

Bacterial Influence on Diatoms from Photoautotrophic Freshwater Biofilms

Dissertation

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Einfluss von Bakterien auf Diatomeen aus photoautotrophen Süßwasser-biofilmen

Photoautotrophe Biofilme sind typische benthische Lebensformen verschiedener Mikroorganismen wie Bakterien, Mikroalgen und Pilzen in der Litoralzone von Meeren, Seen und Flüssen. Dieser grün-braune, schleimige Aufwuchs auf benetzten Oberflächen ist hochproduktiv und bildet eine wichtige Grundlage der aquatischen Nahrungskette. Auf der anderen Seite verursacht sogenanntes Biofouling jährlich hohe Kosten, beispielsweise in der Schifffahrt, indem es den Reibungswiderstand und damit zusammenhängenden Kraftstoffverbrauch erhöht und zu erheblichen Materialkorrosionen führt. Ein fundiertes Wissen über Mechanismen, die die Entwicklung solcher Biofilme beeinflussen, kann daher zu ihrer Kontrolle sehr nützlich sein. Diatomeen (Kieselalgen) sind, bei ausreichendem Licht, zusammen mit Bakterien Pioniere in der Besiedlung benetzter Oberflächen und wesentlicher Bestandteil des ausgereiften Biofilms. In der vorliegenden Arbeit wurden Interaktionen zwischen Diatomeen- und Bakterienisolaten aus photoautotrophen, epilithischen Biofilmen des Bodensees untersucht, die einen wesentlichen Einfluss auf die Entwicklung solcher Biofilme haben können. Es wurde ein Protokoll entwickelt, das die Gewinnung von Diatomeenreinkulturen (axenische Kulturen) aus Mischkulturen mit Bakterien (xenische Kulturen) ermöglicht. Langzeitbeobachtungen von axenischen und xenischen Kulturen zeigten, dass Bakterien einen wesentlichen Einfluss auf den Formwechsel der Algen haben können, der im Verlauf des Entwicklungszyklus vieler benthischer Diatomeen stattfindet. Zwei der drei untersuchten Diatomeenarten wiesen dabei in Reinkultur deutlich kleinere Zellgrößen sowie Anomalien der Schalen auf, während die xenischen Kulturen weitestgehend unverändert blieben, bzw. verhältnismäßig geringere Zellverkleinerungen aufwiesen. Es wurden zwei Modellsysteme entwickelt, mit deren Hilfe die zellulären Mechanismen der Interaktionen zwischen Diatomeen und Bakterien untersucht werden können, die das Kulturwachstum und die Biofilmbildung der Algen deutlich beeinflussen. Es konnte gezeigt werden, dass Zellen der Diatomee *Achnanthydium minutissimum* in Anwesenheit von Bakterien Oberflächenadhärenz aufweisen und Biofilme bilden, wohingegen Zellen der axenischen Kultur planktonisch sind. Die xenische Kultur bildet vermehrt zellgebundene, extrazelluläre Substanzen in Form von Kapseln, während in der axenischen Kultur überwiegend gelöste extrazelluläre Substanzen gemessen wurden. Kapsel- und Biofilmbildung konnten in der axenischen Kultur durch ein *Bacteroidetes*-Bakterium induziert werden und weitere Untersuchungen zeigten, dass die Interaktion zwischen *A. minutissimum* und dem Bakterium durch gelöste bakterielle Substanzen vermittelt werden. Dasselbe Bakterium zeigte in einem zweiten Modellsystem einen wachstumsstimulierenden Einfluss auf die Diatomee *Fragilaria brevistriata*. Diese Diatomee bildet in Ko-Kultur mit dem Bakterium lange Zellketten bis hin zu makroskopisch sichtbaren Kolonien. Mit Hilfe einer Transposonmutagenese und Identifizierung von bakteriellen Mutanten, die keinen Einfluss mehr auf das Wachstum von *F. brevistriata* aufwiesen, konnte gezeigt werden, dass zwei Gene, deren Produkte homolog zu Transkriptionsregulatoren der MarR Familie bzw. zu Calcium-ATPasen anderer Bakterien sind, an dem wachstumssteigernden Effekt des Bakteriums beteiligt sind. Die Ergebnisse dieser Arbeit zeigen, dass Bakterien einen starken Einfluss auf den Formwechsel, das Kulturwachstum und die Bildung von extrazellulären Substanzen von Diatomeen haben können, Eigenschaften, die die Biofilmbildung wesentlich beeinflussen können. Diese Arbeit trägt zu einem tieferen Verständnis der chemischen und genetischen Grundlagen der Interaktionen zwischen Diatomeen und Bakterien bei.

