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Taxonomic and genetic analysis of *Marinobacter* sp. HP15 and its chemotactic behaviour towards the diatom *Thalassiosira weissflogii*

by

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Abstract

Aggregation of micro-algae, mainly of diatoms, is an important process in marine pelagic systems leading to the sinking of particulate organic matter in form of marine snow. This process has been studied extensively, but the specific role of heterotrophic bacteria, their genes, gene products, and secondary metabolite signals for this process has largely been neglected. A bilateral model system consisting of the diatom, *Thalassiosira weissflogii*, and the bacterial strain, *Marinobacter* sp. HP15, was found suitable for an in-depth molecular analysis by attachment assays, TEP production determination, and aggregation experiments. Following the taxonomic and genomic description of the bacterial model strain, its genetic accessibility was demonstrated and the interaction has been studied by molecular and bioinformatics investigations. Chemotaxis- and motility-deficient mutants were generated and their phenotypes were described in appropriate assays in order to investigate the particular role(s) of these processes in diatom-bacteria interactions. HP15 was attracted to diatom homogenate in soft agar assay and chemotaxis-deficient mutants showed a decreased ability to attach to *T. weissflogii* supporting the importance of chemotaxis in diatom-bacteria interactions. These and future results based on the model system will improve our understanding of bacteria-phytoplankton interactions and how they influence the flux and cycling of carbon during marine snow formation.

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I want to express my gratitude to Prof. Dr. Matthias Ullrich for supervising this thesis work, the constant support and scientific freedom.

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Never-ending gratitude to my parents and my sisters. The world does not turn without you.

List of Abbreviations

ALA 5-aminolevulinic acid

CCMP Center for Culture of Marine Phytoplankton

CFU Colony forming units

CLSM Confocal laser scanning microscopy

CIP Collection de l'Institut Pasteur

Cm Chloramphenicol

DAPI 4´,6-diamino-2-phenylindole

DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

DOC Dissolved organic carbon

DOM Dissolved organic matter

ESD Equivalent spherical diameter

EPS Exopolysaccharides

GFP Green fluorescent protein

LB Luria-Bertani broth

MB Marine broth

MIC Minimal inhibitory concentrations

POC Particulate organic carbon

POM Particulate organic matter

SOC Super optimal broth with catabolite repression medium

TEM Transmission electron microscopy

TEP Transparent exopolymer particles

Contents

Chapter 1

Introduction

1.1 Biological pump

Worldwide primary production is on a quantity basis equally conducted on land as well as in the ocean (Smetacek, 1999; Lalli and Parsons, 1993). Compared to benthic macroalgae, phytoplankton organisms are responsible for more than 90 % of carbon dioxide fixation and thus significantly contribute to the global climate system. The organic matter fixed in phytoplankton biomass is transfered to lower trophic levels by direct exudation (Hellebust, 1965), indirect production through "sloppy feeding" by zooplankton (Lampert, 1978), dissolution of fecal pellets, marine snow and other aggregates (Alldredge et al., 1993), and cell lysis (Aluwihare and Repeta, 1999) (Fig. 1.1). Heterotrophic bacteria transform the organic matter by respiration and production of new biomass, where members of the *Cytophaga-Flavobacteria-Bacteriodes* cluster and the γ-*Proteobacteria* were suggested to be the main actors (Grossart and Ploug, 2001; Giovannoni and Rappé, 2000). Earlier studies have indicated that bacterial biomass in the sea is directly related to phytoplankton concentration and that bacteria utilize 10 to 50 % of the carbon fixed by photosynthesis (Azam et al., 1983). Furthermore, it

was suggested that bacteria increase aggregation probabilities of phytoplankton and other particles and act as stabilizers of already existing aggregates as marine snow (Decho, 1990). Hence, bacteria either loosely or tightly associated with phytoplankton may efficiently increase sedimentation and removal of organic matter from the water column. However, most of the carbon fixed by phytoplankton is converted back through consumers to CO_2 released to the atmosphere. Some finds its way to the deep ocean where it is stored on average for 1,000 years (Chisholm, 2000). Even though the principle of the biological pump is well understood, little is known on the specific biological interactions driving the aggregation of organic matter to marine snow and thereby influencing the global climate system.

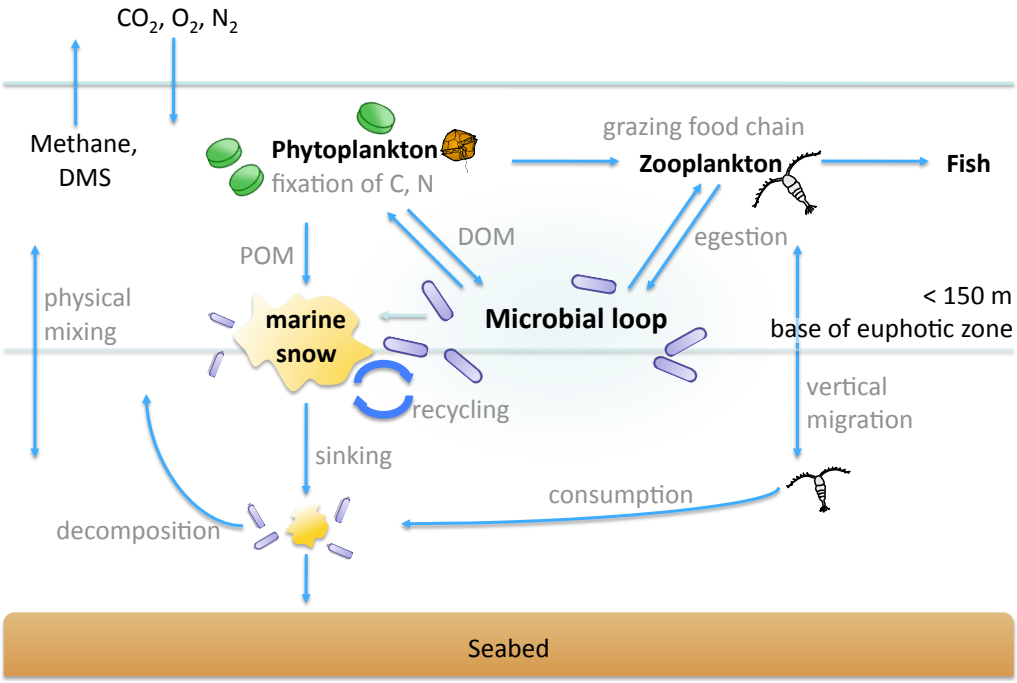


Figure 1.1: Schematic presentation of the biological pump describing the transport of atmospheric CO_2 fixed by autotrophs from the upper ocean to deep waters by various processes; DMS = dimethyl sulfide, DOM = dissolved organic matter, POM = particulate organic matter. Modified after `http://www.msrc.sunysb.edu` and Azam (1998).

1.2 Marine snow

The term ”marine snow” describes marine aggregates larger than 0.5 mm formed from phytoplankton, fecal pellets, inorganic particles, and zooplankton feeding structures (Alldredge et al., 1998). They originate by two major pathways in the ocean (Alldredge and Silver, 1988). First, zooplankton such as larvaceans and pteropods produce novel aggregates as discarded mucus feeding webs or flocculent fecal pellets. Next, marine snow is formed primarily via the collision and subsequent attachment of smaller particles present in the water column (McCave, 1984), often glued by a matrix of sticky, gel-like transparent exopolymer particles (TEP) (Alldredge et al., 1993). The abundance of marine snow is often linked to the occurrence of phytoplankton blooms (Alldredge and Gotschalk, 1989). Marine snow aggregates are enriched in carbon, nitrogen and other nutrients, and harbor concentrated communities of microorganisms at abundances several orders of magnitude higher than those found free-living in the surrounding seawater (Alldredge and Silver, 1988).

1.3 TEP

Transparent exopolymer particles (TEP) have been described to play an important role in phytoplankton-bacteria interactions and marine snow formation (Passow, 2002b). TEP are formed from exopolysaccharides released by these microorganisms (Passow, 2000). They primarily contain acidic polysaccharides and can therefore be visualized by alcian blue staining (Alldredge et al., 1993). Dominant single sugars of TEP are fucose, rhamnose and arabinose (Myklestad and Haug, 1972). Nitrogen-rich substances like amino acids (Schuster et al., 1998) and proteins (Hoagland et al., 1993; Mopper et al., 1995), trace elements, and heavy metals (Engel and Passow, 2001) are easily absorbed. The size of the discrete,

transparent particles ranges from 2 to 500 µm in diameter (Passow and Alldredge, 1994). They are abundant in marine and freshwater systems (Alldredge et al., 1993; Grossart and Simon, 1997) at concentrations of 1 to 10^4 particles ml^{-1} (Passow and Alldredge, 1994; Passow, 2002a; Mari and Burd, 1998). TEP are highly surface-active (Mopper et al., 1995) and easily aggregate with themselves and with other particles. Therefore, they are thought to act as precursors to form larger particles (Passow, 2002a) and act as the main vehicles for fast downward flux of organic matter (Fowler and Knauer, 1986) and sedimentation (Passow et al., 2001). Further, they have an important role in the food web, because they aggregate nanometer-sized particles making them available as food source for larger zooplankton (Dilling et al., 1998; Passow and Alldredge, 1999; Prieto et al., 2001).

1.4 Bacteria-diatom interactions

Diatoms represent the largest group of phytoplankton organisms. They are unicellular eukaryotic algae, which are characterized by their silica cell walls. Diatoms are distributed worldwide – in marine and freshwater – and occur as solitary cells or in chains. They are responsible for more than 50 % of marine primary production (Nelson et al. 1995). The concept of the 'phycosphere' was introduced as a pelagic analogy to the rhizosphere (Bell and Mitchell, 1972; Cole, 1982). It describes the zone extending outward from an algal cell or colony for a variable distance, in which bacterial growth is stimulated by extracellular products of the algae. The bacterial communities in the phycosphere of a diatom species are distinct (Grossart et al., 2005). Representatives of these communities mainly belong to the *Cytophaga-Flavobacteria-Bacteriodes* group in contrast to typical free-living bacterial communities, which were shown to be mainly α- and γ-*Proteobacteria* (Fandino et al., 2001; Grossart et al., 2005). *In situ*, bacteria are often virtu-

ally absent in early diatom bloom stages, whereas late bloom stages coincide with increased colonization by attached bacteria (Azam, 1998).

Laboratory experiments revealed four major modes of diatom-bacteria interactions were identified (Cole, 1982; Grossart, 1999; Caron et al., 2000; Steinert et al., 2000): 1) Bacteria benefit from phytoplankton exudates while phytoplankton cells profit from bacterial products such as remineralized nutrients, vitamins, and other growth factors (mutualism) (Bell et al., 1974; Cole, 1982). 2) Bacteria may parasitize on algal cells, which may lead to cell lysis and death (Cole, 1982; Imai et al., 1993). To prevent this, many algae have evolved the ability to produce antibiotics (Sieburth and Jensen, 1968; Cole, 1982; Kellam and Walker, 1989; Sastry and Rao, 1994). 3) Commensalic bacteria benefit from phytoplankton without having any negative effect on it. This mode is transient and may change to parasitism under stress conditions (Barbeyron and Berger, 1989). 4) Bacteria and diatoms are only loosely associated and compete for limited nutrients (Rhee, 1972). The type of interaction is highly depended on the environmental conditions and on the bacteria present. Diverse and multiple interactions may exist simultaneously (Cole, 1982; Grossart, 1999). The pathways of diatom-bacteria interactions are influenced by factors such as inorganic nutrients, dissolved organic matter (DOC), antagonistic substances, and functional traits such as motility, attachment, and chemotaxis (Fig. 1.2) (Cole, 1982).

1.5 Bacterial chemotaxis

Chemotaxis describes the process of an organism sensing and reacting to a chemical gradient by movement. Depending on the effect of the chemical on the organism, chemotaxis is positive to attractants or negative to repellents such as toxins. In bacteria, chemotaxis is regulated by a histidine–aspartate phosphorelay (HAP)

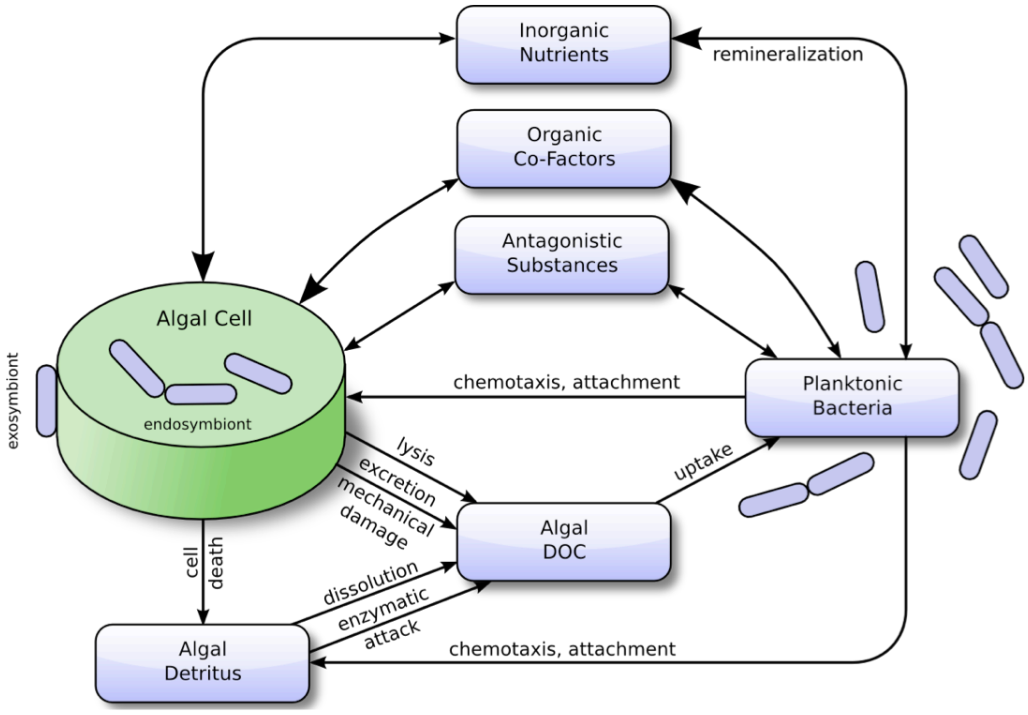


Figure 1.2: A simplified presentation of the pathways that occur in the phycosphere. An array of indirect interactions such as bacterial metabolism of chelators or allelopathic substances are omitted from this diagram. The arrows with the large heads denote the predominant direction of a process. Modified after Cole (1982).

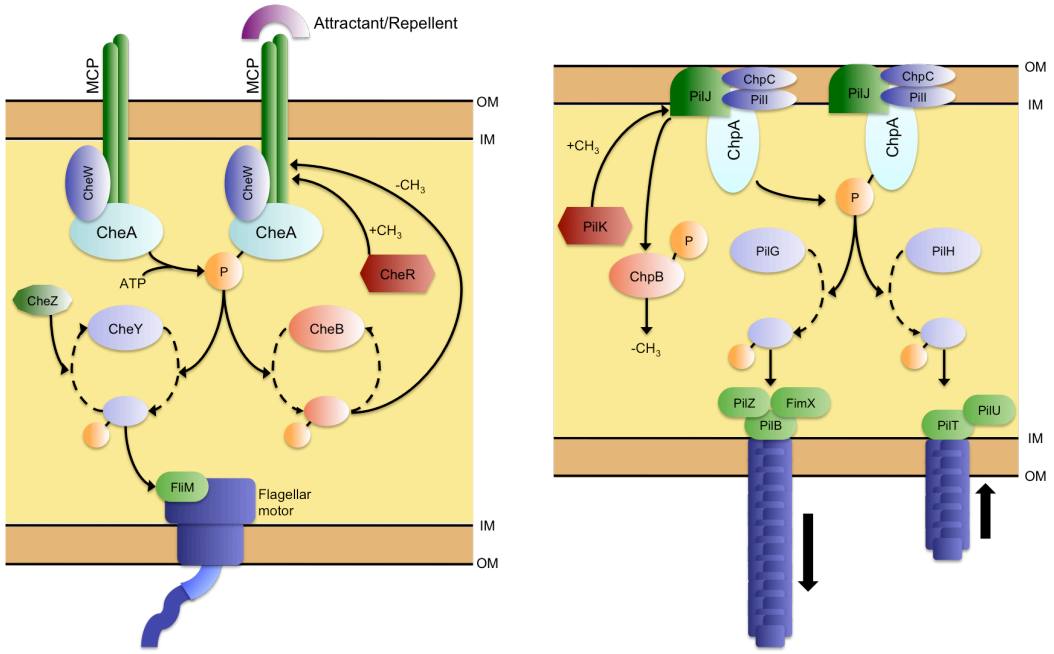
system with at least three components - a methyl-accepting chemotaxis proteins as receptor, a usually dimeric histidine protein kinase (HPK), and a response regulator (RR) (West and Stock, 2001). It has been most extensively studied in the enteric bacteria *Escherichia coli* (Wadhams and Armitage, 2004) and *Salmonella enterica serovar Typhimurium* (Stecher et al., 2004). Chemical signals are detected by transmembrane chemoreceptors - methyl-accepting chemotaxis proteins (MCPs) (Fig. 1.3 a). The MCPs are linked to the cytoplasmic HPK, CheA, by the help of an adaptor protein, CheW. Two RRs compete for the binding to CheA – the flagellar-motor-binding protein CheY and the methylesterase CheB, which controls the adaptation of the MCPs. The flagella generally rotate counterclockwise caus-

ing the cell to swim in one direction (Armitage 1992). When the phosphorylated CheY (CheY-P) binds to the switch protein FliM on the flagellar motor, it causes the reversal of rotation to clockwise resulting in cell tumbling and direction change. CheZ is required to determine the signal of CheY–P by spontaneous dephosphorylation. Adaption to the stimulus is established by the phosphorylation of CheB by CheA–P. Increased methylesterase activity of CheB results in increased demethylation of the MCPs and thereby, a decrease of autophosphorylation of CheA and the direction changing returns to pre-stimulus level. Increasing attractant concentration inhibits the autophosphorylation of CheA and therefore, the motility towards the positive direction. CheB phosphorylation is also reduced, which allows further methylation of the MCPs via the activity of the methyltransferase CheR. In turn, this stimulates CheA autophosphorylation, even in the continued presence of a chemoattractant, and returns CheA autophosphorylation to the pre-stimulus level. Consequently, the bacterium continues with a normal frequency of direction changing.

A similar model was hypothesized for the regulation of type IV pilus activity, the so-called twitching motility, by the Chp chemosensory system (Bertrand et al., 2010) (Fig. 1.3 b). The histidine kinase ChpA is linked to the MCP, PilJ, by one of the CheW homologous PilI or ChpC. The CheY-like proteins PilG and PilH interact with the motor complex of the pili causing extension and retraction of the latter one.

