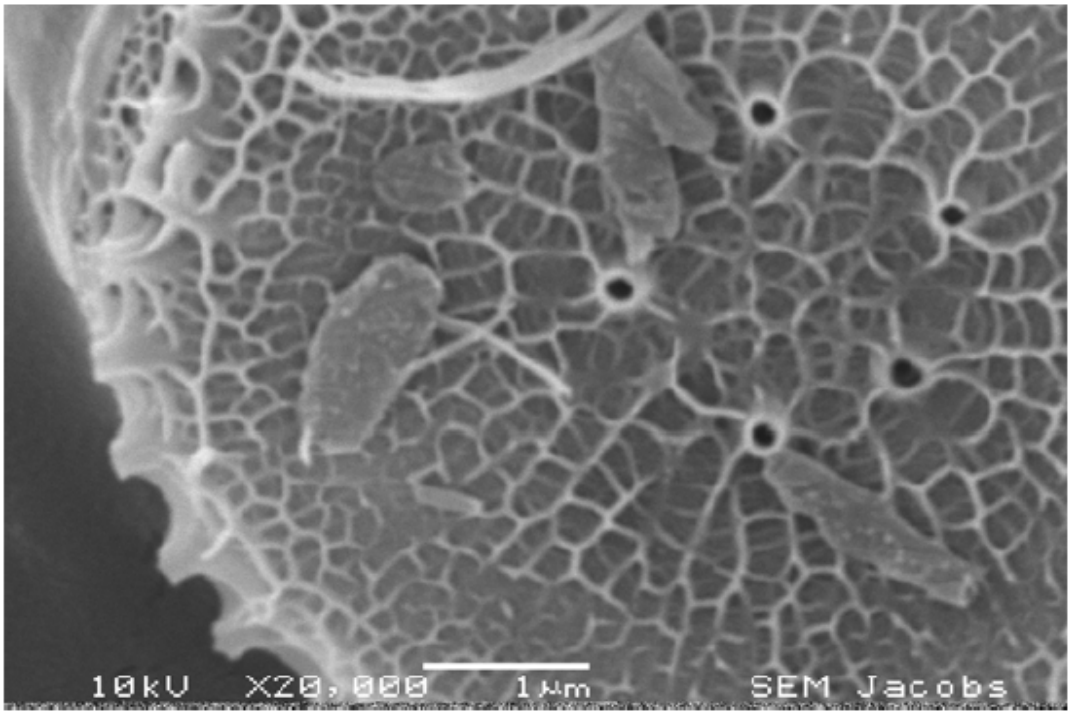

Figure 11. Scanning electron microscopy of *M. adhaerens* HP15 attached to *T. weissflogii* within 24 h of incubation. Scale bar: 1 μm (Gärdes et al. 2012).

1.4.3. Interaction between *T. weissflogii* and *M. adhaerens* HP15

The interaction between *T. weissflogii* and *M. adhaerens* HP15 has been characterized to some detail (Gärdes et al. 2011, Gärdes et al. 2012, Sonnenschein et

al. 2012). As mentioned above, *M. adhaerens* HP15 induce TEP production and aggregate formation of *T. weissflogii* (Gärdes et al. 2011). Cultures of photosynthetically active and inactive *T. weissflogii* either alone or in co-culture with *M. adhaerens* HP15 were analyzed in rolling tank experiments (Gärdes et al. 2011). It was demonstrated that *M. adhaerens* HP15 induce TEP production and aggregate formation only in photosynthetically and metabolically active diatom cells (Gärdes et al. 2011).

To evaluate the composition and amount of released polysaccharides and DOC by *T. weissflogii* under different nutrient conditions, the diatom was incubated in phosphorous- or nitrogen-limited media with *M. adhaerens* HP15 added or not (Gärdes et al. 2012). Under nutrient-balanced conditions *M. adhaerens* HP15 induced TEP formation, cell growth, and DOC accumulation of *T. weissflogii*. These results and the fact that the bacteria can grow only in sterile seawater in the presence of *T. weissflogii* using its exudates as sole carbon source suggested a rather mutualistic relationship between these two organisms. On the other hand, under nutrient-deficient conditions the bacterial effect on *T. weissflogii* was dramatically impaired, suggesting a shift from mutualism to commensalism between *M. adhaerens* HP15 and *T. weissflogii* as a result of nutrient stress. In addition, by the use of lectin staining of exopolymers it was shown that the bilateral interaction may result in either modification or *de novo* synthesis of exopolymers (Gärdes et al. 2012).

The established genetic accessibility techniques (Sonnenschein et al. 2011) allowed the analysis of genes, potentially important for the interaction of *M. adhaerens* HP15 with diatom cells. For example, flagellum-deficient mutants *fliG*::Tn*5* and *ΔfliC,* were generated by transposon and site-directed mutagenesis, respectively (**Figure 12**). As expected, the *fliG*::Tn*5* mutant was unable to form the flagellum, since this gene is required for the hook formation as described in *Salmonella enterica* (Thomas 2001) (**Figure 12B**). In contrast, the *ΔfliC* mutant (**Figure 12A**) exhibited the flagellar hook but was missing the flagellar filament. In addition, a type IV mannose-sensitive haemagglutinin (MSHA) pilus-deficient mutant was generated by gene-specific mutagenesis lacking the *mshB* gene (Seebah 2012). To study the impact of the cellular appendages during the interaction, the attachment behavior towards *T. weissflogii* cells was studied for the flagellum- and pili-deficient mutants (Δ*fliC*, *fliG*::Tn5 and Δ*mshB*) compared to the wild-type strain. The mutant strains, showed a significant reduction in attachment to diatom cells, suggesting that a functional flagellum and MSHA type-IV

pilus are required for the attachment of *M. adhaerens* HP15 to *T. weissflogii* cells (**Figure 13**, Seebah 2012).

Chemotaxis is an important mechanism during the attraction of marine bacteria by phytoplankton cells or exudates (Seymour et al. 2010, Paerl & Pinckney 1996, Willey & Waterbury 1989, Bell & Mitchell 1972). Chemotaxis by *M. adhaerens* HP15 towards *T. weissflogii* was studied by the generation and analysis of chemotaxis-deficient *M. adhaerens* HP15 mutants during the interaction with the diatom (Sonnenschein et al. 2012). Genes in two chemotaxis signaling cascades were mutagenized, *cheA* coding for a central histidine kinase and *chpB* coding for a methyltransferase. The mutant strains exhibited a decreased diatom attachment, microscopic analysis of bacterial and diatoms stained with carbol fuchsin and TEP stained with alcian blue showed more wild-type cells attached to diatoms or to TEP compared to those observed form the mutants (**Figure 14**). These results suggest that chemotaxis plays an important role during the interaction (Sonnenschein et al. 2012)

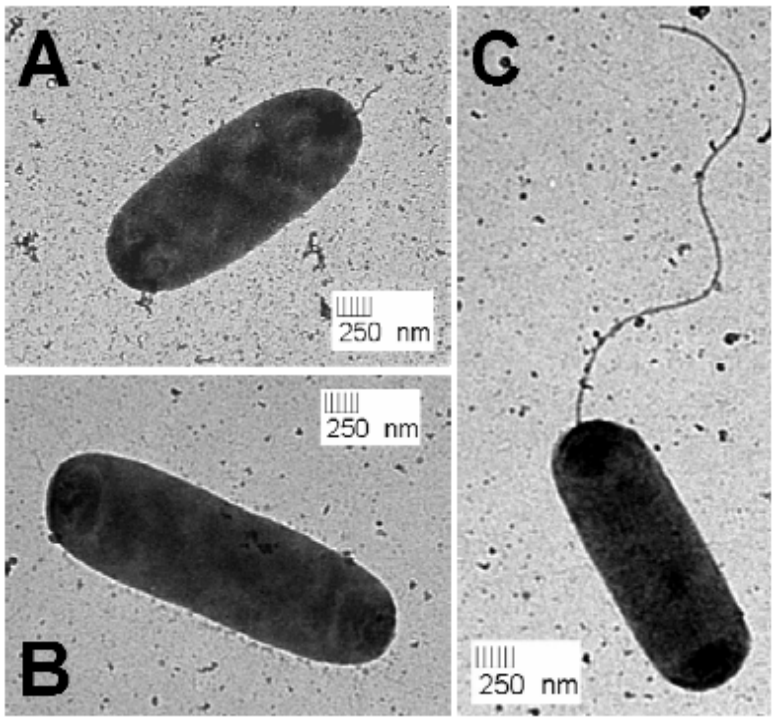

Figure 12. Phenotypic characterization of flagellum-deficient *M. adhaerens* HP15 mutants by transmission electron microscopy: A, *M. adhaerens* HP15 mutant Δ*flfC*; B, *M. adhaerens* HP15 mutant *flfG*::Tn5; C, *M. adhaerens* HP15 wild-type. (Sonnenschein et al. 2011).

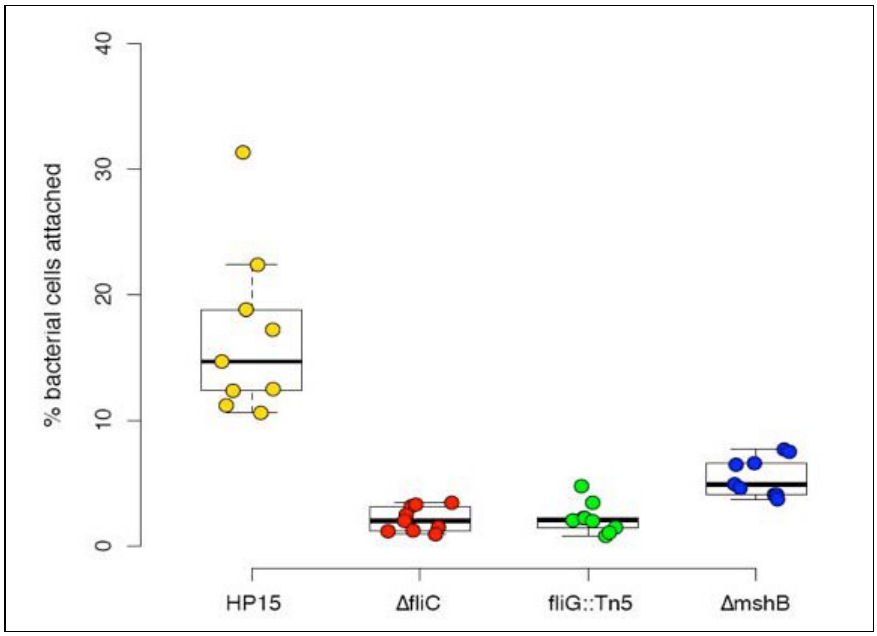

Figure 13. Percentage of *M. adhaerens* HP15 cells, wild-type and mutants Δ*fliC*, *fliG*::Tn5, Δ*mshB* attached to *T. weissflogii* after 48 hrs of incubation in f/2 medium (Seebah 2012).

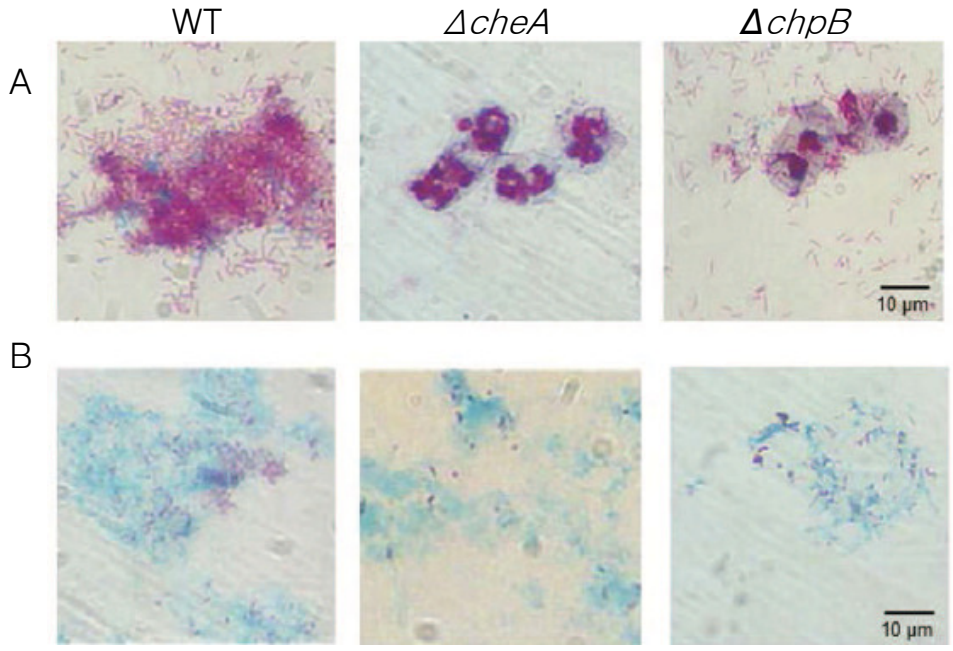

Figure 14. Microscopic analysis of *M. adhaerens* wild-type or the mutants Δ*cheA* and Δ*chpB* attached to *T. weissflogii* cells after 24 h of incubation. Bacterial and diatom cells were stained with carbol fuchsin and TEP with alcian blue. **A**. Bacterial cells attached to diatom cells; **B.** alcian blue-stained aggregates populated by carbol fuchsin-stained bacterial cells (Sonnenschein et al. 2012).

1.5. Methods for the analysis of differential gene expression during cell-to-cell interactions

Different methods have been established for the study of bacterial gene expression in the last years. These can either focus on individual genes of interest, or in the study of all the genes that are expressed under a particular condition. It can be done for environmental samples or on an isolated organism. It is important to note that bacteria growing under *in vitro* conditions might not express genes in a similar manner as they would under conditions of their natural environment. For this reason, it is important to study the role of mixed communities in their natural environment. The "omics" methods allow studies to be conducted at all biological levels including DNA, RNA, proteins and metabolites in a mixed community (Raes & Bork 2008, Siggins et al. 2012). A summary of the advantages and the disadvantages of the methods described here for the analysis of differential gene expression during cell-to-cell interactions are shown in **Table 1**.

1.5.1. Promoter trap techniques

The use of reporter genes has helped to study the expression of genes in bacteria isolated and cultivated in the laboratory. Examples of these reporters genes are the *lacZ*, encoding for ß-galactosidase (Silhavy & Beckwith 1985), *gusA*, encoding for ß-glucoronidase (Jefferson 1989), and *gfp*, encoding for the green fluorescent protein (Chalfie et al. 1994). With the use of reporter genes, some techniques were developed to study bacterial genes expressed during growth under specific conditions including the so-called promoter-trapping techniques (Wilson et al. 1995 Chiang & Mekalanos 1998, Ullrich et al. 2000, Goyer & Ullrich 2006). For example, in the transposon mutagenesis coupled with reporter genes strategy, a genetic fusion between a promoterless reporter gene and a transposon is generated. The transposon insertions allow the reporter gene to be randomly placed into the genome. When the reporter gene has been inserted downstream of a promoter involved in or influenced by the condition tested, this reporter gene is then differentially expressed (Chiang & Mekalanos 1998, Ullrich et al. 2000,

Goyer & Ullrich 2006). Other promoter-trapping techniques were progressively developed. For example: differential fluorescence induction (DFI) (Valdivia & Falkow 1996) and the so-called in vivo expression technology (IVET) (Mahan et al. 1993). DFI (Valdivia & Falkow 1996) uses the green fluorescent protein (GFP) as a selection marker to study the promoter activity. In this strategy, those genes that are specifically expressed under a certain condition are screened with the help of fluorescent-activated cell sorting (FACS).

1.5.1.1 *In vivo* expression technology (IVET)

The majority of bacterial virulence genes are induced during infection of the host organism. In the laboratory certain host environmental parameters can be mimicked inducing a subset of virulence genes including changes in temperature, oxidative stress or pH (Rediers et al. 2005). However, the full combination of virulence genes is only expressed *in vivo*. In order to identify these genes a genetic system termed *in vivo* expression technology (IVET), was developed (Mahan et al. 1993). In this genetic approach, a live host, with tissue barriers and immune system, is used to signal induction of virulence genes. Briefly, IVET (**Figure 15**) is a promoter-trapping technique used for the selection of bacterial promoters that are specifically induced *in vivo*. The *in vivo*-induced promoters are identified by the ability to drive expression of a promoterless selection marker gene that is essential for survival. The expression *in vitro* of a promoterless reporter gene cloned downstream the selection marker allows the exclusion of constitutive promoters (Rediers et al. 2005, Lee & Cooksey 2000). Therefore, this strategy allows the identification of genes that are specifically expressed under the conditions found in the host tissue but are minimally expressed under laboratory conditions (Mahan et al. 1993).

This strategy was originally developed to identify virulence genes induced when *Salmonella typhimurium* infects their host (Mahan et al. 1993). The *purA* gene was mutated in *S. typhimurium* generating a purine auxotrophic mutant, which was not able to survive in the host unless it was complemented *in trans*. Random genomic fragments were cloned upstream of a promoterless *purA-lacZ* operon present on a suicide vector. This library was transformed into the *S. typhimurium* Δ*purA* mutant and was integrated by homologous recombination into the chromosome. Once the entire pool of recombinant mutant transformants was injected into the peritoneal cavity of mice, the bacterial growth

in the mouse was used as a positive selection for strains, in which a promoter of a virulence gene was driving expression of *purA–lacZ*. Strains expressing the gene fusion *in vivo* became prototrophic and survived, whereas strains not expressing the fusion remained auxotrophic and died. Finally, after re-isolation of the bacterial population from the mice a screen for *lacZ* expression was applied to discard constitutively active fusions (Mahan et al. 1993). A total of 5 *in vivo*-induced genes (*ivi*) were identified by this method, two of them showed no significant homology to previous reported sequences in the GenBank, suggesting that the technique allowed the identification of new and thus far uncharacterized genes specifically induced during infection (Mahan et al. 1993).

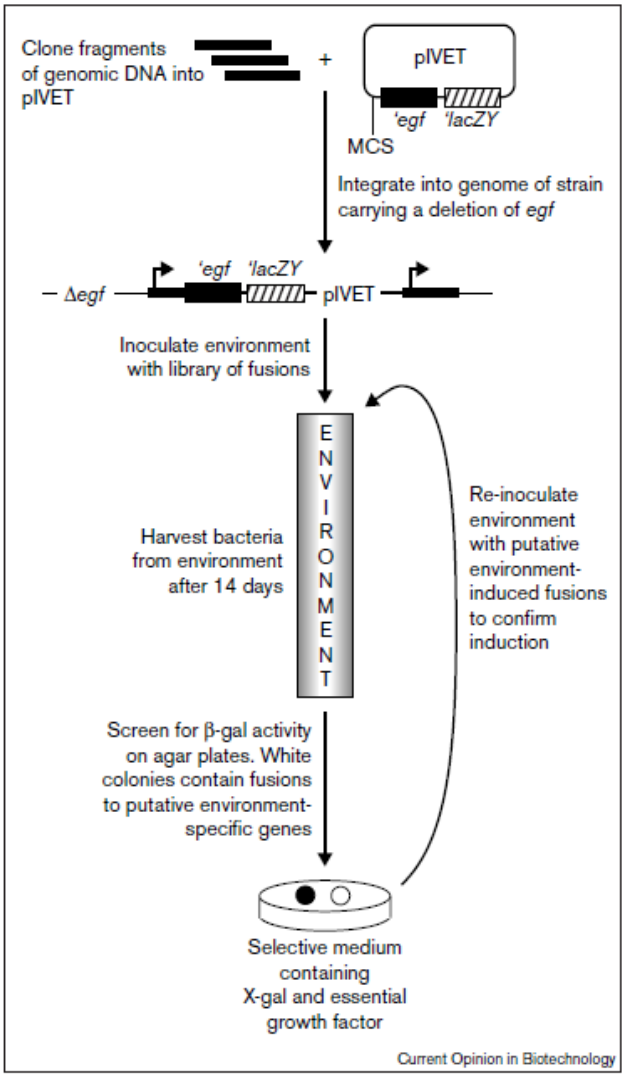

Figure 15. Schematic representation of a typical IVET strategy. MCS: multiclonal site. egf: essential growth factor (Reiney & Preston 2000).

In the past years, this technique has been applied to study gene expression in a wide range of microorganisms living in different environments (Rediers et al. 2005). IVET has been successfully used for studying bacterial genes involved in different interactions. For instance, plant pathogenic bacteria such as: *Pseudomonas putida* KT2440 (Fernández et al. 2013), *P. syringae* pv tomato (Boch et al. 2002), *Erwinia chrysanthemi* (Yang et al. 2004), and *Ralstonia solanacearum* (Brown & Allen 2004). Human pathogenic bacteria that have been studied using IVET include *Helicobacter pylori* (Singh et al. 2012), *Staphylococcus aureus* (Lowe et al. 1998), *Klebsiella pneumoniae* (Lai et al. 2001), *Porphyromonas gingivalis* (Wu et al. 2002), *Shigella flexneri* (Bartoleschi et al. 2002), *Vibrio vulnificus* (Lee et al. 2007), *Burkholderia pseudomallei* (Shalom et al. 2007), *Streptococcus pneumonae* (Meng et al. 2008), and *Enterococcus faecalis* (Hanin et al. 2010). In addition, IVET was also applied to study environmental microorganisms such as *Rhizobium meliloti* (Oke & Long 1999), *Bacillus cereus* (Brillard et al. 2010), *R. leguminosarum* biovar *viciae* (Barr et al. 2008), *P. putida*, a biological control bacterium (Lee & Cooksey 2000) and *Lactobacillus lactis* which is an important industrial bacterium (Bachmann et al. 2008). The later studies principally demonstrated that IVET can be applied to virtually any *in vivo* situation and that it shall not be restricted to virulence-associated gene identification.

This strategy has many advantages: the selected genes are isolated during a strong positive selection (Angelichio & Camilli 2002), the analysis can be carried out with standard molecular techniques and has low costs, the genome information of the organism is not required, and the approach can be applied to study a wide variety of organisms under different conditions (Rediers et al. 2005). However, it also entails disadvantages which include, for instance, the difficulty of generation of mutants in some strains. However, modifications of the original IVET have been developed to overcome such difficulties, for example the recombinase-based *in vivo* expression technology (RIVET) (Lee et al. 1999, Camilli & Mekalanos 1995). In addition, IVET cannot be used to isolate weakly expressed or repressed promoters, the identified genes depend on the strength of the selection *in vivo*, proteins expressed in a constitutive manner that are just activated *in vivo* are not detected, and the genes identified depend on the *in vitro* growth conditions (Rediers et al. 2005).

1.5.2. Transcriptomics

The transcriptome is defined as the complete set of RNA molecules of a cell in a specific stage or physiological condition (Cox & Mann 2011, Morozova et al. 2009, Wang et al. 2009). It can change when external conditions are different, and reflects the genes that are being actively expressed. The aim of transcriptomics is to quantify the expression levels of genes and operons during growth under different conditions. The transcriptome can be studied by different approaches; firstly, reverse-transcription quantitative polymerase chain reaction (RT-qPCR) (Noonan et al. 1990, Saleh-Lakha et al. 2011). Secondly, hybridization-based approaches such as microarrays (Schena et al. 1995, Schena 1996). Thirdly, tag-based methods such as cap analysis of gene expression (CAGE, Kodzius et al. 2006), massively parallel signature sequencing (MPSS, Brenner et al. 2000) and serial analysis of gene expression (SAGE, Velculescu et al. 1995). Finally, the most current technology is RNA sequencing (RNA-seq) with high-throughput copyDNA sequencing (Wang et al. 2010).

1.5.2.1. Reverse-Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR is a sensitive method that allows the quantification of the number of mRNA copies of a gene, under different conditions (Saleh-Lakha et al. 2011). This technique involves the isolation and purification of RNA, its reverse transcription into cDNA, which is used as a template during PCR amplification with specific probes. At the end of every amplification cycle, the PCR product is quantified by fluorescent technology (Trevors et al. 2012). RT-qPCR is a highly sensitive technique with a low detection limit (Saleh-Lakha et al. 2011).

1.5.2.2. Microarrays

Microarrays is a chip-based technique in which gene-specific DNA probes are immobilized on a surface by robotics, allowing the hybridization of fluorescent labeled cDNA, the fluorescent intensity provides a measurement of the gene expression (Schena 1996, Watson et al. 1998, Westermann et al. 2012). Microarrays are used for the study

of gene expression in a specific organism under different environmental conditions. With this technique the expression levels of many genes can be carried out simultaneously (Schena 1996, Morozova et al. 2009, Trevors et al. 2012). In addition, microarrays are sensitive and reproducible tools that have broad applicability (Trevors et al. 2012). Nevertheless, this technique has several limitations: the genome information of the organism must be available (Wang et al. 2010), it is a non-quantitative approach where the transcript abundance is obtained indirectly from the hybridization intensity (Cox & Mann 2011, Morozova et al. 2009); background noise during hybridization, due to cross-hybridization (Wang et al. 2010); poor or not signal for low abundant transcripts (Marioni et al. 2008).

1.5.2.3. Tag-based sequencing approaches

Tag-based sequencing approaches are based in the sequencing of expressed sequence tags (ESTs). For example the SAGE (Velculescu et al. 1995) is an approach that uses short nucleotide sequence tag from 9 or 10 bp, these tags have enough information to identify a transcript. The cDNA is cleaved with a restriction enzyme, the 3' tags are isolated and anchored by the restriction site to linkers, and the tags are ligated into a long serial molecule, which is cloned and sequenced (Velculescu et al. 1995). These techniques provide a precise, high throughput data for gene expression levels, having advantages over the microarrays, including the detection of novel transcripts involved in specific biological processes, quantitative information of transcript abundance, easy comparison between samples (Morozova et al. 2009). Nevertheless, tag-based sequencing approaches are still expensive since they are based on Sanger sequencing technology. In addition, some of the short tags (14 or 21 bp) are difficult to map in the reference genome (Wang et al. 2010, Morozova et al. 2009). Some studies have coupled these techniques with next-generation sequencing (NGS) technologies (Cheung et al. 2008, Gowda et al. 2007, Eveland et al. 2008).

1.5.2.4. Next-generation sequencing - RNA sequencing (RNA-seq)

The use of next-generation sequencing (NGS) technologies has increased in the last years. NGS techniques allow the generation of more sequence information in a

shorter time, lower cost and higher resolution than Sanger sequencing, due to the simultaneous sequencing of DNA fragments (MacLean et al. 2009, Heidelberg et al. 2010, Chee-Seng et al. 2010, Dunne et al. 2012). NGS technologies has been used for genomic and diversity analysis (Dunne et al. 2012, MacLean et al. 2009, Maccaferri et al. 2011). Furthermore, NGS technologies can be also used in transcriptomics (Morovoza et al. 2009, Wang et al. 2009, Westermann et al. 2012, Che-Seng et al. 2010), allowing the acquisition of more data compared to transcriptomics based on Sanger sequencing.

In RNA-seq the mRNA of an organism is reverse transcribed into cDNA and fragmented, then a library is created attaching adaptors to one or both fragments ends, each fragment is then sequenced by NGS technology to obtain short sequences from the ends. The resulting short sequences can be aligned to a reference genome to determine the expression state of a transcript in the sample. RNA-seq is a quantitative approach, which determines RNA expression levels in a more accurate and less expensive manner than microarrays. Additionally, the transcriptome of an organism can be sequenced during RNA-seq without having a genome available, it has low background signal and is highly reproducible (Nagalakshmi et al. 2008, Cloonan et al. 2008, Westermann et al. 2012, Wang et al. 2010). However, this technique also presents some disadvantages: during the generation of the cDNA library, a fragmentation step of the RNA is needed and this tends to create biases. Since some of the short reads after sequencing might be identical, they could be the result of RNA abundance or PCR artifacts. Furthermore, the large amount of data demands efficient bioinformatic tools for accurate interpretation (Wang et al. 2010). This technique has been suggested to be used as a so-called “dual RNA-seq”, where the transcriptomes of two symbiotic organisms are analysed in parallel, without the separation of the organism. This idea arises in order to provide new ways to determine virulence factors *in vivo* (Westermann et al. 2012).

1.5.3. Proteomics

Proteomics techniques reveal the entire set of proteins expressed in an organism in a specific state (Cox & Mann 2011). Proteomics provides information on the expressed proteins and not only on the presence of the genes. The differences in protein expression can be evaluated under different conditions. However, to allow for better identification of proteins, genome information should be available (Berlec 2012). Proteomics includes

protein extraction via separation of proteins, their fragmentation into peptides, generation of peptide profile and identification of the protein (Altelaar et al. 2013, Cox & Mann 2011). Several techniques are available in proteomics including reversed-phase liquid chromatography coupled to mass spectrometry (LC-MS). In this approach the peptide mixture is separated in a gradient of aqueous/organic solvent during high-performance liquid chromatography (HPLC) coupled by mass spectrometry (MS). The resulting MS spectra are compared to database search engines and the peptide sequence and the protein is identified (Altelaar et al. 2013, Cox & Mann 2011). Similarly, the protein sample can be separated using one-dimensional or two-dimensional gel electrophoresis (2-DE), excision of protein and in-gel digestion into peptides followed by MS identification (Cox & Mann 2011).

In 2-DE (**Figure 16**) (Rabilloud et al. 2009) the proteins present in the samples are separated in the first dimension by their isolectric points in gel strips with immobilized pH gradients. During the second dimension, the proteins are separated based on their molecular weights in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Ong & Pandey 2001, Trevors et al. 2012). The gels can be stained with silver nitrate or Coomassie Blue, proteins spots are excised then proteolytically digested (Trevors et al. 2012) and analyzed by MS. 2-DE present some disadvantages, proteins with extreme basic or acidic isoelectric points or large or small molecular weight are lost during the analysis. In addition, low concentrated as well as lipophilic proteins are excluded. Furthermore, the reproducibility can be difficult to achieve, normally there are small number of differences between replicates (Ong & Pandey 2001, Siggins et al. 2012),

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has become a widespread analytical tool for proteins, peptides and other bio-molecules, as well as for bacterial identification (Bonk & Humeny 2001, Lay 2001, Emami et al. 2012, Mazzeo et al. 2006). In summary, the sample is co-crystallized with a large molar excess of a matrix compound, such as α-cyano-4-hydroxycinnamic acid (HCCA), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), or 2,5-dihydroxybenzoic acid. Laser radiation of the sample-matrix mixture results in vaporization and thus the sample reaches a high energy state. This energy is transferred from the matrix to the sample molecules, thus the sample is ionizated and desorpted. After ionization and desorption, the ions are accelerated in a magnet-surrounded electric

field towards a detector. Because these ions have the same energy but different masses, they will reach the detector at different times (Time of flight). Smaller ions reach the detector first because of their greater velocity while the larger ions take longer owing to their larger masses. Subsequently, the time of flight is converted to a mass-to-charge ratio and a mass spectrum is obtained (Lewis et al. 2000, Bonk & Humeny 2001). Protein identification by MALDI-TOF is done after the protein samples have been treated with proteolytic enzymes generating characteristic peptide fragments. These enzymes specifically cleave the protein at certain amino acid residues in the sequence. The different peptide fragments generated from the digestion result in a specific peptide pattern. These peptide patterns can be used as fingerprints, which will be compared with known protein sequences from genome sequence information by computer programs and collected in databases (Lewis et al. 2000, Bonk & Humeny 2001). MALDI-TOF-MS is a highly sensitive technique that is used also to study mixtures of peptides and proteins. This technique exhibits a certain level of tolerance of to buffer or salt contaminations in the sample. In comparison to other methods, low amount of sample are needed for MALDI-TOF, which has short measure times (Aitken 2005).