Bacterial influence on diatoms from photoautotrophic freshwater biofilms

Photoautotrophic biofilms are characteristic for the littoral zones of seas, lakes and rivers. These green-brownish, mucous layers on submerged surfaces like stones or macroalgae are a niche of high primary production and play an important role in the aquatic food web. Biofilm formation also causes biofouling and hence immense costs each year for example in shipping and water resource management. Intensive investigations are therefore necessary to understand the complex mechanisms which lead to the development of this particular mode of living. Diatoms and bacteria are early colonizers of wet and illuminated surfaces and dominant members of mature photoautotrophic biofilms. In this thesis, interactions between diatom and bacterial isolates from photoautotrophic, epilithic biofilms from Lake Constance were investigated, that may considerably influence the formation of the whole biofilm. A protocol was established to purify xenic diatom strains from associated satellite bacteria, resulting in axenic diatom cultures. Long-term observations of diatom cultures revealed that bacteria may impact cell size and frustule morphology, features which commonly change during the life cycle of many benthic diatoms. Cell sizes of two of three investigated diatom species were strongly reduced and the frustules exhibited morphological aberrations when cultivated under axenic conditions, whereas the xenic diatoms were well preserved. Two model systems were designed that enable the investigation of cellular mechanisms of diatom/bacteria interactions, which influence biofilm formation and diatom growth. It was shown that the diatom *Achnanthydium minutissimum* was strongly attached to surfaces and formed a biofilm only in the presence of co-isolated bacteria whereas the axenic diatom culture remained planktonic. The xenic culture produced high amounts of bound extracellular polymeric substances (EPS) and formed cellular capsules. In contrast, the axenic diatom culture produced predominantly soluble extracellular carbohydrates and capsules were absent. Capsulation and biofilm formation could be re-induced in the axenic diatom culture by a *Bacteroidetes* bacterium. Further investigations demonstrated that the interaction of *A. minutissimum* with the bacterium was mediated by soluble hydrophobic molecules produced by the bacterium. The same *Bacteroidetes* bacterium exhibited a growth-promoting impact in another model system using the diatom *Fragilaria brevistriata*. The diatom formed long cell chains up to macroscopic visible colonies when co-cultivated with the bacterium. Transposon mutagenesis and identification of bacterial mutants that lost growth-promotion revealed that two genes whose products are homologous to transcriptional regulators of the MarR family and to a calcium ATPase, respectively, are involved in the growth-stimulating potential of the *Bacteroidetes* bacterium. In this study it was shown that bacteria may have strong impacts on different physiological features of diatoms like morphological changes during the algal life cycle, culture growth and formation of EPS, features which may considerably influence the whole biofilm. With the designed bioassays convenient tools are now available which proved to be useful to study the cellular mechanisms of interactions between benthic diatoms and bacteria. This thesis contributes to a deeper understanding of the complex chemical and genetic basics of diatom/bacteria interactions.

Chapter 1

General Introduction

General Introduction

1.1 Biofilms – A highly complex entity and favourable way of life

Biofilms are an accumulation of microorganisms on surfaces, enclosed in a matrix of extracellular polymeric substances (EPS). In nature they are complex communities of mainly bacteria, algae, protozoa and fungi which frequently coexist. It is thought that the majority of microbes are able to form biofilms (Kolter and Greenberg 2006) and it is therefore not surprising that Antony van Leeuwenhoek's "animalcules", which are assumed to be the first description of bacteria, are typical biofilm organisms of a dental plaque (Dobell 1932). Living in a biofilm is a very ancient mode of life, fossils can be dated back to about 3.2 billion years ago (Hall-Stoodley et al. 2004). Biofilms occur nearly everywhere, wherever enough water is available. They can even exist under extreme physical and chemical conditions like in hot springs (Kulp et al. 2008) and it was shown that just such extreme conditions, outside of the individual physiological optimum, may initiate microbes to form such films (LaPaglia and Hartzell 1997; Koerdt et al. 2010). Besides their extensive relevance in the medical field (e.g. as dental plaque (Marsh and Bradshaw 1995), in wound infections (Scali and Kunimoto 2012), in chronic diseases (Costerton et al. 1999), due to their capability for colonization of medical devices (Khardori and Yassien 1995), as well as in the symbiotic microflora of, for example, the intestine (Macfarlane and Dillon 2007)), biofilms gained special interest in both a negative and a positive sense. Biofouling causes significant problems in water resource management, shipping and architecture as biofilms may be a potential contamination source with human pathogens, increase flow resistance of ship hulls and lead to deterioration of human made surfaces (Gaylarde and Morton 1999; Schultz et al. 2011; Wingender and Flemming 2011) (Figure 1.1). However, biofilms also have a huge ecological importance. Photoautotrophic biofilms are an area of high primary production, especially in shallow water bodies with large littoral zones (Wetzel 1964) and may contribute a significant portion to the total amount of carbon fixation. Thus, they play an important role as carbon and energy source for upper trophic levels in aquatic systems. Further, biofilms are an essential component for facilitating sediment stabilisation (Dade et al. 1990; Wigglesworth-Cooksey et al. 2001). Aggregates, like in lake and sea snow, are a main component in the vertical flux of organic and inorganic substances in the water column (Fowler and Knauer 1986) Today, photoautotrophic biofilms have potential for several commercial applications, for example in wastewater treatment, removal of heavy metals, oil degradation, alternative fuel production, and in agri- and aquaculture (Roeselers et al. 2008).