Bacterial chemotaxis in marine planktonic systems

In all kinds of environments, the signalling pathway of bacterial chemotaxis is essential in processes such as pathogenicity, biofilm formation, or symbiotic interactions. In marine environments, *Vibrio fischeri* has been well studied, since chemotaxis guides the free-living bacteria to the squid light organs resulting in



(a) (b)

Figure 1.3: (a) Schematic diagram of the chemosensory system for flagella movement of *Escherichia coli* (modified after Wadhams and Armitage, 2004). (b) Model for the regulation of type IV pilus function by the Chp chemosensory system of *Pseudomonas aeruginosa* (modified after Bertrand et al., 2010).

quorum sensing and thereby, bioluminescence (see Decho, 1999, and references therein). For marine planktonic bacteria, particles provide surfaces for attachment and are a source of nutrients (Shanks and Trent, 1979; Grossart et al., 2003). Aggregates like marine snow as well as phytoplankton cells may provide corresponding surfaces. Many bacterial isolates were obtained from aggregates and phytoplankton cells, and chemotaxis was studied towards marine snow and phytoplankton products (Blackburn, 1998; Kiorboe and Jackson, 2001; Miller and Bassler, 2001; Seymour et al., 2009).

1.6 The interaction model system

The diatom *Thalassiosira weissflogii*

The ubiquitously distributed diatom *Thalassiosira weissflogii* (G. Fryxell and Hasle, 1977) (Fig. 1.4) is a cylindrical cell with numerous long threadlike spines composed of β-chitin. It belongs to the class of *Coscinodiscophyceae* and the phylum of *Bacillariophyta*. Cell diameter range from 12 to 22 µm (Provasoli Guillard National Center for Culture of Marine Phytoplankton (CCMP)). Since it is easy to culture and has a short doubling time, *T. weissflogii* has been used as model species for a long time e. g. for copepod grazing experiments (Koski et al., 2008; Ceballos and Ianora, 2003), gene expression analysis (Armbrust, 1999; Leblanc et al., 1999), and toxicity studies (Casotti et al., 2005; Windust et al., 1997). Essential for this study is that axenic *T. weissflogii* produces TEP, hence in fairly small amounts compared to other diatom species (Passow, 2002a; Crocker and Passow, 1995).

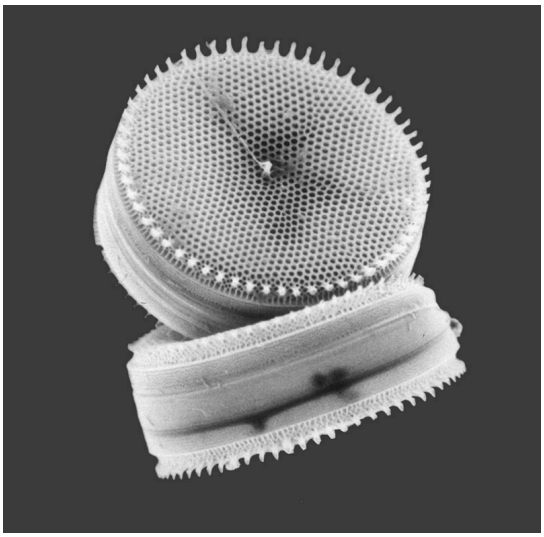


Figure 1.4: The diatom *Thalassiosira weissflogii*, courtesy of F. Hinz, Alfred Wegener Institute, Bremerhaven

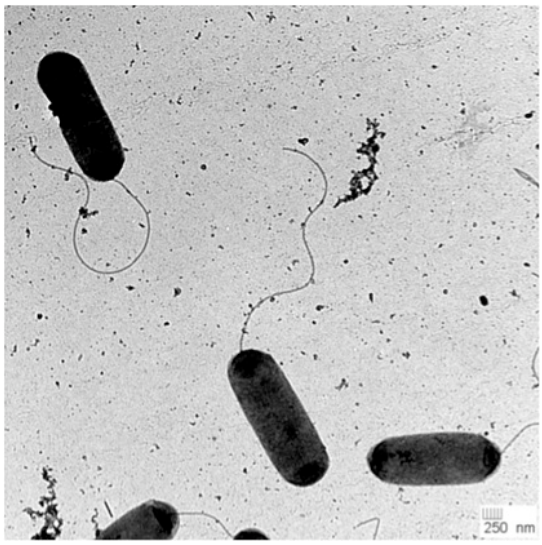


Figure 1.5: The bacterial model strain *Marinobacter* sp. HP15

The bacterium *Marinobacter* sp. HP15

82 particle-associated bacterial isolates from the North Sea (Grossart et al., 2006b; Wichels et al., 2006) were tested for attachment to the diatom *T. weissflogii*. The attaching strains were individually co-incubated with the microalgae and TEP production was measured. Four strains inducing the highest production were further investigated by rolling tank experiments to describe the aggregation behaviour with the diatom (Gärdes et al., 2010a). These strains, Ex5, H14, HP10, and HP15 (Tab. 1.1), belonged to the phyla *Bacteriodetes*, *Proteobacteria*, and *Firmicutes*, respectively, and were all motile. Before testing their genetic accessibility, the sensitivity to various antibiotics as well as their survival in distilled water, which is used for washing of electrocompetent cells, was assessed.

Plasmid transformation was successful for the strain HP15, meeting thereby the last criterium for being a suitable model bacterium. The strain HP15 (Fig. 1.5) was isolated from marine particles (0.1 to 1 mm in diameter) from surface waters of the German Wadden Sea in 2004 (Grossart et al., 2006b). Accord-

Table 1.1: Bacterial strains inducing aggregation with the diatom *T. weissflogii*

Strain, accession no.	Closest relative, %	Class	Phylum
Ex5, AY948365	*Winogradskyella arenosi*, 97	*Flavobacteria*	*Bacteroidetes*
H14, AY277261	*Colwellia aestuarii*, 98	*γ-Proteobacteria*	*Proteobacteria*
HP10, AY172664	*Bacillus pumilus*, 96	*Bacilli*	*Firmicutes*
HP15, AY241552	*Marinobacter flavimaris*, 99	*γ-Proteobacteria*	*Proteobacteria*

ing to 16S rRNA gene analysis (accession no. AY241552), HP15 belongs to the genus of *Marinobacter* (Gauthier et al., 1992) in the order of *Alteromonadales* and the class of *γ-Proteobacteria*. The cells of HP15 are rod-shaped and motile by means of a single polar flagellum and form small translucent-brownish colonies. *Marinobacter* species are tolerant to various conditions as they were isolated from diverse locations - from the sediment (Gorshkova et al., 2003), the water column (Yoon et al., 2004), from coastal (Roh et al., 2008) and deep sea waters (Takai et al., 2005), from the Antarctic (Montes et al., 2008) and from the Red Sea (Antunes et al., 2007). Furthermore, representatives of this genus were isolated from oil-contaminated areas (Huu et al., 1999), hot springs (Shieh et al., 2003), and salines (Martín et al., 2003). Initially, the genus *Marinobacter* has attracted attention due to the ability to degrade complex petroleum-derived hydrocarbons (Huu et al., 1999). Recently, several *Marinobacter* strains have been isolated due to their interactions with phytoplankton organisms (Green et al., 2006; Grossart et al., 2005; Morris et al., 2008). Besides the genome described in this study, three other *Marinobacter* genome sequences are available, namely those of *M. aquaeolei*, *M. aligcola* , and *Marinobacter* sp. ELB17.

Chapter 2

Aims of the work

Although our knowledge on bloom formation and aggregation of organic matter in the ocean has increased in recent years, the exact mechanisms of the interaction and communication between diatoms and bacteria and the way how particle formation occurs are still unknown. By establishing and describing a molecular model system and characterization of the biochemical activities as well as the identification of interaction-relevant genes, results of this study might contribute to our understanding of the role of diatom-bacteria interactions. Hence, the major aims of this thesis were the following:

1. Identification and description of a genetically accessible bacterial model strain interacting with the diatom *Thalassiosira weissflogii*
2. Taxonomic characterization of *Marinobacter* sp. HP15 and description as *Marinobacter adhaerens* nov. sp.
3. Description of the *Marinobacter* sp. HP15 genome with emphasis on genes essential in phytoplankton-bacteria interactions
4. Analysis of the chemotactic behavior towards the diatom *Thalassiosira weissflogii* and its exudates

5. Identification and characterisation of bacterial chemotaxis genes and assessment of their role in the diatom-bacteria interaction

Chapter 3

Results

Results are represented by the following manuscripts originated during this PhD thesis with authors´ contributions:

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

Kaeppel EC, Gärdes A, Seebah S, Grossart HP, and Ullrich MS

ECK conducted all experiments, which were not carried out by DSMZ, and prepared the manuscript. AG, SS, and HPG revised the manuscript. MSU revised and finished writing the manuscript. All authors have read and approved the final manuscript.

(submitted to International Journal of Systematic and Evolutionary Microbiology)

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

Gärdes A, Kaeppel EC, Shezad A, Seebah S, Teeling H, Yarza P, Glockner FO, and Ullrich MS

TEM micrographes were taken by AG and ECK. ECK conducted the chemotax-

onomic experiments. SS prepared the genomic DNA for sequencing. Genome assembly, annotation, and sequence analysis was done by HT. YP conducted the phylogenetic analysis. AS described the COG functional categories of genes. Flagella-associated gene cluster analysis was conducted by AG and ECK. MSU wrote the manuscript. EK, AG, SS and FOG revised the manuscript. All authors have read and approved the final manuscript.

Stand Genomic Sci (2010) vol. 3 pp. 97-107

Genetic accessibility of *Marinobacter* sp. HP15, a marine bacterium interacting with diatoms

Kaeppel EC*, Gärdes A*, Seebah S, Torres-Monroy I, Grossart HP, and Ullrich MS

*these authors contributed equally

ECK conducted the antibiotics susceptibility spectrum analysis, the optimization of an electroporation protocol, the testing and identification of replicating plasmids, established the transposon mutagenesis and *egfp* expression. AG carried out the optimization of conjugation and site-directed mutagenesis. SS cooperated on the site-directed mutagenesis and generated the genomic library. ITM established the *lacZ* expression. The manuscript was prepared by ECK and AG. SS, ITM, and HPG revised the manuscript. MSU conducted the final revision and writing of the manuscript. All authors have read and approved the final manuscript.

(to be submitted to Applied and Environmental Microbiology)

Chemotaxis of *Marinobacter* sp. HP15 and its impact on attachment to the diatom *Thalassiosira weissflogii*

Kaeppel EC, Grossart HP, and Ullrich MS

ECK conducted all experiments and prepared the manuscript. HPG revised the

manuscript. MSU finally revised and finished writing the manuscript. All authors have read and approved the final manuscript.

(to be submitted to Applied and Environmental Microbiology)

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

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

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The Gram-negative, motile, and rod-shaped bacterial strain, HP15^T, was isolated from particles sampled in surface waters of the German Wadden Sea. It was identified among 82 other marine isolates due to its high potential to induce production of transparent exopolymeric particles and aggregate formation while interacting with the diatom, *Thalassiosira weissflogii*. HP15^T grew optimally at a range of 34-38 °C, a pH of 7-8.5, and was able to tolerate salt concentrations between 0.5-20 % (w/v) NaCl. HP15^T was chemotaxonomically characterized by possessing ubiquinone-9 as the major respiratory lipoquinone as well as $C_{16:0}$, $C_{18:1}\omega9c$, $C_{16:1}\omega7c$/iso, and $C_{15:0}$ 2-OH as predominant fatty acids. The G+C content of its DNA was 56.9 mol%. The closest relative by means of 16S rRNA sequence analysis was *Marinobacter flavimaris* with a similarity level of 99 %. The whole-genome relatedness of HP15^T to *M. flavimaris*, *M. salsuginis*, *M. lipolyticus*, and *M. algicola* was determined to be lower than 70 % by DNA-DNA hybridization. On the basis of phenotypic and chemotaxonomic properties as well as phylogenetic analyses, strain HP15^T (=DSM 23420^T = CIP 110141^T) is proposed to represent the novel species, *Marinobacter adhaerens* sp. nov.

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

INTRODUCTION

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

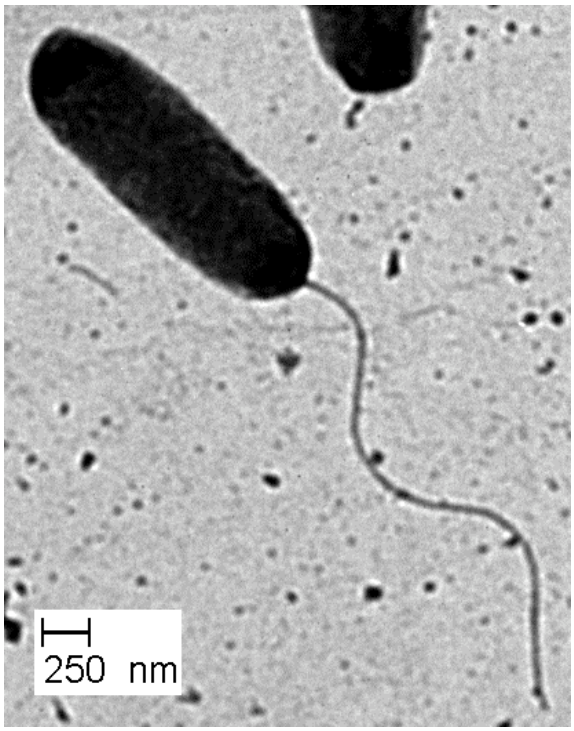

Fig. 1. Transmission electron micrograph of strain HP15^T cultivated in marine broth for 24 hours.

and *M. bryozoorum* derived from Bryozoa (Romanenko *et al.*, 2005).

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

MATERIAL AND METHODS

For phenotypic examination, HP15^T was grown aerobically on Marine Broth (MB) agar plates (5 g peptone, 1 g yeast extract, 0.1 g $FePO_4$, 6 g agar in 750 ml of North Sea water and 250 ml of distilled water, pH adjusted to 7.4) at 28 °C for 48 h. The reference strains *Marinobacter flavimaris* DSM 16070^T , *Marinobacter salsuginis* DSM18347^T, *Marinobacter lipolyticus* DSM15157^T, *Marinobacter algicola* DSM16394^T, and *Marinobacter aquaeolei* DSM11845 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

The Gram staining reaction, cell morphology, and motility were examined by light microscopy and transmission electron microscopy (EM 900, Zeiss). Enzyme activities and carbon utilization were analyzed by using the API 20NE system (bioMérieux) in artificial seawater medium without carbon source (Martinez & Butler, 2007). Determination of carbon utilization patterns using the BIOLOG GN2 was carried out by DSMZ. The analysis of growth conditions were examined in MB medium at 37 °C and 250 rpm. For salinity tests, the indicated NaCl concentration was added to distilled water instead of using sea water. For pH tests, pH was adjusted by using NaOH or HCl. The temperature range analysis (4-60 °C) was performed in Marine Broth (Difco 279110) by DSMZ. The optimal growth temperature was defined by testing bacterial growth at

Table 1. Phenotypic and genotypic differentiation between HP15^T and the closest related *Marinobacter* type strains.

Strains: 1, HP15^T; 2, *M. flavimaris* SW-145^T; 3, *M. salsuginis* SD-14B^T; 4, *M. algicola* DG893^T; 5, M. lipolyticus DSM15157^T; 6, M. aquaeolei DSM11845. +, positive; -, negative; ND, not determined. Additional data for reference strains were taken from Yoon *et al.* (2004), Antunes *et al.* (2007), Green *et al.* (2006), Martin *et al.* (2003), and Huu *et al.* (1999).

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**
Temperature range (°C)	4-45	4-45	10-45	5-40	15-40	13-50
Optimal temperature (°C)	34-38	37	35-37	25-30	37	30
Nitrate reduction to nitrite	-	+	-	-	-	-
Nitrate reduction to N_2	-	-	+	-	-	+
Urease activity	-	-	-	-*	-	-
Gelatinase activity	-	-	-*	-	-	-
β-glucosidase	-	-	-	+	-	-
Utilization of:						
Glycerol	-	+	+	+	-	-
D-Fructose	-	+	-	+	+	-
D-Glucose	-	-	-*	-*	-*	-
Maltose	-	-	-	+	-*	-
D-Mannitol	-	-	-	-*	-*	-
Citric acid	-	-	-	+	-	+
DL-Lactic acid	+	-	-	+	-	+
D-Gluconate	-	+	+	+	+	ND
L-Alanine	+	-	+	+	-	-
L-Phenylalanine	-	-	+	+	-	ND
L-Glutamate	+	-	+	+	-	+
L-Arginine	-	-	-	+	-	ND
Malate	+	+	+	+	-	-
Phenylacetate	+	-	-	-	-	-
G+C content (mol%)	56.9	58.0	55.9	55	57	55.7

*were reported as positive in previous publications

the temperatures of 28, 30, 32, 34, 36, 38, 40, and 42 °C. The salinity range was studied between 0 and 35 % (w/v) NaCl and the pH range between 4 and 11. Cellular fatty acid composition and quinone analysis were carried out by DSMZ. Fatty acid methyl esters were extracted using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988), separated, and analysed using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711, USA) as described previously (Kämpfer & Kroppenstedt, 1996). For quinone analysis, cells were grown in MB at 28 °C and 250 rpm to an OD of 0.3 and harvested. Extraction, separation, and analysis of lipoquinones was conducted as described by Tindall (1990a, b).

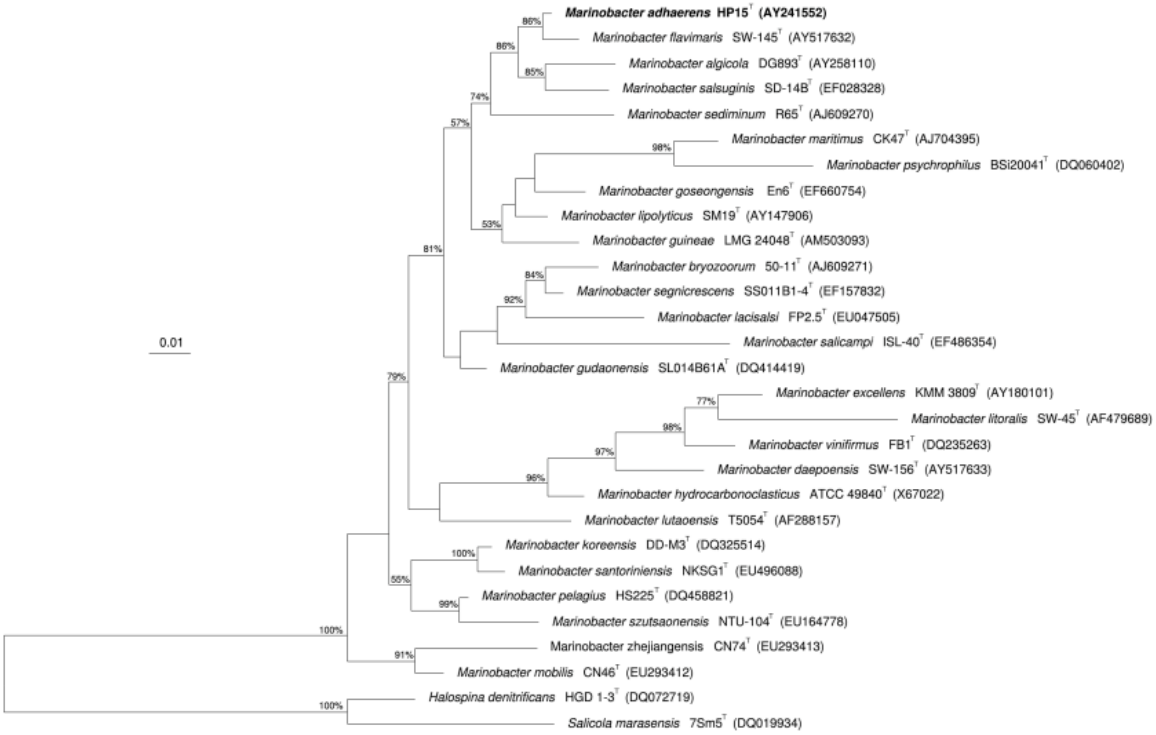

Fig. 2. Maximum likelihood phylogenetic tree based on 16S rRNA sequences of HP15^T, all type strains of the genus *Marinobacter* and the type strains of *Halospina denitrificans* HGD 1-3^T (DQ072719) and *Salicola marasensis* 7Sm5^T (DQ019934) as out groups. The tree was inferred from 1,531 alignment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

DNA-DNA hybridization tests were conducted in duplicates between HP15^T and the closest neighbouring strains derived from 16S rRNA analysis: *M. flavimaris* DSM 16070^T, *M. salsuginis* DSM18347^T, *M. lipolyticus* DSM15157^T, and *M. algicola* DSM16394^T. The hybridization was performed by DSMZ as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) in 2 x SSC with 5% formamid at 68 °C.