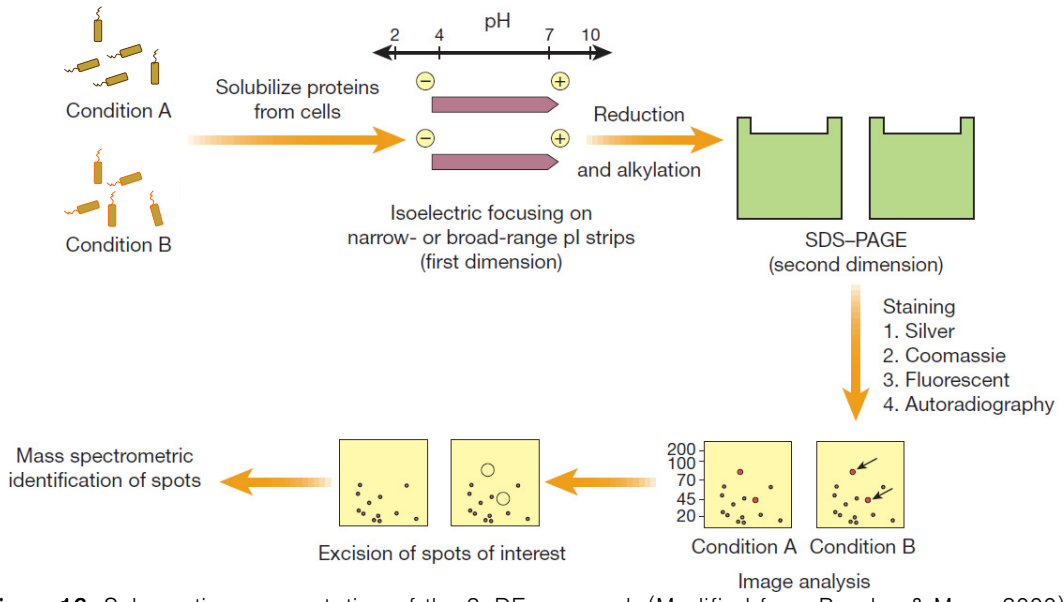

Figure 16. Schematic representation of the 2-DE approach (Modified from Pandey & Mann 2000).

Table 1. Summary of advantages and disadvantages of the methods for the analysis of differential gene expression during cell-to-cell interactions.

	Promoter trap techniques	Microarrays	Next generation sequencing-based transcriptomics	Proteomics (2-DE + MALDI-TOF-MS)
Cost	Low, standard molecular techniques are used	High, equipment is expensive and production of microarrays	Intermediate, less expensive than microarrays but more than promoter trapping or proteomics	Low, equipment is expensive but a long term the costs are lower
Genome availability	Not needed	Needed	Not needed	Not needed
RNA isolation biases	−	+	+	−
Bacterial genetic accessibility	+	−	−	−
Isolate weakly expressed or repressed proteins	−	+	+	−
Reproducibility	+	−	+	−
Sensitiveness	+	+	+	−
Sequencing biases	−	−	+	−
Detection of low abundance transcripts		−	+	
cDNA library generation biases	−	+	+	−
Data handling/analysis complexity	Low	Low, analysis of signal intensities	High, complex annotations and sorting of many sequence reads	Low
Post-translational modifications	Cannot be determined	Cannot be determined	Cannot be determined	Determined
Stability of the molecule use	DNA (very stable)	RNA (not stable)	RNA (not stable)	Proteins (not stable)

2. AIMS OF THIS STUDY

Our molecular knowledge on the bacterial model system, *M. adhaerens* HP15, has increased in the last years and some of the diatom interaction-relevant genes were studied. Specific genes required for and therefore induced during the interaction of this organism with the diatom *T. weissflogii* are, however, still remain unknown. Therefore, the major objective of the thesis was the identification of diatom contact-induced genes in *M. adhaerens* HP15 by two alternative approaches. Firstly, identification of *M. adhaerens* HP15 genes required for the interaction with the diatom by *In vivo* expression technology (IVET). Secondly, identification of *M. adhaerens* HP15 proteins expressed during the interaction with the diatom by comparison of protein profiles and identification of relevant proteins by MALDI-TOF-MS.

In order to implement the IVET screening in *M. adhaerens* HP15, first the LacZ reporter gene expression in this bacterium had to be tested; this experiment was carried out in the context of generating a genetic accessible tool box for *M. adhaerens* HP15.

An additional aim was the description of the tight adherence (*tad*) locus present in the HP15-specific plasmid pHP187 and determination of its role in motility and biofilm formation *in vitro*; as well as during the attachment to *T. weissflogii*.

3. RESULTS

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

3.1. Development of a genetic system for *Marinobacter adhaerens* HP15 involved in marine aggregate formation by interacting with diatom cell

Eva C. Sonnenschein, Astrid Gärdes, Shalin Seebah, Ingrid Torres-Monroy, Hans-Peter Grossart, and Matthias S. Ullrich

(Published in the Journal of Microbiological Methods (2011) 87(2): 97-107)

3.2. Identification of bacterial genes expressed during diatom-bacteria interactions

Ingrid Torres-Monroy, and Matthias S. Ullrich

(To be submitted)

3.3. Identification of *Marinobacter adhaerens* HP15 proteins expressed in response to presence of the diatom *Thalassiosira weissflogii*

Ingrid Torres-Monroy, Antje Stahl, and Matthias S. Ullrich

(To be submitted)

3.4. Identification and possible function of the plasmid-borne *tad* locus in the diatom-associated bacterium *Marinobacter adhaerens* HP15

Ingrid Torres-Monroy, Ania T. Deutscher, and Matthias S. Ullrich

(In preparation)

3.1. Development of a genetic system for *Marinobacter adhaerens* HP15 involved in marine aggregate formation by interacting with diatom cells

(The following manuscript was published in the Journal of Microbiological Methods (2011) 87(2): 97–107)

Development of a genetic system for *Marinobacter adhaerens* HP15 involved in marine aggregate formation by interacting with diatom cells

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Key words: *Marinobacter*, marine aggregates, genetic toolbox, mutagenesis, bacterial motility

Running title: Genetic system for *Marinobacter adhaerens* HP15

ABSTRACT

Diatom aggregation is substantial for organic carbon flux from the photic zone to deeper waters. Many heterotrophic bacteria ubiquitously found in diverse marine environments interact with marine algae and thus impact organic matter and energy cycling in the ocean. In particular, *Marinobacter adhaerens* HP15 induces aggregate formation while interacting with the diatom, *Thalassiosira weissflogii*. To study this effect at the molecular level, a genetic tool system was developed for strain HP15. The antibiotics susceptibility spectrum of this organism was determined and electroporation and conjugation protocols were established. Among various plasmids of different incompatibility groups, only two were shown to replicate in *M. adhaerens*. 1.4 x 10^{-3} transconjugants per recipient were obtained for a broad-host-range vector. Electroporation efficiency corresponded to 1.1 x 10^{5} CFU per μg of DNA. Transposon and gene-specific mutageneses were conducted for flagellum biosynthetic genes. Mutant phenotypes were confirmed by swimming assay and microscopy. Successful expression of two reporter genes in strain HP15 revealed useful tools for gene expression analyses, which will allow studying diverse bacteria-algae interactions at the molecular level and hence to gain a mechanistic understanding of micro-scale processes underlying ocean basin-scale processes. This study is the first report for the genetic manipulation of a Marinobacter species which specifically interacts with marine diatoms and serves as model to additionally analyze various previously reported *Marinobacter*-algae interactions in depth.

INTRODUCTION

Marine 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 marine pelagic systems (Grossart et al. 2006a, Sapp et al. 2008, Geng & Belas 2010). Besides their role in degradation of organic carbon and re-mineralization of nutrients (Cole 1982), these bacteria promote aggregation of phytoplankton cells (Decho 1990) and are thus important for the biological carbon pump (Longhurst & Harrison 1989). Understanding their impact during the interaction with micro-algae is essential to gain knowledge about the ecological relevance of these bacteria on the growth of algae in natural habitats. Bacteria interacting with algal cells might feed on them or their products, or support their growth by re-mineralization of nutrients (Grossart & Simon 2007). Since various scenarios can be envisioned, it remained to be determined whether bacteria enhancing aggregate formation inhibit or promote the metabolism and growth of algae and how they accomplish that. Most previous studies focused on bacterial communities associated with phytoplankton at the ecological level (Grossart et al. 2006b, Sapp et al. 2008), which did not allow to distinguish between the algal and bacterial contribution to specific ecosystem processes. Consequently, very little is known about the genetic characteristics and functional strategies that algae-associated bacteria have adopted to cope with environmental parameters and phytoplankton cells.

The genus *Marinobacter* is one of the most ubiquitous in the oceans and assumed to significantly impact various biogeochemical cycles (Singer et al. 2011, Gauthier et al. 1992, Rotani et al. 2003, Gorshkova et al. 2003). Due to their high functional diversity, different *Marinobacter* species have gained intense attention by the research community. Members of the *Marinobacter* genus were frequently isolated from algal samples, corroborating the hypothesis that several species of *Marinobacter* are frequently associated with phytoplankton (Green et al. 2006, Amin et al. 2009, Alavi et al. 2001, Hold et al. 2001, Gärdes et al. 2011). Genome data of algae-associated *Marinobacter* species suggested tight relationships to their algal partners since a number of genes coding for proteins and secretion systems typical for bacterial pathogens or symbionts have been identified in *M. algicola* DG893 (Amin et al. 2009) and *M. adhaernes* HP15

(Gärdes et al. 2010) as well as in genomes of other algae-associated bacteria (Worden et al. 2006).

For an in-depth molecular analysis of diatom-bacteria interactions and for determining its actual nature and mechanism(s), a bilateral model system consisting of the unicellular diatom, *Thalassiosira weissflogii*, and the bacterial strain, HP15, was established recently (Gärdes et al. 2011, Kaeppel et al. 2011). *M. adhaerens* HP15 had been isolated from marine particles taken from surface water samples of the German Wadden Sea (Grossart et al. 2004). Close and specific interaction of *M. adhaerens* HP15 and *T. weissflogii* was demonstrated *in vitro* by attachment and aggregate formation assays as well as determination of transparent exopolymer particle (TEP) production concluding that strain HP15 plays an important role in *T. weissflogii* aggregation dynamics (Gärdes et al. 2011). Interestingly, this type of interaction required photosynthetic activity of diatom cells and led to improved growth of both interaction partners. This prompted the cautious assumption that the interaction might be symbiotic and not purely saprophytic. Hence, the actual nature of this symbiosis still remains to be elucidated. The genome sequence of *M. adhaerens* HP15 was determined exhibiting interesting features known from other gram-negative bacteria interacting with eukaryotic hosts (Gärdes et al. 2010). *M. adhaerens* HP15 was taxonomically established as a novel member of the *Marinobacter* genus (Kaeppel et al. 2011). Other members of the genus *Marinobacter* were found in various marine habitats (Gauthier et al. 1992, Rotani et al. 2003, 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).

Genetic studies with *M. adhaerens* HP15 have the potential to dissect cell-to-cell interactions of this organism as well as other *Marinobacter* species with phytoplankton cells at the molecular level. This might lead to the identification of novel processes of sensing, cellular communication, and nutrient exchange and might thus help us to better understand globally important processes and biogeochemical cycles such as marine aggregate formation. As previously shown for other environmentally important bacterial species (Bakersmans et al. 2009, Piekarski et al. 2009, Wöhlbrand & Rabus 2008), establishment of the genetic accessibility of individual strains represents the pivotal base for detailed and accelerated research on these organisms.

Herein, for the first time the genetic accessibility of a *Marinobacter* species was comprehensively analyzed. The 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 gene-specific mutagenesis using homologous recombination. Expression of reporter genes such as enhanced green fluorescent protein and β-galactosidase was successfully demonstrated for strain HP15.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The bacterial strains and plasmids used are listed in **Table 1**. Oligonucleotide primers used are listed in **Table 2**. *M. adhaerens* HP15 was isolated from marine particles collected from surface waters of the German Bight (Grossart et al. 2004). *Marinobacter* cells were cultivated in marine broth (MB) medium (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 7.4). For electroporation, cells were cultivated on MB agar medium overnight at 37°C. *Escherichia coli* strains were maintained in Luria-Bertani (LB) agar medium. For conjugation, *M. adhaerens* HP15 cells were grown in 100 ml MB liquid culture at 250 rpm overnight at 28°C. The donor strain *E. coli* ST18 was grown in LB medium containing 50 μg ml^{-1} 5-aminolevulinic acid (ALA). The following antibiotics were added to media when needed (in μg ml^{-1}): chloramphenicol, 25; kanamycin, 500; and ampicillin, 50.

To analyze the antibiotics susceptibility as selection marker for transformation, strain HP15 was grown in MB medium at 28°C to an OD_{600} of 1, and 20 μl of cell suspensions were spotted on MB agar medium containing various concentrations of ampicillin, chloramphenicol, gentamycin, kanamycin, spectinomycin, or tetracycline. The MICs for these antibiotics in MB were determined by the micro-dilution assay as described previously (Burse et al. 2004).

DNA procedures

Plasmids were isolated using the 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 NucleoSpin® Extract kit (Macherey-Nagel). Preparation of genomic DNA was conducted with NucleoSpin® Tissue kit (Macherey-Nagel).

Plasmid conjugation

Recombinant plasmids were introduced to the recipient *M. adhaerens* HP15 by biparental conjugation with *E. coli* ST18 as a donor. Additionally, triparental mating with the plasmid-mobilizing helper strain *E. coli* HB101 (pRK2013) was performed. Bacterial strains were grown as described above overnight and the OD_{600} was adjusted to 0.1 (~3 x 10^7 cells ml^{-1}). 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 re-suspended in 500 µl of LB medium supplemented with ALA, spotted on LB agar plates supplemented with ALA, and incubated for 24, 48, or 72 h at 28°C. After incubation, the cell mass was scraped off the agar plates and re-suspended in MB medium for subsequent dilution plating. Transconjugants were selected on MB agar supplemented with chloramphenicol after incubation for 2–5 days at 28°C. All experiments were conducted in triplicates.

Electroporation

Electro-competent *M. adhaerens* HP15 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 re-suspended in 1 ml of pre-cooled 300 mM sucrose and washed two times with 1 ml of cold 300 mM sucrose using centrifugations at 13,000 rpm and 4°C for 3 min. The final pellet was re-suspended in 200 µl of 300 mM sucrose to obtain a dense suspension (OD_{600} of ~30). 50 µl of cell suspension was mixed with 0.3 to 1.5 µg of plasmid DNA for electroporation (cuvette width 0.2 cm, resistance 200 Ω, capacitance 25 µF, pulse 2.5 kV for ~5 ms). Immediately after the pulse, 950 ml of Super Optimal Broth with Catabolite repression medium (SOC) medium was added to the cuvette. The cell suspension was transferred to a sterile 1.5 ml tube and incubated by shaking for 15–20 h at 37°C. 50 to 400 µl of suspensions were subsequently plated on MB agar medium supplemented with the appropriate antibiotics and incubated at 37°C. Electro-transformation of strain *M. adhaerens* HP15 was tested

in triplicates with the following plasmids: pBBR1MCS, pSUP106, pWeb-Cm, pGEM.Km, pEx18Tc, pK18mob, pLAFR3, pKnock-Cm, pPH1JI, pRK415, and pSU18 (**Table 3**).

Transposon mutagenesis

Plasmids pBK-miniTn7-gfp1, pEP4351, and pRL27 (**Table 1**) containing different transposons were tested for transposon mutagenesis efficiency in *M. adhaerens* HP15 using electroporation. Resulting mutant colonies were grown in MB medium supplemented with kanamycin in 96-well microtiter plates overnight, re-suspended in 15% glycerol, and stored at -80°C. For screening of flagellum-deficient mutants, mutant cells were grown in MB medium containing kanamycin and picked on 10-fold diluted MB soft agar plates (0.3% agar). Swimming-deficient mutants were identified by lack of the typical motility pattern of the *M. adhaerens* HP15 wild-type. The genomic DNA of promising mutants was extracted, treated with the restriction enzyme *Nco*I, re-ligated with T7 DNA ligase, and introduced to *E. coli* DH5α λ-pir by electroporation. Nucleotide sequencing of transposon-flanking regions was conducted with the primers TnF and TnR. The obtained sequence data were aligned with the GenBank sequence database entries using BlastX (Altschul et al. 1990).

Gene-specific mutagenesis by homologous recombination

As a candidate gene for gene-specific mutagenesis, the flagellin-encoding gene, *fliC*, was selected using the *M. adhaerens* HP15 genome sequence (GenBank accession no. CP001978) (Gärdes et al. 2010), GenDB 2.2, and BlastN analysis (Altschul et al. 1990). 1,002 bp upstream and 1,236 bp downstream flanking regions of *fliC* were amplified using the primer pairs FliCupF/FliCupR and FliCdownF/FliCdownR, respectively. Both fragments were sub-cloned to vector pGEM®-T Easy (Promega, Mannheim, Germany) resulting in plasmids, pAS3 and pAS4. A chloramphenicol resistance cassette was excised from pFCM1 with a *Kpn*I restriction digest and inserted into *Kpn*I-treated pAS3 yielding plasmid pAS5. Plasmid pAS5 was treated with the restriction enzymes *Bam*HI and *Spe*I, the fragment was purified, and ligated into the *Bam*HI-*Spe*I-treated plasmid pAS4, resulting in plasmid pAS6, which contained the 6,338bp knock-out fragment consisting of the chloramphenicol resistance gene flanked by *fliC* upstream and downstream fragments. The knock-out fragment was excised with enzyme *Eco*RI and ligated to the *Eco*RI-treated suicide vectors pEX18Ap and pK19mobsacB, respectively,

generating pAS7 and pAS8 as mutagenic constructs. After biparental conjugation and subsequent homologous recombination, correct insertion of knock-out fragments in the *M. adhaerens* HP15 chromosome by double crossover was confirmed by antibiotics selection and PCR with primer pairs FliCF/FliCR and CmF/CmR, respectively.

Determination of mutant phenotype by swimming assay and transmission electron microscopy

Flagellum-deficient mutants and the wild-type strain HP15 were grown overnight in MB medium containing – when needed – kanamycin or chloramphenicol, respectively, inoculated to 10-fold diluted MB soft agar plates (0.3% agar) with a sterile toothpick, and incubated for 48 h. For transmission electron microscopy, cells were grown in MB medium as described above. A 300-µm-mesh carbon-coated copper grid (Plano, 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).

Expression of enhanced green fluorescent protein and β-galactosidase in *M. adhaerens* HP15

Plasmid pBBR.EGFP carries the *egfp* gene encoding enhanced green fluorescent protein in pBBR1MCS downstream of the promoter of *lacZ'*. pBBR.EGFP was introduced to strain HP15 by electroporation. Expression of *egfp* in single cells was visualized using a LSM510 META confocal laser scanning microscope (Zeiss). The wild-type of *M. adhaerens* HP15 carrying the pBBR1MCS vector served as a negative control.

The *E. coli lacZ* gene was amplified from plasmid pMC1871 with primers LacZF and LacZR, each containing a recognition site for *Kpn*I. The resulting 3,057-bp fragment was treated with *Kpn*I and was ligated to *Kpn*I-treated pBBR1MCS in both orientations resulting in plasmids, pITM1 or pITM2. In pITM1, *lacZ* is in opposite direction to the *lacZ'* promoter, whereas in pITM2 it is under the control of the *lacZ'* promoter. Both plasmids were introduced to *M. adhaerens* HP15 via electroporation. Transformants were selected on MB agar plates containing chloramphenicol and X-Gal.

RESULTS

Antibiotics susceptibility of *M. adhaerens* HP15

Growth of *M. adhaerens* HP15 was inhibited by a number of commonly used antibiotics (**Table 3**). Minimal inhibitory concentrations (MIC) were generally higher on agar than those observed in liquid medium. The highest susceptibility of strain HP15 on agar medium with MICs of 25 μg ml^{-1} was observed for ampicillin and chloramphenicol, the later one being further used as selection marker for transformation. Furthermore, dense *M. adhaerens* HP15 cell suspensions with an optical density at 600 nm (OD_{600}) of ~30 were plated on MB agar plates supplemented with either 25 μg ml^{-1} of chloramphenicol and ampicillin, respectively, or with 100 μg ml^{-1} of kanamycin, respectively. Not a single spontaneously resistant colony could be obtained (data not shown) indicating that chloramphenicol, ampicillin, and kanamycin are suitable resistance markers for strain HP15.

Transformation efficiency and expression of reporter genes

From various vectors tested, only plasmids pBBR1MCS and pSUP106 were found to replicate in *M. adhaerens* HP15. Other plasmids, such as pWEB-Cm, pGEM-Km, pLAFR3, pPH1JI, pRK415, and pSU18 (**Table 1**) could not be transformed or did not replicate in strain HP15. Highest conjugation efficiencies were obtained via biparental mating at a donor-to-recipient ratio of 1:2 and after 24 h of mating time (**Table 4**). For plasmid pBBR1MCS, 1.4 x 10^{-3} transconjugants per number of recipients and for plasmid pSUP106 2.7 x 10^{-4} transconjugants per number of recipients were obtained. 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 corresponded to 1.1 x 10^{5} CFU μg^{-1} DNA for pBBR1MCS and 1.6 x 10^{3} CFU μg^{-1} DNA for pSUP106 (**Table 4**).

When plasmid pBBR.EGFP carrying the *egfp* gene encoding enhanced green fluorescent protein was introduced to strain HP15, transformants exhibited fluorescence when excited at a wavelength of 488 nm, thus demonstrating that *egfp* was expressed (**Figure 1A**). In contrast, no fluorescence was observed for strain HP15 carrying vector pBBR1MCS (**Figure 1B**) suggesting that *egfp* is a suitable reporter gene for this bacterium.

Colonies of *M. adhaerens* HP15 wild-type were white-brownish on MB agar. *M. adhaerens* HP15 transformants harboring plasmid pITM1, which contains the ß-galactosidase gene *lacZ* in opposite direction to the Plac promoter, were white-brownish on MB agar containing X-Gal similar to the wild-type (**Figure 2B**). However, transformants containing pITM2, which harbors *lacZ* under control of the Plac promoter, grew in form of blue-colored colonies on MB agar containing X-Gal thus expressing the reporter gene *lacZ* (**Figure 2A**). Next, plasmid pITM2 was isolated from blue transformants of strain HP15. Multiple restriction enzyme treatments of this plasmid extract proved a correction orientation of *lacZ* in the recovered plasmid.

Transposon and gene-specific mutagenesis of *M. adhaerens* HP15

Transposon-carrying plasmids pBK-miniTn7-gfp1, pEP4351, and pRL27 were assayed for their potential to be used for transposon insertion mutagenesis of *M. adhaerens* HP15 via electroporation. Transformation with pBK-miniTn7-gfp1 and pEP4351 did not yield in transposon mutants. In contrast, transformation of strain HP15 with plasmid pRL27 carrying transposon Tn5 resulted in an efficiency of 6.8×10^{2} CFU μg^{-1} DNA (1.8×10^{-7} mutants per number of recipients). A group of 18 randomly chosen mutants was subjected to cloning of the transposon insertion regions. Subsequent nucleotide sequencing of the transposon-flanking regions revealed 18 distinct and unique insertion sites (data not shown) thus confirming the randomness of transposon insertions. Testing a total of 768 transposon mutants by soft agar swimming assay revealed two swimming-deficient mutants. For these *M. adhaerens* HP15 mutants, nucleotide sequencing of the transposon-flanking DNA regions revealed that their phenotype correlated to individual transposon insertions in the motility-associated genes *fliG* and *fliR* (data not shown). A mutant with the transposon insertion in *fliG* termed HP15-*fliG*::tn5 was used for further phenotypic analysis.

Gene-specific mutagenesis was conducted by introducing the suicide plasmids pAS7 and pAS8, respectively, harboring the *fliC* mutagenic construct by biparental conjugation. Transconjugants were selected on MB agar plates supplemented with chloramphenicol, and double crossover of the chloramphenicol resistance cassette in the *fliC* gene of strain HP15 was demonstrated by PCR with primers FliCF and FliCR yielding the expected 1,734-bp fragment. In contrast, PCR with the *M. adhaerens* HP15 wild-

type using the same primer set yielded an intact *fliC* amplification of 2,487 bp. Absence of plasmids pAS7 and pAS8, respectively, was confirmed by lack of recombinant plasmids in extractions from the transconjugants (data not shown). One of the mutants was designated HP15-Δ*fliC*. The results confirmed a successful gene-specific mutagenesis using homologous recombination in *M. adhaerens* HP15. Conjugation of the respective vectors, pEX18Ap and pKmobsaB, without insert DNA homologous to genes of strain HP15 did not yield antibiotics-resistant HP15 transformants.

Phenotypic characterization of *M. adhaerens* HP15 mutants

In contrast to the HP15 wild-type, motility-deficient mutants HP15-*ΔfliC* and HP15-*fliG*::tn5 were not motile on soft agar demonstrating that genes *fliC* and *fliG* were essential for flagellar movement of HP15 (**Figure 3**). Furthermore, transmission electron microscopy revealed that HP15 wild-type possessed one polar flagellum (**Figure 4A**) while mutant HP15-Δ*fliC* did not produce a visible flagellum but retained the flagellar hook (**Figure 4B**) demonstrating the accurate gene-specific mutation. In contrast, transposon insertion in the hook-associated *fliG* gene led to a total loss of the flagellum as seen for mutant HP15-*fliG*::tn5 (**Figure 4C**).

DISCUSSION

In contrast to well-established bacterial model organisms 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 processes in the oceans, genetically accessible model systems are needed. The current study was part of a concerted action, in which the pivotal role of *M. adhaerens* in marine aggregate formation was demonstrated (Gärdes et al. 2011), its genome analyzed (Gärdes et al. 2010), and its taxonomic affiliation as a new species determined (Kaeppel et al. 2011). For the first time, we show that a single marine bacterial species being directly and specifically involved in marine aggregate formation (Gärdes et al. 2011) is genetically accessible in terms of transformation, transposon and gene-specific mutagenesis, as well as reporter gene expression.

This study is distinctive from that of Kato et al. (1998), who established a genetic transformation system for algae-lysing *Alteromonas* strains. These bacteria were shown to lyse different species of diatoms including *Thalassiosira* sp. In the future, a comparative functional analysis of algae-aggregating and algae-lysing bacteria based on mutagenic approaches and gene expression analyses might reveal important new insights into the mechanisms of their interactions with diatoms.

The genus of *Marinobacter* is assumed to contribute significantly to different marine biogeochemical cycles (Singer et al. 2011). Various ubiquitously distributed and environmentally prominent representatives of the *Marinobacter* genus have been under research for almost 20 years in terms of their oil-degrading capacity (Gauthier et al. 1992, Yakimov et al. 2007), wax ester production (Rontani et al. 2003), siderophores (Barbeau et al. 2002, Martinez & Butler 2007), particle colonization (Grossart et al. 2003), and interactions with phytoplankton (Jasti et al. 2005, Sher et al. 2011, Gärdes et al. 2011). The currently available genome sequences of four *Marinobacter* species are highly similar to each other (Gärdes et al. 2010, Singer et al. 2011). Consequently, the herein developed genetic tool box for *M. adhaerens* will assist researchers studying specific functional traits in other *Marinobacter* species.

Essential methods to allow molecular analyses of a given bacterium are plasmid transformation techniques, different types of mutagenesis, and reporter gene expression. Herein, plasmid introduction to *M. adhaerens* HP15 by electroporation and conjugation, random and gene-specific mutagenesis, as well as expression of reporter genes were reported as a first proof-of-principle. With the established techniques, it is now possible to identify the particular role of genes and to quantify gene products important for the interaction of this bacterium with diatom cells. In turn, this might lead to the identification of molecular signals and environmental patterns underlying this interaction.

The current study was conducted with a marine diatom-associated γ-proteobacterium and thus is complementary to but also clearly distinctive from very impressive approaches with representatives of the Roseobacter clade of α-proteobacteria, which are living in symbiosis with heterotrophic dinoflagellates, such as *Pfiesteria piscicida* (Miller et al. 2006, Geng et al. 2008, Geng & Belas 2010). On the one hand – to be highly effective – genetic tools and protocols need to be specific and need to be optimized for bacteria phylogenetically belonging to different proteobacterial sections, i.e. α- and γ-proteobacteria (Davidson 2002). On the other hand, our future

molecular analyses of *M. adhaerens* might reveal fundamentally novel mechanisms of the biotrophic interaction of this bacterium with a photosynthetic marine eukaryote, *T. weissflogii*.

The determined antibiotics susceptibility spectrum of *M. adhaerens* HP15 allowed selection of transformants or mutants by antibiotics resistance markers, i.e. chloramphenicol, ampicillin, and kanamycin. The relative low susceptibility of strain HP15 to other antibiotics might be due to the high salt concentration in the used medium as concluded previously for other marine organisms (Piekarski et al. 2009). Resistance to different antibiotics was earlier claimed to be a suitable taxonomic marker for marine bacteria (Gorshkova & Ivanova 2001). Herein obtained data are comparable to those for *M. aquaeolei* (Huu et al. 1999) 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, could not be introduced to strain HP15, or did not allow for the expression of the respective resistance gene. It remains to be analyzed whether the two native plasmids of strain HP15 with molecular sizes of 42 and 187 kb (Gärdes et al. 2010), respectively, possibly interfere with replication of the latter plasmid groups.

The herein obtained electroporation efficiency was comparable to that of the marine γ-proteobacterium *Pseudoalteromonas* (Kurusu et al. 2001) but was lower than that described for *Alteromonas* (Kato et al. 1998). Plasmid conjugation efficiency for strain 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). The reporter genes *egfp* and *lacZ* were introduced *in trans* to strain HP15 and showed a clear phenotypic expression making both genes suitable for *in vivo* labeling and for reporter gene analyses in future studies.

The transposon delivery plasmid pRL27 (Larsen et al. 2002) was used to generate a library of mutants of *M. adhaerens* HP15. Efficiency of mutagenesis was lower than that for the close relative, *Pseudomonas stutzeri* (Larsen et al. 2002). However, it was sufficient to readily generate a library characterized by a high degree of randomness. For

homologous recombination, derivatives of the mobilizable vectors pEX18Ap and pK18mobsacB were used due to their inability to replicate in non-enterobacterial species (Hoang et al. 1998, Schäfer et al. 1994). As expected, conjugation of these vectors without insert DNA homologous to genes of strain HP15 did not yield HP15 transformants indicating that they could be used as suicide vectors.

To demonstrate the ability to knock-out any specific gene, motility of obtained transposon mutants was screened. The flagellum-deficient transposon mutants HP15-*fliG*::tn5 and HP15-*fliR*::tn5, as well as the gene-specific mutant HP15-Δ*fliC* were non-motile in soft agar in contrast to the HP15 wild-type. As expected, in mutant HP15-*fliG*::tn5 the flagellum was not formed at all since this gene is required for the flagellar hook formation as described earlier for *Salmonella enterica* (Thomas et al. 2001). In contrast, mutant HP15-Δ*fliC* exhibited the flagellar hook but was missing the flagellar filament confirming previous data obtained for *Heliobacter pylori* and other bacteria (Macnab 2003, Seong et al. 1999). These results demonstrated that the flagellar filament of *M. adhaerens* HP15 is encoded by a flagellin gene. The flagellum-deficient mutants will next be tested during their interaction with diatoms to study the role of bacterial motility in chemotaxis and attachment.

CONCLUSIONS

An easy-to-work-with and powerful genetic toolbox for *M. adhaerens* HP15 was established, which renders this bacterium a suitable model organism for molecular analysis of diatom-bacteria interactions. This genetic toolbox can be used for other members of the *Marinobacter* clade involved in phytoplankton interactions and oceanic biogeochemical cycles. Herein tested and established methods and procedures will be applied to knock-out and functionally analyze genes involved in i.e. motility, surface attachment, chemotaxis, biofilm formation, as well as nutrient sensing and acquisition. Use of reporter genes will serve in differential gene expression studies and in a currently being established *in vivo* expression technology screen (Slauch et al. 1994) allowing the identification of novel genes important for the biotrophic interaction of *M. adhaerens* with its diatom host. As shown by previous studies, which established genetic systems for other environmentally important bacteria (Bakersmans et al. 2009, Piekarski et al. 2009, Wöhlbrand & Rabus 2008), the current study has built the technical base for intense

future research on a globally important process: bacteria-induced formation of diatom aggregates and thus their sinking behavior in the ocean. Improving our understanding of specific cell-to-cell interactions at the molecular level provides the basis for a mechanistic understanding of the "biological carbon pump" and is crucial to identify specific environmental parameters and cellular factors contributing to or triggering the ecological consequences of a globally changing world.