What makes a biofilm such a successful mode of life? A biofilm is not a random mixed bunch of organisms, but a highly complex entity, which has been compared to living in a multicultural city (Watnick and Kolter 2000). The biofilm architecture is often not simply planar but rather formed in microcolonies like for example the mushroom-like structures of *Pseudomonas aeruginosa*, and is intersected with pores and channels to facilitate the exchange of substances with the environment (Stoodley and Lewandowski 1994; Stoodley et al. 2002; Kolter and Greenberg 2006). The structure of the mature biofilm is heterogeneous, with different cell types organized in microcolonies or -niches (Stoodley et al. 2002) to perform different tasks. Gradients of light, oxygen, pH and substrates within a biofilm reinforce zonation and niche formation (Okabe et al. 1999; Vroom et al. 1999; Roeselers et al. 2008). Thus, the organisms are not randomly distributed within the biofilm, but rather are arranged in zones with their individual optimal conditions concerning biotic and abiotic factors. Even in single-species biofilms, niche formation may occur, strongly fortified by gradient formation, which consequently may lead to locally well-adapted phenotypes or even different mutants (Kolter and Greenberg 2006). Consequently, the single organism may benefit from the community life through teamwork and division of labour of the plurality of different pheno- or genotypes. This is even more distinct in interspecies communities as shown for nitrifying bacteria where ammonia-oxidizing bacteria and nitrite-oxidizing bacteria live together in the same biofilm in a mutualistic community (Okabe et al. 1999). Even obligate anaerobes are able to live in an aerated environment in consortia with oxygen consuming bacteria for example in oral dental plaques (Bradshaw et al. 1997). It is proposed that biofilms accelerate gene transfer and enable fast adaptation to changes in the environment (Watnick and Kolter 2000; Fux et al. 2005). Of special importance is the biofilm matrix: The main content of the matrix is water (97% in a *Sphingomonas* biofilm (Zhang et al. 1998)). The water retention capacity of the matrix protects the cells from desiccation, which is mainly important in zones with changing water levels for example in the intertidal or littoral zone (Flemming and Wingender 2010). Extracellular polymeric substances (EPS) are, besides water, the major component of the biofilm matrix (for a description of EPS see below). The EPS accomplish surface adhesion, thus initial steps of biofilm formation go hand in hand with EPS production: Studies with *P. aeruginosa* revealed that expression of *algC*, a gene encoding for a key enzyme for the synthesis of the EPS component alginate, was up-regulated in biofilms when compared to planktonic cells and gene expression started when cells were attached to a surface (Davies et al. 1993; Davies and Geesey 1995). The initial reversible binding and loosely attachment of a cell to a surface is strengthening by extracellular polymer

production and is converted into irreversible binding (Stoodley et al. 2002). Organisms living in a biofilm are considerably more resistant to antimicrobial substances than their planktonic counterparts (Ceri et al. 1999) and three possible explanations have been discussed (Hall-Stoodley et al. 2004): The matrix itself may protect the cells from toxic substances; dormant areas in the biofilm, harbouring cells with reduced metabolic activity, are less affected and can be revived once the intoxication is over; and finally the biofilm may contain “persisters”, subpopulations which already exhibit resistant phenotypes. The matrix may serve as a pantry for nutrient storage and thus makes the biofilm inhabitants much more independent from environmental changes in nutrient availability. Several different extracellular enzymes for the degradation of proteins, polysaccharides and lipids were found in biofilms, indicating that the matrix polymers themselves are decomposed to consumable forms and serve as carbon source for the inhabitants (Flemming and Wingender 2010). The EPS matrix may protect the cells from ultraviolet radiation (Ehling-Schulz et al. 1997), which is of great importance for biofilm inhabitants in shallow water areas. Furthermore, it may protect the cells from grazing, either as aggregation alters particle size and thus incorporation by a predator, or the matrix material protects the cells from digestive enzymes to that extent, that some organisms can pass the predators gut and are still viable (Decho 1990). All in all, living in a biofilm has a number of advantages which highly benefits the inhabitants compared to their planktonic counterparts.



Figure 1.1: Formation of photoautotrophic biofilms on various human made surfaces. (The picture was taken in January 2014 by M. Windler).

1.2 EPS – Structure-providing and sticky part of the biofilm matrix with protective functions

Extracellular polymeric substances (EPS) are produced by the biofilm inhabitants themselves and are a feature of bacteria, algae and fungi, among which algae as primary producers may be considered to play a special role. The structure and composition of EPS can be as diverse as their producers and a general statement is hardly possible. Polysaccharides are an important component of EPS (Sutherland 2001; Flemming and Wingender 2010). Exopolysaccharides are both, homo- and heteropolysaccharides. One of the best investigated exogenous heteropolysaccharides is alginate from *P. aeruginosa*. It is a high molecular weight polymer which consists of β -1,4-linked D-mannuronic acid and of its 5-epimer L-guluronic acid (Evans and Linker 1973). Monosaccharide composition of diatom EPS is reported in numerous studies and hexoses like glucose, galactose, mannose, fucose, rhamnose and pentoses like arabinose, xylose and ribose were frequently found, depending on the investigated species (Hoagland et al. 1993; Wustman et al. 1997; Staats et al. 1999; De Brouwer and Stal 2002; Chiovitti et al. 2003; Bellinger et al. 2005; Bahulikar and Kroth 2007; Bahulikar and Kroth 2008). N-acetylglucosamine was also detected (Hoagland et al. 1993; Bahulikar and Kroth 2008) and uronic acids and sulfate residues were frequently found in EPS of diatoms (Hoagland et al. 1993; Staats et al. 1999; Chiovitti et al. 2003; Underwood et al. 2004; Bellinger et al. 2005). A further major group of matrix exopolymers are proteins (Hoagland et al. 1993; Chiovitti et al. 2003), for example polymer-degrading exoenzymes, making the matrix a highly metabolic environment. Further components are lipids and nucleic acids (Flemming and Wingender 2010). Polysaccharides, proteins, and their physical and chemical interactions are considerably involved in the determination of the 3D structure of the biofilm (Flemming and Wingender 2010). Ions, for example Ca^{2+} , may interact with anionic residues such as uronic acids and provide connection of the macromolecules to increase the structural and mechanical stability of the biofilm (Flemming and Wingender 2010).