For the phylogenetic analysis, the com-<plete 16S rRNA gene of HP15^T (AY241552, 1531 bp) was extracted from the genome sequence (GenBank accession no. CP001978) (Gärdes *et al.*, 2010b). The analysis was performed using the ARB software package (Ludwig *et al.*, 2004) and the reference alignment was provided by the Living Tree Project database (Yarza et al., 2008). The phylogenetic tree was based on the HP15^T sequence, all type strains of the genus *Marinobacter*, and the type strains of *Halospina denitrificans* HGD 1-3^T (DQ072719) and *Salicola marasensis* 7Sm5^T (DQ019934) as outgroups. The G+C content of the HP15^T genome was calculated using the complete genomic sequence (GenBank accession nos. CP001978, CP001979, and CP001980).

RESULTS AND DISCUSSION

The cells of strain HP15^T were rod-shaped and motile by one polar flagellum (Fig. 1). HP15^T grew between 4 and 45 °C, at a pH from 5.5 to 10 and between 0.5-20% (w/v) NaCl. The API 20 NE test of HP15^T was negative for nitrate reduction, indole production, arginine dihydrolase, urease, β-glucosidase, gelatinase, β-galactosidase, and the utilization of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, gluconate, capric acid, adipic acid and citrate (Tab. 1). HP15^T utilized malate and phenylacetic acid. The results of the BIOLOG GN2 plate were positive for the utilization of dextrin, Tween 40 and 80, pyruvic acid methyl ester, succinic acid mono-methyl-ester, cis-aconitic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-leucine, and L-proline (Tab. 1). The fatty acid profile of HP15^T was composed of $C_{16:0}$ (21.7 %), $C_{18:1}\omega 9c$ (21.6 %), $C_{16:1}\omega 7c$/iso $C_{15:0}$ 2-OH (14.6 %), $C_{16:1}\omega 9c$ (9.0 %), $C_{12:0}$ 3-OH (7.9 %) and $C_{12:0}$ (6.0 %). Thus, it is similar to that of other *Marinobacter* type strains (Supplementary Tab. S1). The predominant ubiquinone was ubiquinone-9, which is consistent with that of other *Marinobacter* species except *M. lutaeoensis*, which contained ubiquinone-8 (Shieh *et al*., 2003).

Based on its 16S rRNA sequence, strain HP15^T was affiliated to the *Marinobacter* genus of the *Gammaproteobacteria*. It is most closely related to the type strains of *M. flavimaris* (99 %), *M. salsuginis* (98 %), *M. lipolyticus* (98 %), and *M. algicola* (98 %) (Antunes *et al.*, 2007; Green *et al.*, 2006; Martin *et al.*, 2003; Yoon *et al.*, 2004). Beside *M. lipolyticus,* these type strains form a discrete cluster as evident in the phylogenetic tree (Fig. 2).

The G+C content of the genome of HP15^T is 56.9 mol% (**Tab. 1**) and thus is similar to those of other *Marinobacter* species. As determined by DNA-DNA hybridization in duplicates, genomic DNA of HP15^T showed similarities of 63.6 (68.7), 40.0 (38.0), 28.9 (26.0), and 28.2 (24.5) % to those of *M. flavimaris*, *M. salsuginis*, *M. lipolyticus*, and *M. algicola*, respectively. These similarities were below the generally accepted species differentiation limit of 70 % (Wayne *et al.*, 1987). Their order of relatedness was the same as that for the 16S rRNA sequences. Due to DNA-DNA hybridization results, the type strain of *M. flavimaris* seemed to be closely related to the herein proposed species. However, both strains differed significantly in the following distinct characteristics: i) utilization of glycerol, D-fructose, DL-lactic acid, D-gluconate, L-alanine, phenylacetate, L-glutamate; ii) the ability to reduce nitrate to nitrite (**Tab. 1**); and iii) colony pigmentation, for which HP15^T exhibited brownish pigmentation on MB agar whereas colonies of *M. flavimaris* were cream-colored.

Based on the herein determined phenotypic and phylogenetic characteristics and the genomic differences towards other *Marinobacter* type strains, strain HP15^T should be placed in the genus *Marinobacter* and should be considered as a novel species. Due to its unique and characteristic attachment properties in the presence of marine particle surfaces, we propose for HP15^T the name *Marinobacter adhaerens* sp. nov..

Description of *Marinobacter adhaerens* sp. nov.

Marinobacter adhaerens [ad.hae'rens. L. part. adj. *adhaerens*: hanging on, sticking

to].

The cells are motile by means of a single polar flagellum, Gram-negative, and non-spore-forming rods (0.6-0.8 x 1.7-2.4 µm). Colonies on MB agar are brownish translucent and have a circular shape (1-2 mm in diameter) with smooth edges after 2 days of incubation at 28 °C. Colour intensity increased with time of incubation. HP15^T grew between 4 and 45 °C with an optimum at 34-38 °C and at a pH ranging from 5.5 to 10 with an optimum of pH at 7 to 9. No growth was observed at a pH of 5 or lower and 10.5 or higher. The strain grew optimally at NaCl concentrations from 2 to 6 % (w/v). It resisted down to 0.5 % NaCl, but not 0 % and up to 20 % NaCl, but not 25 % NaCl. HP15^T was negative for nitrate reduction, indole production, arginine dihydrolase, urease, β-glucosidase, gelatinase, and β-galactosidase activity. Malate, phenylacetic acid dextrin, Tween 40 and 80, pyruvic acid methyl ester, succinic acid mono-methyl-ester, cis-aconitic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-leucine, and L-proline are utilized as sole carbon source. HP15^T did not utilize α-cyclodextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, m-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, adipic acid, capric acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, p-hydroxy phenylacetic acid, itaconic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-alanyl-glycine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ-amino butyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate, and D-glucose-6-phosphate.

The major fatty acids are $C_{16:0}$ (21.7 %), $C_{18:1}\omega 9c$ (21.6 %), and $C_{16:1}\omega 7c$/iso $C_{15:0}$ 2-OH (14.6 %). The quinone system consists of quinone-9.

The type strain is HP15^T (=DSM 23420^T =CIP 110141^T). The G+C content is 56.9 mol%. The strain was isolated from marine aggregates (0.1-1 mm) of surface waters of the German Bight.

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***Marinobacter adhaerens* sp. nov., prominent in aggregate formation with the diatom *Thalassiosira weissflogii*, by E. C. Kaeppel, A. Gärdes, S. Seebah, H.-P. Grossart, M. S. Ullrich**

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

Strains: 1, HP15^T; 2, *M. flavimaris* SW-145^T; 3, *M. salsuginis* SD-14B^T; 4, *M. algicola* DG893^T; 5, *M. guineae* LMG 24048^T; 6, *M. lipolyticus* SM-19^T; 7, *M. sediminum* R65^T; 8, *M. aquaeolei* VT8^T; 9, *M. hydrocarbonoclasticus* SP.17^T. The data of the reference strains were taken from Yoon *et al.* (2004), Antunes *et al.* (2007), Green *et al.* (2006), Montes *et al.* (2008), Martin *et al.* (2003), Romanenko *et al.* (2005), Huu *et al.* (1999), and Gauthier *et al.* (1992). Values represent the percentages of the total fatty acid content. ND, not determined.

Fatty acid	1	2	3	4	5	6	7	8	9
$C_{10:0}$	0.2	0.5	<0.5	0.4	<1	1.5	ND	0.6	1.0
$C_{12:0}$	6.0	9.1	7.3	7.3	5.2	8.3	4.2	7.9	4.7
$C_{14:0}$	1.2	1.1	1.1	0.5	<1	0	0.9	2.6	2.2
$C_{15:0}$	0	0.7	<0.5	0	<1	1.0	0	2.2	1.4
$C_{16:0}$	21.7	26.7	22.9	25.5	16.9	28.5	21.8	22.6	23.6
$C_{17:0}$	1.1	3.7	0.5	1.7	2.5	3.6	1.3	3.8	3.4
$C_{18:0}$	3.4	3.3	2.9	2.2	2.2	2.7	2.2	1.4	1.9
$C_{16:1}\omega 9c$	9.0	10.2	10.5	4.0	7.1	10.5	13.3	11.6	7.4
$C_{17:1}\omega 8c$	2.0	3.8	3.8	2.6	4.4	2.9	2.8	4.5	3.6
$C_{18:1}\omega 7c$	3.7	1.2	ND	6.2	6.8	2.3	2.9	ND	ND
$C_{18:1}\omega 9c$	21.6	17.4	17.2	10.4	14.0	13.9	16.1	19.8	29.4
$C_{16:1}\omega 7c/C_{15:0}$ iso 2-OH	14.6	6.8	<0.5	19.8	ND	ND	15.9	6.8	8.3
$C_{11:0}$ 3-OH	0.2	<0.4	0.5	0.4	ND	ND	ND	0.6	0.3
$C_{12:0}$ 3-OH	7.9	10.5	9.3	8.7	8.5	11.3	8.0	9.9	7.7
$C_{16:0}$ 10-methyl	0.6	0.2	<0.5	7.7	4.1	4.0	0	2.8	2.8

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

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

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

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Marinobacter adhaerens HP15 is the type strain of a newly identified marine species, which is phylogenetically related to *M. flavimaris*, *M. algicola*, and *M. aquaeolei*. It is of special interest for research on marine aggregate formation because it showed specific attachment to diatom cells. *In vitro* it led to exopolymer formation and aggregation of these algal cells to form marine snow particles. *M. adhaerens* HP15 is a free-living, motile, rod-shaped, Gram-negative Gammaproteobacterium, which was originally isolated from marine particles sampled in the German Wadden Sea. *M. adhaerens* HP15 grows heterotrophically on various media, is easy to access genetically, and serves as a model organism to investigate the cellular and molecular interactions with the diatom *Thalassiosira weissflogii*. Here we describe the complete and annotated genome sequence of *M. adhaerens* HP15 as well as some details on flagella-associated genes. *M. adhaerens* HP15 possesses three replicons; the chromosome comprises 4,422,725 bp and codes for 4,180 protein-coding genes, 51 tRNAs and three rRNA operons, while the two circular plasmids are ~187 kb and ~42 kb in size and contain 178 and 52 protein-coding genes, respectively.

Introduction

Strain HP15 (DSM 23420) is the type strain of the newly established species *Marinobacter adhaerens* sp. nov. and represents one of 27 species currently assigned to the genus *Marinobacter* [1]. Strain HP15 was first described by Grossart *et al.* in 2004 [2] as a marine particle-associated, Gram-negative, gammaproteobacterium isolated from the German Wadden Sea. The organism is of interest because of its capability to specifically attach *in vitro* to the surface of the diatom *Thalassiosira weissflogii*-inducing exopolymer and aggregate formation and thus generating marine snow particles [3]. Marine snow formation is an important process of the biological pump, by which atmospheric carbon dioxide is taken up, recycled, and partly exported to the sediments. This sink of organic carbon plays a major role

for marine biogeochemical cycles [4]. Several studies reported on the formation and properties of marine aggregates [5–8]. Although it was shown that heterotrophic bacteria control the development and aggregation of marine phytoplankton [3], specific functions of individual bacterial species on diatom aggregation have not been explored thus far.

A better understanding of the molecular basis of bacteria-diatom interactions that lead to marine snow formation is currently gained by establishing a bilateral model system, for which *M. adhaerens* sp. nov. HP15 serves as the bacterial partner of the easy-to-culture diatom, *T. weissflogii* [3]. Herein, we present a set of features for *M. adhaerens* sp. nov. HP15 (**Table 1**) together with its annotated complete genomic sequence, and a detailed analysis of its flagella-associated genes.

Classification and features

M. adhaerens sp. nov. strain HP15 is a motile, Gram-negative, non-spore-forming rod (**Figure 1**). Based on its 16S rRNA sequence, strain HP15 was affiliated to the *Marinobacter* genus of *Gammaproteobacteria*. Two other *Marinobacter* species were identified based on their interactions with eukaryotes - *M. algicola* isolated from dinoflagellate cultures [9] and *M. bryozoorum* derived from Bryozoa [10]. Strain HP15's 16S rRNA gene is most closely related to those of the type strains of *M. flavimaris* (99%), *M. salsuginis* (98%) and *M. algicola* (96%). These four type strains form a discrete cluster in the phylogenetic tree (**Figure 2**). In contrast, DNA-DNA hybridization experiments revealed that the genome of *M. adhaerens* sp. nov. HP15 showed about 64% binding to that of *M. flavimaris* [1], which is below the generally accepted species differentiation limit of 70% [11].

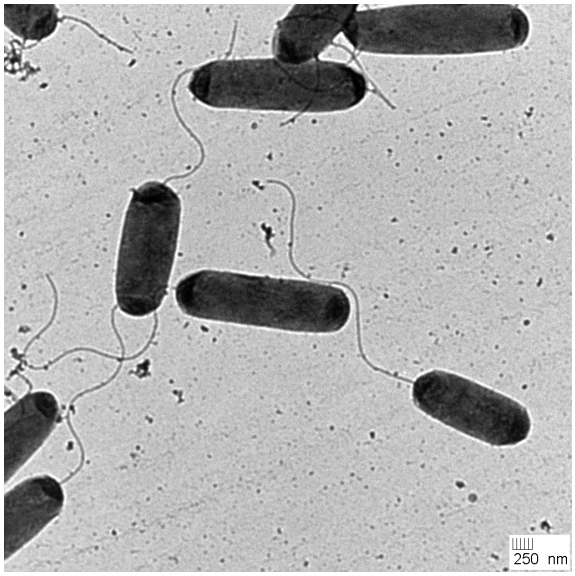

Figure 1. Transmission electron micrograph of *M. adhaerens* sp. nov. strain HP15.

Chemotaxonomy

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

Genome sequencing and annotation

Genome project history

M. adhaerens HP15 was selected for sequencing because of its phylogenetic

position, its particular feature as a diatom-interacting marine organism [3], and its feasible genetic accessibility to act as a model organism. The respective genome project is deposited in the Genome OnLine Database [17] and the complete genome sequence in GenBank. The main project information is summarized in **Table 2**.

Growth conditions and DNA isolation

M. adhaerens sp. nov. HP15 was grown in 100 ml Marine Broth medium [18] at 28°C. A total of 23 µg DNA was isolated from the cell paste using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Genome sequencing and assembly

The *Marinobacter adhaerens* sp. nov. HP15 genome was sequenced at AGOWA (AGOWA GmbH, Berlin, Germany) using the 454 FLX Ti platform of 454 Life Sciences (Branford, CT, USA). The sequencing library was prepared according to 454's instructions from genomic *M. adhaerens* sp. nov. HP15 DNA with a final concentration of 153 ng/µl. Sequencing was carried out on a quarter of a 454 picotiterplate, yielding 258.645 reads with an average length of 405 bp, totaling to almost 105 Mb. These reads were assembled using the Newbler assembler version 2.0.00.22 (Roche), resulting in 253.285 fully and 4.763 partially assembled reads, leaving 932 singletons, 226

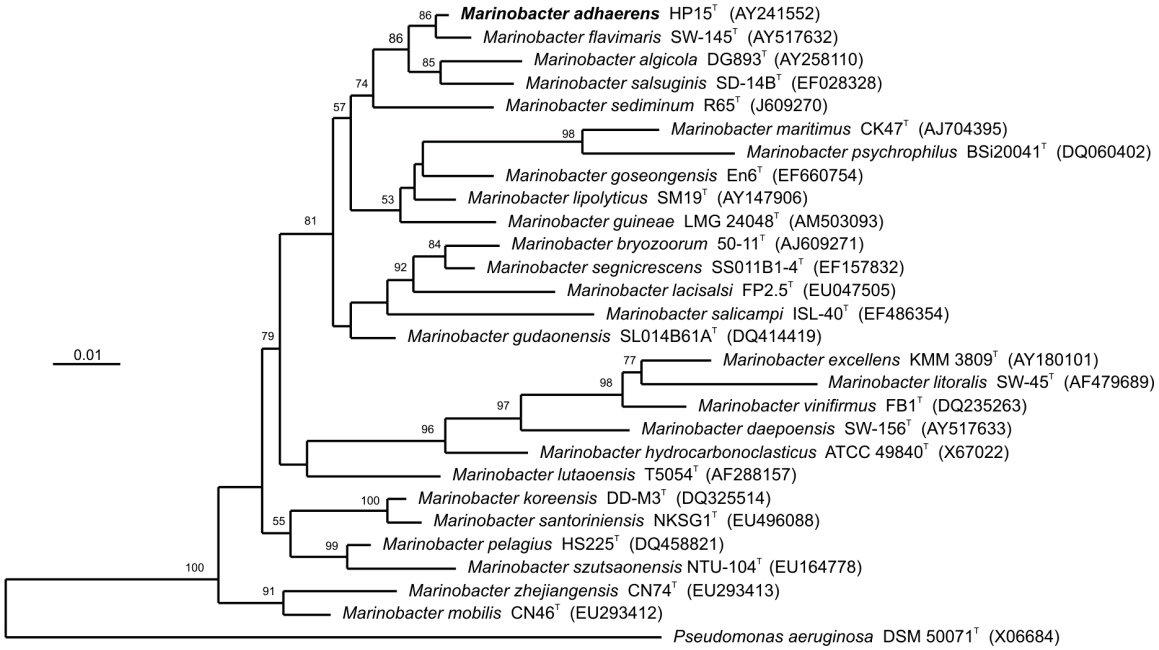

Figure 2. Maximum likelihood phylogenetic tree based on 16S rRNA sequences of *M. adhaerens* type strain (HP15) plus all type strains of the genus *Marinobacter* and the type species of the neighbor order *Pseudomonadales*. Sequence selection and alignment improvements were carried out using the Living Tree Project database [12] and the ARB software package [13]. The tree was inferred from 1,531 alignment positions using RAxML [14] with GTRGAMMA model. Support values from 1,000 bootstrap replicates are displayed above branches if larger than 50%. The scale bar indicates substitutions per site.

repeats and 371 outliers. The assembly comprised 112 contigs, with 40 exceeding 500 bp. The latter comprised more than 4.6 Mb, with an average contig size of almost 116 kb and a longest contig of more than 1.2 Mb. Gaps between contigs were closed in a conventional PCR-based gap closure approach, resulting in a fully closed circular chromosome of 4.421.911 bp, and two plasmids of 187.465 bp and 42.349 bp, respectively. Together all sequences provided 22.5x coverage of the genome. The error rate of the completed genome sequence is about 3 in 1,000 (99.7%).