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Bacterial strains		
Escherichia coli DH5α λpir	Φ80d*lacZ*ΔM15 Δ(*lacZYA*- *argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1/λpir*	Larsen et al. 2002
E. coli ST18	*λpir* Δ*hemA pro thi hsdR*$^+$ Tpr Smr chromosome::RP4-2 Tc::Mu-Kan::Tn7	Thoma & Schobert 2009
E. coli HB101	carrying helper plasmid pRK2013	Figurski & Helinski 1979
Marinobacter adhaerens HP15	wild-type	Grossart et al. 2004
HP15-Δ*fliC*	*fliC* deletion mutant of HP15	This study
HP15-*fliG*::Tn5	transposon insertion mutant in *fliG* of HP15	This study
HP15-*fliR*::Tn5	transposon insertion mutant in *fliR* 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. 1998
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 & Behringer 1984
pRK415	IncP oriT Tet^R	Keen et al. 1988
pSU18	pMB1 Cm^R	Bartolome et al. 1991
pBK-miniTn7-gfp1	pMB1 *mob* Amp^R, miniTn7-*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

pGEM®–T Easy	colE1 *lacZ* Amp^R	Promega, Mannheim, Germany
pFCM1	Amp^R Cm^R	Choi & Schweizer 2005
pK19mobsacB	pMB1 oriT Km^R	Schäfer et al. 1994
pEX18Ap	pMB1 oriT sacB Amp^R	Hoang et al. 1998
pAS3	pGEM®–T Easy containing 1,002 bp upstream *fliC* flanking region of HP15	This study
pAS4	pGEM®–T Easy containing 1,236 bp downstream *fliC* flanking region of HP15	This study
pAS5	FRT sites and Cm^R (1,135 bp) from pFCM1 ligated into *Kpn*I site of pAS3	This study
pAS6	FRT sites, Cm^R and upstream region (2,137 bp) from pAS4 ligated with *Bam*HI/*Spe*I into pAS5	This study
pAS7	knock-out fragment (3,373 bp) from pAS6 ligated with *Eco*RI into pEX18Ap	This study
pAS8	knock-out fragment (3,373 bp) from pAS6 ligated with *Eco*RI into pk18mobsacB	This study
pBBR.EGFP	derivative of pBBR1MCS with insertion of *egfp* by *Cla*I/*Xba*I from pRc/CMV3.EGFP in direction of the *lacZ'* promoter	H. Weingart, Jacobs University Bremen, Germany
pMC1871	*lacZ* Tet^R	Shapira et al. 1983
pITM1	derivative of pBBR1MCS with insertion of *lacZ* by *Kpn*I in opposite direction to the *lacZ'* promoter	This study
pITM2	derivative of pBBR1MCS with insertion of *lacZ* by *Kpn*I under control of the *lacZ'* promoter	This study

Table 2. Oligonucleotide primers used in this study. The underline marks the restriction enzyme recognition sites.

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
LacZF	AGTGGTACCCGTCGTTTTACAACGTC
LacZR	AGTGGTACCTATTATTTTTGACACCA

Table 3. Minimal inhibitory concentration for strain HP15 on 1.2 % MB agar and in MB medium.

Antibiotic	1.2% MB agar (μg ml^{-1})	MB medium (μg ml^{-1})
Ampicillin	25	0.1
Chloramphenicol	25	2
Gentamycin	50	31.3
Kanamycin	100	62.5
Spectinomycin	100	15.6
Tetracycline	250	31.3

Table 4. Conjugation efficiencies for plasmids pBBR1MCS and pSUP106 in Marinobacter adhaerens HP15.

Plasmid	*E. coli* Donor	No. of transconjugants per recipient cell			Recipient to donor ratio	No. of replicates
		24 h	48 h	72 h		
Triparental conjugation						
pBBR1MCS	DH5α	2.0×10^{-4} ($\pm$ 1.6×10^{-6})	5.8×10^{-5} ($\pm$ 2.1×10^{-7})	5.6×10^{-5} ($\pm$ 7.6×10^{-7})	1:3	4
pSUP106	DH5α	2.1×10^{-5} ($\pm$ 1.0×10^{-6})	6.1×10^{-6} ($\pm$ 8.3×10^{-8})	2.5 x 10–5 ($\pm$ 2.3×10^{-6})	1:3	3
Biparental conjugation						
pBBR1MCS	ST18	1.3×10^{-3} ($\pm$ 2.0×10^{-5})	2.2×10^{-4} ($\pm$ 1.7×10^{-5})	6.4×10^{-4} ($\pm$ 3.6×10^{-6})	1:2	2
pSUP106	ST18	2.6×10^{-4} ($\pm$ 1.2×10^{-5})	1.3×10^{-4} ($\pm$ 5.0×10^{-6})	1.2×10^{-5} ($\pm$ 4.1×10^{-6})	1:2	2

Figure 1. Fluorescence microscopy photographs of *M. adhaerens* HP15 harboring the reporter gene-carrying plasmid pBBR.EGFP (A) or the vector pBBR1MCS as control (B) excited at 488 nm.

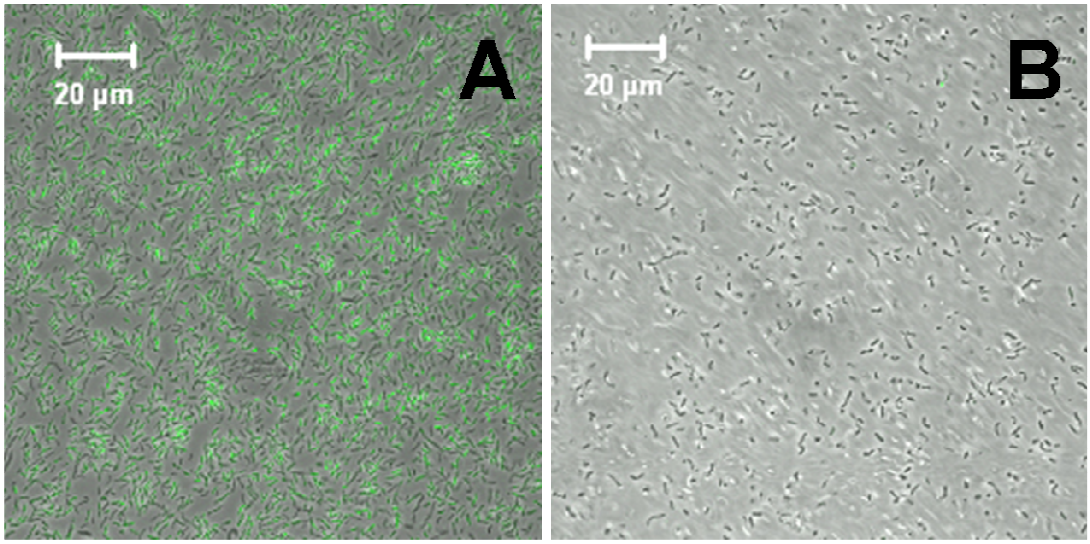


Figure 2. Colony phenotypes of *M. adhaerens* HP15 carrying pITM2 (A) and pITM1 (B) on MB agar supplemented with X-Gal.

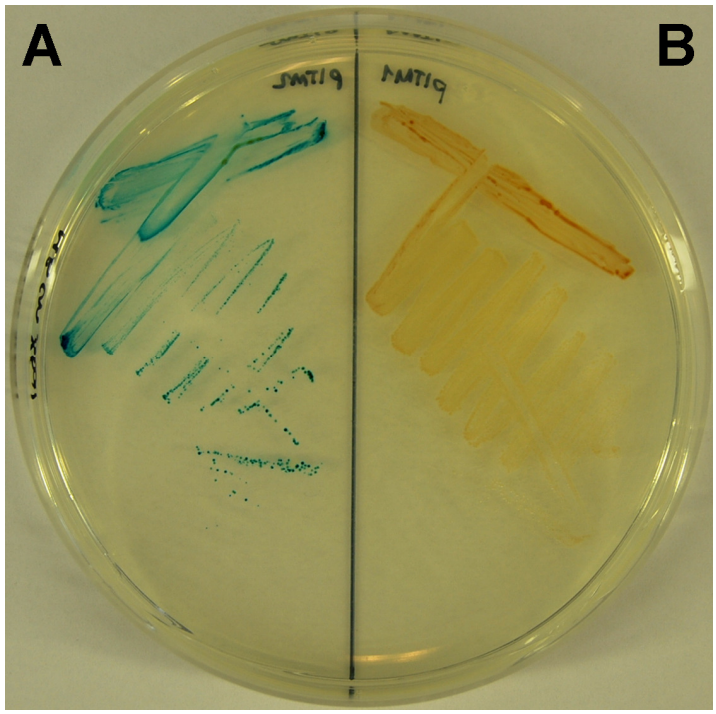


Figure 3. Phenotypic characterization of flagellum-deficient *M. adhaerens* HP15 mutants by 0.3 % soft agar assay after 2 days of incubation: (A) HP15 wild-type; (B) HP15-Δ*fliC*; and (C) HP15-*fliG*::Tn5.

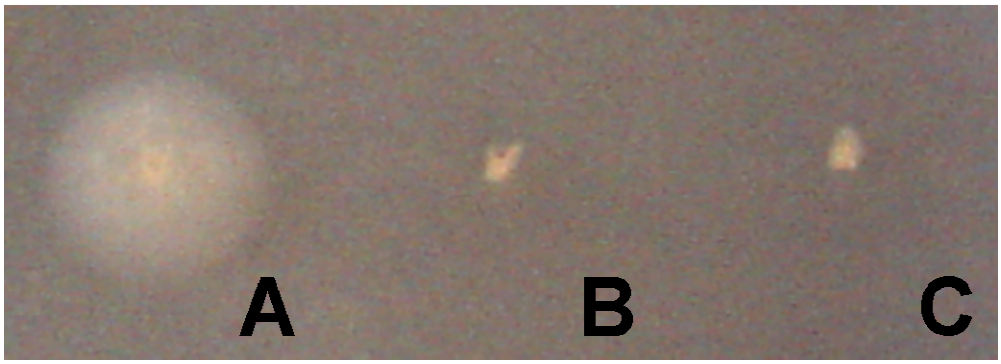
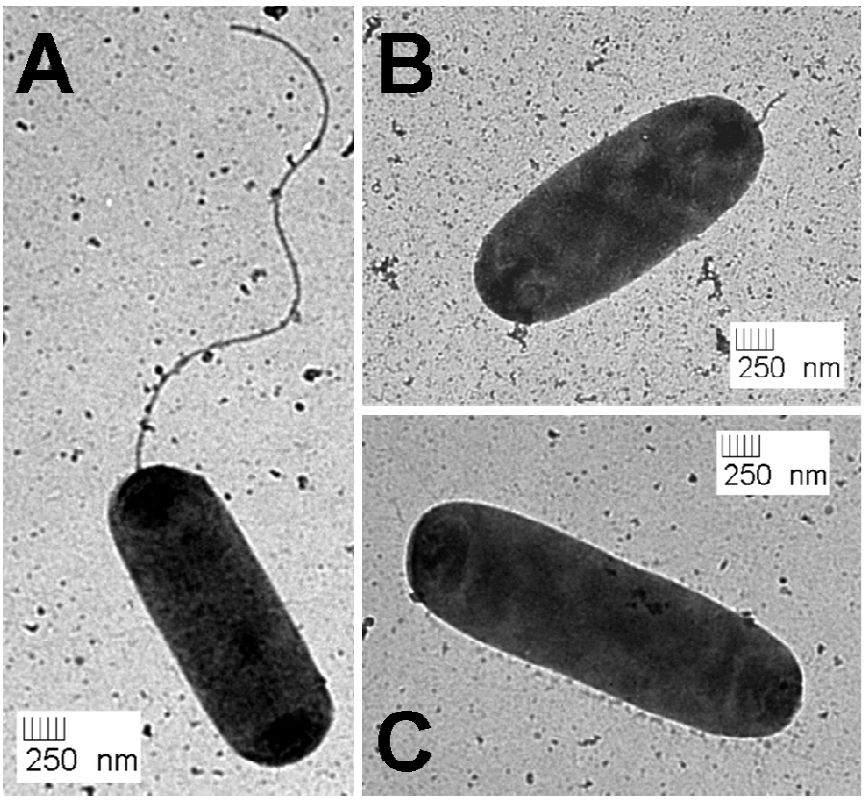


Figure 4. Phenotypic characterization of flagellum-deficient *M. adhaerens* HP15 mutants by transmission electron microscopy: (A) HP15 wild-type showing a full flagellum; (B) HP15-Δ*fliC* carrying the flagellar hook only; and (C) HP15-*fliG*::Tn5 lacking both, flagellar hook and flagellum.



3.2. Identification of bacterial genes expressed during diatom-bacteria interactions

Identification of bacterial genes expressed during diatom-bacteria interactions

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ABSTRACT

Diatom-bacteria interactions play important roles during the aggregate formation leading to the sinking of organic carbon in the oceans. To study diatom-bacteria interactions, a bilateral *in vitro* model system consisting of the diatom *Thalassiosira weissflogii* and the proteobacterium *Marinobacter adhaerens* HP15 had been established. The bacterium was previously shown to attach to *T. weissflogii* cells, to induce transparent exopolymeric particle formation, and to increase marine aggregation formation. Several bacterial genes potentially important during the interaction are currently being investigated. However, genes specifically expressed *in vivo* are still unknown. To identify bacterial genes specifically expressed during this interaction an *In vivo* expression technology (IVET) screen was employed. For that, a promoter-trap vector containing a fusion between a promoterless selection marker gene and a reporter gene was constructed. Construction of a library of plasmids carrying genomic fragments upstream of the fusion and its subsequent transformation into a selection marker mutant allowed the selection of bacterial promoters specifically expressed during the interactions with *T. weissflogii*. Thirty-four genes expressed during the co-cultivation with diatoms but not expressed in culture media were identified. Sequence analyses of these genes showed that they are required for i.e. central intracellular metabolism, cell envelope structure, nutrient scavenging, regulation, chemotaxis, protein secretion, and DNA transfer. These genes may play a role in the interaction between *M. adhaerens* HP15 and *T. weissflogii*.

INTRODUCTION

In the ocean, aggregate formation by living cells, organic matter, fecal pellets, debris, etc, in form of marine snow is an essential mechanism that mediates the sinking of organic carbon to depth (Alldredge & Silver 1988). This process is important for marine biogeochemical cycles and the efficiency of the biological pump (Fowler & Knauer 1986, Jahnke 1996). Diatom–bacteria interactions play an important role during this process by secretion of different extra-cellular polysaccharides, which increase the size of aggregates (Decho 1990, Alldredge et al. 1993, Logan et al. 1995, Passow 2002). To better understand the molecular basis of diatom–bacteria interactions, a bilateral *in vitro* model system was established, consisting of the diatom *Thalassiosira weissflogii* and the marine proteobacterium *Marinobacter adhaerens* HP15. Strain HP15 (Kaeppel et al. 2012) was previously shown to specifically attach to *T. weissflogii* cells, to induce transparent exopolymeric particle (TEPs) production, and to increase aggregation (Gärdes et al. 2011). Its genome was fully sequenced and annotated (Gärdes et al. 2010). Furthermore, *M. adhaerens* HP15 has been shown to be a genetically accessible bacterium and several genes potentially important during the interaction have been investigated by mutagenesis (Sonnenschein et al. 2011). For instance, chemotaxis-deficient mutants were generated to study the chemotactic behavior of *M. adhaerens* HP15 towards *T. weissflogii*; corresponding mutants exhibited a decreased diatom attachment suggesting that chemotaxis plays an important role during the interaction (Sonnenschein et al. 2012). Bacterial genes that are specifically induced during the interaction remained unknown. However, information about such genes might shed light on the nature of the diatom–bacteria interaction and its underlying biochemical and cellular mechanisms.

In this study an *in vivo* expression technology (IVET) screen (Mahan et al. 1993) was applied to identify bacterial genes that are specifically induced during the interaction between *M. adhaerens* HP15 and *T. weissflogii*. IVET is a promoter-trapping technique that allows the selection of genes that are specifically expressed *in vivo* but not under laboratory conditions (Angelichio & Camilli 2002, Rediers et al. 2005). This technique has been applied to study gene expression in a wide range of microorganisms living in different environments (Rediers et al. 2005). For the first time, the current approach applied the IVET strategy to identify bacterial genes induced during diatom–bacteria interactions.

For this, a promoterless selection marker gene of *M. adhaerens* HP15, *pyrB*, which is essential for survival and is encoding aspartate transcarbamoylase (ATCase; EC 2.1.3.2) needed for biosynthesis of pyrimidines (Schurr et al. 1995), was cloned upstream of a promoterless ß-galactosidase gene (*lacZ*) to generate the IVET plasmid. The availability of pyrimidines in many natural environments is limited, which made *pyrB* a successful selection marker in previous studies (Lee & Cooksey 2000). Subsequently, a herein generated auxotrophic *pyrB* mutant of *M. adhaerens* HP15 was complemented with a library of IVET plasmids containing random genomic DNA fragments of *M. adhaerens* HP15 upstream of the *pyrB-lacZ* fusion. The transformant pool was co-inoculated with *T. weissflogii* thereby selecting for constitutive or *in vivo* expressed promoters which allowed *pyrB* expression and therefore survival. After cell recovery, constitutive expression of the *pyrB-lacZ* fusion was tested on culture media containing a pyrimidine source on which LacZ-negative (white) colonies indicated non-constitutive promoter sequences active only during the interaction with the diatom cells (**Figure 1**). A number of interesting genetic loci of *M. adhaerens* HP15 could be identified by this approach and are discussed herein.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used are listed in **Table 1**. *M. adhaerens* HP15 was isolated from particles collected from surface waters of the German Bight (Grossart et al. 2004). This bacterium was grown in marine broth (MB) medium (ZoBell 1941) (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 7.6) at 28°C. MB medium was supplemented with uracil (5 µg ml^{-1}), as a pyrimidine source, for growth of *M. adhaerens* HP15 pyrimidine-deficient auxotrophs. During the *in vitro* screening, MB medium was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (50 µg ml^{-1}) allowing for blue/white colony selection. f/2 GLUT medium was made with f/2 medium (Guillard & Ryther 1962) supplemented with 5 g l^{-1} glutamate and was used as minimal medium to confirm auxotrophy of the *M. adhaerens* HP15 mutant Δ*pyrB*.

Escherichia coli strains were maintained in Luria-Bertani (LB) agar medium supplemented with the appropriate antibiotics. *E. coli* DH5α was used for maintaining the *M. adhaerens* HP15 genomic library generated in the IVET vector. *E. coli* ST18 (Thoma & Schobert 2009) was used as a donor strain during biparental conjugation and was grown in LB medium containing 50 µg/ml 5-aminolevulinic acid (ALA). *E. coli* HB101 (pRK2013) was used as a helper strain during triparental conjugation (Figurski & Helinski 1979). The following antibiotics were added to media when needed (in µg ml^{-1}): chloramphenicol, 25; kanamycin, 500; and ampicillin, 50.

Diatom culture conditions

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, with a 12:12 h photoperiod at 115 µmol photons $m^{-2}s^{-1}$. Diatom cell numbers were determined by cell counts in a Sedgewick Rafter Counting Chamber S50 (SPI Supplies, West Chester, PA). f/2 medium was prepared with pre-filtered (0.2 µm pore size) and autoclaved North-Sea water.

DNA techniques

Plasmid preparation, total DNA extraction, agarose gel electrophoresis, PCR, and other standard DNA techniques were performed as previously described (Sambrook et al. 1989). Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturer (Fermentas, St. Leon-Rot, Germany). DNA sequencing was carried out at Eurofins MWG (Ebersberg, Germany). The DNA sequences analysis and oligonucleotide primers were designed using the Vector NTI® Software 10.3.0 (Invitrogen Corporation, Carlsbad, CA, USA) and DNA sequences analyzed using Basic Local Alignment Search Tool BLAST provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1990) and InterProScan Sequence Search provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Zdobnov & Apweiler 2001). The oligonucleotides used in this study are listed in **Table 2**.

Creation of a pyrimidine auxotrophic mutant of *M. adhaerens* HP15

A gene-specific mutagenesis based on homologous recombination was conducted

to generate a Δ*pyrB* mutant in *M. adhaerens* HP15 according to Hoang et al. (1998). A mutagenic plasmid was constructed in which a chloramphenicol resistance (Cm^R) cassette was flanked by DNA fragments obtained from upstream and downstream regions of the *pyrB* gene as follows: 960 bp upstream and 984 bp downstream of the *pyrB* gene were PCR amplified using the primers pyrB_upF/pyrB_upR and pyrB_down2F/pyrB_down2R, respectively. Both flanking fragments were sub-cloned into the vector pGEM®-T Easy (Promega, Mannheim, Germany) resulting in plasmids, pGEM_pyrBup and pGEM_pyrBdown, respectively. A DNA fragment of 1,129 bp carrying a Cm^R cassette was amplified with the primers cm2R/cm2F from pFCM1. The fragment was treated with *Nde*I and sub-cloned into *Nde*I-treated pGEM_pyrBdown resulting in plasmid pGEM_pyrB_down_cm. From this plasmid, the 2,168-bp fragment containing the downstream region and the Cm^R cassette was obtained by restriction with *Kpn*I and *Nhe*I and ligated into *Kpn*I/*Nhe*I-treated pGEM_pyrB_up, resulting in plasmid pGEM_pyrB_down_cm_up. From this plasmid, a 3,134-bp fragment containing both flanking regions and the Cm^R cassette was then excised with *Hind*III and ligated into the *Hind*III-treated pEX18Tc generating the conjugable mutagenic construct pEX_pyrBcdu, which was transformed into *E. coli* ST18 and subsequently transferred to *M. adhaerens* HP15 by conjugation. For this, both bacterial strains were grown overnight on LB ALA and MB agar plates, respectively, and cell mass was scraped off the agar with the OD_{600} adjusted to 1 (corresponds to ~3×10^9 cells ml^{-1}). Cells of donor and recipient were mixed in a ratio of 1:2, spotted on LB agar plates, and incubated for 24 h at 28°C. The cell mass was then scraped off the agar plates and re-suspended in MB medium for subsequent dilution plating. The resulting mutants were selected on chloramphenicol-containing MB agar plates. A successful double cross-over event for the *ΔpyrB* mutant was confirmed by PCR using the primers mut_pyrBF/mut_pyrBR resulting in the expected fragments for the wild-type (1,205 bp) and the mutated *pyrB* gene (1,410 bp).

To confirm the auxothrophy of Δ*pyrB*, this mutant was grown in f/2 GLUT medium with and without uracil. Subsequently, to determine whether the *pyrB* gene is a suitable selection marker for promoters expressed during the interaction with *T. weissflogii*, mutant Δ*pyrB* and the wild-type were indep co-cultivated with the diatom in the absence of uracil. Bacteria were grown overnight in MB liquid medium at 18°C and the cells were harvested by centrifugation at 4,000 rpm for 15 min. To avoid carrying over of nutrients from MB medium the cells were washed twice with f/2 media. The cell numbers were

adjusted to 1 x 10^5 CFU ml^{-1} and mixed with 10,000 cells ml^{-1} of *T. weissflogii* in exponential growth stage. The cultures were incubated for 6 days at 16°C, 12 h photoperiod at 115 µmol photons $m^{-2}s^{-1}$ and shaking at 50 rpm. Dilutions series were done daily in appropriate media, and CFU numbers were determined.

IVET vector construction

The broad-host-range plasmid pBBR1MCS-4 (Kovach et al. 1995) was used as a backbone to construct the IVET vector (**Figure 2**). The full-sized *E. coli* promoterless *lacZ* gene was amplified from plasmid pMC1871 with the primers LacZF/LacZR (Smirnova & Ullrich 2004). The resulting 3.1-kb PCR fragment was treated with *Kpn*I and ligated into *Kpn*I-treated pBBR1MCS-4 in opposite direction of the vector-borne lac promoter resulting in plasmid pITM3. A second plasmid, pITM4, was generated with the 3.1-kb PCR fragment containing *lacZ* cloned in the same orientation as that of the lac promoter of pBBR1MCS-4. Plasmid pITM3 and pITM4 were transformed into *M. adhaerens* HP15 by biparental conjugation with *E. coli* ST18 as donor strain. Transconjugants were selected on MB agar supplemented with ampicillin and X-Gal after incubation at 28°C for 2-3 days.

Primers pyrBF and pyrBR were designed to amplify the promoterless *pyrBC* locus from *M. adhaerens* HP15. This 2,285-bp PCR product was restricted with *Hin*dIII and *Xho*I and cloned into *Hin*dIII/*Xho*I-treated pITM3 to generate pITM3_pyrB. Plasmid pITM3_pyrB was transformed into *M. adhaerens* HP15 wild-type and Δ*pyrB* mutant by biparental conjugation with *E. coli* ST18, as described above. The transconjugants were selected in MB media containing X-Gal and ampicillin.

Complementation of the *pyrB* mutant

A functional native promoter from the *M. adhaerens* HP15 genome was cloned upstream the *pyrBC-lacZ* fusion in pITM3_pyrB. Such a native promoter was selected by partial restriction of the genomic DNA of *M. adhaerens* HP15 with *Sau3*AI and cloning of a pool of restriction fragments into *Bam*HI-treated plasmid pITM1 (Sonnenschein et al. 2011). The resulting genomic library was transformed into *M. adhaerens* HP15 wild-type by biparental conjugation. Five blue colonies were selected and the region upstream the *pyrBC-lacZ* fusion from one of those clones (pITM1_Sau3A_3) was sequenced thereby

identifying the native promoter. This DNA region was excised from pITM1_Sau3A_3 with *Xba*I and *Hin*dIII and cloned into *Xba*I/*Hin*dIII-treated pITM3_pyrB, resulting in the plasmid pITM3_pyrB_prom, which was transformed into Δ*pyrB M. adhaerens* HP15 by biparental conjugation with *E. coli* ST18. Transconjugants were selected on f/2 GLUT agar supplemented with ampicillin and X-Gal without uracil after incubation at 28°C for 2-3 days.

M. adhaerens HP15 genomic IVET library construction

A genomic IVET library of HP15 was constructed in pITM3_pyrB as follows: total genomic DNA of *M. adhaerens* HP15 was partially digested with *Sau*3AI. DNA fragments with sizes ranging from 0.5 to 1.5 kb were selected and ligated into the *Bam*HI site of dephosphorylated vector pITM3_pyrB. The IVET library was then transformed into *E. coli* DH5α. To verify the randomness of inserts, 24 clones of *E. coli* DH5α were randomly picked and the region upstream the *pyrBC-lacZ* fusion was amplified with the primers T7 and PyrB_out by colony PCR. The IVET library was then transferred into the mutant Δ*pyrB* by triparental conjugation. Briefly, *E. coli* DH5α carrying the IVET library was used as a donor and *E. coli* HB101 (pRK2013) served as a helper strain. Bacterial strains were grown overnight on plates, cell mass was scraped off the agar plates and the OD_{600} was adjusted to 10 (~1 $\times 10^{10}$ CFU ml^{-1}). Cells of donor, helper, and recipient were mixed in a ratio of 1:1:2 and spotted on LB ALA agar plates and incubated for 24 h at 28°C. Cell mass was scraped off the agar plates and re-suspended in MB medium for subsequent dilution plating. The resulting mutants were selected on MB agar plates containing chloramphenicol, ampicillin, uracil and X-Gal. To verify the randomness of plasmids transformed into mutant Δ*pyrB*, 24 clones were randomly picked and subjected to colony PCR as described above.

Screening of promoters expressed during interaction with *T. weissflogii*

For *in vivo* selection of promoters, co-cultivation of mutant Δ*pyrB* transconjugants carrying the IVET library with diatom cells was conducted. Briefly, 10,000 cells ml^{-1} of *T. weissflogii* in exponential growth stage were mixed with the transconjugants (1×10^7 CFU ml^{-1}) in cell culture flasks with a final volume of 35 ml of f/2 media and incubated at room light and temperature conditions for 24 h. Next, 5 ml of the co-cultures were transferred to 30 ml fresh f/2 medium containing *T. weissflogii* in exponential growth

stage (10,000 cells ml^{-1}) and incubated at room temperature for additional 24 h. This procedure was repeated five times in order to enrich for positive clones. After 6 days transconjugants were recovered and subjected to *in vitro* selection. For this, cultures initiated on day 4 and 5 were individually centrifuged at 4,000 rpm for 20 min and dilution series were performed with recovered cells in MB agar plates containing chloramphenicol, ampicillin, uracil and X-Gal at 28°C. After 3-4 days of incubation brownish colonies (non-blue) were selected while blue colonies, indicating a constitutive expression of *lacZ,* were discarded. Brownish (non-blue) colonies represented transconjugants with IVET plasmids carrying an *in vitro* inactive promoter sequence. However, since these transconjugants had survived five passages of diatom co-cultures, their promoter sequences should have been active *in vivo*. A control experiment was performed to observe the growth pattern of the mutant Δ*pyrB* carrying plasmid pITM3_pyrB without any insertion upstream the *pyrBC-lacZ* fusion (empty IVET vector). 10,000 cells ml^{-1} of *T. weissflogii* in exponential growth stage were mixed with Δ*pyrB* transconjugants carrying pITM3_pyrB (1 x 10^5 CFU ml^{-1}) in cell culture flasks in f/2 medium and incubated at room temperature for 24 h for 5 days. Dilution series were carried out daily from the culture and the number of CFU was calculated.

Identification of diatom-induced genes

Brownish colonies of Δ*pyrB* transconjugants were selected after co-inoculation with *T. weissflogii*, and subjected to colony PCR with the primers T7/pyrB_out to check for the presence of an insert upstream of the *pyrBC-lacZ* fusion in pITM3_pyrB. Transconjugants giving individually different PCR products of sizes larger than 570 bp were selected and their PCR product sequences determined. The obtained sequences were analyzed by BLAST and potential promoters regions as well as downstream located genes identified. The positive transconjugants were individually co-inoculated with *T. weissflogii* to confirm their survival in presence of diatoms. For this, bacteria were grown overnight in MB liquid cultures at 18°C, the cells were harvested by centrifugation at 4,000 rpm for 15 min, washed twice with f/2 medium, their concentration adjusted to 1 x 10^5 CFU ml^{-1}, and mixed with 10.000 cells ml^{-1} of *T. weissflogii* in exponential growth stage. The co-cultures were incubated for 8 days at 18°C, with a 2 h photoperiod. Dilutions series were done in appropriate media and CFU numbers were determined.

RESULTS

Pyrimidine auxotrophic mutant of *M. adhaerens* HP15

To verify the potential of the *pyrBC* locus as a selection marker, a *pyrB* deletion mutant in HP15 was created. This mutant should be unable to grow in the absence of a pyrimidine source. A mutagenic construct was transformed into HP15 wild-type and the *pyrB* gene was replaced by a Cm^R cassette via homologous recombination. Seventeen Cm^R clones were obtained, of which 10 had undergone a double crossover of the Cm^R cassette as demonstrated by PCR. Subsequently, the auxotrophy of the Δ*pyrB* mutant was confirmed by testing its growth on agar minimal medium with or without uracil as the sole pyrimidine source. Mutant Δ*pyrB* was not able to grow without uracil thus proving its expected phenotype (data not shown). In addition, growth of the Δ*pyrB* mutant and the *M. adhaerens* HP15 wild-type were indistinguishable from each other when uracil was supplemented indicating that the mutation did not affect bacterial growth in general.

To determine whether lack of *pyrB* expression is a suitable selection criterion during the interaction with *T. weissflogii* and to test whether the diatom provides sufficient pyrimidines to functionally complement the mutant's genotype. The Δ*pyrB* mutant was cultivated together with the diatom in f/2 medium without any uracil. Δ*pyrB* cell numbers dramatically decrease during the incubation time when compared to the growth of the wild-type under the same conditions (**Figure 3**). This result clearly demonstrated that the diatom is not providing any source of pyrimidines proving *pyrB*'s suitability as a selection marker gene for this study.