The exopolymers can be divided into soluble and bound EPS. The soluble portion is directly released into the surrounding environment, whereas bound EPS remain attached to the cell surface. The last named are formed by diatoms in well defined structures known as stalks, tubes, fibrils, pads and capsules (Hoagland et al. 1993; Bahulikar and Kroth 2008). Another form of diatom EPS are the so called transparent exopolymer particles (TEP), filterable, individual EPS particles (Passow 2002).

Besides the above described functions as matrix components, EPS have additional functions. EPS secretion is involved in diatom locomotion (Edgar and Pickett-Heaps 1983)

and it is thought that EPS production in algae may serve as an overflow mechanism when nutrients are limited (Staats et al. 2000). In benthic diatoms, EPS production is often closely associated with sexual reproduction as mating cells are encased by a “jelly” (Geitler 1932).

1.3 Diatoms – A huge and diverse phylum of considerable ecological relevance

Diatoms (Bacillariophyceae) are unicellular algae belonging to the phylum of the Heterokontophyta. They represent a huge and diverse class comprising of an estimated number of at least 200 000 species (Mann and Droop 1996). Taxonomically, they are divided into two orders: the radially symmetrical Centrales and the bilaterally symmetrical Pennales. Diatoms occur nearly ubiquitously in marine and freshwater habitats, where centric diatoms are mainly located in the pelagic zone and pennate are mostly found attached to surfaces in the littoral zone. Accordingly, Bahulikar (2006) attributed more than 99% of the identified diatom community of epilithic biofilms of Lake Constance to the Pennales. The conspicuous feature of diatoms is their silicified cell wall, the so called diatom frustule, which is mainly composed of silica (SiO_2) (Kröger and Poulsen 2008). Diatoms are traditionally classified according to the morphological characteristics and fine structures of their frustules which are species-specific. The frustules are composed of two thecae (epi- and hypotheca), which are unequal in size and overlap partly like a petri dish (Figure 1.2). A consequence of the rigid frustule is the characteristic morphological change within the life cycle of many diatoms: During a mitotic cell division within the vegetative phase each daughter cell inherits one theca of the mother cell, which becomes the new epitheca of the daughter cell. The hypotheca is newly formed by each daughter cell and, as a consequence, one daughter cell is smaller. This leads to a gradual decrease of the cell size during the vegetative phase, known as the MacDonald-Pfitzer rule (MacDonald 1869; Pfitzer 1871; Chepurnov et al. 2004). The initial cell size is restored by sexual reproduction once the cells have reached a minimum size threshold. Such small cells become sexually potent and with the appropriate external trigger determined for example by light, temperature, osmotic and nutrient conditions, sexual reproduction is induced (Drebes 1977; Chepurnov et al. 2004). After gamete fusion, a specialized cell, the so called auxospore is formed, which expands to the original size of the mother cell and the life cycle is completed. Storage materials of diatoms are chrysolaminarin, a β -1,3- and β -1,6-linked glucose polymer, lipids and polyphosphate (Beattie et al. 1961; Round et al. 1990). Carotenoids like fucoxanthin mask the chlorophyll pigments and lead to the characteristic brownish colour of the diatoms (Round et al. 1990).

The ecological relevance of the diatoms becomes obvious when considering that nearly half of the globally primary production is allocated to the oceans, conducted mainly by phytoplankton, among which diatoms are a major group (Field et al. 1998; Falciatore and Bowler 2002). It is estimated that they are responsible for 40-45% of the oceans primary production (Mann 1999) and are further key players in the biogeochemical cycle of silica as they incorporate Si into their cell wall (Treguer et al. 1995).