Genome annotation

Potential protein-coding genes were identified using GLIMMER v3.02 [19], transfer RNA genes were identified using tRNAScan-SE [20] and ribosomal RNA genes were identified via BLAST searches [21] against public nucleotide databases. The annotation of the genome sequence was performed with the GenDB v2.2.1 system [22]. For each predicted gene, similarity searches were performed against public sequence databases (nr, SwissProt, KEGG) and protein family databases (Pfam, InterPro, COG). Signal peptides were predicted with SignalP v3.0 [23, 24] and transmembrane helices with TMHMM v2.0 [25]. Based on these observations, annotations were derived in an automated fashion using a fuzzy logic-based approach [26]. Finally, the predictions were manually checked with respect to missing genes in intergenic regions and putative sequencing errors, and the annotations were manually curated using the Artemis 11.3.2 program and refined for each putative gene [27].

Genome properties

The genome of strain HP15 comprises three circular replicons: the 4,422,725 bp chromosome and two plasmids of ~187 kb and ~42 kb, respectively (**Table 3A** and **Figure 3**). The genome possesses a 56.9% GC content (**Table 3B**). Of the 4,482 predicted genes, 4,422 were protein coding genes, and 60 RNAs; 391 pseudogenes were also identified. The majority of the protein-coding genes (67.5%) were assigned with a putative function, while those remaining were annotated as hypothetical proteins. The distribution of genes into COGs functional categories is presented in **Table 4**.

Table 1b. Classification and general features of *M. adhaerens* sp. nov. HP15 in accordance to the MIGS recommendations [15]

MIGS ID	Property	Term	Evidence code
		Domain *Bacteria*	
		Phylum *Proteobacteria*	
		Class *Gammaproteobacteria*	TAS [16]
	Current classification	Order *Alteromonadales*	TAS [16]
		Family *Alteromonadaceae*	TAS [16]
		Genus *Marinobacter*	TAS [1]
		Species *Marinobacter adhaerens*	TAS [1]
		Type strain HP15	TAS [1]
	Gram stain	negative	IDA

Table 1b. Classification and general features of *M. adhaerens* sp. nov. HP15 in accordance to the MIGS recommendations [15]

MIGS ID	Property	Term	Evidence code
	Cell shape	rod-shaped	IDA
	Motility	motile, single polar flagellum	IDA
	Sporulation	non-sporulating	NAS
	Temperature range	mesophilic	IDA
	Optimum temperature	34-38°C	IDA
	Salinity	0.4-10 g NaCl/l (optimum/growth within 1 day)	IDA
MIGS-22	Oxygen requirement	strictly aerobic	IDA
	Carbon source	dextrin, Tween 40 and 80, pyruvic acid methyl ester, succinic acid mono-methyl-ester, cis-aconitic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-keto glutaric acid, α-keto valeric acid, D,L-lactic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-leucine and L-proline	IDA
	Energy source	chemoorganoheterotrophic	IDA
MIGS-6	Habitat	sea water	IDA
MIGS-15	Biotic relationship	free-living and particle-associated	TAS [2]
MIGS-13	Culture deposition no.	DSM 23420	IDA
MIGS-14	Pathogenicity	none	NAS
	Biosafety level	1	NAS
	Isolation	marine aggregates (0.1-1 mm)	TAS [2]
MIGS-4	Geographic location	German Wadden Sea	TAS [2]
MIGS-4.1	Latitude	53°43'20''N	TAS [2]
MIGS-4.2	Longitude	07°43'20''E	TAS [2]
MIGS-4.3	Depth	surface waters	TAS [2]
MIGS-4.4	Altitude	sea level	TAS [2]
MIGS-5	Sample collection time	15 June 2000	TAS [2]

Evidence codes – IDA: inferred from Direct Assay (first time in publication); TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property of the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [17]. If evidence code is IDA, then the property was directly observed for a live isolate by one of the authors or an expert mentioned in the acknowledgements.

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

MIGS ID	Property	Term
MIGS-31	Finishing quality	Finished
MIGS-28	Library used	454 pyrosequencing standard library
MIGS-29	Sequencing platforms	454 FLX Ti
MIGS-31.2	Sequencing coverage	22.5x pyrosequencing
MIGS-30	Assemblers	Newbler version 2.0.00.22
MIGS-32	Gene calling method	GLIMMER v3.02, tRNAScan-SE
		CP001978 (chromosome)
	Genbank ID	CP001979 (pHP-42)
		CP001980 (pHP-187)
	Genbank Date of Release	September 18, 2010
	GOLD ID	Gi06214
	NCBI project ID	46089
	Database: IMG	pending
	Project relevance	Marine diatom-bacteria interactions

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

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

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

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

Attribute	**Genome (total)**	
	Value	% of total[a]
Genome size (bp)	4,651,725	
DNA Coding region (bp)	4,178,502	89.8
DNA G+C content (bp)	2,644,970	56.9
Number of replicons	3	
Extrachromosomal elements	2	
Total genes[b]	4,410	
tRNA genes	51	1.16
5S rRNA genes	3	0.07
16S rRNA genes	3	0.07
23S rRNA genes	3	0.07
Protein-coding genes	4,355	98.66
Genes assigned to COGs	3,027	67.54
Genes with Pfam domains	2,918	65.1
1 Pfam domain	2,041	45.54
2 Pfam domains	598	13.34
3 Pfam domains	194	4.33
4 or more Pfam domains	85	1.9
Genes with signal peptides	765	17.07
Genes with transmembrane helices	1,043	23.27
1 transmembrane helix	341	7.61
2 transmembrane helices	154	3.44
3 transmembrane helices	72	1.61
4 or more transmembrane helices	476	10.62
Genes in paralogous clusters	570	12.72
Genes with 1 paralog	364	8.12
Genes with 2 paralogs	63	1.41
Genes with 3 paralogs	26	0.58
Genes with 4 or more paralogs	117	2.61
Pseudo/hypothetical genes	391	8.72
Conserved hypothetical genes	668	14.90
Genes for function prediction	3,363	75.03

a)The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.

b) Also includes 54 pseudogenes and 5 other genes.

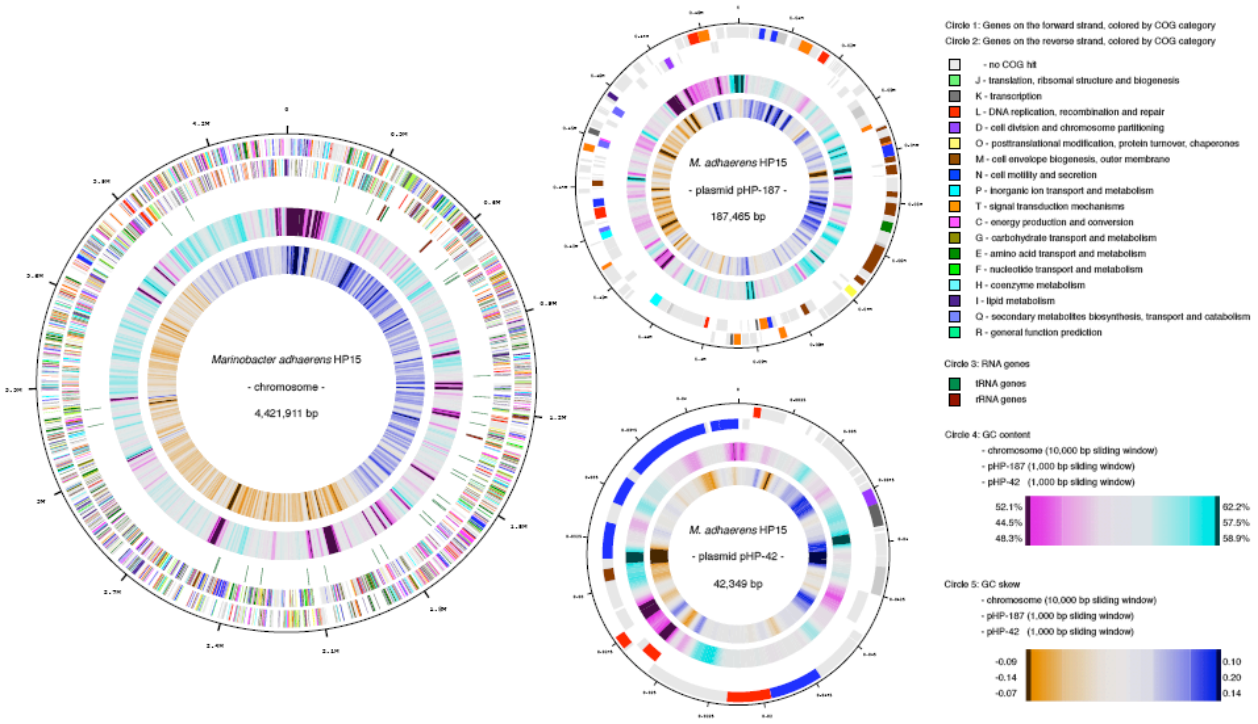

Figure 3. Graphical circular maps of the genome and the two plasmids of HP15. From outside to the center: Genes on forward strand (color by COG categories), Genes on reverse strand (color by COG categories), RNA genes (tRNAs green, rRNAs red, other RNAs black), GC content, GC skew.

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

Code	Value	% of total[a]	Description
J	162	3.7	Translation
A	0	0	RNA processing and modification
K	161	3.6	Transcription
L	132	3	Replication, recombination and repair
B	0	0	Chromatin structure and dynamics
D	32	0.7	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	0	0	Defense mechanisms
T	199	4.5	Signal transduction mechanisms
M	151	3.4	Cell wall/membrane biogenesis
N	166	3.8	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	0	0	Intracellular trafficking and secretion
O	127	2.9	Posttranslational modification, protein turnover, chaperones
C	192	4.3	Energy production and conversion
G	82	1.9	Carbohydrate transport and metabolism
E	254	5.7	Amino acid transport and metabolism
F	51	1.1	Nucleotide transport and metabolism
H	97	2.2	Coenzyme transport and metabolism
I	141	3.2	Lipid transport and metabolism
P	138	3.1	Inorganic ion transport and metabolism
Q	76	1.7	Secondary metabolites biosynthesis, transport and catabolism
R	330	7.5	General function prediction only
S	251	5.7	Function unknown
multiple COGs	285	6.4	
	3,027	68.6	Total
-	1,383	31.4	Not in COGs

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

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

Because *M. adhaerens* HP15 was experimentally shown to adhere to diatom cells, gene clusters coding for secretion, assembly, and mechanistic function of the polar flagellum were analyzed in detail (**Figure 4**). Besides several other chemotactic mechanisms and various cell surface interactions, bacterial flagella and other cell appendages had previously been shown to be instrumental for chemotactic movement towards and adhesion to biotic surfaces [28, 29]. The amino acid sequences of proteins encoded by the three identified gene clusters showed significant similarities to orthologous and experimentally well-described gene products of *P. aeruginosa* PAO1 and various other bacterial species as determined by BLASTP algorithm comparison using the Blosum 62 substitution matrix [21]. Not surprisingly, hook and motor switch complex components were most conserved. However, gene products involved in flagellar filament formation encoded by Cluster II also showed 53 to 78% similarity to the respective PAO1 proteins. Mutagenesis of flagella-

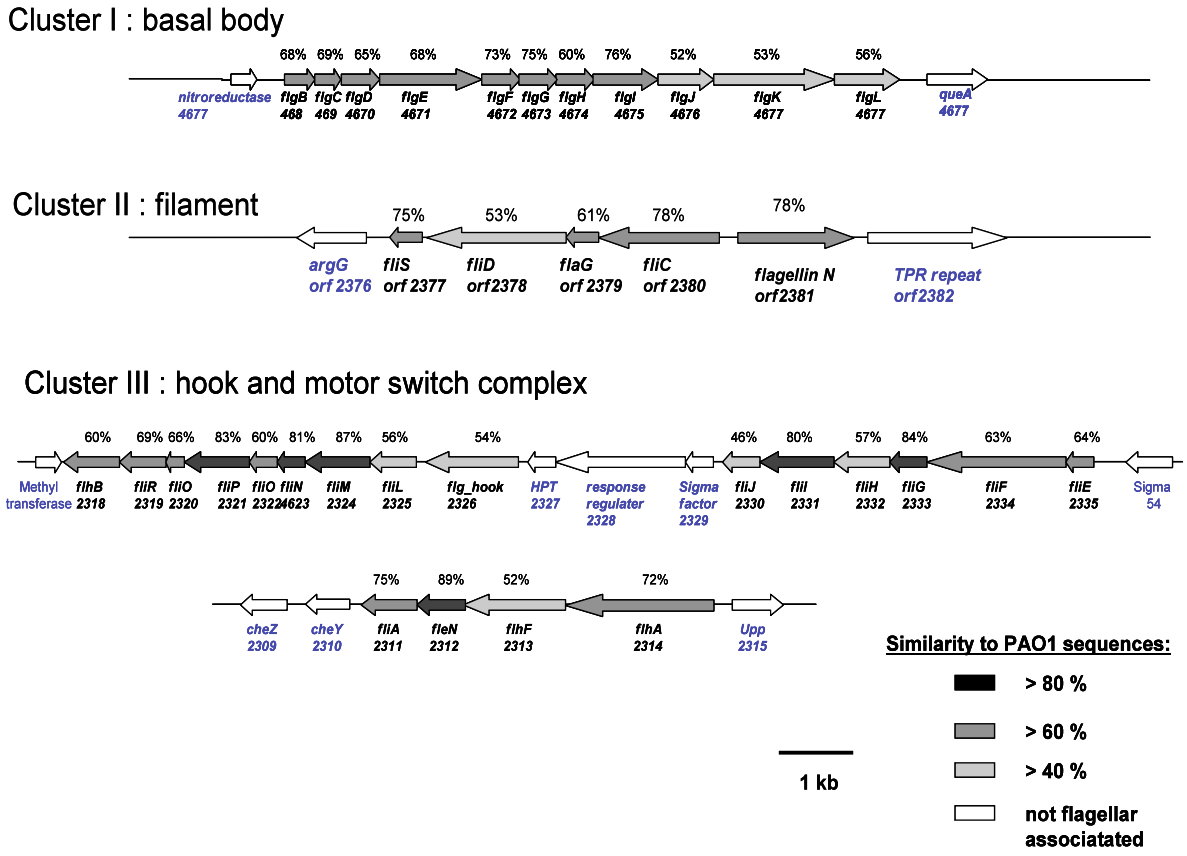

Figure 4. Schematic presentation of the three flagella-associated gene clusters of *M. adhaerens* HP15 coding for the basal body, the filament, and the hook and motor switch complex. Identities to the respective orthologs in the genome of *P. aeruginosa* PAO1 are indicated by gray-scale code. Numbers of CDS are shown below gene names.

associated genes of *M. adhaerens* HP15 will be carried out in the near future to study the role of flagella in bacteria-diatom interactions and to further our understanding of the cell-to-cell communication between those organisms.

Acknowledgements

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3.3 Genetic accessibility of *Marinobacter* sp. HP15, a marine bacterium interacting with diatoms

Genetic accessibility of *Marinobacter* sp. HP15, a marine bacterium interacting with diatoms

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Marinobacter species are ubiquitously found in diverse marine environments. *Marinobacter* sp. HP15 was isolated from marine aggregates and shown to induce aggregate formation while interacting with the diatom, *Thalassiosira weissflogii*. Diatom aggregation is substantial for the flux of organic carbon from the photic zone to deeper waters and the sea bed. To analyze this effect at the molecular level, a genetics tool system was developed for strain HP15. The antibiotics susceptibility spectrum of this organism was determined and transformation protocols using both, electroporation and plasmid conjugation, were established. Among various plasmids of different incompatibility groups, only plasmids pBBR1MCS and pSUP106 were shown to replicate in *Marinobacter* sp. HP15. For conjugation, 1.4 x 10^{-3} and for electroporation 5.1 x 10^{-5} transformants per number of recipients were obtained for the broad-host-range vector pBBR1MCS. The electroporation efficiency corresponded to 1.1 x 10^{5} CFU per µg of DNA. Transposon mutagenesis using the suicide plasmid pRL27 and site-directed mutagenesis using homologous recombination were conducted for flagellum biosynthetic genes. The resulting mutant phenotypes were confirmed by soft agar assay and transmission electron microscopy. A genomic cosmid library was constructed to screen for genes and heterologous gene expression. Expression of the reporter genes encoding for enhanced green fluorescent protein and β-galactosidase was successfully demonstrated in HP15 revealing a useful tool for gene expression studies. This study is the first report for the genetic manipulation of a member of the *Marinobacter* genus.

INTRODUCTION

Heterotrophic bacteria interacting with micro-algae play an important role in the formation of marine snow particles and are thus important for the carbon cycling in marine pelagic systems (20, 42). Besides their role in degradation of organic carbon and re-mineralization of nutrients (9), these bacteria promote aggregation of phytoplankton cells (13). For this specific micro-environment, the concept of

the ”phycosphere“ was described as the area surrounding micro-algae, in which microorganisms interact with algae via diverse cell-to-cell mechanisms (6). Bacteria interacting with algal cells might feed on them or their products, or support their growth by re-mineralization of nutrients (23). Since various scenarios can be imagined, it remains to be determined whether bacteria enhancing aggregate formation inhibit or promote the growth of algae and how they accomplish that. Most previous studies focused on bacterial communities associated with phytoplankton at the ecological level (21). Consequently, virtually nothing is known about the specific role of single bacterial strains, their genes, and gene products for this interaction.

For an in-depth molecular analysis of diatom-bacteria interactions, a bilateral model system consisting of the diatom, *Thalassiosira weissflogii*, and the bacterial strain, *Marinobacter* sp. HP15, was established (15). Close interaction of the two organisms was demonstrated by attachment assays and determination of transparent exopolymer particle (TEP) production concluding that HP15 plays an important role in *T. weissflogii* aggregation dynamics. Generally, members of the genus *Marinobacter* were found in various marine habitats (18) as well as in interactions with eukaryotic organisms such as *Bryozoa* or dinoflagellates (19, 41).

As previously shown for other environmental bacterial strains, establishment of the genetic accessibility forms the base for detailed and accelerated research of the organisms (3, 4, 11, 33, 39, 44, 53). Herein, for the first time the genetic accessibility of a *Marinobacter* species was comprehensively analyzed. The suitability of *Marinobacter* sp. HP15 for molecular studies was demonstrated by transfer of plasmids via electroporation and conjugation and by two types of mutagenesis. As proof-of-principle, motility-deficient mutants were generated by transposon insertion as well as by site-directed mutagenesis using homologous recombination. A genomic cosmid library of HP15 was constructed and can be used for gene screening and heterologous gene expression. Expression of reporter genes such as enhanced green fluorescent protein and β-galactosidase was successfully demonstrated in HP15 and will be a useful tool for further studies on promoter activities and transcriptional regulation.