Generation of the IVET library

The suitability of *lacZ* as an *in vitro* reporter gene in *M. adhaerens* HP15 had previously been described (Sonnenschein et al. 2011) thus this gene was used for the construction of the IVET vector. As *in vivo* selection marker the promoterless *pyrBC* locus of *M. adhaerens* HP15 was chosen since previous studies had shown that the *pyrB* gene alone was not able to complement *pyrB* deletion mutants and that the *pyrC* gene located downstream of *pyrB* was required for this purpose (Schurr et al. 1999, Lee & Cooksey 2000). The Δ*pyrB* mutant carrying the IVET plasmid pITM3_pyrB did not grow in f/2 GLUT medium without uracil. In contrast, this transconjugant grew well in form of brownish

colonies in f/2 GLUT medium supplemented with uracil and X-Gal (data not shown) confirming that the *pyrBC-lacZ* fusion present in pITM3_pyrB is not expressed. Furthermore, the introduction of a functional promoter derived from the genome of *M. adhaerens* HP15 into the IVET plasmid and its subsequent transformation into the Δ*pyrB* mutant allowed its full complementation. Corresponding transformants grew on agar medium without uracil and formed LacZ-expressing (blue) colonies when X-Gal was provided (data not shown). In summary these tested parameters demonstrated that the IVET plasmid and the Δ*pyrB* mutant were suitable tools for the identification of diatom contact-induced genes of *M. adhaerens* HP15.

A genomic library of *M. adhaerens* HP15 was constructed in pITM3_pyrB and transformed into *E. coli* DH5α. The randomness of inserts was confirmed by PCR showing that 85% of the analyzed *E. coli* DH5α transformants had an insert in the IVET plasmid. Importantly, all of the insert sizes were different from each other (data not shown) demonstrating that the IVET library contained a random number of different DNA fragments. After conjugation of the IVET library into mutant Δ*pyrB*, a total of 4.5×10^6 CFU mL^{-1} were obtained, with 40% of the transconjugants forming LacZ-positive blue colonies on agar medium containing X-Gal. To demonstrate the randomness of IVET plasmids in the transconjugant pool, a second round of PCR tests was done. In this case 82% of the analyzed clones had an insert, and all inserts had different sizes (data not shown) suggesting that the roughly one fifth of all transferred IVET plasmids did not contain an insert.

In vivo selection of promoters during interaction with *T. weissflogii*

In order to test whether mutant transconjugants with empty IVET plasmids could survive the *in vivo* selection, a control experiment in which a Δ*pyrB* transconjugant carrying an empty IVET plasmid was grown together with the diatom was conducted first. From an initial inoculum of 1.2×10^5 CFU ml^{-1} only 395 CFU ml^{-1} were recovered after two days and 22 CFU ml^{-1} after three days; however after day 4 no colonies were recovered. These results showed that Δ*pyrB* (pITM3_pyrB) is not able to survive for more that four days in the presence of the diatom.

Consequently, the following main diatom-bacteria co-cultures were performed in such a way that positive clones were enriched while negative clones or clones with empty IVET plasmids were eliminated. To achieve this, co-cultures of Δ*pyrB* mutant carrying the

IVET library with diatom cells were diluted every 24 h by transferring 5 ml of the co-culture into f/2 medium containing fresh diatom cells. Determination of CFUs was carried out daily (data not shown), and randomly chosen brownish colonies were checked for inserts by PCR. All analyzed brownish colonies from 4- or 5-day-old cultures had an insert thus proven the suitability of the selection regime. Co-cultures were repeated several times to increase the number of positive clones.

Potential *in vivo* induced promoters

In total, 148 brownish colonies were obtained which carried a DNA insert, confirmed by PCR, of apparently variable sizes upstream of the *pyrBC-lacZ* fusion in pITM3_pyrB. These transconjugants were subsequently cultivated in f/2 GLUT solid medium without uracil to distinguish promoters solely active during the diatom co-culture from those active *in vitro* but not expressing *lacZ*. Surprisingly, 62 colonies were able to grow under these conditions indicating that their cloned promoter sequences were active irrespective of diatom cell presence. From the remaining 86 transconjugants carrying IVET plasmids with potential diatom-induced promoter, PCR products for 74 were sequenced. Nucleotide sequence analysis indicated that almost one third of the sequences occurred in duplicates so that a total of 45 individual and unique insert DNAs could be obtained. The corresponding IVET plasmids were isolated from the transconjugants and used for further characterization.

Interestingly, from the 45 insert DNAs, 34 inserts were in the same 5'-3' orientation in terms of their downstream reading frames and the *pyrBC-lacZ* fusion of the IVET plasmid, while 11 were found in the opposite orientation suggesting that the later cannot drive the reporter genes. However, this has been a common finding during previous IVET screens (Jackson & Giddens 2006). In addition to the *in vivo* induced clones, a randomly chosen blue colony representing a constitutively expressed *pyrBC-lacZ* phenotype was sequenced and used as a control in the following experiments. The insert of this transconjugant showed sequence identities to the upstream sequence of a gene encoding aldehyde dehydrogenase (Locus tag: HP15_943). Furthermore, 12 brownish colonies able to grow without uracil *in vitro* were also sequenced since they might represent 'false-positive' sequence loci – whose expression is required during but not restricted to the diatom presence.

To confirm that the 45 cloned insert sequences were specifically allowing expression of *pyrBC-lacZ* during the interaction with *T. weissflogii,* the growth patterns of 10 randomly chosen *in vitro* uracil-deficient brown transconjugants (colonies 1, 10, 15, 22, 26, 30, 68, 100, 161 and 168), three brown colonies without uracil deficiency (colonies 28, 90 and 194), and the blue transconjugant were analyzed in individual co-cultures with the diatom in f/2 medium without uracil (**Figure 4**). A transconjugant carrying the empty IVET plasmid was used as a negative control. All 10 positive and the three false-positive transconjugants were able to survive the diatom co-incubation reaching cell numbers comparable to that of the wild-type or the blue transconjugant, which harbors a constitutively expressed promoter sequence. After 4 days of incubation there was a more than three orders of magnitude difference between the growth of the positive clones and the negative control demonstrating that the identified promoter sequences were induced and enabled the Δ*pyrB* mutant to survive during the interaction with *T. weissflogii*.

The false-positive transconjugants had the following characteristics: i) they were not able to express *pyrBC-lacZ* in medium containing uracil (brownish colonies); ii) they must have been able to express *pyrBC-lacZ* under *in vivo* conditions since they grew in presence of the diatom but without uracil; and iii) they were able to grow in minimal media without uracil. These results suggested that the corresponding IVET plasmids could either contain a promoter sequence important during the interaction with the diatom or during uracil deficiency in general, i.e. when pyrimidines are limited and corresponding genes are expressed in response to this limitation.

DNA sequence analysis

M. adhaerens HP15 genes under the control of the promotes identified as specifically induced during interaction with *T. weissflogii* are listed in **Table 3**. The possible functions of these genes were deducted by analysing its amino acid sequence and determine presence of conserved domains or similarities with previous reported proteins. These genes could be grouped depending on their function (Rediers et al. 2005) as follows: genes that encode enzymes required in i) central intracellular metabolism; ii) cell envelope structure and modification; iii) nutrient scavenging; iv) regulation; v) chemotaxis; vi) protein secretion; and vii) DNA transfer.

The majority of identified genetic loci encode for functions in the central cellular metabolism, six of which are associated with conversion or degradation of organic compounds (colonies 15, 19, 26, 136, 238 and 239). Five other genetic loci are involved in different biosynthetic pathways (col. 54, 96, 149, 165 and 207) and two further might play a role in energy metabolism (col. 24 and 30). Finally, two of the identified genetic loci encode for enzymes in intermediary metabolism and pathways such as glycolysis (col. 205 and 211). In addition, four colonies contained cloned promoter sequences associated with genes involved in cell envelope structure and modification (col. 1, 68, 100 and 161). Among the identified genetic loci involved in nutrient scavenging there were genes for uptake of nucleobases (col. 10) and organic acids (col. 130). Additionally, four promoters were associated with genes important for regulation (col. 33, 166, 168 and 204), two in secretion (col. 22 and 32), one in chemotaxis (col. 178), and one in conjugative DNA transfer (col. 188). For the remaining four colonies corresponding associated genetic loci in the *M. adhaerens* HP15 genome did not show putative conserved domains or any significant homologies with any functionally known proteins in the GenBank database (col. 13, 45, 74 and 198).

The functions of genetic loci associated with cloned promoter sequences found in the "false positives" colonies or those where the cloned DNA inserts were in opposite direction to the original gene are listed in **Table 4**. The functions of the genes found associated with "false positives" colonies were involved in central metabolism (amino acid synthesis, lipid/fatty acid degradation and nitrogen metabolism), cell envelope structure, regulation, and unknown functions. For the opposite orientated, the majority were found to be involved in central metabolism, cell envelope biogenesis, nutrient scavenging, or nucleic acid metabolism.

DISCUSSION

Identifying bacterial genes expressed during diatom-bacteria interaction is a fundamental pre-requisite for understanding the functional mechanisms governing bacteria-associated algal aggregation and marine snow formation in the oceans. Therefore, an IVET strategy was employed to identify *M. adhaerens* HP15 genes induced during its interaction with *T. weissflogii*. This strategy allowed the identification of *M. adhaerens* HP15 genes encoding functions such as degradation and biosynthesis of

organic compounds, nutrient scavenging, cell envelope, regulation, secretion and DNA transfer. In addition, conserved hypothetical genes without known functions were found to be expressed during the interaction. With this cultivation-based approach, for the first time and without potential next-generation sequencing biases associated with high-throughput cDNA generation, we suggest the potential importance of some of the following genes for diatom-bacteria interaction.

Central intracellular metabolism

Sixteen of the 34 genes identified as expressed during diatom-bacteria interaction appeared to play a role in central metabolism. This functional group has been previously reported to be the most common class of genes identified by IVET (Rediers et al. 2005). For example, some of these genes were found to be involved in energy metabolism (col. 24, 30, 205, and 211). *M. adhaerens* HP15 might have to adapt to the diatom-based environment and thus might need to undergo physiological changes.

Uptake and degradation of organic compounds was another function found to be potentially important during the interaction of *M. adhaerens* HP15 with *T. weissflogii*. It is known that diatoms secrete organic compounds in the form of dissolved organic carbon (DOC) or extracellular polymeric substances (EPS) (Bhaskar et al. 2005, Grossart et al. 2006), which are then taken up by heterotrophic bacteria (Grossart et al. 2006). Organic compounds released by *T. weissflogii* could be recognized, (partially) degraded, and used as a carbon or nitrogen source by *M. adhaerens* HP15. Accordingly, we identified one genetic locus involved in uptake of organic acids (col. 130). Moreover, genes associated with degradation of organic carbon compounds were also identified: needed for degradation of amino acid (col. 238), protein (col. 239), lipid/fatty acid (col. 26), or diverse compounds degradation processes (col. 15). Some diatom-borne organic compounds degraded by *M. adhaerens* HP15 could then be used as precursors during biosynthesis processes in the bacterial cell. Genes involved in biosyntheses of lipid and fatty acids (col. 207), phospholipids (col. 19 and 136), amino acids (col. 31, 54, and 165), proteins (col. 96 and 149) were also identified.

Nutrient scavenging

Interestingly, a small operon consisting of three genes downstream the promoter sequence found in col. 10 showed high sequence similarities to the *codBA* operon of *E.*

coli previously described to have a putative role in a salvage pathway for nucleobases (Danielsen et al. 1992). The first gene of this operon encoding a purine-cytosine permease (HP15_2770, 57% similarity in the amino acid level to *codB* in *E. coli*) is required for nucleobase transport into the cell (Danielsen et al. 1992); the second gene encoding a purine catabolism regulatory protein (HP15_2771, 31% similarity to *purR*) involved in the induction of the nucleobase degradation pathway (Andersen et al. 1989); and finally a gene encoding cytosine deaminase (HP15_2772, 72% similarity to *codA*) which catalyzes the deamination of cytosine, producing uracil and ammonia (Andersen et al. 1989). The expression of the *codBA* operon is induced by nucleobases starvation (Danielsen et al. 1992). Since *M. adhaerens* HP15 Δ*pyrB* mutant is deficient in pyrimidines biosynthesis and there is no pyrimidine availability during its growth with *T. weissflogii*, this operon might be activated to allow the bacteria to survive under such conditions. Consequently, this operon might not play a role for the diatom-bacteria interaction.

The cloned promoter sequence of col. 130 is associated with a gene encoding a Fe-S oxidoreductase, the last gene in a cluster consisting of four genes in *M. adhaerens* HP15 (**Figure 5A**). This gene cluster resembles the *lutABC* operon (previously called *yvfV*, *yvfW* and *yvbY*) in *Bacillus subtilis* which is involved in lactate catabolism (Chai et al. 2009). In these bacteria, the operon is composed of three genes just as in the *M. adhaerens* HP15 chromosome. However, a fourth gene encoding an iron-sulfur oxidase is sometimes present in or near the *lutABC* operon (Chai et al. 2009). In *M. adhaerens* HP15 the first gene encodes a Fe-S oxidoreductase (HP15_4088) and shared 59% similarity in the amino acid level with *lutA* from *B. subtillis* (YP_006631513.1). The second gene is a 4Fe-4S ferredoxin-type protein (HP15_4089) sharing 59% similarity to *lutB* (YP_006631512.1). The third gene is encoding a conserved hypothetical protein (HP15_4090) with 32% similarity to *lutC* (YP_006631511.1). The *lutABC* operon is regulated by a transcriptional regulator of the GntR family, acting as a repressor when lactate is absent in the media (Chai et al. 2009). The gene encoding for this regulator is present upstream the operon in *B. subtilis*. Interestingly, in the *M. adhaerens* HP15 genome such a regulatory gene is found downstream of the operon and in the opposite orientation (HP15_4087). Even more interestingly, the promoter sequence driving the expression of this GntR-like regulator was also found as one of the *in vivo* expressed genes in col. 33 (**Figure 5A**). It could be speculated that the function of the operon in *M.*

adhaerens HP15 is related with lactate metabolism; however, this function has to be further studied.

Cell envelope structure

Genes involved in cell envelope structure and modification were also found to be expressed during diatom contact. Col. 161 is harboring a promoter sequence upstream of *pyrBC-lacZ* with similarities to *ybgC* (HP15_2039) encoding a Tol-Pal system-associated acyl-CoA thioesterase, the first enzyme in the Tol-Pal system of *E. coli* (Godlewska et al. 2009). The Tol-Pal system consists in five proteins (TolQ, TolR, TolA, TolB and Pal) forming a membrane associated complex, which maintains outer membrane integrity. In *E. coli*, mutations in these genes led to changes in motility and in cell division as well as to the release of periplasmic proteins from the cell (Godlewska et al. 2009). In addition, the operon shows similarity to that coding for the TonB system, which is important for active transport of diverse substrates such as siderophores or vitamin B12 (Moeck & Coulton 1998) and the MotAB proteins in the flagellar motor (Cascales et al. 2001). These previous studies suggest that the system might have different functions or that protein domains found in enzymes encoded by these operons are shared by different processes. During the diatom-bacteria interaction this system could be important for uptake of specific nutrients, motility or integrity of the membrane system.

Additional genes involved in cell envelope structure and modification were found in col. 1, 68, and 100. Col. 1 represents a sequence associated with a gene coding for a putative lipoprotein. Lipoproteins can be structural components of the cell membrane (Nelson & Cox 2000) or enzymes playing roles in nutrient acquisition, cell wall metabolism, cell division, signal transduction and adhesion to host during infection (Nakayama et al. 2012).

Breakdown of diatom products has been previously studied; showing that heterotrophic bacteria associated to phytoplankton or aggregates, mediate the hydrolysis (Arnosti 2011, Sapp et al. 2008, Grossart et al. 2007, Grossart & Simon 1998) or the modification of algal products (Gärdes et al. 2012). Accordingly, the promoter found in col. 68 (**Figure 5B**) is driving the expression of an enzyme belonging to the polysaccharide deacetylases from the carbohydrate esterase family 4 (CE4), which includes chitin deacetylase, acetyl xylan esterase, peptidoglycan deacetylases among others, as defined in the CAZy database (http://www.cazy.org/CE4.html; Cantarel et al.

2008). Two polysaccharide deacetylases (BC1960, NP_831730 and BC3618, NP_833348) were found to be important for protection against host lysozyme in *Bacillus anthracis* (Psylinakis et al. 2005) the same observation was made for a peptidoglycan deacetylase (PgdA) found in *Helicobacter pylori* (Wang et al. 2010). On the other hand, the two polysaccharide deacetylases from *B. anthracis* were active deacetylating both peptidoglycans and chitin substrates, since these two polymers have N-acetylglucosamine residues (Psylinakis et al. 2005). In addition, chitin degradation has been found to be common in the family Vibrionaceae, in this process N-acetylglucosamine-6-phosphate deacetylase, a member of the CE4 family, is involved (Hunt et al. 2008). Diatoms belonging to the genera *Thalassiosira* are known to produce chitin as a component of the cell wall (Durkin et al. 2009, Hert 1979). For this reason, the polysaccharide deacetylases of *M. adhaerens* HP15 identified here might also be involved in the breakdown of chitin or other diatom-borne polysaccharides. Another example is the gene encoding for a glycotransferase found associated to the promoter in col. 100. This enzyme catalyze the synthesis of glycoconjugates, including glycolipids, glycoproteins, and polysaccharides (Kapitonov & Yu 1999, Campbell et al. 1997). Just as for almost any biosynthetic enzyme, the corresponding variant in *M. adhaerens* HP15 might conduct an opposite function, i.e. the breakdown of algal polysaccharides or might be involved in modification of existing polysaccharides as suggested by Gärdes et al. (2012). The role of the genes identified under the control of the promoters in Col. 68 an 100 has to be verified by future studies.

Regulation

M. adhaerens HP15 encounters a novel environment, different from sea water, when interacting with *T. weissflogii*. Thus, the bacteria might need to tightly regulate its gene expression. An example of a regulatory gene was found downstream of the cloned DNA of col. 168 and encodes phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) IIA-like nitrogen-regulatory protein (Cases et al. 2001). The PTS system consists of a group of phosphotransfer proteins involved in the transport of carbohydrates, in chemotaxis towards carbon sources, and in the regulation of other metabolic pathways (Postma et al. 1993). N-Acetylglucosamine/galactosamine PTS IIA, a protein belonging to the PTS system, was previously identified by IVET in *Erwinia chrysanthemi* interacting with tomato plants (Yang et al. 2004). A subsequent mutation of this gene affected the systematic invasion capability of the plant pathogenic

bacterium. This former result indicated that PTS systems might be pivotal for interaction of bacteria with photosynthetic eukaryotic cells.

Two-component signal transduction systems usually consisting of histidine protein kinase and response regulator are used by bacteria to response to different stimuli and to regulate diverse cellular processes (Capra & Laub 2012, Stock et al. 2000). Col. 204 represented a genetic locus encoding a response regulator containing a receiver domain (HP15_3664) and a histidine kinase (HP15_3665). A similar situation was found in the cloned DNA of the "false positive" col. 124. The specific regulatory signals and functions of these gene products have to be elucidated. Likewise, the promoter found in col. 166 is associated to a gene encoding a transcriptional regulator of the LysR family. These regulators are ubiquitous amongst bacteria regulating a diversity of genes involve in virulence, metabolism, quorum sensing, nitrogen fixing, and motility (Maddocks & Oyston 2008). Examples of representatives of this family are *E. coli* LysR regulating lysine metabolism and LrhA regulationg flagella, motility and chemotaxis, and *Rhizobium* spp. NodD regulating nitrogen fixation/symbiosis (Stragier et al. 1983, Lehnen et al. 2002, Schlaman et al. 1992).

Noteworthy, **Tables 3** and **4** show only the first gene found downstream of the promoter region identified in the corresponding IVET plasmid. However, other genes further downstream and potentially co-transcribed could also be specifically expressed by the identified promoter during the diatom-bacteria interaction. This might hold true for colonies 1 and 205 for which the second gene downstream of both promoter sequences might encode for MerR family transcriptional regulators, which mediate responses to environmental stimuli such as heavy metals, drugs, antibiotics, or oxidative stress (Brown et al. 2003). During photosynthesis many reactive oxygen species are released (Asada 2006) and it has been reported that certain bacteria interacting with diatoms detoxify oxygen species (Hünken et al. 2008). *M. adhaerens* HP15 growing with *T. weissflogii* might also face high amounts of reactive oxygen species hence the expression of some proteins countacting oxygen radicals might become necessary.

Protein secretion

The gene identified downstream of the cloned DNA of col. 22 encodes a homologue of Type II secretion system (T2SS) protein C. T2SS is one of several secretory pathways used in bacteria to release proteins into the environment. In *P.*

aeruginosa T2SS genes are arranged as follows: *xcpD, C, D, E, F, G, H, I, J, K, L, M,* and *O*, with *xcpD* in opposite direction to the rest of the gene cluster (Bleves et al. 2010). The T2SS gene encoding protein C found expressed *in vivo* in *M. adhaerens* HP15 is flanked by the T2SS genes for protein N (upstream) and protein D (downstream). Interestingly, no other genes of the T2SS were found in a close proximity suggesting a non-classical gene arrangement (**Figure 5C**). The 'remaining' operon was found 746 locus tag apart in the *M. adhaerens* HP15 genome starting with locus tag HP15_1601 until HP15_1610 and consisted of the genes encoding for proteins C, E, F, G, H, I, J, K, L, and M, with the gene for protein C in opposite orientation to the rest of the cluster. In *P. aeruginosa*, two genes, *xphA* and *xqhA*, homologous to the genes encoding for proteins C and D of the T2SS, respectively, are located distant from the major T2SS operon (Michel et al. 2007). This could be the case for *M. adhaerens* HP15, as well.

The gene associated with the cloned insert of col. 32 was annotated as a conserved hypothetical protein with a putative conserved bacterial Ig-like domain. Proteins carrying this domain are found to be important in host-pathogen interactions (Kelly et al. 1999, Raman et al. 2010). For example, this domain is present in intimin, an outer membrane adhesion protein present in enteropathogenic *E. coli* EPEC or enterohaemorrhagic *E. coli* EHEC (Frankel et al. 2001). Together with the host cell translocated intimin receptor (Tir), this protein mediates intimate attachment to the host cells. Tir is secreted by the type III secretion system into the plasma membrane of the host cell, where it anchors and acts as a receptor for intimin (Kenny et al. 1997). The function of this protein in *M. adhaerens* HP15 has to be further analyzed.

Chemotaxis

Chemotaxis is a mechanism that bacteria use to chemically sense the environment and move towards attractants or away from repellents (Baker et al. 2005). Chemoreceptors interact with those chemicals and together with CheA, a histidine kinase, and CheW form a receptor-signaling complex, which in turn controls the activity of CheY interacting with the flagellar motor thus controlling motility (Eisenbach 1996). Chemoreceptors have a methyl-accepting chemotaxis domain, a homologue of which was found in the putative *M. adhaerens* HP15 protein encoded by the gene downstream of promoter sequence found in col. 178. Two other genes associated with cols. 124 and 204 could also encode for proteins involved in chemotaxis since they share similarities to

CheY-like response regulators (Stock et al.1990). Therefore, our current results support the previous finding that chemotaxis of *M. adhaerens* HP15 is important for the diatom-bacteria interaction (Sonnenschein et al. 2012).

Conjugative DNA transfer

Two additional brownish colonies, 12 and 188, carried DNA sequences from the native *M. adhaerens* HP15 plasmid, pHP-42, which were associated with genes *trbG* and *trbE*, respectively, of the conjugative transfer operon Trb were identified. This operon together with the Tra operon have been described in *Agrobacterium tumefaciens* as genetic determinants of the system used for conjugal transfer between bacteria (Farrand et al. 1996, Cook et al. 1997, Li et al. 1998). Noteworthy, *M. adhaerens* HP15 possess a second, larger plasmid, pHP-187 without tra or trb operons suggesting that the system from the smaller plasmid might control conjugative transfer of the bigger plasmid, which in turn might encode for yet-to-be-determined function(s) important for the diatom-bacteria interaction. Co-regulation of plasmid transfer functions with those involved in host-microbe interactions have been described several times before (Backert & Meyer 2006, Chen et al. 2002, Zupan et al. 2000)

Putrescine metabolism

The second gene downstream of the promoter sequence found in col. 68 (**Figure 5B**) might encode a spermidine/putrescine-binding periplasmic protein. This finding prompted the speculation that uptake and metabolism of putrescine might be important during the interaction of *M. adhaerens* HP15 with *T. weissflogii*. Putrescine (1,4-diaminobutane) is a polyamine present in all kinds of biological materials. Interestingly, diatom cell walls contain high amounts of long-chain polyamines which are organic constituents of diatom biosilica (Kröger et al. 2000, Kröger & Poulsen 2008). In this context, polyamines are a shunt product of silica frustule formation (Sumper & Kröger 2004). Previously, it had been shown that marine bacteria increased the dissolution of silica from lysed diatoms (Bilde & Azam 1999). The bacteria were found to colonize diatom detritus and carried out hydrolytic activities thereby removing the organic matrix and solubilizing the silica. From these reports, we speculate that *M. adhaerens* HP15 might be using the putrescine present in the cell wall of the diatom and transporting it inside the cell as a nitrogen source. Further experiments have to be carried out in order to

test this hypothesis and to determine the actual role of putrescine transport during the interaction.

Conclusion and perspectives

This study revealed an exciting array of genes potentially important during diatom–bacteria interaction. The functions of the identified genes are usually based on similarities or conserved domains found in the proteins encoded by them. However, the true function(s) of such proteins still need to be systematically tested. This is particularly applicable for conserved hypothetical proteins. Consequently, the potential involvement of the identified genes in diatom–bacteria interaction will be thoroughly investigated by site-directed mutagenesis (Sonnenschein et al. 2011) and analysis of the diatom-attachment and marine aggregate formation phenotypes of mutants by attachment assays and rolling tank experiments, respectively (Gärdes et al. 2011). The corresponding mutant analyses shall be done in time- and nutrient-dependent scenarios in order to better understand the actual function of these *in vivo* expressed genes.

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Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
E. coli		
DH5α	Φ80*dlacZ*ΔM15 Δ(*lacZYA*- *argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1/λpir*	Miller & Mekalanos 1988
ST18	λpir Δ*hemA pro thi hsdR*$^{+}$ Trp Smr	Thoma & Schobert 2009
HB101	Carrying helper plasmid pRK2013 KmR	Figurski & Helinski 1979
M. adhaerens		
HP15	wild-type	Grossart et al. 2004
Δ*pyrB*	*ΔpyrB* Cm^{R}	This study
Plasmids		
pGEM®-T Easy	colE1 *lacZ* Amp^{R}	Promega, Mannheim, Germany
pGEM_pyrBup	pGEM®-T Easy containing 960 bp upstream *pyrB* flanking region of HP15	This study
pGEM_pyrBdown	pGEM®-T Easy containing 984 bp downstream *pyrB* flanking region of HP15	This study
pFCM1	Amp^{R} Cm^{R}	Choi & Schweizer 2005
pGEM_pyrB_down_cm	Cm^{R} (1129 bp) from pFCM1 ligated into *Nde*I site of pGEM_pyrBdown	This study
pGEM_pyrB_down_cm_up	Cm^{R} and downstream region (2168 bp) from pGEM_pyrB_down_cm ligated with *Kpn*I/*Nhe*I into pGEM_pyrBup	This study
pEX18Tc	pMB1 oriT *sacB* Tet^{R}	Hoang et al. 1998
pEX_pyrBcdu	Knock-out fragment (3134 bp) from pGEM_pyrB_down_cm_up ligated with *Hin*dIII into pEX18Tc	This study
pBBR1MCS-4	broad-host-range mob Amp^{R}	Kovach et al. 1995
pMC1871	*lacZ* Tet^{R}	Shapira et al. 1983
pITM3	derivative of pBBR1MCS-4 with insertion of *lacZ* by *Kpn*I in opposite direction to the lac promoter	This study
pITM4	derivative of pBBR1MCS-4 with insertion of *lacZ* by *Kpn*I under control of the lac promotor	This study
pITM3_pyrB	derivative of pITM3 with insertion of pyrBC of HP15 upstream *lacZ*	This study
pITM1_Sau3A_3	pITM1 carrying a HP15 native promoter upstream *lacZ*	This study
pITM3_pyrB_prom	derivative of pITM3_pyrB with insertion uppstream *pyrBC-kacZ* fussion of a promoter of HP15	This study
pITM1	derivative of pBBR1MCS with insertion of *lacZ* by *Kpn*I in opposite direction to the lac promoter	Sonnenschein et al. 2011

Table 2. List of primers used in this study

Primer	Sequence 5′ – 3′	Restriction enzyme
pyrBup-F	ATTGGGGTACCGCTAGCTCGTTCGCGGTCATGGGT	*Kpn*I, *Nhe*I
pyrBup-R	ATTGGAAGCTTCGCGTCATGGCGTTCGAT	*Hin*dIII
pyrB_Down2-F	ATTGGAAGCTTGGTACCCCTGCTTGGCGGCGGTAT	*Hin*dIII, *Kpn*I
pyrB_Down2-R	AGGGGCCAGGCATGAGCA	
Cm2 F	AGTGCCATATGGAGCTCGAATTGGGGATCTT	*Nde*I
Cm2 R	AGTGCCATATGGCTAGCGAGCTCGAATTAGCTTCAAA	*Nde*I, *Nhe*I
lacZF	AGT GGT ACC CGT CGT TTT ACA ACG TC	*Kpn*I
lacZR	AGT GGT ACC TAT TAT TTT TGA CAC CA	*Kpn*I
pyrB F	ATTGGCTCGAGCAGATTGCCGGCTTTGGC	*Xho*I
pyrB mutF	CGAATCAGCAGCGCGGTATT	
pyrB mutR	CCATCAACGCGTCAAACTGC	
pyrB out	CGAATCGTCAGCATATCC	
T7	TAATACGACTCACTATAGGG	

Table 3. List of genes expressed during interaction with *T. weissflogii.*

Colony No	Locus tag	Annotation	Possible role
Genes involved in central intracellular metabolism			
15	HP15_3561	Haloacid dehalogenase, type II	Conversion of miscellaneous compounds
19	HP15_802	Glycerol-3-phosphate acyltransferase	Phospholipid metabolism
24	HP15_1623	Putative NADH dehydrogenase	Energy metabolism
26	HP15_267	Acyl-CoA dehydrogenase	Lipid/fatty acid degradation
30	HP15_3716	Putative Na(+)-translocating NADH-quinone reductase subunit B	Energy metabolism
31	HP15_1399	Metal-dependent hydrolase-like protein	multiple funtions
54	HP15_2599	3-isopropylmalate dehydratase, large subunit	Leucine biosynthesis
96	HP15_397	30S ribosomal protein S5	Protein synthesis / Ribosomal synthesis
136	HP15_1598	Glycerol-3-phosphate dehydrogenase	Phospholipid metabolism
149	HP15_623	RNA methyltransferase	Protein synthesis / ribosomal structure
165	HP15_480	3-dehydroquinate synthase	Aromatic amino acid biosynthesis
205	HP15_4	Zinc-dependent alcohol dehydrogenase	Intermediary metabolism
207	HP15_449	Lipid A biosynthesis lauroyl acyltransferase	Lipid synthesis
211	HP15_3399	Glyceraldehyde 3-phosphate dehydrogenase	Intermediary metabolism / Glycolysis
238	HP15_910	3-hydroxyisobutyrate dehydrogenase	Amino acid degradation
239	HP15_1315	Leucyl aminopeptidase	Protein degradation
Genes involved in nutrient scavenging			
10	HP15_2770	Cytosine permease	Uptake of nucelobases
130	HP15_4091	Putative D-lactate dehydrogenase (Fe-S oxidoreductase)	Uptake of organic acids
Genes involved in cell envelope structure and modification			
1	HP15_157	Putative lipoprotein	Outer membrane protein
68	HP15_3542	Polysaccharide deacetylase	Peptidoglycan modification
100	HP15_3966	Glycosyltransferase	Cell envelope biogenesis
161	HP15_2039	Tol-Pal system-associated acyl-CoA thioesterase	Cell envelope integrity
Genes involved in regulation			
33	HP15_4087	Transcriptional regulator, GntR family	Transcriptional regulators
166	HP15_2605	Putative regulatory protein, LysR-like	Transcriptional regulators
168	HP15_2444	PTS IIA-like nitrogen-regulatory protein PtsN	Sugar uptake regulation
204	HP15_3664	Response regulator containing receiver domain	Two-component signal regulation
Genes involved in porotein secretion			
22	HP15_2356	Type II secretion system protein C	Secretion
32	HP15_313	Protein containing bacterial Ig-like domain	Adhesion/invasion
Genes involved in chemotaxis			
178	HP15_2157	Methyl-accepting chemotaxis protein	Chemotaxis
Genes involved in DNA transference			
188	HP15_p42g49	Conjugal transfer Trb operon	Conjugation
Genes of unknown function			
13	HP15_1948	Hypothetical protein	
45	HP15_2904	Conserved hypothetical protein	
74	HP15_154	Hypothetical protein	
198	HP15_p187g86	hypothetical protein	

Table 4. List of genes found in “false positives”, and opposite orientated colonies.