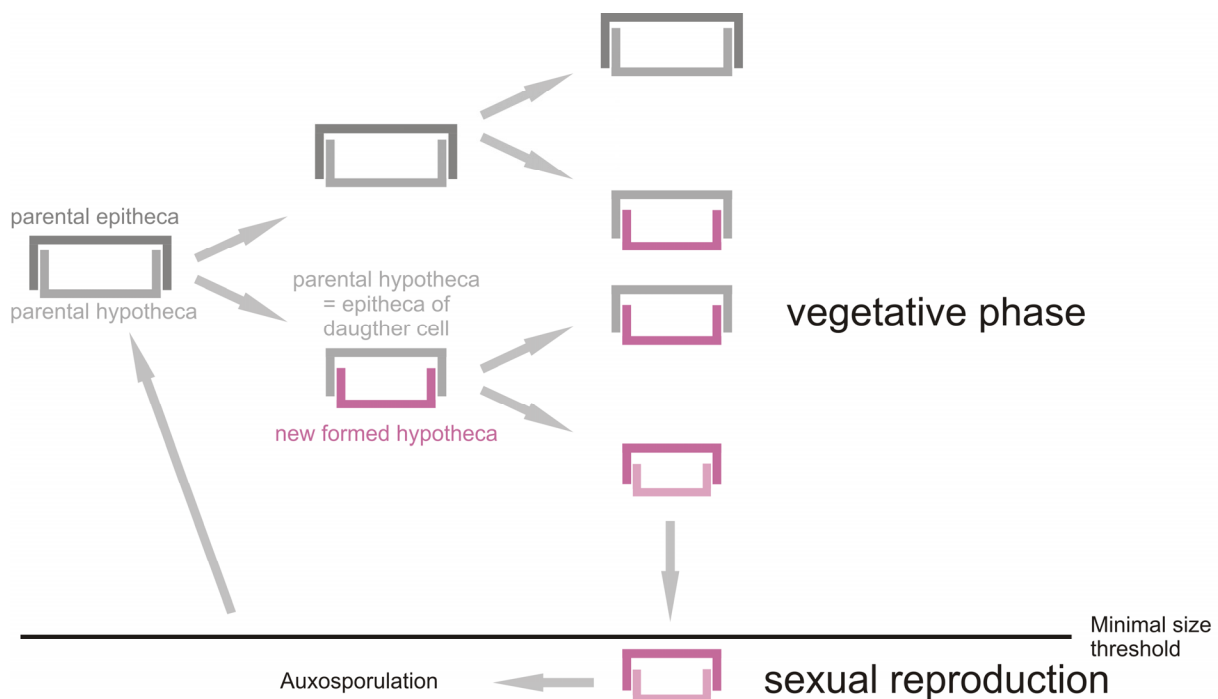


Figure 1.2: Scheme of the typical cell size changes during the diatom life cycle. The diatom frustule consists of a larger epitheca, which covers the smaller hypotheca like the lid of a petri dish. After cell division, each daughter cell inherits one parental theca, which becomes the new epitheca and the hypotheca has to be developed. As a consequence, one daughter cell (which inherits the parental epitheca) retains the same frustule size as the mother cell whereas the other (which inherits the parental hypotheca) is getting smaller. With further cell divisions, the overall cell size of the culture gradually decreases. Cells, which have reached a minimum cell size, become sexually potent and with the appropriate external triggers, the cells reproduce sexually and develop an auxospore. Thecae which retain the same size as those of the initial mother cell, are coloured in grey shades and newly formed, smaller hypothecae are coloured in red shades.

1.4 Interactions between diatoms and bacteria

Interactions of organisms in ecosystems are manifold and can have positive or negative effects for each of the interaction partners. According to that, interactions are divided in mutualism, predation or parasitism, competition, commensalism, amensalism and neutralism (Table 1.1).

Table 1.1: Types of interactions classified based on their influence on each of the interaction partners. Interactions can be divided in mutualism (positive effects for both interaction partners), predation or parasitism (positive effect for one partner and negative effect for the other), competition (negative effects for both partners), commensalism (one partner benefits and the other is not affected), amensalism (one partner has a disadvantage and the other is not affected) and neutralism (both partners are not affected). +, positive, -, negative and 0, no influence on the interaction partner; modified after the lecture script “Aquatische Ökologie” (Prof. Dr. Rothaupt, Limnological Institute, University of Konstanz).

	+	-	0
+	Mutualism		
-	Predation/ Parasitism	Competition	
0	Commensalism	Amensalism	Neutralism

Diatoms in their natural habitats are generally closely associated with bacteria and stable interactions have evolved between them. The close association of diatoms and bacteria is reflected by the diatom genome as several genes are of bacterial origin, received by horizontal gene transfer. Thus, the nuclear genome of the diatom *Phaeodactylum tricoratum* exhibits about 784 genes that are mostly related to bacterial genes and represent 7.5% of the total gene number of *P. tricoratum* (Bowler et al. 2008). The most obvious interaction takes place on the trophic level. Algae as primary producers provide organic substrates; organic matter from dead algae cells and extracellular organic compounds released by vital algae serve as energy and carbon source for heterotrophic bacteria (Cole 1982). Diatoms produce copious amounts of extracellular substances, thus the release of extracellular carbon was estimated to account for more than 50% of the total carbon production in *Chaetoceros affinis* (Myklestad et al. 1989). Growth of different bacteria is much more evident in co-culture with algae (Bell and Mitchell 1972; Grossart et al. 2006). Gärdes et al. (2010) showed that growth of four bacterial isolates was stimulated by photosynthetically active diatoms, suggesting that some bacteria may utilize extracellular algae products rather than dead algae cells. Already in the seventies, Bell and Mitchell (1972) introduced the “phycosphere” concept in conformity to the rhizosphere of the land plants, and described thereby the zone around the algal cell “in which bacterial growth is stimulated by extracellular products of the alga” and showed, that algal products can attract bacteria via chemotaxis. Goto et al. (2001) showed that more than 50% of the extracellular carbon produced by a benthic assemblage and two diatom species, respectively, was mineralized within 24 hrs by a bacterial community. Further, Larsson and Hagström (1982) estimated, that 50% of the bacterial energy requirement is satisfied by extracellular substances of phytoplankton. Bacteria are also known to influence diatom growth either in a positive (Ukeles and Bishop 1975; Riquelm et al. 1988; Fukami et al. 1991; Bruckner et al. 2008; Hünken et al. 2008; Bruckner et al. 2011) or negative way (Fukami et al.