MATERIAL AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used are listed in **Table 1**. Oligonucleotide primers used are listed in **Table 2**. *Marinobacter* sp. HP15 was isolated from marine particulate samples collected from surface waters of the German Bight (22). *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 adjusted to 7.4). For electroporation, cells were cultivated on MB agar medium overnight at 37 °C. *E. coli* strains were maintained in Luria-Bertani (LB) agar medium. For conjugation, *Marinobacter* cells were grown in 100 ml liquid culture shaking at 250 rpm overnight. The donor strain *E. coli* ST18 was grown in LB medium containing 5-aminolevulinic acid (ALA, final concentration: 50 µg ml^{-1}). The following antibiotics were added to media when needed (in µg ml^{-1}): chloramphenicol, 25; kanamycin, 500; and ampicillin, 50.

To analyze antibiotics susceptibility as selection marker for transformation, HP15 was grown in MB medium to an OD of 1, and 20 µl of cell suspensions were spotted on MB agar medium containing various concentrations of ampicillin, chlo

TABLE 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Bacterial strains		
Escherichia coli DH5α λpir	Φ80*dlacZ*ΔM15 Δ(*lacZYA- argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1/λpir*	(38)
Escherichia coli ST18	S17 λ*pir* ΔhemA	(51)
Escherichia coli HB101	carrying helper plasmid pRK2013	(14)
Marinobacter sp. HP15	wild type	(22)
Δ*fliC*	*fliC* deletion mutant of HP15	This study
*fliG::*Tn*5*	transposon insertion mutant in *fliG* of HP15	This study
*fliR::*Tn*5*	transposon insertion mutant in *fliR* of HP15	This study
Plasmids		
pBBR1MCS	broad-host-range *mob* Cm^R	(30)
pSUP106	IncQ *mob cos* Cm^R Tet^R	(40)
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	(25)
pK18mob	pMB1 oriT Km^R	(43)
pLAFR3	IncP *cos* Tet^R	(48)
pKNOCK-Cm	IncX oriT Cm^R	(1)
pPH1JI	IncP Cm^R Gm^R	(24)
pRK415	IncP oriT Tet^R	(28)
pSU18	pMB1 Cm^R	(5)
pBK-miniTn*7*-*gfp*1	pMB1 *mob* Amp^R, miniTn*7*-*gfp*1 with Km^R and Cm^R	(29)
pEP4351	IncX oriT Cm^R, Tn4351 with Em^R and Tet^R	(10)
pRL27	IncX oriT, Tn5 with Km^R	(32)
pGEM®-T Easy	colE1 *lacZ* Amp^R	Promega GmbH, Mannheim, Germany

pFCM1	Amp^R Cm^R	(8)
pK19mobsacB	pMB1 oriT Km^R	(43)
pEX18Ap	pMB1 oriT *sacB* Amp^R	(25)
pAS3	pGEM®-T Easy containing 1002 bp upstream *fliC* flanking region of HP15	This study
pAS4	pGEM®-T Easy containing 1236 bp downstream *fliC* flanking region of HP15	This study
pAS5	FRT sites and Cm^R (1135 bp) from pFCM1 ligated into *Kpn*I site of pAS3	This study
pAS6	FRT sites, Cm^R and upstream region (2137 bp) from pAS4 ligated with *BamH*I/*Spe*I into pAS5	This study
pAS7	knock out fragment (3373 bp) from pAS6 ligated with *EcoR*I into pEX18Ap	This study
pAS8	knock out fragment (3373 bp) from pAS6 ligated with *EcoR*I into pk18mobsacB	This study
pWEB™	colE1 SV40 cos MCS Amp^R Neo^R	Epicentre Technologies, Madison, USA
pBBR.EGFP	derivative of pBBR1MCS with insertion of *egfp* by *Cla*I/*Xba*I from pRc/CMV3.EGFP in direction of the *lac* promoter	H. Weingart, Jacobs University Bremen, Germany
pMC1871	*lacZ* Tet^R	(46)
pITM1	derivative of pBBR1MCS with insertion of *lacZ* by *Kpn*I in opposite direction to the *lac* promoter	This study
pITM2	derivative of pBBR1MCS with insertion of *lacZ* by *Kpn*I under control of the *lac* promoter	This study

ramphenicol, gentamycin, kanamycin, spectinomycin, or tetracycline. The minimal inhibitory concentrations (MICs) for these antibiotics in MB were determined by the micro-dilution assay as described previously (7).

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 *Marinobacter* sp. HP15 by biparental conjugation with *E. coli* ST18 as a donor. Additionally, triparental mating with the plasmid-mobilizing helper strain *E. coli*

TABLE 2. Oligonucleotide primers used in this study. The underline marks the enzyme restriction 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

HB101 (pRK2013) was performed. Bacterial strains were grown as described above overnight and the OD 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 h, 48 h, 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 at 28°C for 2-5 days. As control, the non-replicating cosmid vector pWEB™ was used.

Electroporation. Electrocompetent *Marinobacter* cells were prepared directly before electroporation and kept on ice during all steps of the washing procedure. The cell mass of two fully covered MB agar plates was 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 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 SOC medium was added to the cuvette. The cell suspension was transferred to a 1.5-ml sterile tube and incubated by shaking at 37°C for 15-20 hrs. 50 to 400 µl of suspensions were subsequently plated on MB agar medium supplemented with the appropriate antibiotics and incubated at 37°C. Electrotransformation of HP15 was tested with the following plasmids: pBBR1MCS, pSUP106, pWeb-Cm, pGEM.Km, pEx18Tc, pK18mob, pLAFR3, pKnock-Cm, pPH1JI, pRK415, and pSU18 (**Table 1**).

Transposon mutagenesis. Plamids pBK-miniTn7-gfp1, pEP4351, and pRL27 (**Table 1**) containing different transposons were tested for transposon mutagenesis efficiency in HP15 using electroporation. Resulting mutant colonies were grown in MB medium supplemented with kanamycin in 96 well plates overnight, re-suspended in glycerol (final concentration: 15%), and stored at -80°C. For screening of flagellum-deficient mutants,

mutant cells were grown in MB medium containing kanamycin and inoculated in 10-fold diluted MB soft agar plates (0.3% agar). Swimming-deficient mutants were identified by lack of the typical motility pattern of the 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 (2).

Site-directed mutagenesis. As a candidate gene for site directed mutagenesis, the flagellin-encoding gene, *fliC*, was selected using the *Marinobacter* sp. HP15 genome sequence (GenBank accession no. CP001978) (16), GenDB 2.2 (37), and BlastN analysis (2). An 1,002-bp upstream and an 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 the *Kpn*I-treated pAS3 yielding plasmid pAS5. Plasmid pAS5 was treated with the restriction enzymes *BamH*I and *Spe*I, the insert fragment was purified, and ligated into the *Bam*HI-*Spe*I-treated plasmid pAS4, resulting in plasmid pAS6, which contained the 6,338-bp knock-out fragment consisting of the chloramphenicol resistance gene flanked by *fli*C upstream and downstream fragments. The knock-out fragment was excised with enzyme *Eco*RI and ligated to 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 *Marinobacter* sp. HP15 chromosome by double crossover was confirmed by antibiotics selection and PCR with primer pairs FliCF/FliCR and CmF/CmR.

Determination of mutant phenotype by swimming assay and transmission electron microscopy. Flagellum-deficient mutants and the wild type of HP15 were grown overnight in MB medium containing kanamycin or chloramphenicol, when needed, 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 (TEM), cells were grown in MB medium as described above. A 300-µm-mesh carbon coated copper grid (Plano GmbH, Wetzlar, Germany) was incubated for 30 s in 20 µl of cell suspension, excess liquid was removed, and adhering cells were stained with 1% uranyl acetate, washed with distilled water, and dried. The stained cells were visualized using an EM900 transmission electron microscope (Zeiss, Jena, Germany).

Generation of genomic cosmid library. A genomic cosmid library of *Marinobacter* sp. HP15 was constructed with the pWEB™ cosmid cloning kit following the manufacturer´s protocol (Epicentre Biotechnologies, Madison, USA). *E. coli* cells containing the cosmid library were grown in LB medium in 96 well microtiter plates, and stored at -80°C in 15% glycerol. In order to determine the number of cosmid clones required for full coverage of the genome, the following formula was applied: Genome coverage = [Number of clones x Size of insert] / Genome size. The randomness of the library was checked by randomly picking 15 cosmid clones, ex-

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

Antibiotic	1.2% MB agar (µg ml^{-1})	MB medium (µg ml^{-1})
Ampicillin	> 1000	0.1
Chloramphenicol	25	2
Gentamycin	50	31.3
Kanamycin	100	62.5
Spectinomycin	100	15.6
Tetracycline	250	31.3

tracting their cosmid DNA, and treating it with *EcoR*I followed by agarose gel electrophoresis.

Expression of enhanced green fluorescent protein and ß-galactosidase in *Marinobacter* sp. 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 HP15 by electroporation. Expression of *egfp* in single cells was visualized by use of a LSM 510 META confocal laser scanning microscope (Zeiss). The wild type of HP15 served as a negative control.

The *E. coli lac*Z gene was amplified from plasmid pMC1871 (47) 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 *lac* promoter, whereas in pITM2 it is under the control of the *lac* promoter. Both plasmids were introduced to HP15 via electroporation. Transformants were selected on MB agar plates containing chloramphenicol and X-Gal.

RESULTS

Antibiotics susceptibility. Growth of *Marinobacter* sp. HP15 was inhibited by a number of commonly used antibiotics (**Table 3**). MICs were generally higher on agar than those observed in liquid medium. HP15 showed lowest sensitivity to ampicillin on agar with an MIC > 1000 µg ml^{-1}, but highest in liquid medium with an MIC of 0.1 µg ml^{-1}. The highest susceptibility of HP15 on agar medium with a MIC of 25 µg ml^{-1}was observed for chloramphenicol, which was later used as selection marker for transformation when appropriate.

Transformation efficiency and expression of reporter genes. From various vectors tested, only plasmids pBBR1MCS and pSUP106 were found to stably replicate in *Marinobacter* sp. HP15. Other plasmids could not be transformed or did not replicate in HP15. Highest conjugation efficiencies were obtained via biparental mating at a donor-to-recipient ratio of 1:2 and after 24 hrs 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} for 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 was introduced to HP15, transformants exhibited fluorescence when excited at a wavelength of 488 nm, demonstrating that *egfp* was expressed (**Fig. 1a**). In contrast, no fluorescence was observed for HP15 wild type (**Fig. 1b**) suggesting that *egfp* is a suitable reporter gene in this bacterium.

Colonies of HP15 wild type were white-brownish on MB agar. Transformants containing pITM1 were white-

brownish on MB agar containing X-Gal, formants containing pITM2 grew in form of blue colored colonies on MB agar containing X-Gal thus expressing the reporter gene *lacZ* (**Fig. 2a**). Plasmid pITM2 was isolated from blue transformants of HP15 and multiple restriction enzyme treatments proved that *lacZ* was inserted in the orientation of the *lac* promoter in pBBR1MCS.

Transposon and site-directed mutagenesis of *Marinobacter* sp. HP15. Transposon-carrying plamids pBK-miniTn7-gfp1, pEP4351, and pRL27 were assayed for potential to be used for mutagenesis of *Marinobacter* sp. HP15 via electroporation. Transformation with pBK-miniTn*7*-*gfp*1 and pEP4351 did not yield in transposon mutants. In contrast, transformation of strain HP15 with plasmid pRL27 carrying transposon Tn*5* resulted in an efficiency of 6.8×10^2 CFU μg^{-1} DNA (1.8×10^{-7} mutants per number of recipients). Testing a total of 768 transposon mutants by soft agar swimming assay revealed two swimming-deficient mutants. For these 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 *fliG*::tn*5* was used for further phenotypic analysis**.**

Results of nucleotide sequencing of the transposon insertion loci of 14 additional randomly chosen transposon mutants proved the randomness of transposon insertion in the *Marinobacter* sp. HP15 genome, since each of the 16 determined genotypes was unique (Data not shown).

Site-directed mutagenesis was conducted by introducing the suicide plasmids pAS7 and pAS8, respectively, harboring the *fliC* similar to the wild type (**Fig. 2b**). Trans mutagenic construct by biparental conjugation. Transconjugants were selected on MB Cm and double crossover of the chloramphenicol resistance cassette in the *fliC* gene was shown by PCR with primers FliCF and FliCR yielding in the expected 1,734-bp fragment. One of these mutants was designated Δ*fliC*. In contrast, PCR with the HP15 wild type using the same primer set yielded an intact *fliC* amplification of 2,487 bp. These results confirmed a successful site-directed mutagenesis using homologous recombination in *Marinobacter* sp. HP15. Conjugation of the respective vectors, pEX18Ap and pKmobsaB, without insert DNA homologous to genes of HP15 did not yield antibiotics-resistant HP15 transformants.

Phenotypic characterization of *Marinobacter* sp. HP15 mutants. In contrast to the HP15 wild type, motility-deficient mutants Δ*fliC* and *fliG::*tn5 were not motile on soft agar demonstrating that genes *fliC* and *fliG* were essential for flagellar movement of HP15 (Fig. 3). Furthermore, TEM analysis revealed that HP15 wild type possessed one polar flagellum (Fig. 4c), while the Δ*fliC* mutant did not produce a visible flagellum but retained the flagellar hook (Fig. 4a). In contrast, transposon insertion in the hook-associated *fliG* gene led to a total loss of the flagellum as seen for mutant *fliG*::tn*5* (Fig. 4b).

Generation of the genomic cosmid library. Per bacteriophage λ infection, a total number of ~8,000 cosmid clones could be obtained. For a genome size of ~4.6 MB, this clone number corresponded to a >7-fold genome coverage indicating that the cosmid library may be considered comprehensive. The randomness of the inserts of the genomic cosmid library was assessed by isolating cosmid DNA from 15 randomly picked clones and

TABLE 4. Conjugation efficiencies for plasmids pBBR1MCS and pSUB106 in *Marinobacter* sp. HP15.

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

digestion with restriction enzyme *Eco*RI. The resulting DNA restriction patterns were random, thereby confirming that insertion of genomic DNA fragments of strain HP15 was not biased during cosmid library construction. A fragment of ~8 kb found in every restriction treatment corresponded to linearized pWEB™ vector, since a pair of *Eco*RI restriction sites flanks its multiple cloning site.

DISCUSSION

In contrast to well-established bacterial genetic model systems in medical, veterinary, or plant pathology as well as in microbial biotechnology, environmentally important microbes - particularly of ma rine origin - are often not readily accessible for molecular laboratory work. However, in order to understand the molecular basis of microbial activities in the oceans,

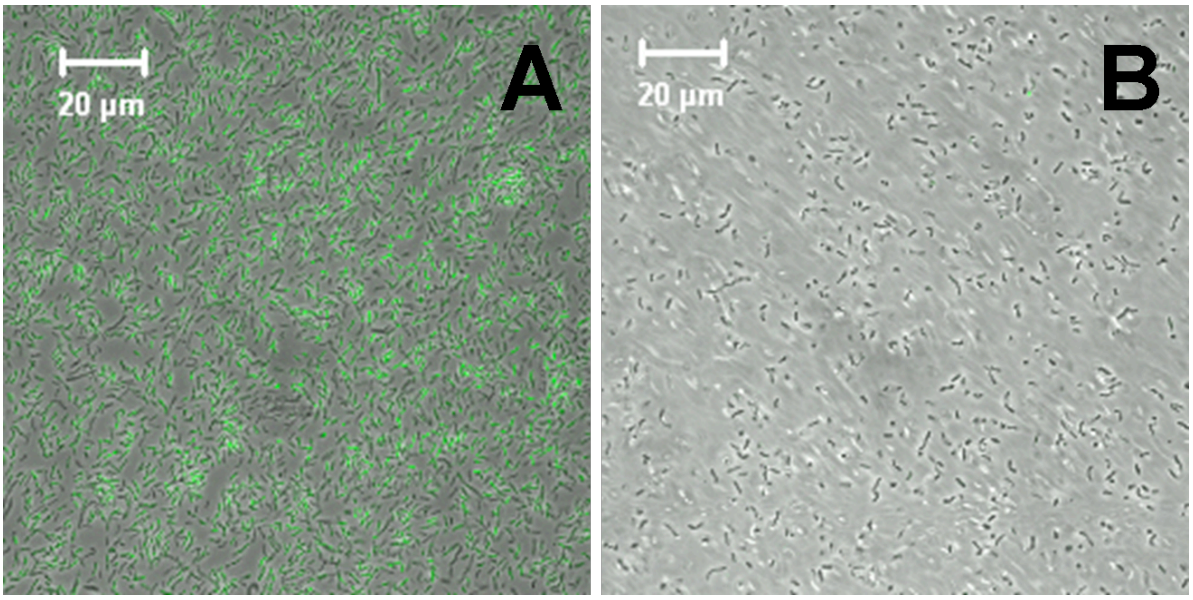

FIG. 1. Fluorescence microscopy photographs of *Marinobacter* sp. HP15 harboring the reporter gene-carrying plasmid pBBR.EGFP (A) and the plasmid-free wild type as control (B) excited at 488 nm.

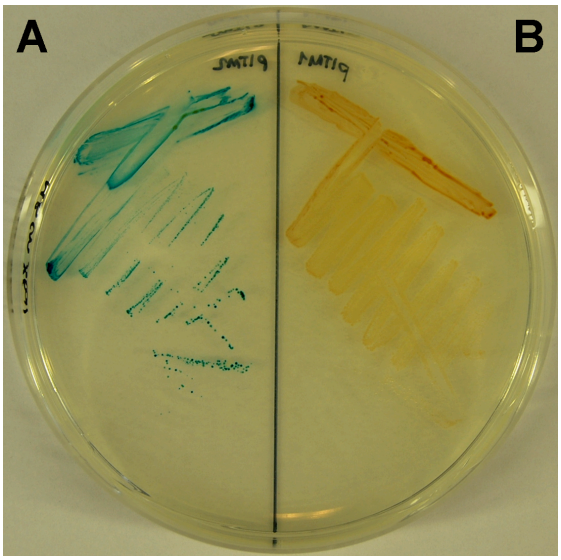

FIG. 2. Colony phenotypes of *Marinobacter* sp. HP15 carrying pITM2 (A) and pITM1 (B) on MB agar supplemented with X-Gal.

genetically accessible model systems are needed. Essential methods to allow molecular analyses of a given bacterium are plasmid transformation techniques, different types of mutagenesis, and reporter gene expression. Herein, transformation of *Marinobacter* sp. HP15 by electroporation and conjugation, random and site-directed mutagenesis, as well as expression of reporter genes were reported for the first time as a proof-of-principle. With the established techniques, it is now possible to identify genes, gene products, and molecular signals important for the interaction of this bacterium with diatom cells.