Colony No	Locus tag	Annotation	Possible role
"FALSE POSITIVES" CLONES			
Genes involved in central intracellular metabolism			
28	HP15_3879	Nitrite reductase (NirD) family	Nitrogen metabolism
90	HP15_3855	Diaminobutyrate--2-oxoglutarate aminotransferase	Salt tolerance
94	HP15_2746	Nitroreductase family protein	Nitrogen metabolism
117	HP15_3110	HNH endonuclease	DNA degradation
118	HP15_1141	Enoyl-CoA hydratase/isomerase	Lipid/fatty acid degradation
Genes involved in cell envelope structure and modification			
162	HP15_3186	Putative porin	Outer membrane protein
Genes involved in regulation			
97	HP15_3711	PAS domain and GGDEF domain	Environmental sensing
124	HP15_3981	Response regulator containing receiver domain	Two-component signal regulation
135	HP15_4168	PAS domain and GGDEF domain	Environmental sensing
194	HP15_1022	EAL domain / GGDEF domain	Environmental sensing
Genes of unknown function			
122	HP15_2818	Conserved hypothetical protein	
128	HP15_3635	Conserved hypothetical protein	
OPPOSITE ORIENTATED CLONES			
Genes involved in central intracellular metabolism			
3	HP15_3495	Haloacid Dehalogenase	Conversion of miscellaneous compounds
5	HP15_802	Glycerol-3-phosphate acyltransferase	Lipid/fatty acid synthesis
18	HP15_1479	Queuine tRNA-ribosyltransferase	Protein synthesis
25	HP15_541	Release Factor 3 protein	Protein synthesis
103	HP15_1330	Oxaloacetate decarboxylase gamma chain	Amino acid synthesis
154	HP15_1911	Beta-glucosidase	Sugar metabolism
190	HP15_1242	Nudix_Hydrolase	Several functions
Genes involved in nutrient scavenging			
93	HP15_829	D-alanyl-D-alanine carboxypeptidase	Cell wall biosynthesis
Genes involved in nutrient scavenging			
51	HP15_351	ClC chloride channels	Ion acquisition
Genes involved in nucleic acid metabolism			
48	HP15_1202	Chromosome segregation protein SMC	Cell division
Genes involved in DNA tranference			
12	HP15_p42g47	Conjugal transfer Trb operon	Conjugation

Figure 1. Schematic representation of IVET strategy.

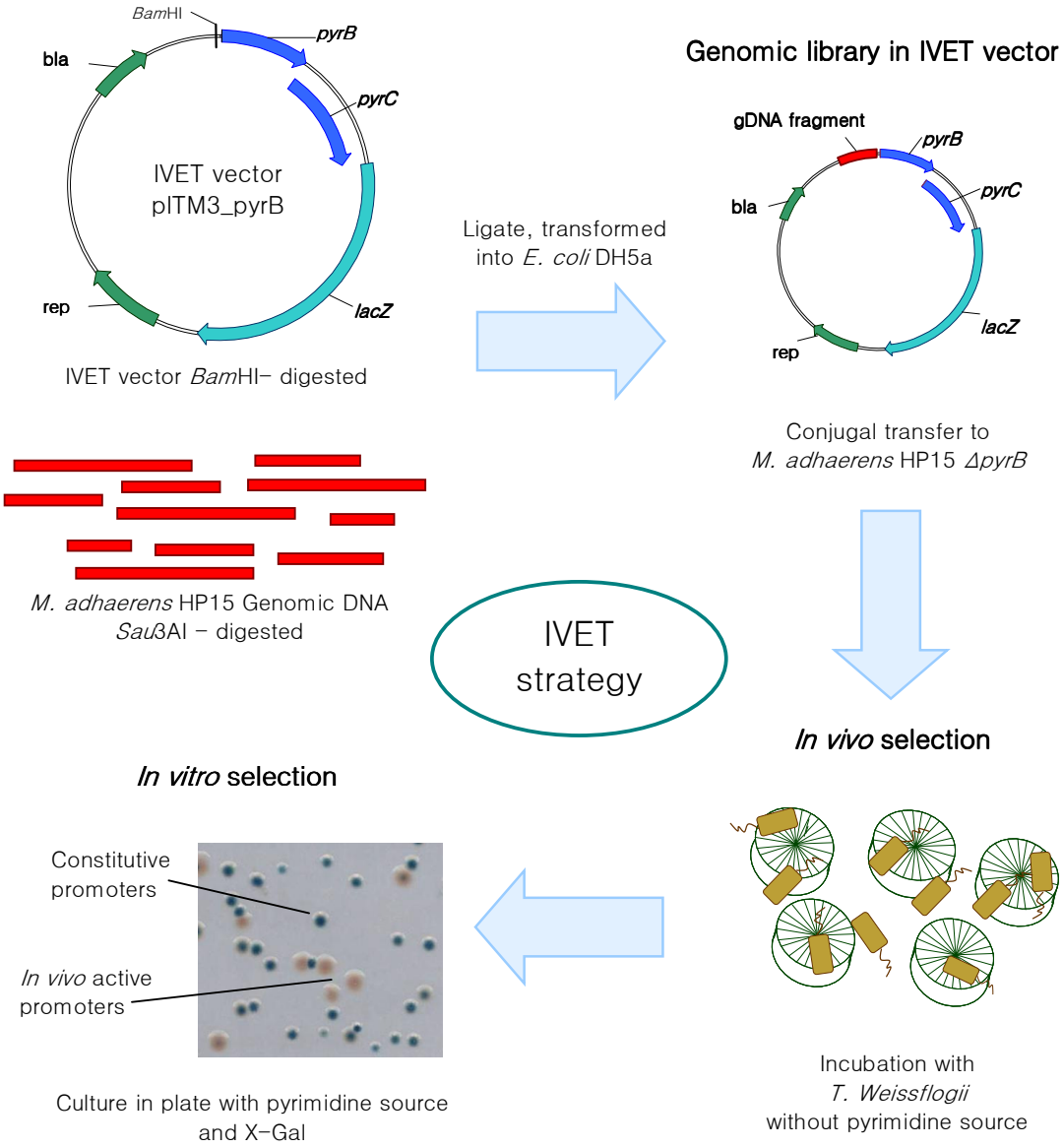


Figure 2. Construction of IVET vector pITM3_pyrB.

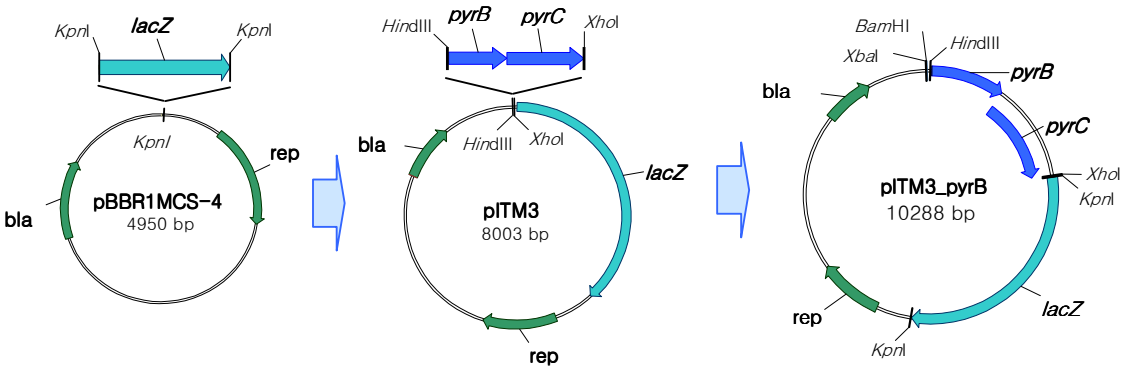


Figure 3. Growth of Δ*pyrB M. adhaerens* HP15 in co-culture with *T. weissflogii* in F/2 media without uracil. *M. adhaerens* HP15 wild-type was used as a positive control.

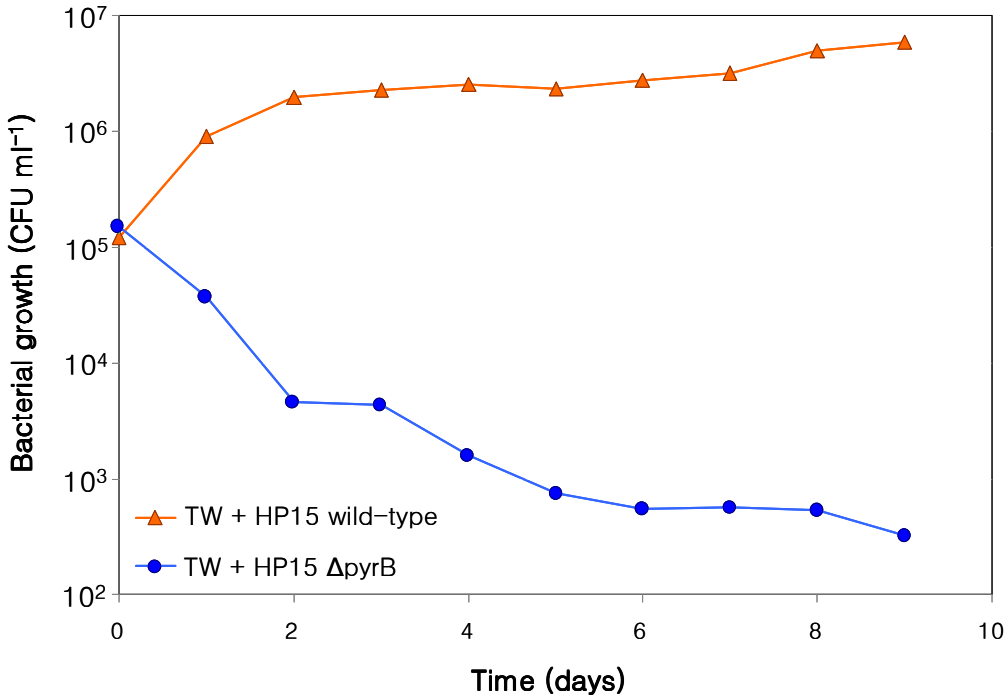


Figure 4. Growth curve of individual clones of *M. adhaerens* HP15 Δ*pyrB* carrying potential *in vivo* induced promoters and *T. weissflogii*. wild-type and a blue colored mutant were used as a positive control. Δ*pyrB* HP15 (pITM3_pyrB) was used as a negative control.

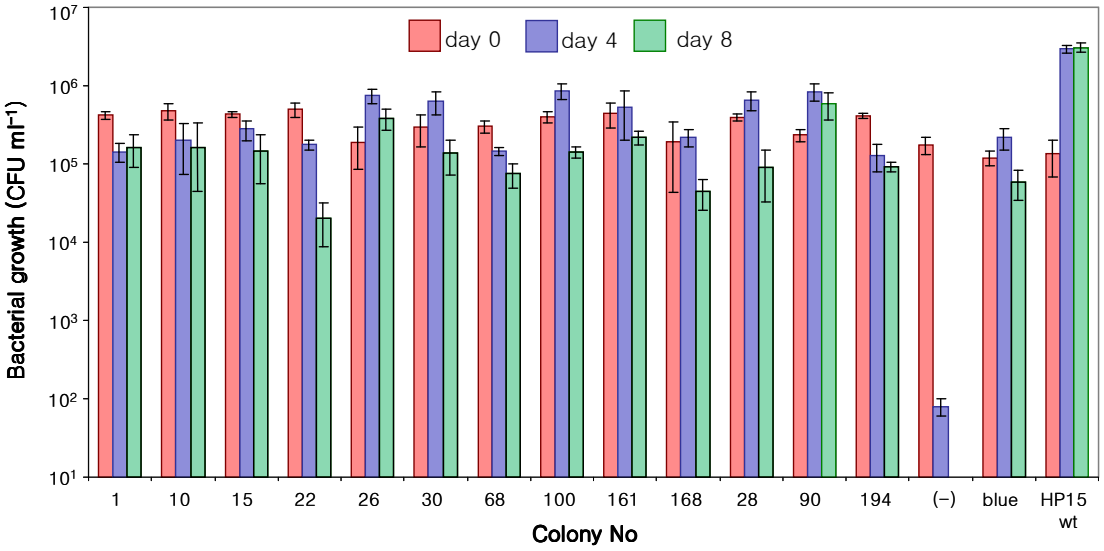
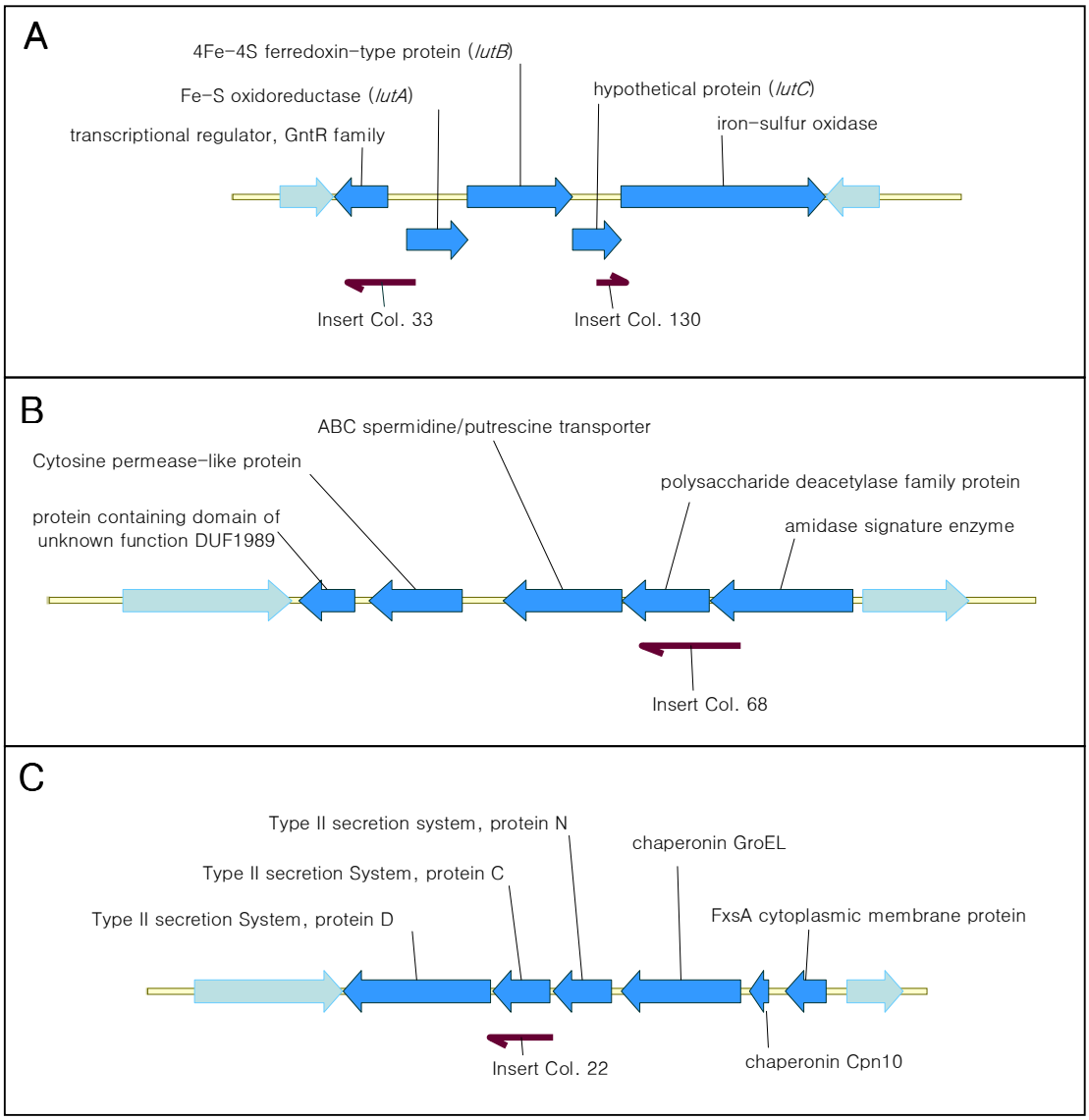


Figure 5. Chromosomal location of the locus associated to the identified promoter for: **A**. Col. 130 and 30; **B**. Col. 68; **C**. Col. 22 in the genome of *M. adhaerens* HP15**.** Purpul arrows represent the DNA inserted upstream the *lacZ-pyrBC* fusion in the IVET vector.



3.3. Identification of *Marinobacter adhaerens* HP15 proteins expressed in response to presence of the diatom *Thalassiosira weissflogii*

Identification of *Marinobacter adhaerens* HP15 proteins expressed in response to presence of the diatom *Thalassiosira weissflogii*

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Keywords: diatom-bacteria interactions, *Marinobacter adhaerens*, *Thalassiosira weissflogii*, marine aggregates, MALDI-TOF-MS, 2D-electrophoresis.

ABSTRACT

Diatom-bacteria interactions play important roles during aggregate formation leading to the sinking of organic carbon in the oceans. A bilateral *in vitro* model system is being used to study the molecular mechanisms underlying diatom-bacteria interactions. This model consists of the marine gamma-proteobacterium, *Marinobacter adhaerens* HP15, and the diatom, *Thalassiosira weissflogii*. The bacterium specifically attaches to *T. weissflogii* cells thereby increasing its aggregation and inducing an increased formation of transparent exopolymeric particles. The bacterial genes specifically induce during the interaction with the diatom still unknown. In this work, bacterial proteins expressed in response to the presence of *T. weissflogii* were identified by comparison of protein profiles of bacterial cultures incubated with or without diatom cells and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Differential expression of *M. adhaerens* HP15 proteins involved in transport and catabolism of different groups of organic compounds, as well as protein synthesis were observed when *T. weissflogii* cells were present. Some of these proteins may be important for the proposed biochemical interaction between *M. adhaerens* HP15 and *T. weissflogii*.

INTRODUCTION

The sinking of organic carbon is an important process in marine biogeochemical cycles and for the efficiency of the biological carbon pump (De La Rocha 2003, Fowler & Knauer 1986, Jahnke 1996). The sinking of carbon is mainly mediated by marine aggregates which consist of living cells, organic matter, fecal pellets, and inorganic compounds. During aggregate formation, diatom–bacteria interactions play an important role by inducing the secretion of different extra–cellular polysaccharides, which increase the size and stickiness of aggregates (Decho 1990, Alldredge et al. 1993, Logan et al. 1995, Passow 2002). The molecular bases of diatom–bacteria interactions are still unclear. Therefore a bilateral *in vitro* model system consisting of the marine gamma–proteobacterium *Marinobacter adhaerens* HP15 described by Kaeppel et al. (2012) and the diatom *Thalassiosira weissflogii* has been established. An increase in aggregation of *T. weissflogii* cells as well as an induction of transparent exopolymeric particles (TEP) production was observed in co–cultures with *M. adhaerens* HP15 (Gärdes et al. 2011, 2012). In addition, the genome sequence of *M. adhaerens* HP15 was determined (Gärdes et al. 2010) and the bacterium's genetic accessibility has been optimized (Sonnenschein et al. 2011). In combination, these findings and tools prompted and allowed to study key genes potentially important during the interaction by site–directed mutagenesis and comparative mutant analyses. For instance, *M. adhaerens* HP15 chemotaxis–deficient mutants exhibited a decrease in diatom attachment, suggesting that chemotaxis may be an essential mechanism during the interaction (Sonnenschein et al. 2012). Most other genes that might be specifically induced during the interaction of *M. adhaerens* HP15 and diatom cells are however still unknown.

The aim of this study was to identify bacterial proteins expressed when *M. adhaerens* HP15 was exposed to but not directly interacting with *T. weissflogii*. The identification was done by comparison of two–dimensional protein profiles combined with matrix–assisted laser desorption/ionization time–of–flight mass spectrometry (MALDI–TOF–MS). MALDI–TOF–MS analysis is a widespread analytical tool for identification of proteins, peptides and other bio–molecules (Bonk & Humeny 2001, Lay 2001). Briefly, the sample is co–crystallized with a large molar excess of a matrix compound, and laser radiation of the sample–matrix mixture results in vaporization and the sample becomes ionized. The ions are accelerated in a magnet–surrounded electric field towards a detector, which they will reach at different times (time–of–flight). The smaller ions reach

the detector first because of their greater velocity while the larger ions take longer owing to their larger masses. Subsequently, the time of flight is converted to a mass-to-charge ratio and a mass spectrum is obtained (Lewis et al. 2000, Bonk & Humeny 2001).

Protein identification by MALDI-TOF-MS is done after individual protein samples have been treated with proteolytic enzymes, such as trypsin, generating characteristic peptide fragments. These enzymes specifically cleave the protein at certain amino acid residues in the sequence. The different peptide fragments generated from the digestion result in a specific peptide pattern. These peptide patterns can be used as fingerprints, which will be compared with known protein sequences from genome sequence information by computer programs and collected in databases (Lewis et al. 2000, Bonk & Humeny 2001).

MATERIALS AND METHODS

Organisms and culture conditions

M. adhaerens HP15 was isolated from particles collected from surface waters of the German Wadden Sea (Grossart et al. 2004). This bacterium was grown in marine broth (MB) agar medium (ZoBell 1941) at 28°C. Liquid cultures were made in MB medium and incubated at 18°C by constant shaking at 250 rpm.

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 (Guillard & Ryther 1962) prepared with pre-filtered (0.2 µm pore size) and autoclaved North Sea water, with a 12 h photoperiod and 115 µmol photons $m^{-2}s^{-1}$. Diatom cell numbers were determined by cell counts in a Sedgewick Rafter Counting Chamber S50 (SPI Supplies, West Chester, PA).

Co-cultivation of *M. adhaerens* HP15 and *T. weissflogii*

To identify bacterial proteins, the following cultivation set-ups were applied: Cultures of *M. adhaerens* HP15 alone and cultures of *M. adhaerens* HP15 with *T. weissflogii* cells residing inside a dialysis hose were grown in f/2 medium. For this, a *M. adhaerens* HP15 overnight pre-culture was prepared by growing the cells in 25 ml of MB medium at 18°C with rotary aeration (250 rpm). Next, the main culture was started by

adding 1 ml of the overnight pre-culture into 50 ml of fresh MB medium and incubated at 18°C until they reached the exponential phase (OD_{600}~1.0). To avoid carry-over of nutrients from MB medium, cells were subsequently washed twice with f/2 medium. Simultaneously, *T. weissflogii* was grown until they reached exponential growth (~80,000 cells ml^{-1}). Diatom cell concentrations were adjusted to ~10,000 cells ml^{-1}, and cells were placed inside a dialysis hose with a molecular weight cut-off of 12-14,000 KDa (Spectra/Por, Spectrum Inc). The dialysis hose was placed into 150 ml of a *M. adhaerens* HP15 cell suspension adjusted to a final OD_{600} of 0.05 in f/2 medium and then incubated at 16°C, in a 12 h photoperiod at 115 µmol photons $m^{-2}s^{-1}$ and 50 rpm for 3-4 days. As a control, the same set up was used with *M. adhaerens* HP15 alone and 15 ml of sterile f/2 medium placed inside the dialysis hose.

Protein isolation

For membrane-associated, periplasmic, or cytoplasmatic protein isolation, an osmotic shock-based cell fractionation protocol was applied according to Boyd et al. (1987) and Manoil & Beckith (1986) with modifications. Briefly, 150 ml cell cultures were centrifuged for 20 min at 4,000 rpm and 4°C. The pellet was then resuspended in 150 µl of cold spheroplast buffer (0.1 M Tris-HCl, pH 8.0, 0.5 mM EDTA and 0.5 mM sucrose) and after incubation for 5 min on ice, the cell resuspension was centrifuged (10 min, 8,000 rpm, 4°C). 100 µl of ice-cold dionized water were added to the pellet and incubated for 30 sec on ice, then 5 µl of 20 mM $MgCl_2$ were added. The osmotically shocked cells were centrifuged (15 min, 8,000 rpm, 4°C), and the supernatant, containing the periplasmic fraction was collected. The pellet was resuspended in 150 µl of cold spheroplast buffer, 15 µl of lysozyme (2 mg ml^{-1}), 1.26 µl of DNAse (2 mg ml^{-1}), 150 µl of cold water, and incubated for 5 min on ice, followed by a centrifugation step (10 min, 8,000 rpm, 4°C). The pellet was then resuspended in 1,000 µl of 50 mM Tris-HCl, pH 7.5 and subjected to sonication 6 times for 15 sec. The lysed spheroplasts were then centrifuged (30 min, 8,000 rpm, 4°C) resulting in the pellet containing the membrane fraction, and the supernatant containing the cytoplasmic fraction.

Two-dimensional protein electrophoresis (2-DE) and analysis of expression patterns

Protein concentrations were measured spectrophotometrically by Nanodrop 2000c (Thermo Scientific, Massachusetts, USA). Depending on the protein concentration a

volume of <25 μl sample was mixed with rehydration buffer (ReadyPrep Rehydration/Sample Buffer, BioRad, Hercules, USA) and applied to ReadyStrip IPG strips (pH gradient of 4 to 7, 12 cm length; BioRad). The strips were incubated for ~16 h at room temperature (RT). Samples were focused in an Iso-electric focusing Apparatus (Multiphor II, Amersham-Pharmacia, Biotech Inc. NJ, USA) at 4°C by stepwise increase of the voltage as follows: 35 V for 10 min, 50 V for 1 h, 150 V for 20 min, 300 V for 15 min, 600 V for 15 min, 1,500 V for 30 min, and 3,000 V for 3.5 h. After isoelectric focusing, strips were equilibrated first in 10 ml equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol and 2% SDS) containing 100 mg of dithiothreitol for 15 min, and secondly in 10 ml equilibration buffer with 0.4 g of iodoacetamide for 15 min. Then the strips were subjected to 12.5% SDS-PAGE using a BioRad Mini-PROTEAN® Tetra cell for 2 hours at 120 V. Gels were then stained with a silver staining kit (Pierce Silver Stain Kit, Thermo Scientific, USA). The comparison of the protein profiles was done visually.

Tryptic in-gel digestion

In-gel digestion was performed as described by Shevchenko et al. (2006), Speicher et al. (2000), and Granvogl et al. (2007) with some modifications. The protease used was modified porcine trypsin (Promega, Madison WI, USA). Nitrile gloves were worn at all times to avoid contamination. Gels were washed in double-distilled water for 2 h under slight agitation. Reaction cups (Nerbe plus, Winsen/Luhe, Germany) were previously rinsed with 150 μl of 50% acetonitrile (ACN; v/v) containing 0.1% trifluoracetic acid (TFA) by shaking ON at 37 °C. Cups were emptied and tried under clean bench afterwards. Protein spots were excised and transferred to rinsed cups. 100 μl of 50 mM ammonium bicarbonate (NH_4HCO_3) buffer containing 50% ACN (v/v) were added, and incubated for 30 min at RT under frequent vortexing (washing step). 500 μl of ACN were added and incubated at RT for 10 min (dehydration step). Supernatant was removed and samples were place on ice and covered with 20-30 μl digestion buffer (13 ng μl^{-1} trypsin in 10 mM NH_4HCO_3 solution containing 10% ACN (v/v)). For complete trypsin saturation, samples were left on ice for 90 min and then covered with 5-20 μl of 100 mM NH_4HCO_3 buffer. The reaction cups were incubated for 12-16 h at 37°C, afterwards the supernatant (peptide sample) was directly used for MALDI-TOF MS.

MALDI-TOF MS

An Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser and operated with FlexControl version 3.0 software was used. α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics) was used as matrix. 1 μl peptide sample was acidified with 1 μl 0.5% TFA. The acidified sample was again mixed with HCCA solution (0.33 mg ml^{-1} in acetone, containing 66% ethanol (v/v). Samples were loaded on a steel manufactured MTB AnchorChip^{TM} target with an anchor diameter of 600 μm (Bruker Daltonics). Loads of 0.5 μl were spotted and left for air-drying. For desalting, dried spots were rinsed twice with 2 μl of 10 mM monobasic ammonium phosphate solution ($NH_4H_2PO_4$) for ~5 s, and then exposed to MALDI-TOF MS. MS was operated in positive-ion reflection mode, laser offset was set to 67% with a range of 15%. Masses were detected in a range of 800-4,000 Da (m z^{-1}). Ion source I was set to a voltage of 19.00 kV, iron source II to 16,90 kV. The iron extraction delay was set to 60 ns. Trypsin autodigestion peaks were used as calibrates. As controls, a blank piece of polyacrylamide gel was used for identification of mass artifacts resulting from sample preparation and bovine serum albumin fraction V (AppliChem, Darmstadt, Germany) was used as a control protein. Data was processed with FlexAnalysis, version 3.0. Peaks assignment used a signal-to-noise (S/N) ratio of 3, peptide masses were derived from moniosotopic peaks. Peptide mass lists were identified with the Mascot search engine (Perkins et al. 1999). Results were serached against the 'National Center of Biotechnology Information' database. The restricting taxonomy frame was set to 'other proteobacteria' for *M. adhaerens* HP15 proteins. A score probability of <0.05 and a mass error of 50 ppm was chosen, accepted missed trypsin cleavages were 1. As variable modifications oxidation of methionine ('Oxidation (M)') was selected, global modifications were carbamidomethylations due to the staining technique applied.

Protein sequence analysis.

Protein sequence analysis, search for conserved domains and similar reported proteins, was done using protein Basic Local Alignment Search Tool (BLASTP) provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1990) and

InterProScan Sequence Search provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Zdobnov & Apweiler 2001).

RESULTS

M. adhaerens HP15 protein profiles

Cultures of *M. adhaerens* HP15 alone, cultures of *M. adhaerens* HP15 with *T. weissflogii* cells residing inside a dialysis hose, and cultures of *M. adhaerens* HP15 with an empty dialysis hose in f/2 medium were started. After 3 to 4 days of incubation the bacterial cells were harvested and proteins were isolated. Membrane associated, periplasmic and cytoplasmatic proteins were separated by osmotic shock fractionation (Boyd et al. 1987, Manoil & Beckith 1986). The protein fractions were then separated by 2-DE, visualized with silver nitrate, and the protein profiles visually compared with each other. Some of the protein fractions yielded low concentration of protein. Therefore, for the appropriate visualization of low concentrated proteins, gels were stained with silver nitrate allowing the detection of proteins with concentrations of 1 to 5 ng (Zhao et al. 2012).

There were no differences in the protein profiles obtained from M. adhaerens HP15 cultures with or without the cell-free dialysis hose (data not shown) indicating that M. adhaerens HP15 did not alter its protein profile in response to the dialysis hose material. In contrast, protein fractions derived from cultures of *M. adhaerens* HP15 alone and from those co-incubated with diatom cells embedded in the dialysis hose showed slightly different protein patterns (**Figure 1**). Protein spots specific to one of the culture conditions were sampled and processed for MADI-TOF MS. Interestingly, several of the proteins designated as membrane-associated and expressed under both conditions were actually cytoplasmic proteins indicating that the applied fractionation method did not yield the expected results and that there was a cross contamination of fractions (data not shown).

Protein identification and sequence analysis

Several bacterial proteins were visually recognized to be differentially expressed in the presence or absence of diatom exudates (**Figure 1**). A total of 22 proteins were

visually observed to be up-regulated (blue numbers in **Figure 1**) when *M. adhaerens* HP15 were exposed to *T. weissflogii* exudates while 26 other proteins seemed to be down-regulated (red numbers). Some of the mentioned protein spots were excised from the gels and subjected to MALDI-TOF-MS analysis. The possible functions of these proteins were deducted by analysing the amino acid sequence and determine presence of conserved domains or similarities with previous reported proteins in the GenBank (**Table 1**).