1991). Auxotrophic diatoms may require essential substances like vitamins from the bacteria. Croft et al. (2005) surveyed 326 different algal species and found vitamin B₁₂ auxotrophy in 171 species and bacteria might supply different diatom species with this vitamin (Haines and Guillard 1974). It was shown that epiphytic bacteria of the diatom *Amphiprora kufferathii* enhance growth of the diatom, most likely by decreasing the reactive oxygen species (ROS) hydrogen peroxide in the xenic culture (Hünken et al. 2008) and metal stress tolerance for copper and tributyltin fluoride of the diatom *Amphora coffeaeformis* was increased by bacteria (Thomas and Robinson 1987). Algicidal activity of the filtrate of the bacterium *Kordia algicida* was shown for three different planktonic diatom strains and exhibited protease activity (Paul and Pohnert 2011), but also the opposite case was reported in which the polyunsaturated fatty acid eicosapentaenoic acid (EPA) acted antibacterial against Gram-positive and Gram-negative bacteria (Desbois et al. 2009). Algae and bacteria may compete for limited nutrients like nitrogen or phosphate (Cole 1982). Further, eight benthic diatoms were shown to be capable of utilization of organic substrates, especially in the dark (Tuchman et al. 2006) making competition with bacteria for such compounds also conceivable. However, their study showed that they apparently used different organic substrata, possibly to avoid strong competition with the bacteria. Further, bacteria may stimulate secondary metabolite production, for example the production of the neurotoxin domoic acid of *Pseudo-nitzschia multiseries* (Bates et al. 1995; Kobayashi et al. 2009b) and may influence sexual reproduction as shown for the diatom *Coscinodiscus wailesii* (Nagai et al. 1994; Nagai and Imai 1998; Nagai et al. 1999). The diatom/bacteria interactions might also directly be of particular importance for biofilm formation. Bacteria are known to influence diatom aggregation (Grossart et al. 2006; Gärdes et al. 2010) which possibly depends on the consumability of EPS (Grossart et al. 2006). Surface attachment and initial steps of biofilm formation of the diatom *Achnanthes longipes* were shown to be affected by bacteria (Gawne et al. 1998) and the diatom *Cymbella microcephala* showed capsules of bound EPS only in the presence of a satellite bacterium or its sterile spent medium while the axenic culture did not exhibit cell bound structures (Bruckner et al. 2008).

The elaborate interplays require sense- and regulatory mechanisms, especially when the organisms have to undergo phenotypic changes to facilitate interaction with each other. In a first step, the interaction partners have to recognize their counterparts. Beside direct cell-to-cell contact (Kobayashi et al. 2009b), this may be accomplished via signalling molecules or chemical cues. Already in the eighties it was shown that soluble molecules of a xenic diatom culture, passing a 0.2 µm pore size filter, changed the lethal doses of toxic metals for a diatom

(Thomas and Robinson 1987), indicating, that soluble molecules are involved in the diatom/bacteria interaction. Relatively little is known about such interkingdom signalling of diatoms and bacteria in aquatic systems. One proposed scenario is that the interaction partners utilize the intraspecific signalling system of their counterpart (Amin et al. 2012). Quorum sensing, for example, is a well known cell density depended, intraspecific communication mechanism of bacteria (Chhabra et al. 2005). Quorum sensing is mediated by autoinducers (AI), molecules which are released by bacterial cells. With increasing cell density, the concentration of AI increases and once it reaches a certain threshold, it induces an intracellular signal cascade and phenotypic changes simultaneously in the whole population. Such an exploitation of intraspecific bacterial signalling via AI was shown for the green seaweed *Ulva*, whose zoospores were attracted by *N*-acylhomoserine lactones (AHLs) (Joint et al. 2007), common AI of Gram-negative bacteria (Chhabra et al. 2005). The red algae *Delisea pulchra* produces halogenated furanones which possess antifouling properties (De Nys et al. 1995) and Hentzer et al. (2002) demonstrated, that synthetically modified furanones, interfered with the AHL-mediated quorum sensing system of *P. aeruginosa* with consequences for the biofilm architecture, indicating an eukaryotic strategy to suppress undesired bacteria. Also the opposite case, where bacteria utilize eukaryotic intraspecific signalling molecules like hormones are known (Hughes and Sperandio 2008) and might also facilitate communication with diatoms. Such extracellular messengers of diatoms might be pheromones, a quite range of C8 and C11 hydrocarbons as they are found in different diatom species (Amin et al. 2012). The polyunsaturated aldehyde (PUA) decadienal DD was shown to increase intracellular levels of NO and Ca^{2+} , which might possibly act as intracellular second messengers in diatoms (Vardi et al. 2006; Leflaive and Ten-Hage 2011) and regulates several mechanisms for example diatom aggregation and biofilm formation (Leflaive and Ten-Hage 2011). Such extracellular, intraspecific molecules of the diatoms might be putative candidates for interkingdom signalling with bacteria (Amin et al. 2012). Growth-stimulating or -reducing properties of diatom PUAs on several different bacterial strains already have been shown (Ribalet et al. 2008), emphasizing a possible dual role of intraspecific signalling molecules in interkingdom interactions.

The extracellular signal has to be transduced in an intracellular response. A well investigated bacterial system of sensing extracellular chemicals which leads to a changed behaviour of cells is chemotaxis, in which bacterial cells sense a chemical gradient and change the rotation of their flagellum to move toward or away from the source of the chemical (Bren and Eisenbach 2000). The intracellular mechanisms of how biofilm organisms

recognize convenient conditions which lead them to give up their planktonic life and to colonize a surface are even in bacteria not fully explained. It is assumed that the small molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is involved as planktonic cells exhibit a low intracellular concentration of this molecule, but the concentration increases when the cells settle down (Kolter and Greenberg 2006).

However the detailed mechanisms of how bacteria and diatoms recognize each other and the intracellular processes which facilitate phenotypic adaptations remained largely unclear and need further research.