The antibiotics susceptibility spectrum of *Marinobacter* sp. HP15 was determined to allow for selection of transformants or mutants by antibiotics resistance markers. In contrast to ampicillin, several other antibiotics were found suitable as markers for transformation or mutagenesis of HP15. However, suscepti

bility of HP15 to some antibiotics was rather low, which might be due to the high salt concentration in the medium as concluded for other marine organisms previously (39). Antibiotics resistances were claimed to be usable taxonomic markers for marine bacteria (17). Herein obtained data are comparable to those for *M. aquaeolei* (26), but not to those of *M. vinifirmus* and *M. alkaliphilus* (34, 50) and thus did not result in a clear genus-specific pattern.

Recombinant plasmids of different incompatibility groups were tested for replication in *Marinobacter* sp. HP15. Interestingly, transformation with plasmids of the incompatibility group IncQ was successful whereas plasmids of incompatibility groups IncP, IncX, colE1, or pMB1 did not replicate or could not be introduced to HP15. It remains to be analyzed whether the two native plasmids of HP15 with molecular sizes of 42 and 187 kb (16), respectively, interfere with replication of the latter plasmid groups.

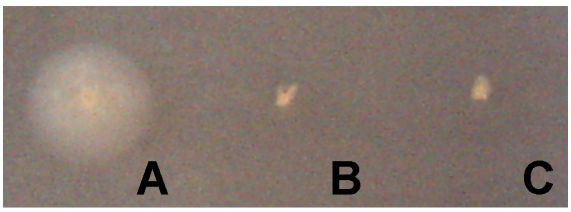

FIG. 3. Phenotypic characterization of flagellum-deficient *Marinobacter* sp. HP15 mutants by 0.3 % soft agar assay after 2 days of incubation: HP15 wild type (A), Δ*fliC* (B), and *fliG::*Tn*5*(C).

For transformation of *Marinobacter* sp. HP15, preparation of competent cells, amount of plasmid DNA, and incubation period following electroporation were optimized. For successful electrotransformation of HP15 a long recovery period post electroporation was needed. This might be due to a prolonged time needed for adequate expression of the antibiotic-resistance gene as described by others (4). The herein obtained electropo-

ration efficiency was comparable to that of the marine γ-proteobacterium *Pseudoalteromonas* (31) but was lower than that described for *Alteromonas* (27). Upon optimization of the mating period, the plasmid conjugation efficiency for HP15 was found to be similar to those of other marine γ-proteobacteria (12) or α-proteobacteria of the *Roseobacter* clade (39).

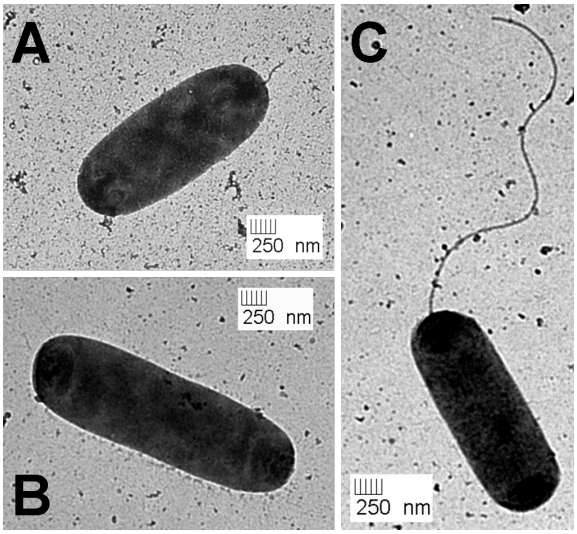

FIG. 4. Phenotypic characterization of flagellum-deficient Marinobacter sp. HP15 mutants by transmission electron microscopy: ΔfliC (A), fliG::Tn5 (B), HP15 wild type (C).

Since random transposon insertion in bacterial genomes is a simple method to produce diverse mutant libraries, the transposon delivery plasmid pRL27 was used to generate random mutants of *Marinobacter* sp. HP15. The efficiency of mutagenesis was lower than that of the close relative, *Pseudomonas stutzeri* (32). However, it was sufficient to readily generate a library of ~2000 mutants. For homologous recombination, derivatives of the mobilizable vectors pEX18Ap and pK18mobsacB were used due to their inability to replicate in non-enterobacterial species (25, 43). As expected, conjugation of these vectors without insert DNA homologous to genes of HP15 did not yield antibiotics-resistant HP15 transformants indicating that they could be used as suicide vectors.

To ensure randomness of transposon insertions and to demonstrate the ability to knock-out specific genes, motility of obtained mutants was screened. The flagella-deficient transposon mutants *fliG*::tn*5*, *fliR*::tn*5*, as well as the site-directed mutant Δ*fliC* were unable to swim in soft agar. As expected, in mutant *fliG*::tn5 the flagellum was not formed at all since this gene is required for the hook formation as described earlier for *Salmonella enterica* (52). In contrast, the Δ*fliC* mutant exhibited the flagellar hook but was missing the flagellar filament confirming previous data obtained for *Heliobacter pylori* and other bacteria (36, 45). These results demonstrated that the flagellar filament of *Marinobacter* sp. HP15 is encoded by a single flagellin gene. Furthermore, the mutational analysis experimentally proofed that motility-associated genetic traits of HP15 are comparable to those of e.g. *Pseudomonas aeruginosa* (35, 49) and thus confirmed our genome sequence data analysis (16). The flagellum-deficient mutants will next be tested during the interaction with diatoms to study the role of bacterial motility in chemotaxis and attachment.

The reporter genes *egfp* and *lacZ* were introduced *in trans* to HP15 and showed a clear phenotypic expression. Thus, *egfp* as well as *lacZ* can be used for *in vivo* labeling of HP15 and for reporter gene analyses in future studies.

The obtained genomic cosmid library of *Marinobacter* sp. HP15 was demonstrated to be comprehensive and adds as a powerful tool for genome-wide analysis.

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

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3.4 Chemotaxis of *Marinobacter* sp. HP15 and its impact on attachment to the diatom *Thalassiosira weissflogii*

Chemotaxis of *Marinobacter* sp. HP15 and its impact on attachment to the diatom *Thalassosira weissflogii*

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Marinobacter sp. HP15 was isolated from aggregates of the German Wadden Sea and has been demonstrated to attach to the diatom *Thalassiosira weissflogii* and to induce transparent exopolymeric particle (TEP) and aggregate formation in co-culture. HP15 possesses one polar flagellum and is highly motile. Three distinct chemotaxis-associated gene clusters were identified in the genome sequence of HP15 in comparison to that of *Pseudomonas aeruginosa* PAO1. Mutants in the genes *cheA*, *cheB*, *chpA*, and *chpB*, encoding for histidine kinases and methylesterases, respectively, involved in either flagellum-associated chemotaxis or pilus-mediated twitching motility were generated and phenotypically analyzed in soft agar assays and biofilm formation tests. Mutants Δ*cheA* and Δ*cheB* were found to be swimming deficient in soft agar. Mutants Δ*chpA* and Δ*chpB* showed a defect in biofilm formation. HP15 wild-type cells were attracted to diatom homogenates in sea water soft agar assay allowing the hypothesis that chemotaxis is important for the interaction of HP15 with diatom cells. When wild-type and the chemotaxis-deficient mutant, Δ*cheA*, were tested for their attachment behavior to *T. weissflogii*, ~26 % of the wild-type cells attached to the diatom, whereas only ~10% of the Δ*cheA* mutant showed attachment. These results therefore highlighted the importance of *Marinobacter* sp. HP15 chemotaxis in initiation and maintenance of its interaction with the diatom *T. weissflogii*.

INTRODUCTION

In the heterogeneous environment of the pelagic ocean, bacterial chemotaxis is considered an important selective advantage to compete for micro-scale nutrient-patches (10). Particles such as phytoplankton cells generate micro-environments supplying bacteria with essential nutrients and surface for attachment to allow these microorganisms to successfully compete with others (6).

Chemotaxis might initiate the interaction of bacterial cells with the phytoplankton organism as it has been postulated within the general idea of the so-called 'phycosphere' introduced by Bell *et al.* (8). Marine bacteria have been demonstrated to be attracted to micro-algae or their exudates and to utilize those compounds (7, 10, 18, 38, 46, 47, 52). Besides the bacterial growth-promoting effect of phytoplankton exudates, the bacteria in turn may fuel algal growth and reproduction by re-mineralization of

nutrients (13, 42).

The four major types of interaction - mutualism, parasitism, commensalism, and competition (23) - are transient and highly depend on micro- and macro-ecological environmental conditions. Various interaction types may exist simultaneously and the balance between stimulatory and inhibitory effects determine the survival of particular organisms (13). The specific mechanisms, by which those organisms interact and how factors such as motility or chemotaxis impact the fate of interactions, are largely unknown. In a symbiotic interaction, bacterial siderophore production was proposed to stimulate algal growth (4). Algicidal activity of *Pseudoalteromonas* sp. strain A28 was referred to an extra-cellular serine protease produced by this bacterium (33). Bioactive polyunsaturated aldehydes (PUAs) produced by several marine phytoplankton organisms were shown to inhibit growth of some bacterial organisms, but to stimulate that of other species (44).

Two different types of bacterial motility were described for *Pseudomonas aeruginosa* which are regulated by different chemotaxis gene clusters (9, 14-16, 19, 29, 51). Flagellar movement is regulated via a histidine–aspartate phosphorelay (HAP) system. Corresponding gene clusters were identified (19) and similar priniciples were also intensively investigated in model organisms as *Escherichia coli* (50) and *Salmonella enterica serovar Typhimurium* (48). Twitching motility describes the movement on surfaces with the help of Type IV pili (26). A similar model as for flagellar chemotaxis was hypothesized for the regulation of twitching motility by the *chp* chemosensory system (9, 51), which controls the motor complex of the pili causing extension and retraction of the latter one. In *P. aeruginosa*, one of the five chemotaxis gene clusters namely the *chp* genes in contrast to the *che* genes of flagellar chemotaxis, is exclusively responsible for the pili chemotaxis (9, 14-16, 51). In the present study, we demonstrate the chemotaxis gene clusters of *Marinobacter* sp. HP15 and the functions of certain individual chemotaxis genes by mutational analysis. This study describes the chemotaxis of a *Marinobacter* strain for the first time.

To obtain more insights on the actual mechanisms by which diatom cells and bacteria interact and to study the effect of distinct bacteria on aggregate formation, a bilateral model system was previously established (20, 28). The bacterial strain *Marinobacter* sp. HP15 was shown to attach to the diatom *Thalassiosira weissflogii* and to induce formation of transparent expolymeric particles (TEP) and aggregates in co-culture with the diatom. Since the bacterium is genetically accessible (28), specific mechanisms of the interaction are now studied on the molecular basis. In the current study, we describe the chemotactic behavior of strain HP15 towards the diatom *T. weissflogii*. By a functional knockout of the gene coding for the central histidinase kinase of the chemotaxis signalling cascade, the importance of bacterial chemotaxis for bacterial attachment to the diatom was demonstrated.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains, plasmids, and primers used are listed in **Table 1**. *Marinobacter* sp. HP15 was routinely grown in marine broth (MB) at 37°C and 250 Luria-Bertani broth (LB) at 37°C and 250 rpm or on LB agar. The following antibiotics were added to the

TABLE 1. Strains, plasmids, and primers used in this study. The underline in sequences marks the restriction enzyme recognition sites.

Strains, plasmid, or primer	Relevant characteristics or sequence (5´ - 3´)[a]	Source of reference
Marinobacter sp. HP15	wild type	(24)
Escherichia coli DH5α λ-pir	Φ80*dlacZ*ΔM15 Δ(*lacZYA- argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/ *λ-pir*	(37)
*chpA::*Tn*5*	*chpA* transposon insertion mutant; Kan^r	(28)
Δ*cheA*	*cheA* deletion mutant; Cm^r	This study
Δ*cheB*	*cheB* deletion mutant; Cm^r	This study
Δ*chpB*	*chpB* deletion mutant; Cm^r	This study
pGEM-T easy	linearized cloning vector; Amp^r	Promega, Mannheim, Germany
pFCM1	carrier of Cm^r with FRT sides	(12)
cheA upstream forward	AAGCTTGGTACCTTCAGAAACCGAGACTGG	This study
cheA upstream reverse	GTGATCTTGGCTTCACCA	This study
cheA downstream forward	ATCCGTCAGCCCTGCCTT	This study
cheA downstream reverse	AAGCTTGCCAGAGAAGGCCGAAG	This study
cheB upstream forward	TACGTTCTCGGCAGGCGC	This study
cheB upstream reverse	AAGCTTTCCTGAGCCTGCTTGGGG	This study
cheB downstream forward	AAGCTTGGTACCTGATCTGGCCGGAGCTGG	This study
cheB downstream reverse	CCACGCCAGGGAACGGTT	This study
chpB upstream forward	AAGCTTGGTACCTTCCCATGAGCGGACGGC	This study
chpB upstream reverse	GGAAGCCTTCGCCGGATG	This study
chpB downstream forward	GCATGGACGTGGTTGCCA	This study
chpB downstream reverse	AAGCTTTCCGGTCAGCCGCTCAAT	This study
Δ*cheA* check forward	AAGCTTCTTCGGCCTTCTCTGGCA	This study
Δ*cheA* check reverse	GAGCTCCCAGTCTCGGTTTCTGAA	This study
Δ*cheB* check forward	TTGACAGCGTTGTCCCGG	This study
Δ*cheB* check reverse	CACCAAGCGGCTCGATG	This study
Δ*chpB* check forward	GATCAGCGGCGAACTGGG	This study
Δ*chpB* check reverse	GGTGCGGGTAAGGGCAGA	This study

[a] Underlined bases indicate the positions of restriction sites. Kan^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant.

media when needed (in mg ml^{-1}): ampicillin (Amp), 50; chloramphenicol (Cm), 25; kanamycin (Km), 500.

Cultures of *T. weissflogii* (CCMP 1336) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, Maine, USA). Diatom cultures were grown at 16°C in f/2 medium (24, 25) using a 12 h photo period 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, USA).

DNA procedures. Restriction enzyme reactions, electrophoresis, purification of DNA from agarose gels, electroporation, PCR, and plasmid DNA preparation were performed by standard techniques (45). Electroporation of *Marinobacter* sp. HP15 was conducted as described previously (28).

Chemotactic behavior of *Marinobacter* sp. HP15 towards diatom homogenates. For preparation of diatom cell homogenates, 100-ml cultures of *T. weissflogii* were grown to exponential phase (approx. 60,000 cells ml^{-1}) and harvested by centrifugation of 2 x 50 ml at 4°C and 4,000 rpm for 20 min. The supernatant was removed, and the cell pellets were separately re-suspended in 1 ml of sterile ice-cold deionized water. Cells were homogenized by sonication, and successful cell disruption was assessed by microscopy. The homogenates were stored at -20°C. For the chemotaxis plate assay, HP15 was grown on MB agar plate overnight at 37°C and inoculated in triplicates in soft agar plates (25 ml of sea water supplemented with 0.3% agar) containing 100 µl of diatom homogenate. As control, plates were prepared without diatom homogenates. Plates were incubated at room temperature. The diameter of swimming was measured daily for 9 days.

Mutagenesis of HP15 chemotaxis genes. Identification of chemotaxis gene clusters in *Marinobacter* sp. HP15 (GenBank accession no. CP001978 (21)) was conducted by comparison to those of *Pseudomonas aeruginosa* PAO1 using Blastp (2). PAO1 is a model organism for chemotaxis (29) and shares 90% 16S rRNA similarity to that of HP15 (53). Conserved domains of CheA, CheB, ChpA, and ChpB were analyzed using the CDD (Conserved Domain Database) (35). Transposon mutants of HP15 were created as described previously (28), and mutant *chpA::*Tn*5* was identified by the soft agar swimming assay. Site-directed disruption mutants in genes *cheA*, *cheB*, and *chpB* were generated by homologous recombination. For this, up- and downstream regions of the respective genes using the primer pairs *cheA*_upstream_forward/reverse, *cheA*_downstream_forward/reverse, *cheB*_upstream_forward/reverse, *cheB*_downstream_forward/reverse, and *chpB*_upstream_forward/reverse, and *chpB*_downstream_forward/reverse, respectively, were PCR amplified, ligated separately into pGEM-T Easy and transformed into *E. coli* DH5α λ-pir. The chloramphenicol resistance cassette (Cm^r) was excised from pFCM1 with *Kpn*I and ligated into the upstream region vectors. The upstream region vectors containing Cm^r was brought into the pGEM-T easy vector containing the downstream regions by *Hind*III/*Spe*I. The thus-by generated knockout constructs were introduced to HP15 by electroporation, and colonies were screened on MB agar supplemented with chloramphenicol. Double crossover events were confirmed by PCR using the primer pairs Δ*cheA*_check_forward/reverse, Δ*cheB*_check_forward/reverse, and

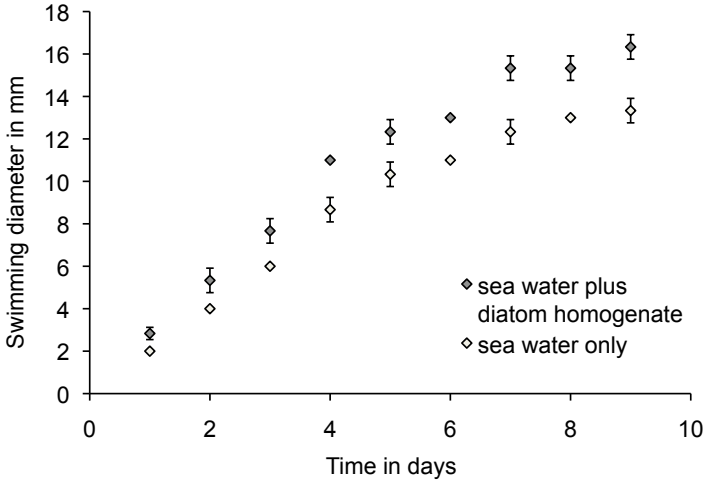

FIG. 1. Chemotactic behavior of *Marinobacter* sp. HP15 in sea water soft agar (0.3%) supplemented with diatom homogenate.

Δ*chpB*_check_forward/reverse, respectively.

Since PCR fragments for the wild type and Δ*cheB*, and the wild-type and Δ*chpB* were of similar size, respectively, restriction digests with *Kpn*I were used to confirm the successful mutagenesis.

Phenotypic characterization of HP15 chemotaxis mutants. The chemotactic phenotypes were confirmed by light and transmission electron microscopy, soft agar swimming assay (28), and biofilm formation assay (41). For soft agar swimming, an overnight plate culture of HP15 was inoculated with a toothpick in freshly prepared plates containing 10fold diluted MB and 0.3% agar. For the biofilm formation, cells were pre-cultured overnight in 5 ml MB at standard conditions and inoculated at a final OD of 0.1 in 150 µl MB in microtiter plates in five replicates per strain. After overnight incubation at 37°C without shaking, optical densities of the cultures were determined and attached cells were visualized by crystal violet staining as described previously (41) with elution in 150 µl 96% ethanol. Absorbance was measured at 600 nm. One-tailed Student´s t-test was applied to access significance of differences between strains.