We successfully identified four **up-regulated proteins** (**Table 1**). Firstly, protein 88 in the *M. adhaerens* HP15 genome was annotated as a translation elongation factor TU, which plays a role in protein synthesis (Andersen et al. 2003). In addition, this protein shared 98% similarity with the translation elongation factor TU in *M. algicola* (ZP_01894447). These results suggested that protein 88 might be involved in protein synthesis.

Secondly, protein 185 annotated as methylmalonate-semialdehyde dehydrogenase (MMSDH) and sharing 83% similarity to its homologue in *P. aeruginosa* PAO1 (NP_252260.1). MMSDH is encoded by *mmsA* which belongs to the *mmsAB* operon involved in valine metabolism (Steele et al. 1992). The gene *mmsB* encodes a 3-hydroxyisobutyrate dehydrogenase and is located downstream *mmsA,* while *mmsR* encodes a positive regulator and is located upstream oriented in opposite direction. Homologous of all three genes are present in *M. adhaerens* HP15 (**Figure 2B**). However, *mmsA* (locus tag: HP15_906) and *mmsB* (HP15_910) are separated from each other by three genes involved in lipid and fatty acid degradation: acyl-coA dehydrogenase (HP15_907) and two enoyl-CoA hydratase/isomerase (HP15_908 and 909). The amino acid sequence of the protein encodes by *M. adhaerens* HP15 *mmsB* shared 79% similarity and *mmsR* shared 62% with the genes found in *P. aerugionosa* PAO1, which prompted to suggest that protein 185 is possibly involved in the catabolism of valine.

Thirdly, protein 218 annotated as a conserved hypothetical protein; however, analysis of its amino acid sequence showed that it shared 51% similarity to a sigma E-type regulatory protein, MucB/RseB of *Pseudomonas* sp. CHOI1 (ZP_11247052.1) and 52% to its homologue in *Camamonas testosterone* KF-1 (ZP_03544426.1), suggesting that protein 218 might play a role in stress response (Alba & Gross 2003).

Finally, protein 219 annotated as a lipoprotein belonging to the NlpA family, shared 89% similarity with a D-methionine-binding lipoprotein (MetQ) found in *P. stutzeri*

CCUG 29243 (YP_006459889.1) and 66% to the corresponding homologue of *E. coli* K-12 (NP_414739.1). The gene *metQ,* encoding MetQ, is part of the *metD* locus (*metNIQ*) of *E. coli* K-12 (Gál et al. 2002). Interestingly, genes homologous to *metN* and *metI* are also present in *M. adhaerens* HP15 (**Figure 2A**). *M. adhaerens* HP15 protein YP_005887595.1 shared 66% similarity with MetN and YP_005887596.1 shared 68% similarity with MetI from *E. coli* K-12. These results prompted to assume that this lipoprotein might be involved in methionine transport.

Nine proteins (Proteins: 154, 179, 189, 190, 198, 200, 215, 216 and 217) **down-regulated** in the presence of *T. weissflogii* exudates were identified (**Table 1**). Protein 154 was annotated as an ATPase, F1 complex, delta subunit, and shared 93% similarity to the ATP synthase delta chain in *M. algicola* DG893. ATPases are involved in energy metabolism by producing ATP, and play diverse functions within the cell i.e. transport of substances, cell motility (Cross & Müller 2004, Rappas et al. 2004)

Protein 179 was annotated as a tripartite ATP-independent periplasmic (TRAP) dicarboxylate transporter (DctP subunit), a periplasmic C4-dicarboxylate-binding protein essential for high-affinity transport of C4-dicarboxylates malate, succinate and fumarate (Shaw et al. 1991). The gene *dctP* is part of the *dctRSPQM* locus described in *Rhodobacter capsulatus* (Forward et al. 1997). A similar locus was found in *M. adhaerens* HP15 with the following genes *dctP* (HP15_4076), *dctQ* (HP15_4075), and *dctM* (HP15_4074), its amino acid sequence shared 47%, 45% and 54% similarity respectively with the homologue found in *R. capsulatus*. However no homologous for the genes *dctR* and *dctS* were found, these genes encode a two-component sensor-regulator system for the control of the operon expression (Forward et al. 1997). The results prompted to assume that protein 179 migth play a role in transport of C4-carboxylates; however due to the absence of the genes *dctR* and *dctS* in *M. adhaerens* HP15 chromosome the functionality of this locus has to be further analyzed.

Protein 189 was annotated as an aconitate hydratase 1, an iron-sulphur enzyme that catalyzes the reversible isomerization of citrate and isocitrate in the citric acid and glyoxylate cycles (Tang et al. 2004). In addition, this protein shared 62% and 60% similarity with its homologous in *E. coli* K-12 (NP_414660.1) and *P. aeruginosa* PAO1 (NP_250253.1), suggesting that this protein plays a role in the citric acid and glyoxylate cycles.

Protein 190 was annotated as a dihydropicolinate synthase (DHDPS); furthermore it shared 77% to the homologue found in *P. aeruginosa* PAO1 (NP_249701.1). These results suggest that this protein might be involved in the lysine biosyntesis catalyzing a key step with the condensation of aspartate beta-semialdehyde and pyruvate to dihydrodipicolinate (Laber et al. 1992).

Protein 198 was annotated as a hypothetical protein; however, analysis of its amino acid sequence demonstrated the presence of conserved domains found in a lipid/polyisoprenoid-binding YceI-like protein such as the lipid-binding protein YceI found in *E. coli* K-12 (Stancik et al. 2002) and the polyisoprenoid-binding protein TT1927b of *Thermus thermophilus* (Handa et al. 2004). In addition, it shared 76% to the YceI like-family protein of *Alcanivorax* sp. DG881 (ZP_05041223.1). The function of the *E. coli* protein is still unknown; however, TT1927b crystal structure studies suggest that YceI-like proteins play a role in the electron transport system by binding polyisoprenoid molecules, a part of isoprenoid quinones (Handa et al. 2004). The function of protein 198 needs to be further analyzed.

Protein 200 was annotated as an inorganic diphosphatase (PPase). This enzyme is involved in the hydrolysis of inorganic pyrophosphate and in different biosynthetic reactions such as amino acid, nucleotide, polysaccharide, and fatty acid biosynthesis (Lahti 1983).

Protein 215 annotated as a TonB-dependent receptor, part of the TonB-dependent regulatory systems which sense the environment and transmit signals leading transcriptional activation of target genes (Koebnik 2005). The first TonB-dependent receptor identified was the FhuA in *E. coli* B834, which is a receptor for siderophore ferrichrome (Locher et al. 1998). Subsequently, many FhuA homologues were described, many of them involved in uptake of iron-siderophore complexes and vitamin B12 (Koebnik 2005). In addition, protein 215 shared 44% similarity with FhuA found in *E. coli* K-12 (NP_414692.1), suggesting that this protein might play a role in the transport of iron or vitamin B12.

Protein 216 annotated as an amino acid ATP-binding cassette (ABC) transponder or periplasmic substrate-binding ABC transporter protein. Analysis of its amino acid sequence confirmed the presence of conserved domains for a periplasmic solute-binding protein part of an ABC transporter. However these transporters are involved in the

transport of a variety of compounds such as amino acids, sugars, peptides or ions (Tam & Saier 1993, Schneider & Hunke 1998).

Protein 217 was annotated as an electron transfer flavoprotein subunit alpha (ETF). ETFs are divided in two subfamilies: Group I housekeeping ETF involved in the oxidation of fatty acids; and some amino acids including lysine, an example is the ETF in *Paracoccus denitrificans* (Husain & Steenkamp 1985). Group II ETFs are required under special environment conditions (Weidenhaupt et al. 1996), for example, *fixB* and *fixA* in *Rhizobium meliloti* and other nitrogen fixers which play a role in a specific electron transport essential for nitrogen fixation (Earl et al. 1987, Weidenhaupt et al. 1996). Protein 217 shared 71% similarity to the ETF alpha subunit from *P. denitrificans* (AAA03072.1) and 51% to its homologue in *R. meliloti fixB* (AAK65104.1), suggesting that protein 217 might play a role in the oxidation of fatty acids.

DISCUSSION

Identifying bacterial proteins needed for diatom–bacteria interaction is important for understanding the mechanisms underlying aggregate and marine snow formation in the oceans. In this study a number of proteins were identified which were expressed by *M. adhaerens* HP15 during the interaction with *T. weissflogii* exudates. These proteins are listed in **Table 1**. The functions can be divided into: i) transport, biosynthesis or catabolism of carbon compounds; ii) stress response; and iv) central metabolism.

Transport, biosynthesis or catabolism of carbon compounds

Two proteins (185 and 219) involved in transport and catabolism of organic compounds were found to be up-regulated during the interaction. It is hypothesized that *T. weissflogii* might release organic compounds, which are taken up by *M. adhaerens* HP15 for subsequent use. Accordingly, protein 219 identified as a D-methionine-binding lipoprotein (MetQ) which is part of the methionine transport system encoded by the *metD* locus (*metNIQ*) of *E. coli* K-12 (Gál et al. 2002). Another example is the MMSDH (protein 185) involved in catabolism of valine (Steele et al. 1992). The gene encoding this protein is part of the operon *mmsAB*. Interestingly, in a parallel study the promoter driving the expression of the *mmsB* gene was found to be specifically induced in *M. adhaerens*

HP15 during its interaction with *T. weissflogii* (Torres-Monroy et al. in preparation), suggesting that the bacterial utilization of valine produced by the diatom might play an important role during diatom-bacteria interactions.

Two possible scenarios might explain the *M. adhaerens* HP15 proteins identified as being down-regulated in the presence of *T. weissflogii*. Firstly, the expression of such proteins could be inhibited by the presence of a diatom compound. For instance, the activity of the purified DHDPS (protein 190), involved in lysine biosynthesis, was inhibit by the end product of the pathway, lysine (Laber et al. 1992, Mirwaldt et al. 1995). Other examples could be the aconitate hydratase (protein 189), the PPase (protein 200), and the alpha subunit of the ETF (protein 217).

Secondly, these proteins might be expressed when *M. adhaerens* HP15 is growing in the absence of diatoms compounds to efficiently scavenge the low concentrated nutrients that might be present in the media. Although no carbon sources were added, the minor concentrations of nutrients in the seawater used cannot be excluded. Some of the proteins identified might support this hypothesis. The periplasmic C4-dicarboxylate-binding protein (DctP, protein 179), essential for transport of C4-dicarboxylates malate, succinate and fumarate (Shaw et al 1991). The periplasmic substrate-binding protein (Protein 216) associated to an amino acid ABC transponder. Finally, the TonB-dependent receptor (protein 215) possibly involved in uptake of iron-siderophore complexes and vitamin B12 (Koebnik 2005).

Stress response

Protein 218 shared high sequence similarity to sigma E-like regulatory proteins MucB/RseB of *Pseudomonas* sp. CHOI1 (Holert et al. 2013). This protein might play a role during a stress response mechanism induced by unfolded proteins. To reduce the accumulation of the later, chaperones and proteases are synthesized (Jin et al. 2008) following a well-understood interplay between regulatory proteins such as MucA and MucB with the alternative sigma factor E (De Las Peñas 1997, Alba & Gross 2003). Relevant stressors include changes in temperature, pH or osmolarity, contact with ethanol, heavy metals, or antibiotics, as well as carbon starvation or oxidative stress (Bukau 1993). Whether or not there is a stress and if so which type of stress *M. adhaerens* HP15 might be exposed to in presence of diatom exudates remains to be elucidated. However, protein 218 might also play a role in the regulation of

exopolysacchride synthesis of *M. adhaerens* HP15 as shown for its homologous in *Vibrio vulnificus* (Brown & Gulig 2009) and *P. aeruginosa* (Damron & Goldberg 2012). It is puzzling that mutations of the corresponding genes in *V. vulnificus* and *P. aeruginosa*, respectively, result in dramatically opposing effects: Knock-out of *mucB* in *P. aeruginosa* led to overproduction of the exopolysaccharide alginate and increased virulence (Martin et al. 1993) while mutation of *rseB* of *V. vulnificus* resulted in decreased exopolysaccharide synthesis and virulence (Brown & Gulig 2009). It remains to be analyzed whether polysaccharide synthesis by *M. adhaerens* HP15 plays a role during diatom-bacteria interactions.

Central metabolism

Protein 88 which was identified to be up-regulated in the presence of diatom exudates is a homologue of the translation elongation factor TU (EF1A) that is responsible for the correct placement of tRNA species into the ribosome (Andersen et al. 2003, Nilsson & Nissen 2005). It is hypothesized that *M. adhaerens* HP15 interacting with *T. weissflogii* needs to coordinate the expression of novel proteins; therefore the up-regulation of an elongation factor might help to increase the accuracy of their translation.

The remaining proteins might play diverse roles within the cell, for example protein 154, identified as a subunit of the ATPase, and protein 198, identified as a protein of the YceI-like family. These proteins might play a role in the electron transport system. However, the true function and the importance during the interaction of *M. adhaerens* HP15 need to be determined. The same is true for the protein 189 identified as an aconitate hydratase 1 playing a role in some important pathways including the Citric acid cycle.

Conclusion and perspectives

This study revealed an interesting combination of proteins potentially important during diatom-bacteria interaction. The functions of the identified proteins were based on similarities with previously reported proteins, but the actual function(s) have to be determined. For that reason the potential involvement of the identified proteins in diatom-bacteria interaction will be confirmed in future experiments. The expression levels of the identified genes in the presence or absence of *T. weissflogii* will be determined using i.e., reverse-transcription quantitative real-time PCR (RT-qPCR). In addition, these

genes will be mutagenized and the corresponding mutants analyzed for their diatom-attachment phenotype by attachment assays and rolling tank experiments as described by Gärdes et al. (2011).

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Table 1. List of proteins expressed during interaction with *T. weissflogii.*

Protein No.	Accession No.	Annotation	Possible role
Up-regulated in presence of diatom exudates			
88	YP_005884070.1	Translation elongation factor TU	Protein synthesis
185	YP_005884598.1	Methylmalonate-semialdehyde dehydrogenase (MMSDH)	Valine metabolism
218	YP_005886113.1	Putative sigma E regulatory protein	Stress response
219	YP_005887597.1	D-methionine-binding lipoprotein (MetQ)	Methionine transport
Down-regulated in presence of diatom exudates			
154	YP_005887374.1	ATPase, F1 complex, delta subunit	Energy metabolism
179	YP_005887768.1	TRAP dicarboxylate transporter, DctP subunit	Transport of C4-dicarboxylates
189	YP_005885622.1	Aconitate hydratase 1	Citric Acid cycle
190	YP_005885719.1	Dihydrodipicolinate synthase (DHDPS)	Lysine synthesis
198	YP_005886215.1	Putative lipid/polyisoprenoid-binding, YceI-like protein	Energy metabolism
200	YP_005886908.1	Inorganic diphosphatase (PPase)	Diverse compounds biosynthesis
215	YP_005885102.1	TonB-dependent receptor	Iron and vit B12 uptake
216	YP_005885888.1	Amino acid ABC transporter, periplasmic substrate-binding protein	Transport of amino acids
217	YP_005885447.1	Electron transfer flavoprotein subunit alpha (ETF)	Oxidation of fatty acids

Figure 1. Protein profiles of *M. adhaerens* HP15 grown in the presence of *T. weissflogii* residing inside a dialysis hose or alone in f/2 media. Proteins were separated by 2-DE and visualized by Silver nitrate staining. Blue numbers symbolize bacterial proteins up-regulated in the presence of *T. weissflogii* and red numbers proteins down-regulated under the same condition. The X symbolizes proteins that were not excised from the gels.

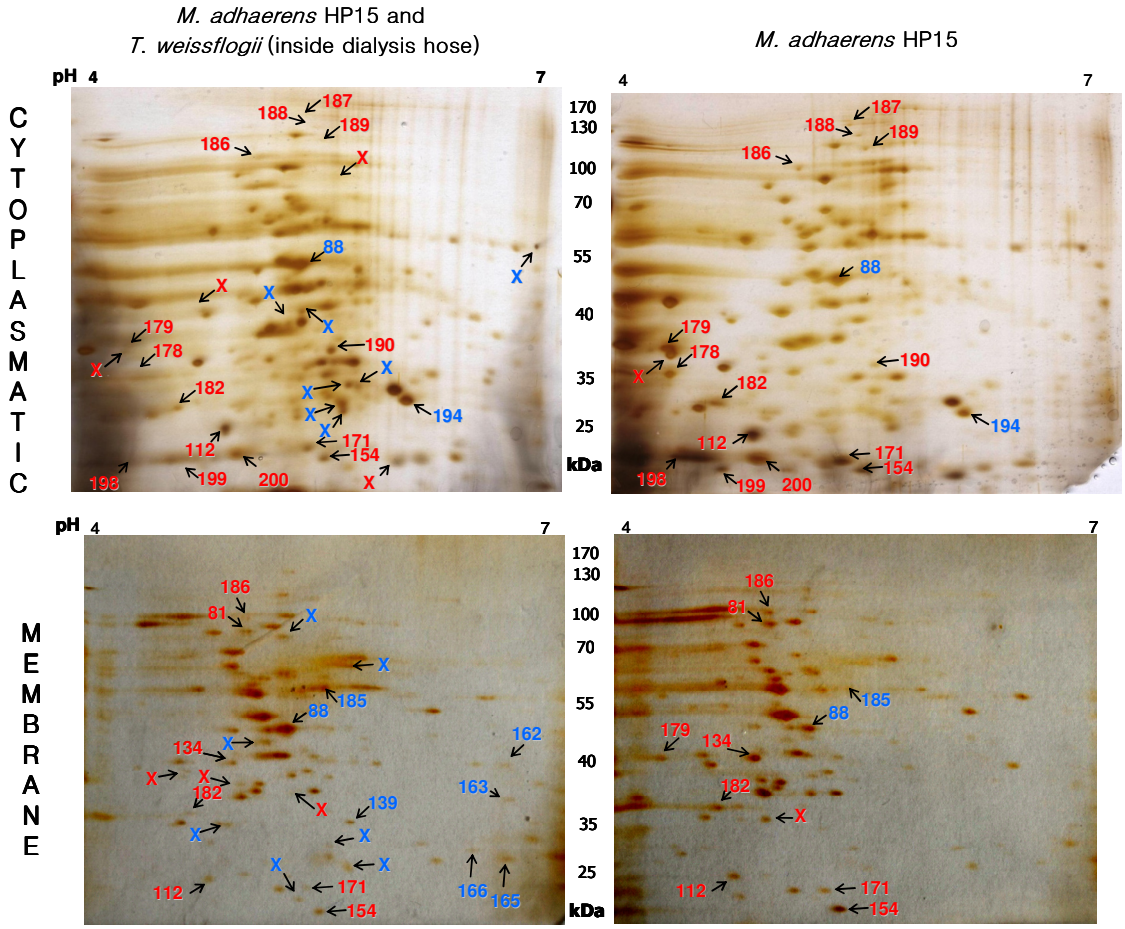
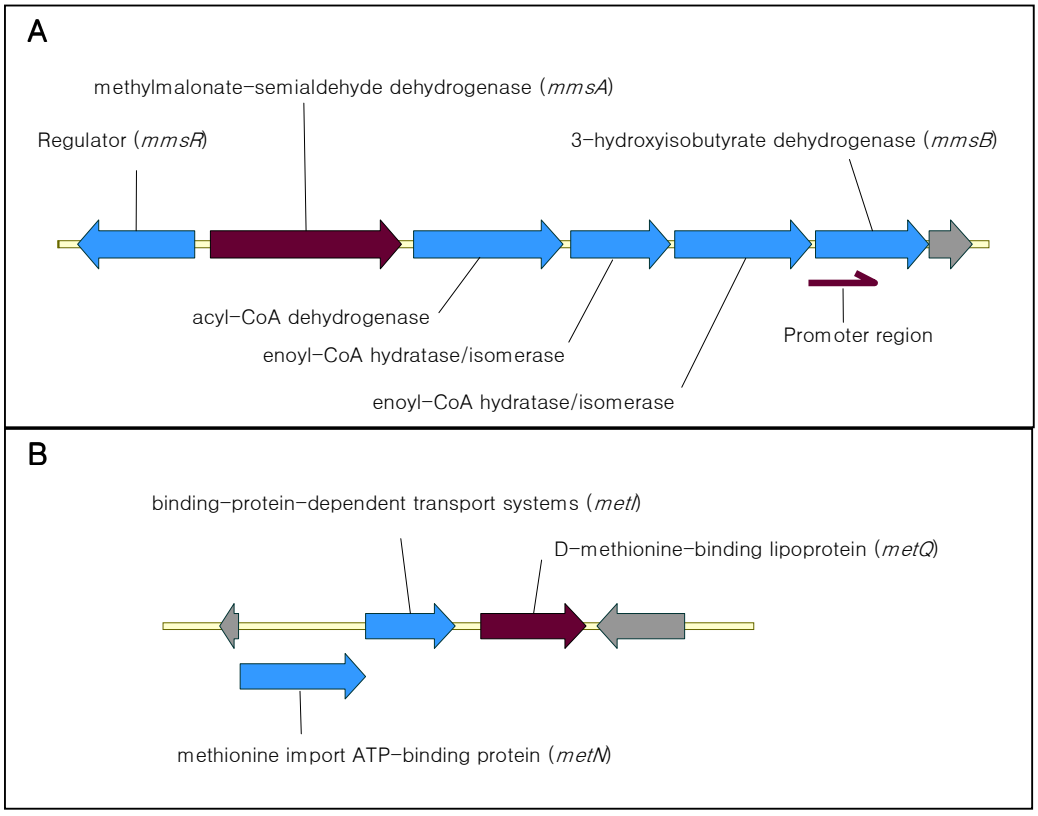


Figure 2. Chromosomal location of the genes identified (purple) encoding for proteins 185 and 219 in the genome of *M. adhaerens* HP15. A. locus *mmsRAB*, *mmsA* encodes MMSDH (prot 185); and B. Locus *metNIQ*, metQ encodes for MetQ (Prot. 219). The purple arrow represents the promoter region identified by IVET.



3.4. Identification and possible function of the plasmid-borne *tad* locus in the diatom-associated bacterium *Marinobacter adhaerens* HP15

Identification and possible function of the plasmid-borne *tad* locus in the diatom-associated bacterium *Marinobacter adhaerens* HP15

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ABSTRACT

Interactions between bacteria and phytoplankton play an important role in the formation of marine snow leading to the sinking of organic carbon in the oceans. Not much is known about the molecular mechanisms responsible for the diatom-bacteria interaction. To address this issue, a bilateral model system consisting of *Marinobacter adhaerens* HP15 and the diatom *Thalassiosira weissflogii* has been established. *M. adhaerens* HP15 specifically attaches to *T. weissflogii* cells increasing its aggregation and inducing transparent exopolymeric particle formation. In this study, the *tight adherence* (*tad*) gene locus present on the 187-kb HP15 plasmid, pHP-187, was analyzed. This locus consists of 9 genes (*flp*, *rcpCA*, *tadZABCDG*), which share high sequence similarities to corresponding genes found in other Gram-negative bacteria. In the later, this locus encodes for a type IVb fimbrial low-molecular-weight (Flp) pilus, which plays a role in adherence and biofilm formation. A constitutively active promoter upstream the *flp* gene of *M. adhaerens* HP15 was identified suggesting the *tad* locus to be transcribed. To test whether the *tad* locus plays a role in adherence or motility in *M. adhaerens* HP15, a *flp-rcpCA* deletion mutant was generated and analyzed in terms of its attachment to abiotic and biotic surfaces. Under the experimental conditions used herein, the mutant did neither show a phenotype in terms of surface attachment nor motility. However, the preliminary results of the current study encourage an in-depth analysis of the role of the *tad* locus in *M. adhaerens* HP15.

INTRODUCTION

In the oceans, the sinking of organic carbon, as described by the biological pump, is mainly due to marine aggregates termed marine snow (De la Rocha 2003). These aggregates consist of living cells, organic matter, fecal pellets, and inorganic compounds (Alldredge & Silver 1988, Alldredge & Gotschalk 1989). Diatom-bacteria interactions play an important role during the formation of aggregates by inducing the secretion of different extracellular polysaccharides, which increase the size of aggregates (Decho 1990, Alldredge et al. 1993, Logan et al. 1995, Passow 2002). Currently little is known about the molecular basis of diatom-bacteria interactions. For that reason, a bilateral model system consisting of the marine proteobacterium *Marinobacter adhaerens* HP15 (Kaeppel et al. 2012) and the diatom *Thalassosira weissflogii* was established (Gärdes et al. 2011). *M. adhaerens* HP15 specifically attaches to *T. weissflogii* stimulating the aggregation of diatom cells as well as the production of transparent exopolymeric particles (TEPs) (Gärdes et al. 2011). In addition, *M. adhaerens* HP15 is a genetic accessible bacterium (Sonnenschein et al. 2011), and its genome sequence is available (Gärdes et al. 2010). These tools have allowed the study of key genes potentially important during the interaction (Sonnenschein et al. 2012). For example, a type IV mannose-sensitive haemagglutinin (MSHA) pilus-deficient *M. adhaerens* HP15 mutant (Seebah 2012) and a flagellum-deficient mutant (Sonnenschein et al. 2011) were generated. The biofilm formation capacities on propylene surfaces were impaired for both mutants compare to those of the wild-type (Seebah 2012). In addition, both mutants showed a statistically significant reduction in attachment to diatom cells, suggesting that a functional flagellum and MSHA type-IV pilus are required for the attachment of *M. adhaerens* HP15 to *T. weissflogii* cells (Seebah 2012).

The tight adherence (*tad*) locus encodes the type IVb fimbrial low-molecular-weight (Flp) pili and was first identified in *Aggregatibacter (Actinobacillus) actinomycetemcomitans,* a Gram-positive human periodontal pathogen by a transposon mutagenesis approach (Kachlany et al. 2000). Since then *tad* loci have been described in a wide variety of organisms, including pathogenic and non-pathogenic bacteria, as well as archaea (Kachlany et al. 2000). In most analyzed organisms the *tad* locus plays an important role in the colonization of respective environmental niches (Kachlany et al. 2001). In *Pseudomonas aeruginosa,* Flp pili contribute to adherence to abiotic surfaces and surfaces of eukaryotic cells (de Bentzmann et al. 2006). A homologous locus was

also identified in *Caulobacter crescentus,* a non-pathogenic aquatic bacterium, and designated as *cpa* (*Caulobacter* pilus assembly). The *cpa* locus encodes PilA pili, which are homologous to Flp, are present at the flagellar pole of the swarmer cells, and also play a role in surface attachment (Bodenmiller et al. 2004). Due to its wide spread distribution and presence on a mobile genomic island the *tad* locus has been considered as the so-called Widespread Colonization Island (WCI) (Planet et al. 2003). In *A. actinomycetemcomitans* this locus is composed of the genes *flp1-flp2-tadV-rcpCAB-tadZABCDEFG* encoding proteins that constitute a fibril secretion system (Kachlany et al. 2000), which in turn is responsible for tight adherence, autoaggregation, biofilm formation, and the production of bundled fimbria-like fibers of individual pili (Kachlany et al. 2000, 2001, Planet et al. 2003, Perez et al. 2006, Tomich et al. 2007). In comparison, transposon insertions in these genes resulted in smooth instead of rough mutant colony morphology, lack of pili or fibrils, and impaired surface adherence and cell-to-cell aggregation, respectively (Kachlany et al. 2000, 2001).

In this study we describe for the first time the presence of a tight adherence (*tad*) locus in *M. adhaerens* HP15. Furthermore, the functionality of the locus and the possible role during the interaction with *T. weissflogii* was assessed using different *in vitro* assays.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in **Table 1**. *M. adhaerens* HP15 was isolated from particles sampled in surface waters of the German Bight (Grossart et al. 2004). This bacterium was grown in marine broth (MB) medium (ZoBell 1941) (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 7.6) at 28°C. *Escherichia coli* strains were maintained in Luria-Bertani (LB) agar medium supplemented with the appropriate antibiotics. *E. coli* DH5α (Larsen et al. 2002) was used for carrying intermediate plasmids constructs, while *E. coli* ST18 (Thoma & Schobert 2009) was used as a donor strain during biparental conjugation and was grown in LB medium containing 50 μg ml^{-1} 5-aminolevulinic acid (ALA). Chloramphenicol, ampicillin and 5-bromo-4-chloro-3-

indolyl-β-D-galactopyranoside (X-Gal) were added to media when needed at a concentration of 25, 50 and 50 μg ml^{-1}, respectively.

Axenic cultures of *T. weissflogii* (CCMP 1336), obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine, USA), were grown at 16°C in f/2 medium (Guillard & Ryther 1962), prepared with pre-filtered (0.2 μm pore size) and autoclaved North Sea water, with a 12:12 h photoperiod and light intensity of 115 μmol photons $m^{-2}s^{-1}$. Diatom cell numbers were determined by cell counts in a Sedgewick Rafter Counting Chamber S50 (SPI Supplies, West Chester, PA).

DNA techniques

Plasmid preparation, PCR, and other standard DNA techniques were performed as previously described (Sambrook et al. 1989). Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturer (Fermentas, St. Leon-Rot, Germany). Oligonucleotide primers were designed using the Vector NTI® Software 10.3.0 (Invitrogen Corporation, Carlsbad, USA) and synthesized by Eurofins MWG (Ebersberg, Germany). The oligonucleotide primers used in this study are listed in **Table 2**.

In silico description of the tad locus in *M. adhaerens* HP15

The complete *M. adhaerens* HP15 plasmid pHP-187 sequence deposited at GenBank under accession number CP001980.1 (Gaerdes et al. 2010), was searched for genes encoding for *tad* locus proteins using Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1990). To determine sequence similarities of the *tad* locus of *M. adhaerens* HP15, the amino acid sequences of the encoded proteins were compared to those encoded by the genes of the *tad* loci of *A. actinomycetemcomitans* D11S-1, *Pseudomonas aeruginosa* PAO1, and *Caulobacter crescentus* CB15.

Identification of promoter region

To determine whether the DNA region separating genes HP15_p187g3 and *flp* in *M. adhaerens* HP15 contains a functional promoter, this region was scanned for putative SigmaA binding sites using Prokaryotic Promoter Prediction (PPP)

(http://bioinformatics.biol.rug.nl/websoftware/ppp) (Zomer er al. 2007). Additionally, a 335-bp region upstream of the coding sequence of *flp* was PCR amplified using the primer pair tad_promF/tad_promR. The PCR fragment was restricted with *Xba*I and *Hin*dIII and cloned into the *Xba*I/*Hin*dIII-treated vector pITM3_pyrB (Torres-Monroy et al. in preparation) thereby generating the plasmid pITM3_pyrB_tad_prom. This plasmid was transformed into *E. coli* ST18 and next introduced to the pyrimidine-auxotroph mutant of *M. adhaerens* HP15, Δ*pyrB*, by conjugation. For this, both bacterial strains were grown overnight on LB and MB agar plates, respectively, and cell mass was scraped off the agar with an OD_{600} adjusted to 1 (corresponded to ~1×10^9 cells ml^{-1}). Cells of donor and recipient were mixed in a ratio of 1:2, spotted on LB ALA agar plates, and incubated for 24 h at 28 °C. The cell mass was then scraped off the agar plates and re-suspended in MB medium for subsequent dilution plating. The transconjugants were selected on MB agar plate containing ampicillin, chloramphenicol and X-Gal.