1.5 Sampling site and organisms – Diatoms and bacteria of photoautotrophic, epilithic biofilms from Lake Constance

Lake Constance is the third largest lake in Central Europe (<http://www.igkb.org/start/>) and is located at the northern of the Alps, bordered by Germany, Switzerland and Austria. Renaturation measures since the mid seventies, after a period of eutrophication, have resulted in an oligotrophic state of the lake with a current phosphor concentration of $8 \mu\text{g l}^{-1}$ (<http://www.lubw.baden-wuerttemberg.de/servlet/is/3603/>). The substratum of the large littoral zone of the lake is composed of sand and rocks with macrophytes, mainly Potamogetonaceae and Characeae, and expanded reed stands during the summer (<http://www.lubw.baden-wuerttemberg.de/servlet/is/3603/>) (Figure 1.3A). Submersed stones in the littoral zone are covered with a green-brownish mucous layer of biofilms (Figure 1.3B). Such photoautotrophic, epilithic biofilms represent a fascinating small world when observed with a microscope (Figures 1.3C, D and E). Diatoms and bacteria are early colonizers of wet and illuminated surfaces (Cooksey and Wigglesworth-Cooksey 1995; Wetherbee et al. 1998; Sekar et al. 2004) and, besides green algae and cyanobacteria, significant part of mature, photoautotrophic, epilithic biofilms of Lake Constance. Bahulikar (2006) investigated the composition of the diatom and bacterial community and found the diatom genera *Fragilaria*, *Cymbella* and *Achnanthes* as well as bacteria of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, High GC content gram positive (HGC) bacteria and β -*Proteobacteria* to be dominant in these biofilms. The bacterial community coincidences with those from other studies, which demonstrated that *Proteobacteria* and CFB group bacteria are frequently found to be associated with diatoms (Knoll et al. 2001; Schäfer et al. 2002; Sapp et al. 2007b,c; Bruckner et al. 2008).

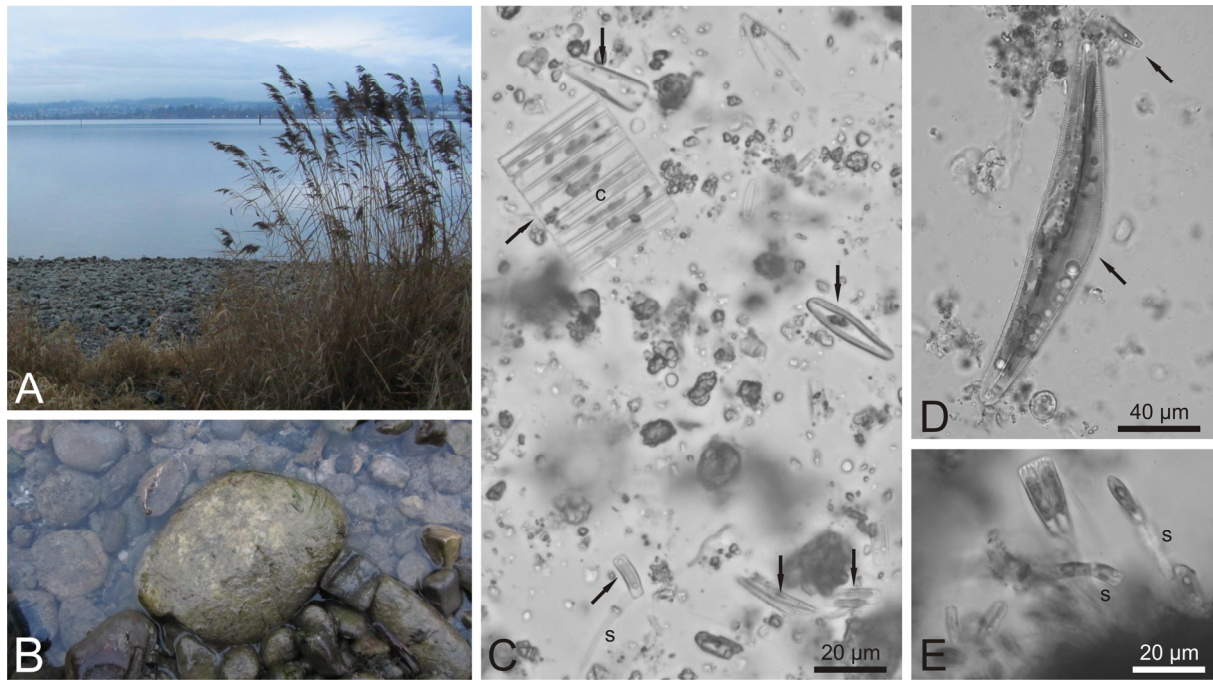


Figure 1.3: Photoautotrophic biofilms of the littoral zone of Lake Constance. A) Typical lakeshore area of the Lake Constance with reed stands and rocky ground. B) Stones are substantial substrata of the littoral zone of the lake. They are covered with green-brownish mucous layers of photoautotrophic biofilms. C) The microscopic observation of such biofilms reveals the frequency of various diatom species (marked with arrows), which are manifold in shape, size and colony formation. Some of them form chains (c) in which the cells are attached to each other by EPS pads. Some form stalks (s), EPS sticks with various lengths, by which cells are attached to the substratum. D) Diatom species can vary remarkably in size and shape; small and large diatom species are marked by arrows. E) Several diatoms attached to a substratum with long stalks (s). (Pictures were taken in January 2014 by M. Windler).

1.6 Objectives of this thesis

The main goal of this study was to investigate interactions between diatoms and bacteria with consequences for the development of photoautotrophic biofilms. For this purpose model systems should be established which would allow to research bacterial influences on the physiology of benthic freshwater diatoms. Once an appropriate diatom/bacterium pair is identified, we intended to investigate the underlying cellular mechanisms which mediate such interkingdom interactions. To accomplish that, transposon tagged bacterial mutants should be generated to characterize the genetic background of the respective diatom/bacteria interaction. We further planned to isolate soluble bacterial molecules which mediate signalling and communication between the organisms. This study aims to contribute to a deeper understanding of the complex diatom/bacteria interactions within photoautotrophic biofilms.