Attachment assay with the diatom *T. weissflogii*. HP15 wild type and its Δ*cheA* mutant were grown in MB at 18°C to an OD of 0.6 (exponential growth phase). *T. weissflogii* cells were grown for six days to a cell concentration of approx. 43,000 cells ml^{-1} (exponential growth phase). Aliquots of 10 ml f/2 medium were inoculated with bacterial and diatom cells at a final OD of approx. 0.0001 corresponding to 1 x 10^5 bacterial cells ml^{-1} and a final cell concentration of approx. 22,000 diatom cells ml^{-1}. Total bacterial cell numbers were determined by dilution plating on MB agar plates and the suspensions were incubated at room temperature for 1 hour without shaking. Attaching and non-attaching bacterial cells were separated by sieving through 10-µm gaze (20). Both fractions were collected and bacterial CFU ml^{-1} were determined by dilution plating on MB agar plates. One-tailed Student´s t-test was applied to access the significance of differences between strains.

RESULTS

Chemotaxis of *Marinobacter* sp. HP15 towards the diatom *T. weissflogii*. Bacterial cells that are inoculated to the center of a soft agar plate consume nutrients and generate a concentration gradient that increases outward from the point of inoculation. By sensing the generated gradient, bacterial cells swim outwards following the increase in

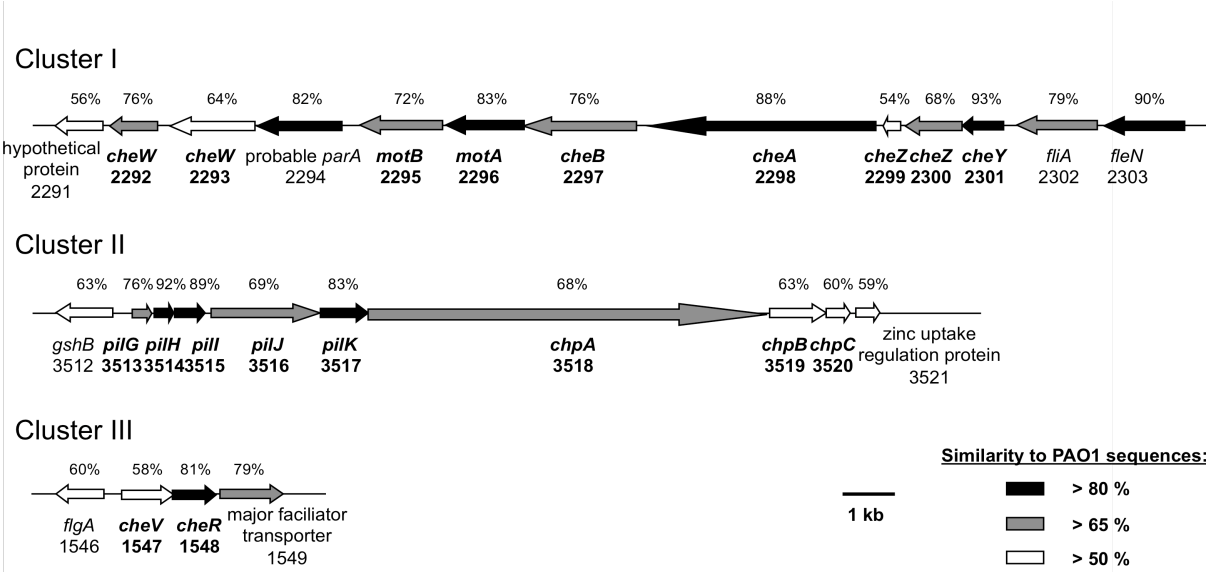

FIG. 2. Genetic maps of chemotaxis gene cluster of *Marinobacter* sp. HP15 based on protein similarities to *P. aeruginosa* PAO1. Similarities: in black, > 80%; in grey, > 65%; in white, > 50%. Size marker: 1 kb.

nutrient concentration. In sea water soft agar supplemented with diatom homogenate, HP15 showed a swimming behavior, which was significantly stronger than in respective soft agar without the diatom homogenate (**Fig. 1**) indicating that the cells were attracted to components of the diatom homogenate.

Chemotaxis gene clusters in *Marinobacter* sp. HP15. Three distinct chemotaxis gene clusters were identified in the genome sequence of *Marinobacter* sp. HP15 when compared with the genome sequence information of P. aeruginosa PAO1 (**Fig. 2**). The respective protein sequences of both strains were closely related to each other with a general similarity higher than 65 % and reaching up to 93 %. Chemotaxis gene clusters I and III comprise the chemotaxis genes responsible for the regulation of flagella movement and were found to be homologous to clusters I and V of *P. aeruginosa* PAO1 (19, 49), respectively. The HP15 chemotaxis gene cluster II corresponded to cluster IV of strain PAO1, which controls twitching motility via pili (9, 30, 36). No significant homologs of the chemotaxis gene cluster II and III of PAO1 were identified in HP15. All three chemotaxis clusters of strain HP15 were found to be located in close vicinity to genes encoding for the respective motility appendages (Data not shown).

By comparison to the CDD, CheA contained a CheA regulatory domain acting as a histidine kinase, which has further been proposed to interact with the regulator CheW, a histidine kinase-like ATPase, a signal transducing histidine kinase, and a histidine phosphotransfer domain. The same domains were found in ChpA. However, additionally, a signal receiver domain and in total six histidine phosphotransfer domains were detected. As expected, a methylesterase domain was present in CheB as well as in ChpB. However, CheB contained a CheY-like receiver domain, which was absent in ChpB.

Chemotactic behavior and surface attachment of HP15 mutants.

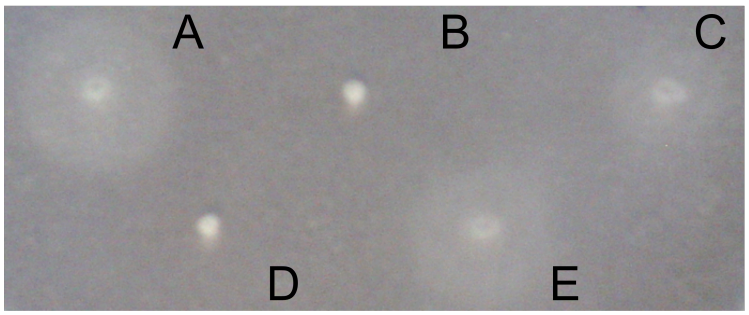

FIG. 3. Phenotypes of HP15 wild type and its chemotaxis mutants in 0.3 % soft agar swimming assay. (A) wild type, (B) Δ*cheA*, (C) *chpA::*Tn*5*, (D) Δ*cheB*, (E) Δ*chpB*.

Mutants were generated for the genes *cheA*, *cheB*, *chpA*, and *chpB*. The HP15 mutants Δ*cheA* and Δ*cheB* carry mutations in chemotaxis gene cluster I and were generally non-chemotactic in MB/10 soft agar (**Fig. 3**). Transmission electron pictures showed that the cells formed proper flagella as the wild type (Data not shown) thereby supporting previous results of Masduki *et al.* (36) and Kato *et al.* (30) for *P. aeruginosa* PAO1. In contrast, HP15 mutants *chpA::*Tn*5* and Δ*chpB* carrying mutations in chemotaxis gene cluster II exhibited a wild type swimming behavior, thus demonstrating that these genes are not essential for flagellum-mediated chemotaxis.

Furthermore, the ability of all four HP15 mutants to attach to an abiotic surface and form biofilms was assayed in micro-titer plates. The wild type and all four mutants reached the exponential growth phase (OD of 0.4 - 0.8) overnight (Data not shown). Staining with crystal violet revealed that the four chemotaxis mutants showed a significantly decreased ability to attach to the wells of the microtiter plate in contrast to the wild type of HP15 (**Fig. 4**) (n = 5, $p_{WT\ vs.\ mutant} < 0.001$). These results indicated that chemotaxis via both, the polar flagellum and putative pili, were required for surface attachment and biofilm formation of *Marinobacter* sp. HP15.

Attachment of HP15 chemotaxis mutants to diatom cells. The role of chemotaxis for the interaction of HP15 with diatom cells was tested by comparing its wild type and the Δ*cheA* mutant regarding the ability to attach to cells of the diatom *T. weissflogii*. Mutant Δ*cheA* was used for this experiment, since it showed no chemotaxis in the soft agar assay and *cheA* is coding for the histidine kinase that initiates the signaling cascade required for flagellum-mediated chemotaxis. The total cell numbers of HP15 wild type and Δ*cheA* mutant used in this assay were on average $5.8 \times 10^4 \pm 9.9 \times 10^3$ (standard deviation) and 1.0×10^5 CFU $ml^{-1} \pm 8.2 \times 10^3$ (SD), respectively. The sum of attached and non-attached cells of wild type and Δ*cheA* mutant after filtration were in average $2.3 \times 10^4 \pm 4.3 \times 10^3$ (SD) and 1.5×10^5 CFU $ml^{-1} \pm 2.6 \times 10^4$ (SD), respectively. When numbers of attaching cells were compared with those of non-attaching cells, 26 % ± 1.4 (SD) of the wild type cells were found to be attached to the diatom surface, whereas only 10 % ± 0.2 (SD) of the Δ*cheA* mutant cells adhered to the diatom surface (**Fig. 5**) (n = 3, p = 0.003). This result demonstrated that flagellum-mediated chemotaxis of HP15 seems to play a role in attachment to the surface of *T. weissflogii*.

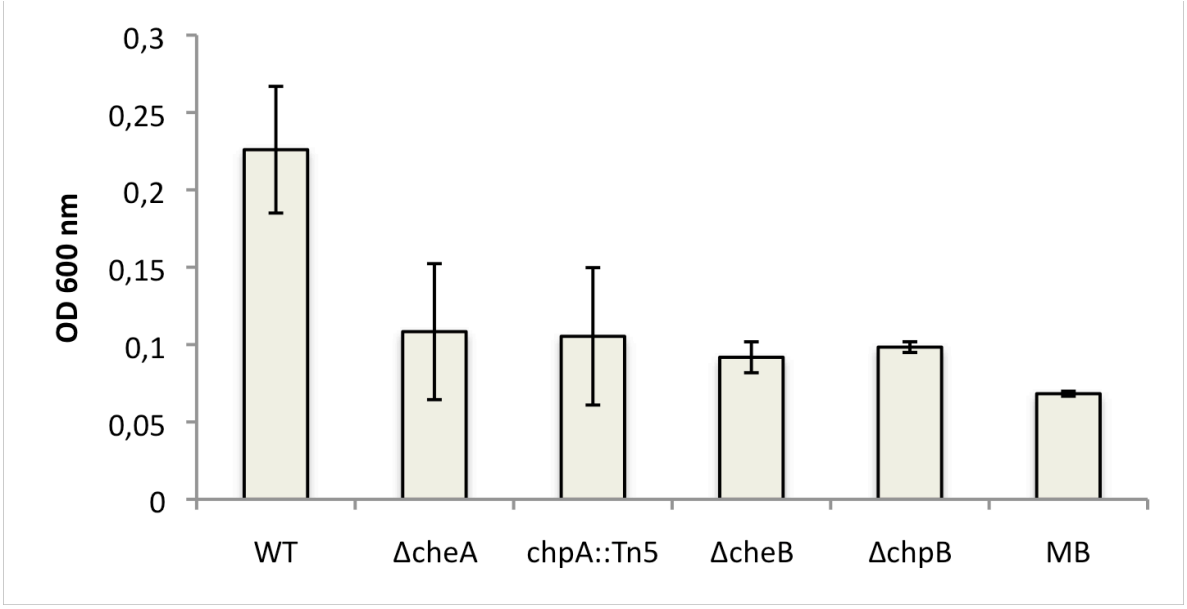

FIG. 4. Bacterial surface attachment of HP15 wild type and its chemotaxis mutants visualized with crystal violet staining after washing off planktonic cells determined by absorbance measurement 600 nm, n = 5, $p_{WT\ vs.\ mutant} < 0.001$. Wells filled with MB served as controls. WT = wild type.

DISCUSSION

The phycosphere of a phytoplankton cell is an important area of nutrient uptake for bacteria in the oceans (22). Interaction processes between those two groups of organisms can be various and might be beneficial or detrimental for one or both partners. To identify genes and gene products, which are essential for this interactions, a bilateral model system consisting of the diatom *T. weissflogii* and the bacterial strain *Marinobacter* sp. HP15 was established (20, 28). Under rich nutrient conditions (f/2 medium), this interaction promotes the growth of both, the bacterium and the diatom: *Marinobacter* sp. HP15 attached to the diatom and the co-culture resulted in increased TEP synthesis and aggregation of the diatom cells (20). Processes required for initiation of this interaction might be bacterial motility, chemotaxis, and surface attachment as proposed previously (13). In this study, it was hypothesized that bacterial chemotaxis may be required for this diatom-bacteria interaction since HP15 is highly motile and possesses distinct chemotaxis gene clusters. Interestingly, strain HP15 indeed was found to be attracted to diatom homogenates. Moreover, various chemotaxis-deficient mutants showed a decreased ability to attach to an abiotic surface and to diatom cells thereby supporting the hypothesis. In the future, fractions of the diatom homogenate will be tested in the chemotaxis assay and chemically described to identify the key molecules important for the diatom-bacteria interaction. Interestingly, HP15 does not metabolize any sugars that *T. weissflogii* has been shown to produce (3, 27). To our knowledge, the specific amino acid pattern that *T. weissflogii* is secreting has not been characterized in detail, thus it will be focus of our studies in the future since HP15 is able to grow on specific amino acids only (27). Further, testing

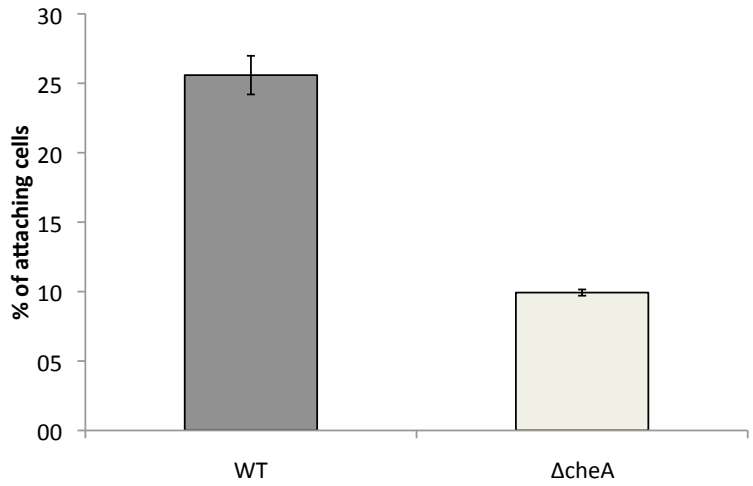

FIG. 5. Diatom surface attachment assay of HP15 wild type and its Δ*cheA* mutant with *T. weissflogii* by determination of the percentage of attaching cells in comparison to non-attaching cells for three replicates per experimental setup. WT = wild type, n = 3, p = 0.00002.

aggregate formation and TEP synthesis during co-cultures in so-called rolling tank experiments will potentially reveal the actual effects of flagella- or pili-mediated chemotaxis during the diatom-bacteria interaction.

The signalling cascade of bacterial chemotaxis is initiated by a two component system. More precisely, it is a histidine-aspartate phosphorelay (HAP) system (50). Thereby, an autophosphorylating histidine protein kinase serves as a phosphoryl donor to a response regulator protein. This response regulator protein is modulated by phosphorylation of a aspartic acid residue. In HP15, two chemotaxis related HAP systems were identified. In comparison to *P. aeruginosa* PAO1, the associated gene clusters could be correlated to the chemotaxis of flagella and pili, respectively. In general, high similarities to the chemotaxis genes of PAO1 were identified making PAO1, a model organism for chemotaxis research, a suitable comparison tool for this study. Even though the signalling cascade of flagellar chemotaxis has been well defined, the regulation of twichting motility is hardly understood. The histidine kinase ChpA represents a complex CheA homolog, but carrying more Hpt domains. The Hpt domains containing a histidine residue act as phosphoreceiver and phosphodonor and can therefore be integrated in complex multistep regulatory systems (50). In *P. aeruginosa*, some of the Hpt domains have been shown to be essential for twitching motility (9, 34). ChpB is homologous to the CheB methylesterase in the flagellar chemotaxis system and was therefore hypothesized to serve for the demethylation of the predicted methyl-accepting chemotaxis protein PilJ as part of a sensory adaption system (51). Besides *chpA*, *chpB*, and *chpC*, two further genes, *chpD* and *chpE*, were identified in the chemotaxis cluster II of PAO1 and predicted to be associated with the regulation of twitching motility. However, in HP15, there are no homologs in the respective region of the genome. Concluding, the flagellar chemotaxis system is well understood in various organisms and even regulated by homologous genes. With this study, we were able to describe genes and their functions in comparison to *P. aeruginosa* PAO1 and therefore could demonstrate that the same patterns are valid for *Marinobacter* sp. HP15. However, more biochemical as well as genetic analysis will be required to understand the signalling cascade regulating pili movement.

The soft agar plate assay implies that the bacteria are able to sense the self-made nutrient gradient and therefore,

swim outwards following the increase in nutrient concentration (38). The flagella movement of the chemotaxis mutants is not impaired as has been shown by microscopy. So, in soft agar, *chpA* and *chpB* mutants of HP15 showed a swimming phenotype indistinguishable from that of the wild-type, whereby mutants in *cheA* and *cheB* showed a clear defect in swimming behaviour. These observations indicate that the Chp chemosensory pathway might not be intersecting with the swimming chemotaxis system of HP15. These findings are supported by the proposed functions of the corresponding genes in *P. aeruginosa* (19, 30, 36, 51). The used agar matrix might require utilization of the flagellum for motility, but not that of putative pili by HP15. As shown for PAO1, twitching motility mutants can be observed on very thin agar plates (17). However, this method was not successful to demonstrate twitching motility of HP15. In future experiments, additional techniques to investigate the phenotype of *chp* mutants will be applied, i.e. biofilm formation assays by flow cells (32, 37, 40).

Previously, the impact of bacterial chemotaxis on attachment to abiotic surfaces has been described for *Aeromonas* spp. and the plant pathogenic bacterium, *Agrobacterium tumefaciens* (31, 37). For both, the attachment of chemotaxis-deficient mutants to abiotic surfaces was notably compromised. In *Vibrio cholerae*, chemotaxis was proposed to play a stage-specific role in attachment to a polystyrene microtiter dish, since a *cheY*-3 mutant was deficient in generating a monolayer although not in biofilm formation in general (39). Chemotaxis-deficient mutants of *E. coli* appear to form biofilms indistinguishable from the *E. coli* wild type (43). The opportunistic human pathogen, *P. aeruginosa*, has a very complex chemotaxis system, in which one of the chemotaxis gene clusters is exclusively regulating twitching motility (9, 29). Even though sequence analysis revealed homologies of the twitching motility gene cluster of *P. aeruginosa* in HP15, respective *P. aeruginosa* mutants form a more robust biofilm than the wild type (12), whereas *chp* mutants in HP15 have a decreased ability to attach to an abiotic surface. In summary, our results and results of others suggested that the impact of chemotaxis on attachment is highly species-specific. Noteably, all of the previous reported results were based on tests with abiotic surfaces. The current study described the chemotaxis-mediated attachment of a single bacterial strain to an environmentally relevant biotic surface.