Creation of *tad* locus mutants in *M. adhaerens* HP15

Gene-specific mutagenesis based on homologous recombination according to the procedure of Zumaquero et al. (2010) was used to knock-out the *flp-g5-rcpCA* genes within the *tad* locus on the 187-Kb plasmid of *M. adhaerens* HP15 thereby generating the *M. adhaerens* HP15 Δ*tad* mutant (**Figure 1**). Plasmids were constructed, in which a chloramphenicol resistance (Cm^R) cassette was flanked by DNA fragments obtained from upstream and downstream of the *flp-g5-rcpCA* gene region. Briefly, a 652-bp fragment from upstream of the *flp* gene and a 698-bp fragment from downstream of the *rcpA* gene were PCR-amplified using the primer pairs tad_upF/tad_upR and tad_down2F/tad_down2R, respectively. The primers tad_upR and tad_down2F included a complementary sequence (5'-GACTCACTATAGGG-3') at their 5' end followed by a *Bam*HI restriction site in such a manner as to provide homology and a cloning site between the fragments. The PCR products were fused together by a polymerization step conducted at 94°C for 2 min followed by 12 cylces of 94°C for 30 sec, 55°C for 60 sec and 72°C for 30 sec, and 5 min finally at 72°C. The resulting PCR product was gel purified and used as a template for a second PCR with the primers tad_upF/tad_down2R. The PCR product was then sub-cloned into the pJET1.2/blunt Cloning Vector (CloneJET PCR Cloning Kit, Thermo Scientific, USA), according to manufacturer's instructions, resulting in the plasmid pJET_tad_down_up_2. A DNA

fragment of 1,153 bp carrying a Cm^R cassette was excised with *Bam*HI from the plasmid pFCM1 and then sub-cloned into *Bam*HI-treated pJET_tad_down_up_2 resulting in plasmid pJET_tad_down_cm_up_2. From this plasmid, a 2,529-bp fragment comprising the regions flanking *flp-g5-rcpCA* and the Cm^R cassette was excised with *Hind*III and ligated into the *Hind*III-treated pEX18Tc generating the conjugable mutagenic construct pEX_taddcu_2. This construct was transformed into *E. coli* ST18 and subsequently transferred to *M. adhaerens* HP15 by conjugation as described above. A successful double cross-over event was confirmed by PCRs performed with the primers In_tad_smallF/In_tad_smallR resulting in no amplified PCR products in the mutant compared to the presence of a PCR product in the wild-type (~800 bp), and with the primers Tad_mut_smallF/cat_out2 and cat_out5/in_tad_bigR specific for the mutant. Primers Tad_mut_smallF and in_tad_bigR anneal outside the mutated area, whereas cat_out2 and cat_out5 inside the Cm^R cassette.

Mutant characterization by swarming, swimming and twitching

Colony morphologies of the mutant *M. adhaerens Δtad* and the wild-type were compared by growth on MB agar plates. To determine whether the *tad* locus gene products are involved in the motility of *M. adhaerens* HP15, swimming, swarming, and twitching motility of the wild-type and *Δtad* mutant were compared by soft-agar assays with 0.25%, 0.5% and 1% agar, respectively. *M. adhaerens* HP15 *ΔfliC,* impaired in the production of the flagellum (Sonnenschein et al. 2011), was used as a control. Wild-type and mutant strains were grown overnight in MB liquid medium at 18°C with constant shaking (250 rpm). Subsequently, bacterial concentrations were adjusted to an optical density (OD_{600}) of 0.1 and 2 µl were spotted in soft-agar plates in triplicates of each incubated at two different temperatures, 18 and 28°C. The level of motility was determined by quantitative estimation of the obtained diameters of bacterial growth. One-tailed Student's t test was applied to assess the significance of differences.

Biofilm assays

In vitro biofilm assays were performed according to O'Toole (2011). Briefly, *M. adhaerens* HP15 wild-type and its mutants *ΔfliC* and *Δtad* were grown overnight as described above. Then, 600 µl of bacterial culture with an OD_{600} of 0.1 were added to 1.5 ml polypropylene tubes (Nerbe, Winsen/Luhe, Germany) and incubated at 37°C for

24 h without shaking. To remove non-attached cells, the microtubes were washed in distilled water, air-dried, and attached cells were subsequently stained with 700 µl of 0.1% crystal violet for 20 min. After staining, tubes were washed in distilled water, air-dried and 900 µl of 95% ethanol were added. The thereby stained biofilm cells were quantified spectrophotometricaly at 600 nm. The assay was performed 3 times with 4 replicates each. One-tailed Student's t test was applied to assess the significance of differences between the biofilms formed by wild-type versus mutants.

Attachment assay with *T. weissflogii*

To determine the attachment of *M. adhaerens* HP15 wild-type and its *Δtad* mutant to *T. weissflogii* cells, attachment assays were performed according to Sonenschein et al. (2012). Briefly, diatom cells were grown in f/2 medium until stationary growth phase. Diatoms at a final concentration of 5,000 cells ml^{-1} were incubated with approximately 1 x 10^5 CFU ml^{-1} in a final volume of 20 ml f/2 medium. Triplicates co-cultures were incubated for 24 h at room-temperature in darkness without shaking. The diatom-attached and non-attached bacterial cell fractions were separated by passing the co-culture through a 10 µm pore size sieve (Sefar, Heiden, Switzerland). Diatom-attached and non-attached bacterial cells were collected and bacterial colony forming units (CFU ml^{-1}) were determined by dilution plating on MB agar plates. One-tailed Student's t test was applied to assess the significance of differences.

RESULTS

Bioinformatic analysis of the *tad* locus of *M. adhaerens* HP15

The *tad* locus in *M. adhaerens* HP15 is present on one of the indigenous plasmids, pHp-187, starting with the gene HP15_p187g4 and ending with gene HP15_p187g13. BLASTP searches with the proteins encoded by these genes against the ones in *A. actinomycetemcomitans* D11S-1, *C. crescentus* CB15 and *P. aeruginosa* PAO1 (**Table 4**) suggested that the *tad* locus in *M. adhaerens* HP15 consists of *flp* followed by a gene that does not show significant similarity (gene HP15_p187g5) to any *tad* locus proteins and then *rcpC/cpaB, rcpA/cpaC, tadZABCDG* (**Figure 2**). Interestingly, the protein encoded by gene HP15_p187g5 share high sequence similarity (50%) with a

TadE-like protein in *Alcanivorax* sp. DG881, which was isolated from a dinoflagellate (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA19279). Unfortunately, the genome sequence of this bacterium is not fully available with only some genes and not their location being annotated. Consequently, it is unknown as to whether the matching genes of this bacterium are found within a locus. No homologous proteins in *M. adhaerens* HP15 were found for the proteins RcpB, TadV, TadE or TadF. In *A. actinomycetemcomitans* RcpB is an outer membrane protein possibly required for the stability of the Tad secretion system (Perez et al. 2006), TadV functions as a prepilin-specific peptidase which removes the leader peptide from the prepilin necessary for pilus assembly, whereas TadE and TadF are pseudopilins (Tomich et al. 2006). Even after performing BLASTP searches with the prepilin-specific peptidase sequences from *A. actinomycetemcomitans* (TadV) or *P. aeruginosa* (FppA) or *C. crescentus* (CpaA), we did not find a homologue within, up or downstream of *M. adhaerens* HP15 *tad* locus. Sequence analysis of Flp from *M. adhaerens* HP15 indicated that the conserved G↓XXXXEY amino acid sequence cleavage motif characteristic for Flp prepilins is present within the protein (Tomich et al. 2007). The gene upstream or downstream of *flp*, HP15_p187g3, and HP15_p187g5, respectively, did not show any significant similarity to proteins of known *tad* loci.

Functional analysis of the *tad* locus promoter region

A promoter was identified upstream the *flp* gene in the *tad* locus of *M. adhaerens* HP15 as previously demonstrated for *A. actinomycetemcomitans* (Haase et al. 2003). The region upstream *flp* was searched for promoter-specific sequences by the program PPP. This tool predicted two promoter regions 5'-TTGCTTAACAAACTTTTGCAATCTAAT-3' and 5'-TTTACGGTGTAAGCTATCAAAGACAAAAT-3', with expected values (E-value) of 0.12 and 0.95, respectively. To test whether the region indeed exhibits promoter activity, the region upstream *flp* was PCR amplified and cloned upstream a *lacZ-pyrBC* fusion in the vector pITM3_pyrB. This vector was designed in a parallel study to establish an *in vivo* expression technology (IVET) assay for *M. adhaerens* HP15 (Torres-Monroy et al. in preparation). The thereby generated plasmid, pITM3_pyrB_tad_prom, allowed the determination of promoter activity based on the *flp* upstream sequence. The plasmid was subsequently transformed into *M. adhaerens* HP15 Δ*pyrB*, an auxotroph strain unable to grow without a pyrimidine source (uracil). The transconjugants obtained grew in medium

lacking uracil and exhibited a blue (LacZ-positive) phenotype in medium containing XGal. These results indicated that the region upstream of *flp* contained a functional promoter. In addition, the identified promoter is expressed *in vitro* and thus might mediate a constitutive expression of the *tad* locus genes.

Mutagenesis of the genes *flp-rcpCA* from the tad locus in *M. adhaerens* HP15

To investigate the role of the *tad* locus of *M. adhaerens* HP15 in bacterial motility and attachment to abiotic and biotic surfaces, a *flp-g5-rcpCA* deletion mutant of *M. adhaerens* HP15 was created (*Δtad*). For this, the *flp-g5-rcpCA* genes were replaced by a Cm^R cassette via homologous recombination. Fifteen transformants were obtained on MB agar plates supplemented with chloramphenicol. Two of the transformants had undergone a double crossover of the Cm^R cassette as confirmed by PCR: The primer pair In_tad_F/In_tad_R amplified a 800-bp region in the to-be-deleted region only in the wild-type of *M. adhaerens* HP15. As expected, no PCR product was obtained with the mutant. To further confirm the mutation event, primers annealing outside the mutated area (Tad_mut_smallF and in_tad_bigR) and inside the Cm^R cassette (cat_out2 and cat_out5) were combined in subsequent PCRs. Only in a successful double cross over event these two PCR reactions should yield corresponding products in contrast to no products to be obtained with cell samples of the wild-type. As expected, primers Tad_mut_smallF/cat_out2 amplified a 1,299-bp product in the *Δtad* mutant but not in the wild-type. Similarily, primer pair cat_out5/in_tad_bigR allowed amplification of a 2,288-bp fragment in the mutant but not in the wild-type. The results indicated a successful generation of the *M. adhaerens* HP15 *Δtad* mutant.

Phenotypic characterization of *Δtad* mutant

Analysis of colony morphology of the Δ*tad* mutant was carried out by plating the strain in MB agar plates. The colony morphology of the *Δtad* mutant was not differing from that of the wild-type (data not shown). Light microscopic analysis furthermore showed that mutant cells were indistinguishable from wild-type cells (data not shown). Subsequently, the swimming ability of Δ*tad*, Δ*fliC* and the wild-type, were analyzed on MB agar plates with soft agar (0.25% agar). As expected, the swimming ability of the Δ*tad* mutant was not affected and resembled that of the wild-type while swimming behavior of Δ*fliC* was dramatically reduced (data not shown). In addition, the swarming

and twitching motility were analyzed on MB agar plates with 0.5% and 1% agar, respectively. However, under none of the conditions tested these types of motility were observed for the wild-type or mutant.

Moreover, *in vitro* biofilm assays were carried out for *M. adhaerens* HP15 the wild-type of and its mutants Δ*tad* and Δ*fliC* using polypropylene tubes as abiotic surfaces (**Figure 3A**). The biofilm formation capacity of the Δ*tad* was slightly lower than that of the wild-type but this difference was not statistically significant. In contrast, mutant Δ*fliC* showed a dramatically lower biofilm formation compared to the wild-type. When biofilm formation was quantified by staining of attached cells with crystal violet and subsequent spectrophotometry (**Figure 3B**) the attachment of the three bacterial samples was significantly different from the stain resulting from MB medium ($p < 0.01$). Aside of this, the results of quantification confirmed the previous visual observations. The wild-type was attaching slightly more to the surface than the Δ*tad* mutant without statistically significant differences ($p > 0.01$). In contrast and as expected, Δ*fliC* biofilm formation was significantly lower as compared to the wild-type ($p < 0.01$) (**Figure 3B**).

Interaction of the Δ*tad* mutant with *T. weissflogii*

To study the potential role of gene products encoded by the *tad* locus during the interaction with *T. weissflogii*, the attachment of *M. adhaerens* HP15 Δ*tad* to diatom cells was tested. *M. adhaerens* HP15 Δ*tad* and the wild-type were separately incubated with *T. weissflogii* cells for 24 h. The CFU values for the mutant and wild-type did not statistically differ at the start of the experiment (t=0) (**Table 3**). After the incubation time of 24 h the diatom-attached and non-attached bacterial cells were fractionated and quantified by dilution plating. *M. adhaerens* HP15 Δ*tad* showed an increase in the attachment towards diatom cells (30.8 ± 16.3%) compared to the attachment observed for the wild-type (10.1 ± 8.2%). However, the differences between the attachment of Δ*tad* and the wild-type were not statistically significant with a $P > 0.01$.

DISCUSSION

In this study the presence of the tight adherence (*tad*) gene locus, known for encoding type IVb fimbrial low-molecular-weight (Flp) pili, was demonstrated for the

indigenous plasmid pHP-187 of *M. adhaerens* HP15. The *tad* locus was found in other bacteria that interact with protozoa like diatoms or dinoflagellates, such as α-proteobacteria belonging the *Roseobacter* clade or *Vibrio cholerae* (Geng & Belas 2010, Slightom & Buchan 2009, Tomich et al. 2007), although no study had characterized or investigated the function of the *tad* locus within these species. BLASTp searches with the amino acid sequences of the Flp pilin, Flp1 from *A. actinomycetemcomitans,* PilA from *C. crescentus* and Flp from *P. aeruginosa* against the *Marinobacter* protein sequences available in the NCBI protein database did not identify a homologue of similar size other than in *M. adhaerens* HP15.

The numbers of genes that constitute a *tad* locus, as well as the amino acid sequecuence of the protein encoded by them, differ between species suggesting that the functional role of the *tad* locus may differ between species as well. In *A. actinomycetemcomitans* D11S-1 the *tad* locus is composed by 13 genes (*flp-1-tadV-rcpCAB-tadZABCDEFG*). The *flp-1* gene encodes the Flp1 prepilin and is post-translationally modified by a prepilin protease (TadV) (Perez et al. 2006). In *C. crescentus* the *cpa* locus consists of a gene encoding the pilin subunit PilA, a Flp prepilin homologue, *cpaA* encoding a prepilin peptidase, and five adjacent genes *cpaBF* required for PilA pili assembly, with *cpaABCEF* being homologous to the *A. actinomycetemcomitans tadV-rcpCA-tadZA* genes (Skerker & Shapiro 2000, Tomich et al. 2007). In contrast to the *tad* and *cpa* loci in *A. actinomycetemcomitans* and *C. crescentus*, respectively, the arrangement of the genes within the *tad* locus is different in *P. aeruginosa*. Here the gene encoding the prepilin protease FppA is transcribed in the opposite direction to all other genes of the *tad* locus apart from *flp* and is found at that C-terminal end of the locus (Bernard et al. 2009). In *M. adhaerens* HP15 the *tad* locus consists of *flp* followed by a gene that does not show significant similarity to any *tad* loci genes and then *rcpCA*, *tadZABCDG, tadZABCDG* (**Table 4**). Interestingly, no prepilin peptidase homologue was found up or downstream of *M. adhaerens* HP15 *tad* locus. Identification of such a prepilin protease in the *M. adhaerens* HP15 *tad* locus requires further investigation.

A promoter region was *in silico* predicted and experimentally identified to be present upstream the *flp* gene (HP15_p187g4). The region upstream *flp* was sufficient to express a *lacZ* reporter gene under laboratory conditions suggesting that this promoter activity is rather constitutive. This functional promoter might furthermore indicate that the

tad locus is actually expressed and its gene products used in *M. adhaerens* HP15. For this reason, to determine the functionality and possible role of the *tad* locus in *M. adhaerens* HP15 a mutant lacking the genes *flp-g5-rcpCA* (Δ*tad*) was constructed.

Several experiments were carried out to characterize potential mutant phenotypes. However, under none of the conditions used here could we demonstrate a differential phenotype for the mutant compared to the wild-type. For example, the ability to form a biofilm on a propylene surface did not show significant differences compared to the significantly lower level of surface attachment observed for mutant Δ*fliC* lacking the flagellum (Sonnenschein et al. 2011). These results suggested that experiments performed in this study were not suitable to demonstrate a potential function of gene products encoded by the *tad* locus. Interestingly, Schilling et al. (2010) created a Δ*flp* mutant in *Yersinia enterocolitica* and showed that the attachment capacities of the Δ*flp* mutant to plastic surfaces as well as to eukaryotic cells were not impaired. However, the *Y. enterocolitica* Δ*flp* mutant was impaired in microcolony formation compared to the wild-type. For this reason, experiments to assess the microcolony formation in *M. adhaerens* Δ*tad* and wild-type will have to be performed in the future. Additionally, the conditions to assess twitching motility have to be standardized and then tested under different growth conditions and in different media. Furthermore, to test the biofilm formation on abiotic surfaces, the assays should be repeated with different other surface materials and with different bacterial concentrations.

Another potential explanation for the lack of a mutant phenotype could be a lack of expression of the genes forming the *tad* locus in *M. adhaerens* HP15, maybe due to the lack of some of the genes reported in other organisms such as the prepilin peptidase. Demonstration of an active promoter sequence upstream of *flp* counter-argues here, but future experiments have to be carried out to determine if the Flp pili are actually being formed in *M. adhaerens* HP15. This will be analyzed by transmission electron microscopy of cells incubated under pilus-inducing conditions and by quantification of *flp* expression using RT-qPCR.

The Δ*tad* mutant was used to determine a possible role during the interaction of *M. adhaerens* HP15 with the diatom *T. weissflogii.* For that, the attachment of the bacterial mutant cells towards diatom cells was compared to that of the wild-type. Surprisingly, some yet-to-be further analyzed differences were observed between the mutant and the wild-type with the mutant showing a better attachment. A repetition of

the assay and certain modifications of the experimental conditions might reveal whether the Flp pilus actually impacts attachment or not.

Even though the experimental conditions used in this study were not suitable for determine the role of the *tad* locus in *M. adhaerens* HP15, the results shown here represent a good basis for further experiments to better understand and characterize the role of this locus in *M. adhaerens* HP15.

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Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
E. coli		
DH5α	Φ80d*lacZ*ΔM15 Δ(*lacZYA- argF*) U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1/λpir	Larsen et al. 2002
ST18	S17 λpir Δ*hemA*	Thoma & Schobert 2009
M. adhaerens		
HP15	wild-type	Grossart et al. 2004
Δ*fliC*	Δ*fliC* Cm^R	Sonnenschein et al. 2011
Δ*tad*	Δ*flp-g5-rcpCA* Cm^R	This study
Δ*pyrB*	Δ*pyrB* CmR	Torres-Monroy, in preparation
Plasmids		
pJET1.2/blunt Cloning Vector	Eco47IR, Amp^R	Thermo Scientific
pJET_tad_down_up_2	pJET1.2/blunt Cloning Vector containing 1376 bp of the polymerized downstream and upstream *flp-g5-rcpCA* flanking region of HP15	This study
pFCM1	Amp^R Cm^R	Choi & Schweizer 2005
pJET_tad_down_cm_up_2	CmR (1153 bp) from pFCM1 ligated into *Bam*HI site of pJET_tad_down_up_2	This study
pEX18Tc	pMB1 oriT *sacB* Tet^R	Hoang et al. 1998
pEX_taddcu_2	Knock-out fragment (2529 bp) from pJET_tad_down_cm_up_2 ligated with *Hin*dIII into pEX18Tc	This study
pITM3_pyrB	derivative of pBBR1MCS with insertion of *lacZ* gene from *E. coli* and *pyrBC* from HP15	Torres-Monroy et al. in preparation
pITM3_pyrB_tad_prom	derivative of pITM3_pyrB with insertion upstream *pyrBC-lacZ* fusion of a 335 bp upstream the gene *flp* in tad locus of HP15	This study

Table 2. List of primers used in this study

Primer	Sequence 5′ – 3′	Restriction enzyme
Tad_down2_F	GGATCC**GACTCACTATAGGG**CGAGGGGACGATTGGTAA	*Bam*HI
Tad_down2_R	ATTGGAAGCTTAAACGCCTCACTGGATGC	*Hin*dIII
Tad_prom_F	ATTGGTCTAGAGAGCAAATCAAGACCACA	*Xba*I
Tad_prom_R	ATTGGAAGCTTCATTTGCTTCATTTTTCA	*Hin*dIII
tad_mut_F	CGCATTCTTTCGCTCAGA	
tad_mut_R	CGTTGATGCGATCGTCAG	
tad_mut_small_F	GGTCACTGTCCGTTTCCT	
tad_mut_big_F	TTACACGCTTGCGGAACC	
in_tad_small_F	TTCGTAAGCGTCCTAGCC	
in_tad_small_R	TCCCGACAACGCTTCAAG	
in_tad_big_R	CGGCACATACTGGCTCAA	
cat_out2	CTTACGTGCCGATCAACG	
cat_out5	GTGATGGCTTCCATGTCG	

Table 3. Cell dynamics of *M. adhaerens* HP15 wild-type and Δ*tad* mutant in the *T. weissflogii* attachment assay observed after dilution plating and quantification of bacterial cells, at the start of the experiment (t=0), after 24 h (t=1), and those attached to diatom cells or free-living.

Bacteria cell abundance (x 10^6 CFU ml^{-1})	*M. adhaerens* HP15	
	wild-type	Δ*tad*
t=0	1.26 ± 0.27	2.29 ± 0.27
t=1	3.58 ± 2.27	1.55 ± 0.29
Attached	0.25 ± 0.03	0.45 ± 0.21
Free-living	3.34 ± 2.27	1,1 ± 0.47
% attached	10,1 ± 16.3%	30,8 ± 8.2%

Table 4. Similarity of *M. adhaerens* HP15 *tad* locus proteins to proteins of homologous tad loci in *P. aeruginosa* PAO1, *C. crescentus* CB15 and *A. actinomycetemcomitans* D11S- 1. In brackets are the tag locus numbers.

M. adhaerens HP15 locus tag	Putative Protein	*A. actinomycetemcomitans* D11S-1	Query Cover (%)	Similarity (%)	*C. crescentus* CB15	Query Cover (%)	Similarity (%)	*P. aeruginosa* PAO1	Query Cover (%)	Similarity (%)
HP15_p187-g4	Flp	Flp1 (ACX82161.1)	67	56	PilA (CC_2948)	73	58	Flp (PA4306)	35	83
HP15_p187-g5	?	-								
HP15_p187-g6	RcpC	-			cpaB (CC_2946)	34	50	RcpC (PA4305)	59	44
HP15_p187-g7	RcpA	RcpA (ACX82158.1)	64	49	cpaC (CC_2945)	45	61	RcpA (PA4304)	87	60
HP15_p187-g8	TadZ	-						TadZ (PA4303)	65	40
HP15_p187-g9	TadA	TadA (ACX82155.1)	89	69	CpaF (CC_2942)	91	68	TadA (PA4302)	98	73
HP15_p187-g10	TadB	TadB (ACX82154.1)	63	39	TadB (CC_2941)	69	40	TadB (PA4301)	59	53
HP15_p187-g11	TadC	TadC (ACX82153.1)	43	53	H. prot. (CC_2940)	63	46	TadC (PA4300)	57	50
HP15_p187-g12	TadD	-			-			TadD (PA4299)	80	47
HP15_p187-g13	TadG	-			-			TadG (PA4297)	69	40
HP15_p187-g14	?	-			-			two-component response regulator (PprB) (PA2798)	28	43

Figure 1. Schematic presentation of the strategy applied for the generation of a gene-specific *tad* locus mutant in *M. adhaerens* HP15. **A**. Upstream and downstream flanking regions were PCR amplified with primers A1 and B1 (small black arrows) which had a homologous sequence (white rectangles). **B**. A polymerization step was done to linked the two previous PCR products. **C**. A second PCR with primers A2 and B2 was carried out. **D**. The PCR product containing the polymerized upstream and downstream flanking regions was cloned in a high copy number vector. **E**. A chloramphenicol resistance (Cm^R) cassette was cloned into the previous vector using *Bam*HI. **F**. the fragment containing the up, downstream and the chloramphenicol resistance cassette was cloned into a conjugable plasmid.

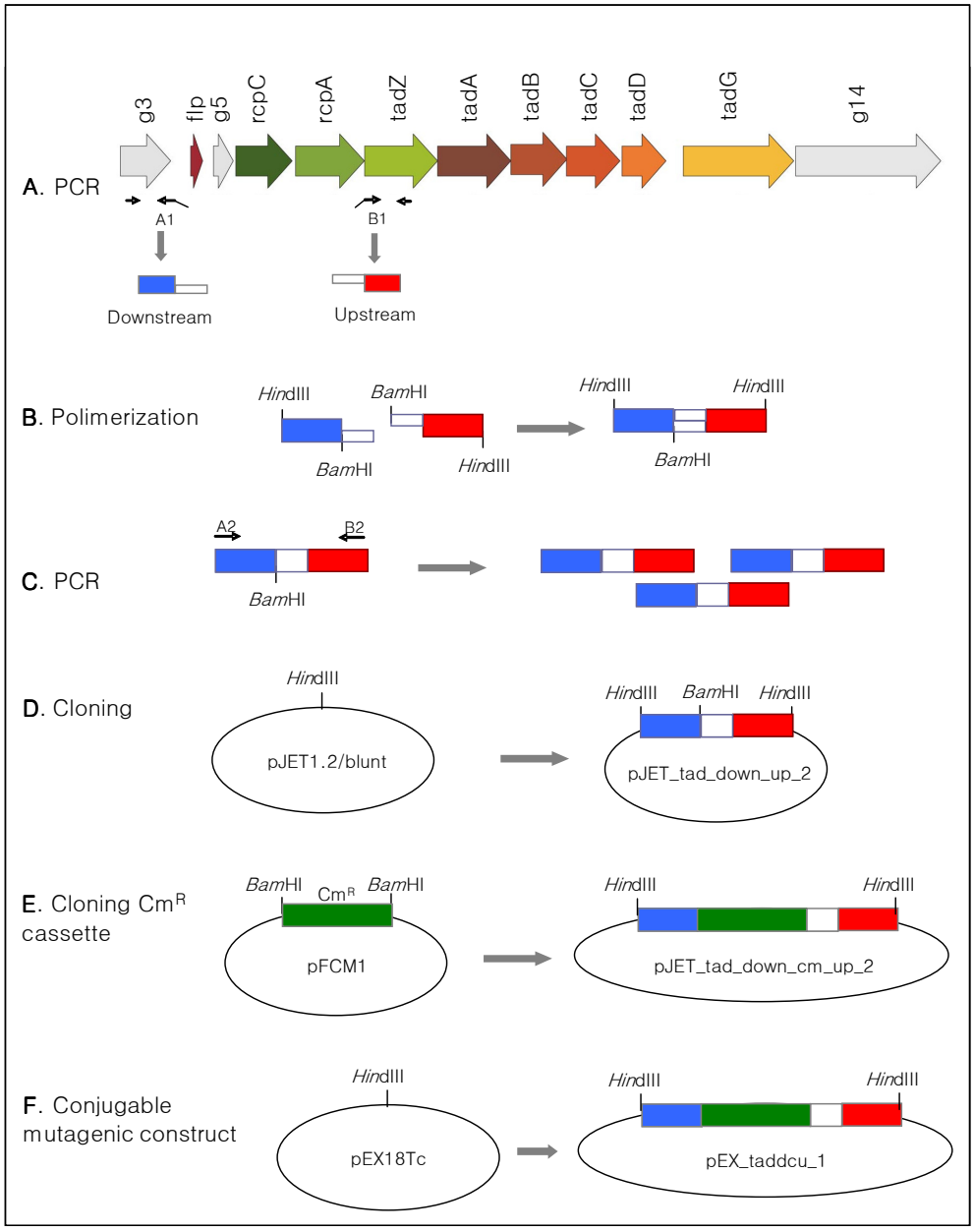


Figure 2. Schematic diagram of the *tad*/*cpa* loci from selected Gram-negative bacteria. Arrows indicate the open reading frames (ORFs) and the direction of transcription. ORFs that encode homologous products are shaded in the same color. Shaded in grey are genes that encode proteins with no homology to *tad*/*cpa* gene products. From BLASTP searches (**Table 4**) the predicted products of *M. adhaerens tad* genes were determined.

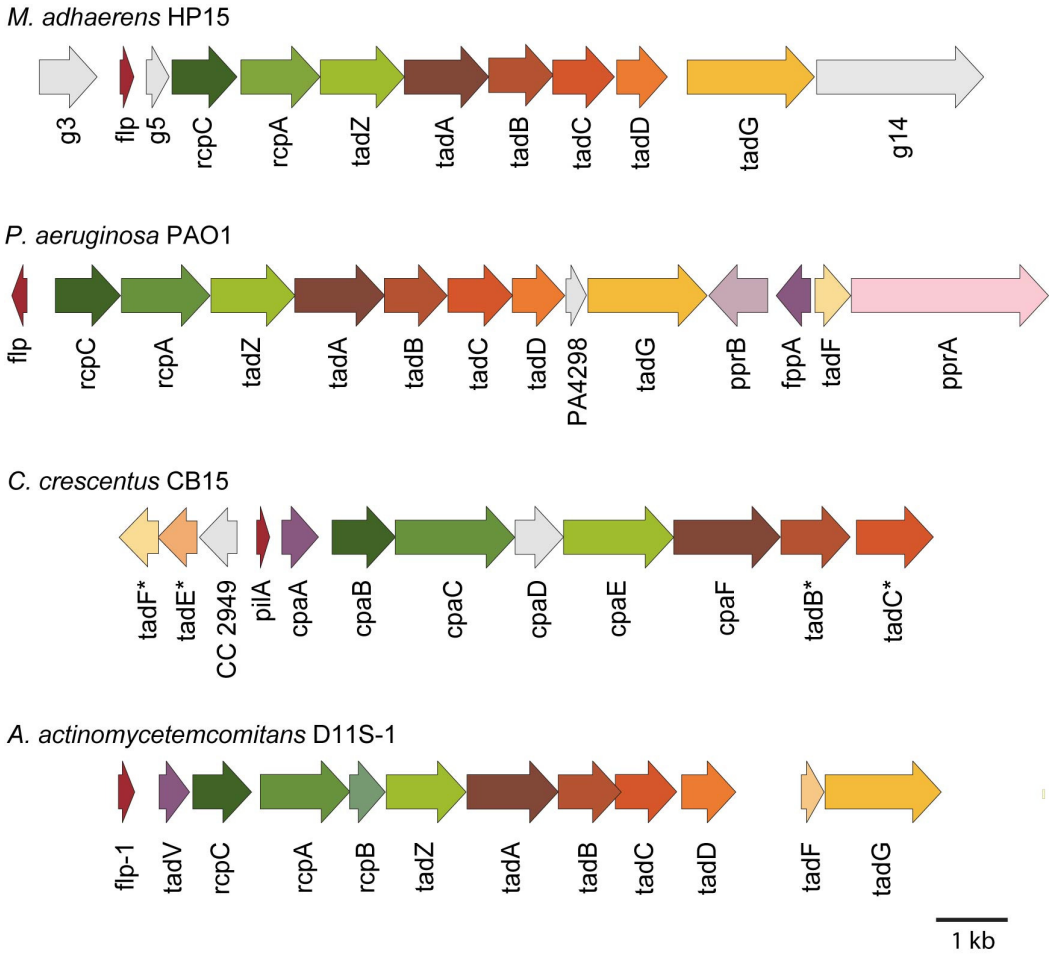
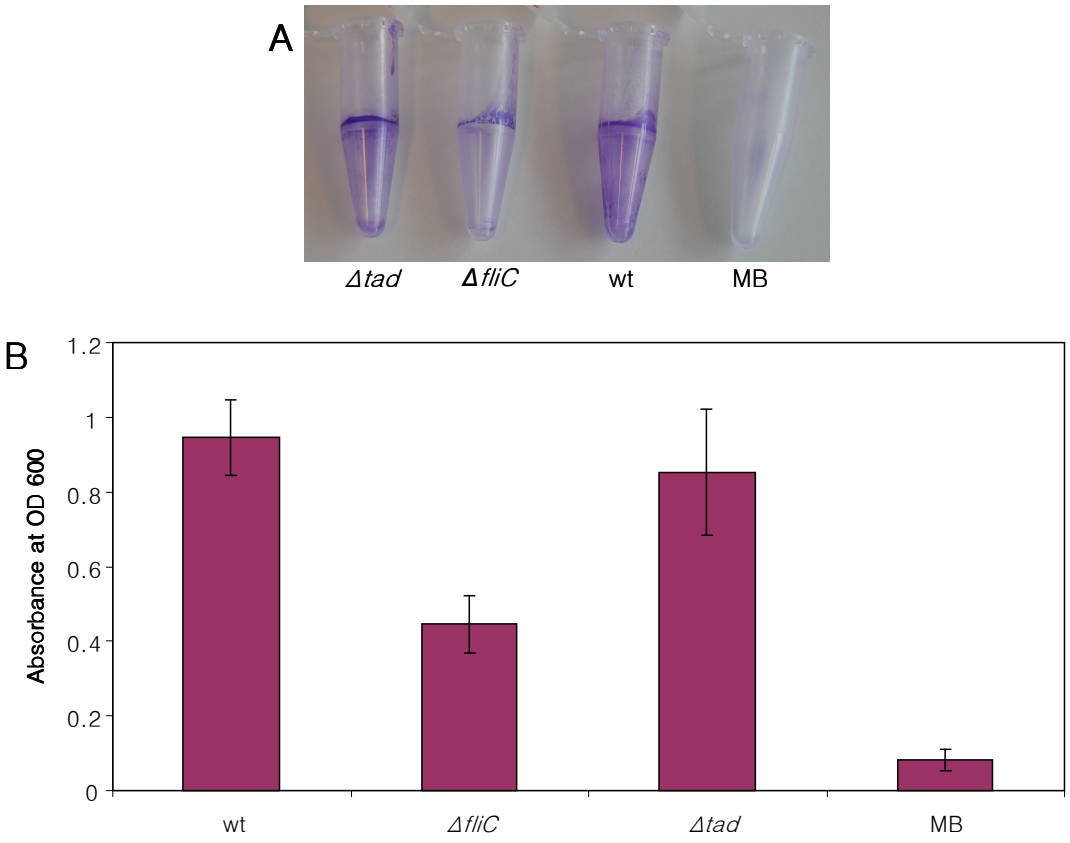


Figure 3. *In vitro* biofilm formation of *M. adhaerens* HP15 wild-type, Δ*tad* and Δ*fliC* mutants. MB medium was used as a negative control. **A**. Visualization of bacterial cells attached to polypropylene surface stained with 0.1% crystal violet. **B**. Quantification of crystal violet stained attached cells dissolved in 95% ethanol by absorbance at a 600 nm wavelength. Data are from the average of 8 samples from two independent experiments. Bars represent standard errors.