In conclusion, although a wealth of knowledge on mechanisms of chemotaxis in various bacterial species has been acquired *in vitro* (1, 5, 11, 29, 50), the individual effects of chemotaxis in environmental habitats have been hardly understood. For the first time, this study demonstrated the importance of chemotaxis in phytoplankton-bacteria interactions using genetic tools. Future research will focus on the further effects of chemotaxis on TEP formation and aggregate production in co-incubation studies with the diatom *T. weissflogii*.

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Chapter 4

Summary and concluding remarks

Phytoplankton blooms, particularly composed of diatoms, are proposed to be a major source of marine particulate organic carbon (POC) (Simon et al., 2002). After fixation of atmospheric CO_2 by phytoplankton, the biomass is transformed by zooplankton grazing, sedimentation in form of aggregates, and consumption via the microbial loop (Smith et al., 1995). The importance of heterotrophic bacteria in decomposition of diatoms and their silicate frustules as well as in formation of diatom aggregates has been highlighted in many studies (Bidle and Azam, 2001; Smith et al., 1995; Grossart, 1999). However, the specific role of heterotrophic bacteria in the aggregation processes as well as in the formation of transparent exopolymer particles (TEP) is still unclear since previous studies have been conducted with non-axenic algae cultures (Grossart et al., 2006a). Thus, the aim of this study was to establish a bilateral model system consisting of a diatom and a bacterial species to investigate the processes leading to aggregate formation in-depth at the molecular level.

Herein, we studied the interaction between the diatom *T. weissflogii* and the bacterial strain *Marinobacter* sp. HP15. As one of four inducing the production of TEP and aggregate formation with the diatom (Gärdes et al., 2010a) strain

HP15 could be demonstrated to be genetically accessible (Kaeppel et al., b). The biochemical characteristics and phylogenetic position of HP15 were described and it was proposed as a novel species of the genus *Marinobacter* (Kaeppel et al., a). The genome of this organism was sequenced and analyzed (Gärdes et al., 2010b). Further, we hypothesized that chemotaxis may be an important process in diatom-bacteria interactions and verified this proposition by showing a decreased ability of chemotaxis-deficient mutants of HP15 to attach to *T. weissflogii*.

4.1 Chemotaxonomic and phylogenetic characterization of *Marinobacter* sp. HP15

Besides the well-studied diatom species *T. weissflogii* (Grossart, 1999; Passow, 2002a; Aluwihare and Repeta, 1999; Armbrust, 1999; Leblanc et al., 1999; Koski et al., 2008), the bacterial strain HP15 (Grossart et al., 2006b) was found suitable for the bilateral model system (Gärdes et al., 2010a). In accordance with its 16S rRNA analysis, HP15 (GenBank accession no. AY241552) belongs to the genus *Marinobacter* of *γ-Proteobacteria*. The fatty acid profile, the predominant ubiquinone and G+C content supported this assignment. The closest relatives according to 16S rRNA comparison were the *Marinobacter* species *M. flavimaris*, *M. salsuginis*, *M. lipolyticus*, and *M. algicola* (Antunes et al., 2007; Green et al., 2006; Martín et al., 2003; Yoon et al., 2004). These strains formed a discrete cluster in the phylogenetic tree (Kaeppel et al., a). The respective strains were isolated from various environments, i.e. from sea water of the Yellow Sea (Korea), the brine–seawater interface of the Red Sea, hypersaline habitats of southern Spain, and laboratory cultures of dinoflagellates. Common features of all these strains were the wide growth range in terms of temperature (approx. 4-45 °C), salinity (approx. 1-20% (w/v) NaCl), and pH (approx. 5-10). The chemotaxo-

nomic pattern of HP15 was unique regarding the utilization of monosaccharides and amino acids as well as enzyme activities. Average DNA-DNA hybridization values to *M. flavimaris* (66%), *M. salsuginis* (39%), *M. lipolyticus* (28%), and *M. algicola* (26%) were below the generally accepted species differentiation limit of 70% (Wayne et al., 1987). Based on these results, HP15 was proposed as a novel species, *Marinobacter adhaerens* nov. sp. (=DSM 23420^T = CIP 110141^T) with the attribute *adhaerens* referring to its ability to attach to the diatom *T. weissflogii*.

4.2 The genome sequence of *Marinobacter* sp. HP15

To study the interaction of *Marinobacter* sp. HP15 and *T. weissflogii* at the molecular level, the genome of HP15 was fully sequenced and annotated (Gärdes et al., 2010b). HP15 possesses three replicons: the chromosome (~4.4 Mb, GenBank accession no. CP001978), and two circular plasmids (~187 kb and ~42 kb, GenBank accession nos. CP001979 and CP001980, respectively). In total, the genome comprises 4410 genes including 4,355 coding for proteins. The size of the genome is in the range of that of the other *Marinobacter* species, i.e. *M. aquaeolei* (~4.3 Mb), *M. algicola* (~4.4 Mb), and *Marinobacter* sp. ELB17 (~4.9 Mb), for which the complete genome sequence (*M. aquaeolei*) or whole genome shotgun sequences (*M. algicola*, *Marinobacter* sp. ELB17) are available. As in HP15, two plasmids, pMAQU01 and pMAQU02, were identified in *M. aquaeolei* (~240 kb and ~213 kb, respectively). Sequence analysis of the plasmids of HP15 and *M. aquaeolei* using blast searches (Altschul et al., 1990) revealed no highly similar sequences, but two more similar sequences (E value = 2 x 10^{-90}) between pHP187 and pMAQU01, i.e. one encoding for a protein of the phage integrase family and

an initiator RepB protein.

The sequence of the phage integrase family protein (accession no. ADQ00114 and YP957008 in HP15 and *M. aquaeolei*, respectively) showed a similarity of 85%. With a similarity of 70%, a homolog of the HP15 protein was also identified in the genome sequence of *Marinobacter* sp. ELB17 and with less than ~50% similarity in the genomes of other *Proteobacteria* such as *Altermonas*, *Pseudoalteromonas*, *Coxiella*, and *Burkholderia*.

The initiator RepB protein sequence of HP15 (accession no. ADQ00167 and YP956944 in HP15 and *M. aquaeolei*, respectively) has a similarity of 70% to its homolog in *M. aquaeolei*. RepB was shown to be an initiator of plasmid replication and possesses nicking-closing (topoisomerase I) like activity (Moscoso et al., 1997). The closest sequence similarities with approx. 50% where found to *Clostridium* spp., *Bacillus* spp. and the *Escherichia-Pseudomonas* shuttle vector pUCP18 (Schweizer, 1991).

Motility is an important selective force in bacterial communities of the pelagic ocean. The micro-environments of pelagic bacteria, considered to be homogenous for a long time, are actually heterogenously structured in terms of the abundance and spatial distribution of organic matter (Grossart et al., 2001). Particles such as diatom cells or marine snow may serve as "hot spots" for heterotrophic bacteria (Azam, 1998). Chemical gradients generated by particulate organic matter may cause an evolutionary selection for those bacteria that adapted to the heterogenous habitat by responding to such gradients. The motility in natural assemblages may vary from 10 to 70% depending on the environmental conditions (Mitchell et al., 1995; Grossart et al., 2001). Therefore, we proposed that motility is an important process in the interaction of the motile bacterium HP15 with the diatom *T. weissflogii*. The gene clusters coding for secretion, assembly, and mechanistic function of the polar flagellum of HP15 were analyzed *in silico* with emphasis

on the comparison to those of the well-studied model bacterium *Pseudomonas aeruginosa* PAO1, which is closely related to HP15. The hook and motor switch complex components were found to be highly conserved and three flagella gene clusters identified in HP15 showed significant similarities to orthologous and experimentally well-described gene clusters of PAO1. Besides their role in motility, flagella are essential for the initial attachment of bacteria to surfaces as demonstrated for *sad* (surface attachment defective) mutants of PAO1, which lack the flagellum (O'Toole and Kolter, 1998). The same was shown for HP15. Besides the swimming defect in soft agar plates, mutants in flagella-associated genes were unable to attach to abiotic surfaces (unpublished data).

4.3 The genetic accessibility of *Marinobacter* sp. HP15

After identification of four strains that induce production of TEP and aggregate formation with the diatom *T. weissflogii* (Gärdes et al., 2010a), the next requirement for a suitable bacterial model strain was its genetic accessibility. In contrast to well-established bacterial genetic model systems in medical, veterinary, or plant pathology as well as in microbial biotechnology, environmentally important microbes — particularly of marine origin — are often not readily accessible for molecular laboratory work. After determination of the antibiotics susceptibility spectrum of the organism in liquid medium and on agar plates, transformation protocols for electroporation were established. Among various plasmids of different incompatibility groups, only two plasmids, pBBR1MCS and pSUP106 (incompatibility group *rep*) (Kovach et al., 1994; Priefer et al., 1985), replicated in *Marinobacter* sp. HP15. Additionally, transposon mutagenesis using the suicide plasmid pRL27 (Larsen et al., 2004) and site-directed mutagenesis using homolo-

gous recombination were conducted for flagellum biosynthetic genes. The resulting mutant phenotypes were confirmed by soft agar assay and transmission electron microscopy. Expression of the reporter genes encoding for enhanced green fluorescent protein (*egfp*) and β-galactosidase (*lacZ*) was successful in HP15 revealing useful tools for gene expression studies. In this study, we reported the genetic manipulation of a member of the genus *Marinobacter* for the first time. Having a model system with a genetically accessible bacterium on hand, it is now possible to identify genes, gene products, and molecular signals important for the interaction of this bacterium with diatom cells.

Mutants in flagellum biosynthetic genes were generated for future tests in the interaction. With mutation of different flagellum-associated genes, we could particularly demonstrate their function. In the mutants *fliG::*tn5 and *fliR::*tn5, the flagellum was not assembled, since these genes are required for the hook formation. In contrast, the Δ*fliC* mutant exhibited the flagellar hook but was missing the flagellar filament confirming previous data obtained for other bacteria (Macnab, 2003). These results demonstrated that the flagellar filament of HP15 is encoded by a single flagellin gene as it was shown, e.g., for *Pseudomonas aeruginosa* (Lillehoj et al., 2002) and therefore confirmed the annotation of corresponding genes (Gärdes et al., 2010b).

4.4 Chemotaxis of *Marinobacter* sp. HP15 and its importance in diatom-bacteria interactions

Based on results of previous studies (Miller et al., 2004; Seymour et al., 2009, 2010; Willey and Waterbury, 1989) and due HP15's attraction to diatom homogenates in sea water soft agar, it was hypothesized that chemotaxis may play an important role for in the interaction with the diatom. By nucleotide sequence analysis, three distinct chemotaxis gene clusters were identified in comparison to *Pseudomonas aeruginosa* PAO1 (Kaeppel et al., c). Mutants in the genes *cheA*, *cheB*, *chpA*, and *chpB*, corresponding to the histidine kinase and the methyltransferase in flagella chemotaxis and twitching motility, were generated and tested for their phenotype to verify the *in silico* findings. In the interaction with *T. weissflogii*, the chemotaxis deficient mutant, $\Delta cheA$, was tested in comparison to the wild-type. A decreased ability in attaching of this mutant to the diatom was observed. This result underlined the importance of bacterial chemotaxis in diatom-bacteria interactions on the basis of the herein described model system.

Even though proposed for a long time, only few studies have reported that specific bacterial strains are attracted by phytoplankton organisms and their exudates (Miller et al., 2004; Seymour et al., 2009, 2010; Bell and Mitchell, 1972; Willey and Waterbury, 1989). Modeling and simulation of chemotaxis in nutrient patches predicted that chemotactic bacteria grow 50% faster when gathered in loose clusters within nutrient patches suggesting that foraging strategies enabling bacteria to respond to DOM gradients could be adaptive (Blackburn and Fenchel, 1999; Blackburn, 1998; Blackburn et al., 1997).

The role of chemotaxis for attachment, even though emphasized (Grossart et al., 2007; Jackson, 1989), has been studied in the environment only for *Aeromonas*

spp. and the plant pathogenic bacterium *Agrobacterium tumefaciens* (Kirov et al., 2004; Merritt et al., 2007). For both, the attachment of chemotaxis-deficient mutants to abiotic surfaces were notably compromised. In *Vibrio cholerae*, chemotaxis was proposed to play a stage-specific role in attachment, since a *cheY*-3 mutant was deficient in generating a monolayer but not in biofilm formation (Moorthy and Watnick, 2005). On the other hand, for *E. coli* no effect of chemotaxis on biofilm formation had been observed (Pratt and Kolter, 1998). *P. aeruginosa* has a very complex chemotaxis system, in which one of the chemotaxis gene clusters is exclusively regulating pili movement (Bertrand et al., 2010; Kato et al., 2008). *P. aeruginosa* mutants defective in twitching motility genes were effected in terms of biofilm formation (Caiazza et al., 2007). In summary, these results suggested that the impact of chemotaxis on attachment is highly species-specific. Interestingly, all of the previous tests have been conducted with abiotic surfaces. Herein and for the first time, we describe the impact of chemotaxis on attachment of a single bacterial strain to an environmentally relevant biotic surface.

In conclusion, there is significant knowledge about the molecular mechanisms of chemotaxis in various bacterial species. However, the role of this process for most of the environmentally important microbial processes has hardly been touched. This current study describes the importance of chemotaxis in phytoplankton-bacteria interactions by molecular means for the first time.

Chapter 5

Future scope

Based on our taxonomic work, the close relatedness of HP15 to *Marinobacter algicola* is of special interest to our study. Strains of the species *M. algicola* were isolated from cultures of the toxic dinoflagellates *Gymnodinium catenatum* and *Alexandrium tamarense* (Green et al., 2006), which draws an interesting analogy to our own model system. *M. algicola* represents a unique marine bacterium, which has the ability to produce and use the siderophore vibrioferrin (VF) in contrast to numerous closely related organisms (Amin et al., 2007). The structure of vibrioferrin originally isolated from *Vibrio parahaemolyticus* (Yamamoto et al., 1994) was elucidated and its chemical properties were described (Amin et al., 2009b). Further, the biosynthetic genes were identified (Tanabe et al., 2003) and this siderophore was shown to be produced and utilized by several *Marinobacter algicola* strains isolated from dinoflagellate cultures (Amin et al., 2009a). This functional conservation has been hypothesized as an indication for specific selective processes operating between bacteria and dinoflagellates. In algae, iron is essential for photosynthetic carbon fixation. Fixed carbon subsequently fuels growth and reproduction of the associated bacteria such as *M. algicola*. In bacteria-algae mutualism, iron scavenged by vibrioferrin production may in turn be provided to the

algae. These aspects may have far reaching implications for the biogeochemical cycling of iron and carbon, and the overall influence of phytoplankton and bacteria on each others' evolution. As for other photosynthetic organisms, iron is essential for the growth of the diatom *T. weissflogii* (Anderson and Morel, 1982; Harrison and Morel, 1986). Knowing that *T. weissflogii* promotes growth of HP15 and vice versa (Gärdes et al., 2010a; Gärdes, 2010), siderophore production might be one of the key factors in this interaction, too. In order to substantiate or preclude this option, it would be an interesting future project to test siderophore production in *Marinobacter* sp. HP15. So far, sequence comparisons of the biosynthetic genes of vibrioferrin, the specific siderophore proposed to be relevant for the interaction between *M. algicola* and dinoflaggelates, could not reveal any homologs in HP15 (unpublished data). However, siderophore production can easily be observed on CAS agar (Schwyn and Neilands, 1987). Further, siderophore analysis is elaborative, but contact to scientists with experience in *Marinobacter* siderophores has been established (Martinez and Butler, 2007; Homann et al., 2009; Butler, 1998; Challis, 2008). Siderophore-deficient mutants would then be tested regarding their effects on diatom growth, formation of TEP, and aggregation during the interaction.

Having the genome sequence of *Marinobacter* sp. HP15 on hand, a wide range of bioinformatics analyses has become possible. Of specific interest might be a future flux-balance analysis (FBA) (Thiele and Palsson, 2010; Edwards and Palsson, 2000). By simulation of the HP15 metabolic activities based on genetic information and in combination with experimentally measured flux data, more insights could be obtained on the metabolites essential for the interaction with the diatom. With the genome sequence data of *T. weissfloggi*, prediction from a combined FBA (Bordbar et al., 2010) of HP15 and the diatom would reveal the synergistic interface between those two organisms, as it was recently shown for interactions

between *E. coli* mutants (Wintermute and Silver, 2010). Unfortunately, the sequence data of this diatom is not available yet. Therefore, it would be relevant to study HP15 in the interactions with *T. pseudonana*, for which sequence data is published (Armbrust et al., 2004).

Having a genetically accessible bacterium on hand, mutational analysis of any gene of interest is possible and will facilitate expression studies. Thus, *Marinobacter* sp. HP15 can be observed intensively under various conditions with and without the diatom. Expression studies on polysaccharide, secondary metabolites, or exo-enzymes production might be important for the understanding of the interaction. On the background of the global climate change, the effect of temperature and pH changes on the interaction might be of particular interest.

Based on results of the genetic accessibility tests, plasmids carrying the origin of replication *rep* seem to replicate in HP15. Nucleotide sequence analysis of the HP15 indigenous plasmids revealed sequence homologies to an existing cloning vector carrying the repB origin of replication, pUCP18 (Schweizer, 1991). pUCP18 combines the properties of the widely used cloning vector pUC, such as small size, high copy number, and a versatile MCS (Vieira and Messing, 1982) with the ability to replicate in organisms other than *E. coli*, such as *Pseudomonas aeruginosa*. Therefore, pUCP18 might be a further useful vector for gene cloning for HP15, since the currently used plasmid pBBR1MCS is replicated only at a mid copy number.

For a better understanding of chemotaxis in *Marinobacter* sp. HP15, the genetic information of methyl-accepting chemotaxis proteins should be analyzed *in silico*, *in vivo* and *in vitro*. Besides the biochemical identification of those compounds in the diatom homogenate (Paul et al., 2009), to which HP15 is attracted to, this information will help to elucidate the chemotactic behavior of HP15 towards the diatom in more detail and might reveal algae products, which can be

utilized by HP15.

Testing other *Marinobacter* species, e.g., *M. algicola* and *M. aquaeolei*, as well as other genera, e.g., *Pseudomonas*, *Escherichia*, or *Vibrio*, will help to reveal the species specificity of the interaction. In this context, organisms with a known genome sequence such as *M. algicola* and *M. aquaeolei* will be of great interest, since they allow genomic comparison to HP15 to support experimental findings. The same approach would be important on the diatom's side. As mentioned above, model diatoms such as *Thalassiosira pseudonana*, *T. rotula*, or *Skeletonema marinoi* should be tested in the interaction to reveal if HP15 generally induces aggregation or if it is specific for *T. weissflogii*.

All these future sub-projects combined would lead to a comprehensive analysis of bacteria-diatom interactions on the basis of the herein proposed bilateral model system. Knowledge on the important environmental factors as well as on the essential gene products will allow to specify the influence of bacteria-diatom interactions on nutrient flux during marine snow formation.

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I hereby confirm that the thesis presented here has not been submitted at another university for the conferral of a degree, and was written by me, Eva Käppel, using only the cited sources.

Bremen, January 26, 2011