4. DISCUSSION

4.1. Identification of genes specifically expressed during the interaction between *M. adhaerens* HP15 and *T. weissflogii*

Identifying bacterial genes expressed during diatom-bacteria interaction is essential for understanding the functional mechanisms governing algal aggregation and marine snow formation in the oceans. Therefore, the *in vitro* model system consisting of *M. adhaerens* HP15 and *T. weissflogii* was used to identify bacterial genes specifically induced during the interaction of *M. adhaerens* HP15 with *T. weissflogii*. For this, two different strategies were used, firstly, genes were identified by *in vivo* expression technology (IVET), and secondly, proteins were identified by comparison of proteins profiles and identification by MALDI-TOF-MS. These strategies allowed the identification of genes encoding functions such as degradation and biosynthesis of organic compounds, central intracellular metabolism, nutrient scavenging, cell envelope structure, regulation, chemotaxis, secretion, stress response, and DNA transfer. For the purpose of this discussion the genes and proteins identified by the independent methods will be mixed and generally be named as proteins (**Table 2**).

4.1.1. Uptake, degradation and biosynthesis of organic compounds

We hypothesized that *M. adhaerens* HP15 induces genes to recognize, (partially) degrade, and use organic compounds secreted by *T. weissflogii* as carbon and nitrogen source. Once inside the cell, this carbon and nitrogen can be used to synthesize cellular compounds. Previously it has been shown that diatoms excrete organic carbon in the

form of DOC or EPS (Bhaskar et al. 2005, Grossart et al. 2006). In addition, it has been observed that heterotrophic bacteria can use these compounds (Grossart et al. 2006). This hypothesis is supported by a combination of proteins identified during this study.

Firstly, proteins involved in the **transport of substances** were identified. We found two lipoproteins; one putative lipoprotein, whose function is still not defined; and one D-methionine-binding lipoprotein, which is part of the methionine transport system (*metD* locus: *metNIQ*) (Gál et al. 2002). On the other hand, a protein down regulated, when *M. adhaerens* HP15 was exposed to but not directly interacting with *T. weissflogii*, a dihydropicolinate synthase (DHDPS) which is involved in lysine biosynthesis was identified. The activity of the purified DHDPS, was inhibit by the end product of the pathway, lysine (Laber et al. 1992, Mirwaldt et al. 1995), which prompts us to hypothesize that the availability of new organic carbon sources produced by *T. weissflogii,* might inhibit the expression of certain genes, as in this case. In contrast, other transport systems in *M. adhaerens* HP15 might be expressed in the absence of *T. weissflogii,* as a strategy to efficiently scavenge the low concentrated nutrients that might be present in the media. Although no carbon sources were added in the media when the diatom was absent, the minor concentrations of nutrients in the seawater used during the media preparation cannot be excluded. Three of the proteins identified here might support this hypothesis. Firstly, a protein of the TRAP dicarboxylate transporter (DctP), which a periplasmic C4-dicarboxylate-binding protein essential for high-affinity transport of C4-dicarboxylates malate, succinate and fumarate (Shaw et al. 1991). Secondly, a periplasmic substrate-binding protein associated to an amino acid ABC transponder (Tam & Saier 1993, Schneider & Hunke 1998). Finally, a TonB-dependent receptor, which might be involved in uptake of iron-siderophore complexes or vitamin B12 (Koebnik 2005).

Secondly, we identified enzymes involved in the **degradation** of organic compounds including amino acids (3-hydroxyisobutyrate dehydrogenase, and methylmalonate-semialdehyde dehydrogenase), proteins (leucyl aminopeptidase) lipid/fatty acid (acyl-CoA dehydrogenase), or diverse compounds degradation processes (haloacid dehalogenase, type II). An interesting example is the one for the enzymes involved in amino acid degradation; the two genes encoding for these enzymes are part of a locus (*mmsAB*) involved in the catabolism of valine (**Figure 17A**). This locus is comprised of the *mmsA* gene which encodes a methylmalonate-semialdehyde

dehydrogenase, *mmsB* gene, encoding for 3-hydroxyisobutyrate dehydrogenase and *mmsR* which encodes for a positive regulator (Steele et al. 1992). *mmsB* was found to be specifically induced in the presence of diatoms by the IVET screening strategy, whereas the *mmsA* was found to be an up-regulated protein when *M. adhaerens* HP15 was growing in contact of diatom exudates during the protein approach. These results strongly suggest that the catabolism of valine might play an important role during diatom-bacteria interactions. In addition, we identified a Fe-S oxidoreductase present in a genetic locus involved in lactate utilization, with homology to the one present in the *lutABC* operon of *Bacillus subtilis* (Chai et al. 2009). The *lutABC* operon is regulated by a transcriptional regulator of the GntR family, acting as a repressor when lactate is absent in the media (Chai et al. 2009). The promoter sequence driving the expression of such a regulator was also found as one of the *in vivo* expressed genes (**Figure 17B**), suggesting that this locus is indeed used during the interaction of *M. adhaerens* HP15 with *T. weissflogii.*

Finally, enzymes for **biosynthesis** processes were also identified, involved in biosyntheses of lipids and fatty acids (lipid A biosynthesis lauroyl acyltransferase), phospholipids (glycerol-3-phosphate acyltransferase and glycerol-3-phosphate dehydrogenase), amino acids (metal-dependent hydrolase-like protein, 3-isopropylmalate dehydratase, large subunit, and 3-dehydroquinate synthase) and proteins (30S ribosomal protein 5S, RNA methyltrasnferase and translational elongation factor TU). On the other hand, three other proteins (aconitate hydratase 1), an inorganic diphosphatase (PPase), and the alpha subunit of an electron transfer flavoprotein were expressed in the absence of *T. weissflogii*. These proteins might be inhibited by the presence of a *T. weissflogii* by-product.

4.1.2. Breakdown or modification of algal products

Heterotrophic bacteria associated to phytoplankton or aggregates mediate the hydrolysis or the modification of algal products (Arnosti 2011, Sapp et al. 2008, Grossart et al. 2007, Grossart & Simon 1998, Gärdes et al. 2012, Bilde & Azam 1999). Consequently, we found three proteins that might support these observations. Firstly, a polysaccharide deacetylase from the carbohydrate esterase family 4 (CE4) was

identified. This enzyme could be involved in the breakdown of chitin or other diatom-borne polysaccharides, since this family of proteins also includes chitin deacetylases (http://www.cazy.org/CE4.html; Cantarel et al. 2008). The genera *Thalassiosira* is known for producing chitin as a component of the cell wall (Durkin et al. 2009, Hert 1979), being a perfect target for the action of this enzyme. Secondly, a glycotransferase, which catalyzes the synthesis of glycoconjugates including glycolipids, glycoproteins, and polysaccharides (Kapitonov & Yu 1999, Campbell et al. 1997). Just as for almost any biosynthetic enzyme, the corresponding variant in *M. adhaerens* HP15 might conduct an opposite function, i.e. the breakdown of algal polysaccharides or might be involved in modification of existing polysaccharides as suggested by Gärdes et al. (2012). Thirdly, a spermidine/putrescine-binding periplasmic protein was also identified, which prompted us to speculate that uptake and metabolism of putrescine might be important during the interaction of *M. adhaerens* HP15 with *T. weissflogii*. The cell wall of diatoms contains high amounts of long-chain polyamines as part of the biosilica (Kröger et al. 2000, Kröger & Poulsen 2008) and putrescine (1, 4-diaminobutane) is a kind of polyamine. Previously, it had been shown that marine bacteria increased the dissolution of silica from lysed diatoms (Bilde & Azam 1999). The bacteria were found to colonize diatom detritus and carried out hydrolytic activities thereby removing the organic matrix and solubilizing silica. Therefore, we hypothesised that *M. adhaerens* HP15 might be using and transporting the polyamines present in the diatom cell wall inside the cell and use them as nitrogen source.

4.1.3. Proteins involved in other processes

Some proteins were identified to play roles in other processes of the **central metabolism**, including proteins involved in energy metabolism (putative NADH dehydrogenase, putative Na(+)-translocating NADH-quinone reductase subunit B, zinc-dependent alcohol dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase). *M. adhaerens* HP15 might have to adapt to the diatom-based environment and thus might need to undergo physiological changes. In addition, proteins involved in ribosom/protein synthesis were identified, such as a translation elongation factor TU (EF1A), a 30S ribosomal protein S5 and a RNA methyltransferase. It is hypothesized that

M. adhaerens HP15 interacting with *T. weissflogii* needs to coordinate the expression of novel proteins.

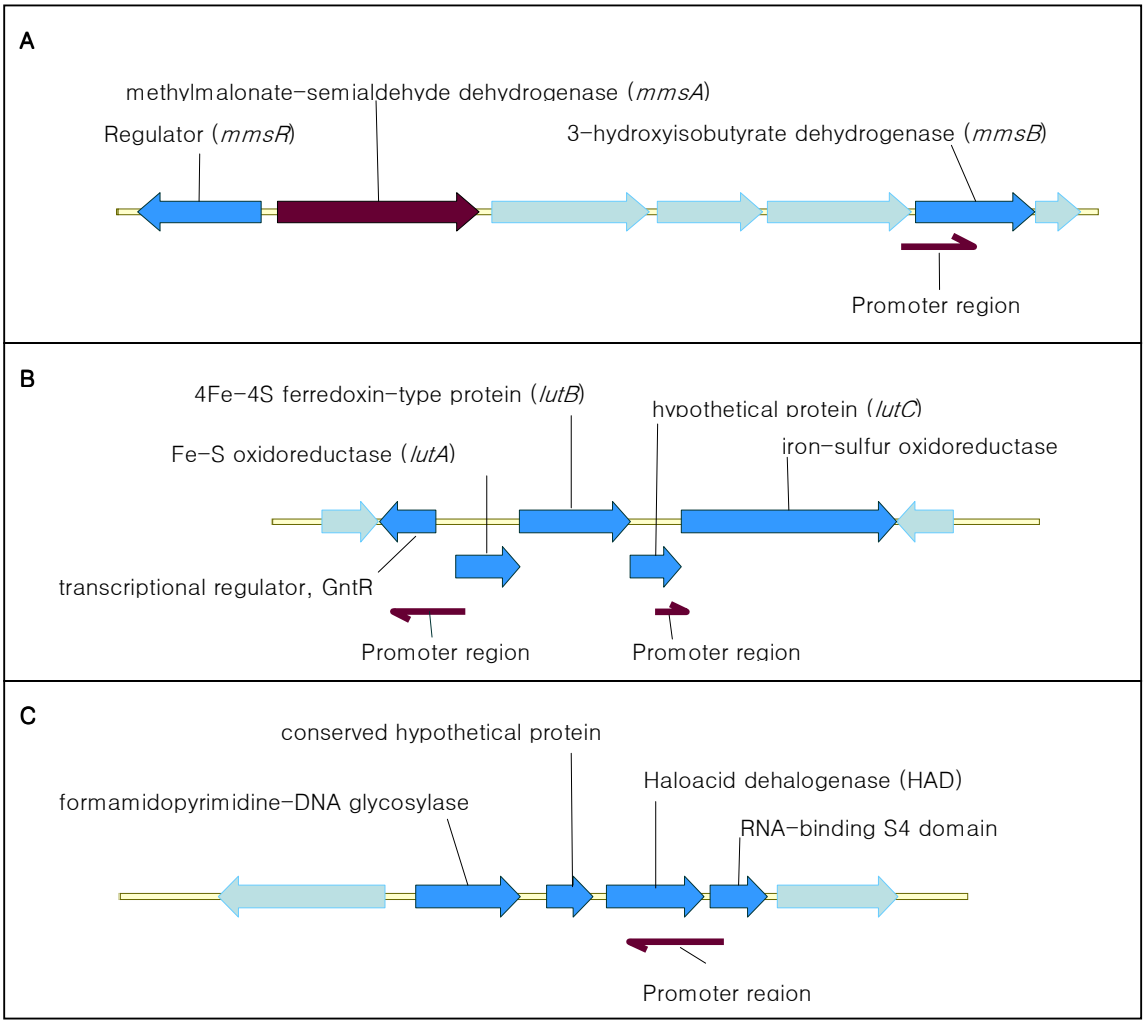


Figure 17. Chromosomal location of the genes encoding for the identified proteins in the genome of *M. adhaerens* HP15. The purple thin arrows represent HP15 genomic DNA inserts found upstream the *lacZ-pyrB* fusion by IVET. **A**. locus involved in the catabolism of valine; **B**. locus involved in lactate utilization; and **C**. an example for transcriptional fusions that are orientated in the antisense strand.

A Tol-Pal system-associated acyl-CoA thioesterase was identified, this protein is part of the Tol-Pal system which forms a membrane associated complex that maintains **outer membrane integrity** (Godlewska et al. 2009). However, this system has been reported to play other roles, for example in *E. coli* is involved in motility, cell division as well as in the release of periplasmic proteins from the cell (Godlewska et al. 2009). In addition, the operon shows similarity to that coding for the TonB system, which is important for active transport of diverse substrates such as siderophores or vitamin B12

(Moeck & Coulton 1998) and the MotAB proteins in the flagellar motor (Cascales et al. 2001). These previous studies suggest that the system might have different functions or that protein domains found in the enzymes encoded by these operons are shared by different processes. During the diatom–bacteria interaction this system could be important for uptake of specific nutrients, motility or integrity of the membrane system.

From the proteins identified to be involved in **regulation**, we speculate that *M. adhaerens* HP15 encounters a novel environment, different from the sea water, when interacting with *T. weissflogii.* Therefore, under these new conditions, the bacteria might need to regulate its gene expression. A PTS IIA–like nitrogen–regulatory protein, PTS system consists of a group of phosphotransfer proteins involved in the transport of carbohydrates, chemotaxis towards carbon sources, and in the regulation of other metabolic pathways (Postma et al. 1993). Secondly, a genetic locus consisting of two genes, a response regulator containing a receiver domain and a histidine kinase were identified. Thirdly, a transcriptional regulator of the LysR family was identified. This family of regulators is ubiquitous amongst bacteria regulating a diversity of genes involved in virulence, metabolism, quorum sensing, nitrogen fixing, and motility (Maddocks & Oyston 2008). Examples of representatives of this family are the *E. coli* LysR regulating lysine metabolism and LrhA regulating flagella, motility and chemotaxis, and *Rhizobium* spp. NodD regulating nitrogen fixation/symbiosis (Stragier et al. 1983, Lehnen et al. 2002, Schlaman et al. 1992). Fourthly, a putative MerR transcriptional regulator, which mediates responses to environmental stimuli such as heavy metals, drugs, antibiotics, or oxidative stress was identified (Brown et al. 2003). During photosynthesis many reactive oxygen species are released (Asada 2006) and it has been reported that certain bacteria interacting with diatoms detoxify oxygen species (Hünken et al. 2008). *M. adhaerens* HP15 growing with *T. weissflogii* might also face high amounts of reactive oxygen species hence the expression of some proteins counteracting oxygen radicals might become necessary.

A homologue of Type II **secretion** system (T2SS) protein C was identified to be expressed in *M. adhaerens* HP15 interacting with diatom cells. T2SS is a secretory pathway used in bacteria to release proteins into the environment. The gene encoding protein C is flanked by the T2SS genes for protein N (upstream) and protein D (downstream) in the chromosome of *M. adhaerens* HP15. Interestingly, the 'remaining' operon was found 746 locus tag apart in the *M. adhaerens* HP15 chromosome. A similar

scenario is found in *P. aeruginosa*, where two genes, *xphA* and *xqhA*, homologous to the ones encoding proteins C and D, respectively, are located distant from the major T2SS operon (Michel et al. 2007).

Chemotaxis has been shown to be important in the marine ecosystem as a bacterial strategy to reach the phycosphere (Barbara & Mitchell 2003, Mitchell et al. 1995, Stocker et al. 2008). In addition, *M. adhaerens* HP15 mutants in the genes *cheA,* coding for a central histidine kinase, and *chpB,* coding for a methyltransferase, exhibited a decreased diatom attachment (Sonnenschein et al. 2012). A protein containing a methyl-accepting chemotaxis domain was identified in this study. Additionaly, the response regulator containing a receiver domain mentioned in the regulation section above might be also involved in chemotaxis since it shares similarities to CheY-like response regulators (Stock et al. 1990). Therefore, our current results support the previous finding that chemotaxis of *M. adhaerens* HP15 is important for the diatom-bacteria interaction.

Additionally proteins (encoded by the genes *trbG* and *trbE*) in the **conjugative transfer** operon Trb found in the native plasmid of *M. adhaerens* HP15 (pHP-42) were identified. This operon together with the Tra operon have been described in *Agrobacterium tumefaciens* as genetic determinants of the system used for conjugal transfer between bacteria (Farrand et al. 1996, Cook et al. 1997, Li et al. 1998). Noteworthy, *M. adhaerens* HP15 possess a second, larger plasmid, pHP-187 without tra or trb operons suggesting that the system from the smaller plasmid might control conjugative transfer of the bigger plasmid. Co-regulation of plasmid transfer functions with those involved in host-microbe interactions have been described several times before (Backert & Meyer 2006, Chen et al. 2002, Zupan et al. 2000). Furthermore, it has been observed the existence of diatom genes with bacterial origin in the genome of the diatoms *Phaeodactylum tricornutum* (Bowler et al. 2008) and *Thalassiosira pseudonana* (Armbrust et al. 2004), indicating that the transfer of genes from bacteria to diatoms is a recognized mechanism.

A homologue sigma E-like regulatory protein was identified. This protein might play a role during a **stress response** mechanism induced by unfolded proteins. Relevant stressors include changes in temperature, pH or osmolarity, contact with ethanol, heavy metals, or antibiotics, as well as carbon starvation or oxidative stress (Bukau 1993). Whether or not there is a stress and if so which type of stress *M. adhaerens* HP15 might

be exposed to in presence of diatom exudates remains to be elucidated. However, this protein might also play a role in the regulation of exopolysacchride synthesis of *M. adhaerens* HP15 as shown for its homologous in *Vibrio vulnificus* (Brown & Gulig 2009) and *P. aeruginosa* (Damron & Goldberg 2012) both involved in exopolysaccharide synthesis important for virulence. It remains to be analyzed whether polysaccharide synthesis by *M. adhaerens* HP15 plays a role during diatom-bacteria interactions.

Table 2. List of proteins identified in this study, black proteins were identified by IVET screening, the blue proteins by the protein approach.

Identified proteins	Possible role
Central intracellular metabolism	
Haloacid dehalogenase, type II	Conversion of miscellaneous compounds
Glycerol-3-phosphate acyltransferase	Phospholipid metabolism
Putative NADH dehydrogenase	Energy metabolism
Acyl-CoA dehydrogenase	Lipid/fatty acid degradation
Putative Na(+)-translocating NADH-quinone reductase	Energy metabolism
Metal-dependent hydrolase-like protein	Multiple funtions
3-isopropylmalate dehydratase, large subunit	Leucine biosynthesis
30S ribosomal protein S5	Protein/ribosomal synthesis
Glycerol-3-phosphate dehydrogenase	Phospholipid metabolism
RNA methyltransferase	Protein/ribosomal synthesis
3-dehydroquinate synthase	Aromatic amino acid biosynthesis
Zinc-dependent alcohol dehydrogenase	Intermediary metabolism
Lipid A biosynthesis lauroyl acyltransferase	Lipid synthesis
Glyceraldehyde 3-phosphate dehydrogenase	Intermediary metabolism / Glycolysis
3-hydroxyisobutyrate dehydrogenase	Valine catabolism
Leucyl aminopeptidase	Protein degradation
Translation elongation factor TU	Protein synthesis
Methylmalonate-semialdehyde dehydrogenase	Valine catabolism
Nutrient scavenging	
D-methionine-binding lipoprotein (DHDPS)	Methionine transport
Putative lipoprotein	Outer membrane protein
Cytosine permease	Uptake of nucelobases
Putative D-lactate dehydrogenase (Fe-S oxidoreductase)	Lactate utilization
Cell envelope structure and modification	
Polysaccharide deacetylase	Peptidoglycan modification
Glycosyltransferase	Cell envelope biogenesis
Spermidine/putrescine-binding periplasmic protein	Spermidine/putrescine transport
Tol-Pal system-associated acyl-CoA thioesterase	Cell envelope integrity
Regulation	
Transcriptional regulator, GntR family	Transcriptional regulators
Putative regulatory protein, LysR-like	Transcriptional regulators
PTS IIA-like nitrogen-regulatory protein PtsN	Sugar uptake regulation
Response regulator containing receiver domain	Two-component signal regulation
MerR transcriptional regulator	Transcriptional regulators

Protein secretion	
Type II secretion system protein C	Secretion
Protein containing bacterial Ig-like domain	Adhesion/invasion
Chemotaxis	
Methyl-accepting chemotaxis protein	Chemotaxis
DNA transfer	
Conjugal transfer Trb operon	Conjugation
Stress response	
Putative sigma E regulatory protein	Stress response
Down-regulated in presence of diatom exudates	
ATPase, F1 complex, delta subunit	Energy metabolism
Dihydrodipicolinate synthase	Amino acid synthesis / lysine
Lipid/polyisoprenoid-binding YceI-like protein	Energy metabolism
Inorganic diphosphatase (PPase)	Central metabolism
TRAP dicarboxylate transporter, DctP subunit	Transport of C4-dicarboxylates
Aconitate hydratase 1	TCA cycle
TonB-dependent receptor	Iron and vitamin B12 uptake
Amino acid ABC transporter, periplasmic substrate-binding protein	Transport of amino acids
Electron transfer flavoprotein (ETF) subunit alpha	Oxidation of fatty acids

During the IVET screening, four conserved hypothetical proteins were identified; for them a possible role could not be defined, since they share no significant similarities with any previously reported protein in the GenBank, additionally no putative conserved protein domain could be indentified. These proteins might play an important role during diatom-bacteria interactions, and its role should be elucidated in the future. In addition, we identified 11 transcriptional fusions that are orientated in the wrong direction, on the antisense strand of an annotated coding region, an example is shown in **Figure 18C**. The proteins associated to these transcriptional fusions are listed in **Table 3**. Such antisense transcripts are commonly identified during IVET screens (Silby et al. 2004, Reiders et al. 2005, Jackson & Giddens 2006), they could be artifacts of the IVET screening. However, the amount of these kind of transcripts found in different IVET systems and studies suggest that they are indeed positive hits and not just artifacts derived from the technique. They might drive the expression of small non-coding RNA molecules which are important during adaptation to the environment, by regulation of protein synthesis affecting transcription, translation and stability or altering protein activity by protein binding (Repoila et al. 2003).

The diverse functions of the identified genes or proteins were postulated based on their similarities to previously reported genes or proteins or by the presence of individual

conserved protein domains. However, the actual functions of such proteins and their potential roles during the diatom–bacteria interaction still need to be determined experimentally. This particularly applies to the so–called 'conserved hypothetical proteins' and to transcripts which showed the wrong orientation. Therefore, the expression levels of the identified genetic loci or bacterial proteins should be determined by culturing *M. adhaerens* HP15 in presence and absence of *T. weissflogii* cells, followed by isolation of mRNA and quantification of expression levels by RT–qPCR with individual and specific primers. Once expression levels are determined, the differential expressed proteins can then be studied further. Ultimately, the potential involvement of the those genetic traits in diatom–bacteria interaction should be systematically investigated by knocking out the corresponding gene(s) by site–directed mutagenesis (Sonnenschein et al. 2011) and analysing the phenotypes of mutants in terms of attachment to diatom cells and marine aggregate formation using attachment assays and rolling tank experiments, respectively (Gärdes et al. 2011). Simultaneously, the potential role postulated in this work for proteins involved in uptake, transport and degradation of organic compounds could be analysed by growing the corresponding mutants in media containing such organic compounds. For example, a mutant strain unable to synthesise the D–methionine–binding lipoprotein is expected to be impaired in growth on D–methionine–containing media.

Table 3. List of proteins identified during IVET screening, with transcriptional fusions that are orientated in the antisense strand.

Identified proteins	Possible role
Central intracellular metabolism	
Haloacid Dehalogenase (HAD)	Conversion of miscellaneous compounds
Glycerol–3–phosphate acyltransferase	Lipid/fatty acid metabolism
Queuine tRNA–ribosyltransferase	Protein synthesis
Release Factor 3 (RF3) protein	Protein synthesis
Oxaloacetate decarboxylase gamma chain	Amino acid synthesis
Beta–glucosidase	Sugar metabolism
Nudix_Hydrolase	Several functions
Cell envelope structure and modification	
D–alanyl–D–alanine carboxypeptidase	Cell wall biogenesis
Nutrient scavenging	
ClC chloride channels	Ion acquisition
Nucleic acid metabolism	
Chromosome segregation protein SMC	Cell division
DNA transfer	
Conjugal transfer Trb operon	Conjugation

Over all, it is important to exactly define the type of interaction between *T. weissflogii* and *M. adhaerens* HP15. In previous studies it has been shown that *M. adhaerens* HP15 together with *T. weissflogii* show a synergistic association, in which the growth of both organisms is enhanced (Gärdes et al. 2010). In addition, the results obtained during this work indicated that *M. adhaerens* HP15 might benefit from compounds released by diatoms, which the later produced during photosynthesis or needed during cell wall synthesis. However, the benefit that the diatom cells botains during the interaction is still unknown. For this reason, the type of interaction between these organisms has to be further analyzed. Specifical studies on the molecular response of *T. weissflogii* towards the co-inoculation with *M. adhaerens* HP15, have to be carried out. Microscopic observations of *T. weissflogii* at different growth stages, i.e. exponential, early and late stationary grown with different concentrations of *M. adhaerens* HP15 cells can help to see changes in diatom morphology. Furthermore, expression analysis on the genes induced in *T. weissflogii* while interacting with *M. adhaerens* HP15 will further help to elucidate the mechanisms behind this interaction. The current study provided a number of interesting hypotheses for novel experimental approaches and thoughts.

4.2. Identification of the *tad* locus in *M. adhaerens* HP15

The tight adherence (*tad*) gene locus, found in several bacteria and archaea, and known for encoding type IVb fimbrial low-molecular-weight (Flp) pili, was demonstrated to be present in the native plasmid pHP-187 of *M. adhaerens* HP15. In most organisms the *tad* locus plays an important role in the colonization of environmental niches. It was first identified in *Aggregatibacter actinomycetemcomitans,* a Gram-positive human periodontal pathogen, being responsible for tight adherence, autoaggregation, biofilm formation, and the production of bundled fimbria-like fibers of individual pili (Kachlany et al. 2000, 2001, Planet et al. 2003, Perez et al. 2006, Tomich et al. 2007). In this bacterium the *tad* locus is composed by 13 genes (*flp-1-tadV-rcpCAB-tadZABCDEFG*). The *flp-1* gene encodes the Flp1 prepilin and is post-translationally modified by a prepilin protease (TadV) (Perez et al. 2006). In contrast, the *tad* locus present in *M.*

adhaerens HP15 consists of 9 genes, starting with the *flp* followed by a gene with non significant similarity to any *tad* loci genes and then the genes *rcpCA* and *tadZABCDG*. The proteins encode by these genes share high amino acid similarities to the ones found in *A. actinomycetemcomitans* D11S-1, *Caulobacter crescentus* CB15 and *Pseudomonas aeruginosa* PAO1. Interestingly, no homologous proteins in *M. adhaerens* HP15 were found for the proteins RcpB, TadV, TadE or TadF. In *A. actinomycetemcomitans* RcpB is an outer membrane protein possibly required for the stability of the Tad secretion system (Perez et al. 2006), TadV functions as a prepilin-specific peptidase which removes the leader peptide from the prepilin necessary for pilus assembly, whereas TadE and TadF are pseudopilins (Tomich et al. 2006).

A promoter region upstream the *flp* gene in *M. adhaerens* HP15 was *in silico* predicted and experimentally identified. This region was able to express a *lacZ* reporter gene under laboratory conditions, suggesting a constitutive activity. This functional promoter might indicate that the *tad* locus is being expressed in *M. adhaerens* HP15. Consequently, a *M. adhaerens* HP15 mutant lacking the genes *flp-g5-rcpCA* (Δ*tad*) was constructed to determine the functionality and possible role of the *tad* locus. Different experiments, such as colony morphology, motility, *in vitro* biofilm formation, attachment to diatom cells, were carried out to characterize potential mutant phenotypes. However, under none of the conditions tested a differential phenotype for the mutant compared to the wild-type could be verified. For example, the ability to form biofilm on abiotic surface did not show significant differences compared to the wild-type strain. A similar observation was done during the attachment of *M. adhaerens* HP15 Δ*tad* mutant to diatom cells, where the wild-type showed better attachment; however, not statistically significant. These results might suggest; first, that performed experiments were not suitable to demonstrate a potential function of the *tad* locus; or second, a lack of expression of the genes forming the *tad* locus, maybe due to the absence of some of the genes previously reported to be essential for pilus assembly, such as the prepilin peptidase. However, a Δ*flp* mutant in *Yersinia enterocolitica* showed no differences in the biotic or abiotic attachment capacities compared to the wild-type. However, this mutant was impaired in microcolony formation (Schilling et al. 2010). For this reason, experiments to assess the microcolony formation in *M. adhaerens* Δ*tad* and wild-type will have to be performed in the future. In addition, the conditions of the experiments carried out here have to standardize. Furthermore, further experiments have to be carried out to

determine whether the Flp pili are actually being formed in *M. adhaerens* HP15. For example by transmission electron microscopy of cells incubated under pilus-inducing conditions and by quantification of *flp* expression using RT-qPCR.

The results shown in this study represent a good basis for further experiments to better understand and characterize the role of the *tad* locus in *M. adhaerens* HP15.

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