Chapter 2

Purification of benthic diatoms from associated bacteria using the antibiotic imipenem

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Chapter 2: Purification of benthic diatoms from associated bacteria using the antibiotic imipenem

2.1 Abstract

Benthic diatoms and bacteria often co-operatively build up phototrophic, epilithic biofilms. Studying the properties and contributions of the individual partners requires the establishment and maintenance of axenic cultures of the involved organisms. Axenification of biofilm organisms is often challenging, because bacteria as well as diatom cells are embedded in a matrix of extracellular polymeric substances (EPS). Due to this mucilage, the cells stick together and also are less affected by antimicrobial substances. Here we describe a short and feasible protocol for culture axenification, which was successfully applied to cultures of the benthic diatoms *Achnanthydium minutissimum*, *Cymbella affinis* and *Nitzschia palea*. Our protocol includes treatment of the cultures with the antibiotic imipenem and might also be useful for the purification of other cultivated diatom strains. Once axenified, diatom cultures often decay after a certain life span. Our protocol is especially useful to re-establish axenic cultures from co-cultures of diatoms with their accompanying bacteria (also referred to as xenic cultures).

Keywords: diatom · biofilm · benthic · purification · antibiotic · imipenem

2.2 Introduction

Epilithic biofilms consist of a complex community of algae and bacteria, which thrive within a shared matrix of extracellular polymeric substances (EPS) that, together with the cells, makes up the biofilm (Callow and Callow 2006; Flemming and Wingender 2010). Among the eukaryotic algae found in such biofilms, diatoms are particularly abundant (Callow and Callow 2006). To understand the organismic interactions that lead to the formation of biofilms, the generation and maintenance of axenic diatom cultures from environmental samples is a basic prerequisite. However, biofilm-forming benthic diatoms often are difficult to separate from their bacterial partners. Purification via simple application of antibiotics often turned out to be ineffective, because microorganisms in biofilms often are less sensitive to antimicrobial agents compared to their planktonic counterparts (Ceri et al. 1999). A reduced penetration of substances into the cells due to the diffusion barrier of the biofilm matrix could be one possible explanation for this phenomenon (Stewart 1996; Stewart and William Costerton 2001). The EPS formation of diatoms is influenced by the associated bacterial community (Bruckner et al. 2008). This implies that the effectiveness of a purification method depends not only on the diatom species itself, but also on the composition of the associated bacterial community.

A variety of methods for establishing axenic diatom cultures have been reported, applying a diverse range of techniques, like mechanical separation of cells from the surrounding biofilm matrix, filtration, treatment with detergents, substitution of the associated bacteria with cultivated bacteria that are sensitive against antibiotics and subsequent removal of these bacteria (Bruckner and Kroth 2009; Shishlyannikov et al. 2011). All these axenification techniques either start from environmental samples, or from cultures of diatoms that still contain the associated bacteria that have been co-isolated along with the diatoms (so called xenic cultures) (Bruckner and Kroth 2009). Depending on the species, after a certain time of axenic cultivation, some diatom cultures lose their viability. Xenic cultures are not only easier to maintain than axenic cultures, but also more stable during long-term cultivation. It is therefore a good strategy to maintain a xenic culture and to derive axenic sub-cultures as needed for experimental reasons. In this study we present a protocol for the purification of axenic cultures out of xenic cultures which was successfully applied to three different benthic diatom species.

2.3 Materials/Methods and Results

2.3.1 Initial cultivation of diatom strains

Achnantheidium minutissimum (Kützing) Czarnecki isolate B-13, *Cymbella affiniformis* Krammer isolate B-16 and *Nitzschia palea* (Kützing) W. Smith isolate B-01 were isolated in January 2009 from phototrophic, epilithic biofilms taken from the littoral zone of Lake Constance (47° 41' N; 9° 11' E, Germany) with the methods described in Bruckner and Kroth (2009). Biofilms were scraped from stone surfaces and about 200 µl of the material was diluted in 1-1.5 ml Bacillariophycean Medium (BM). This medium (Schlösser 1994) was modified by the following changes: 236 µM Na₂CO₃, 27 µM iron(III) citrate hydrate, 214 µM citric acid, 60 nM MnSO₄, 200 µg l⁻¹ thiamine-HCl and 10 µg l⁻¹ biotin were used. The biofilm suspension was vortexed for 10 min, diluted and 100 µl were streaked on BM agar plates containing 1.5% Bacto™ Agar (Becton, Dickinson and Company, USA). After cultivation at standard conditions for diatoms (16°C, 12:12 light:dark cycle with light intensities of 20-50 µmol m⁻² s⁻¹), the emerging diatom colonies were picked and transferred into liquid medium. The diatom strains were taxonomically identified based on morphological aspects and via 18S rDNA sequence analysis. The diatom isolates were maintained with their naturally occurring bacterial community in xenic cultures.

2.3.2 Strain axenification

Starting from the xenic cultures, we purified the diatoms roughly following the strategies described by Bruckner and Kroth (2009). Our protocol combines mechanical cell separation, removal of loosely attached EPS and treatment with antibiotics as outlined in the protocol box (BOX1-Axenification protocol).

In order to overcome the protective effect of the extracellular carbohydrate matrix we loosened the biofilm structure via sonication (Brown and Bischoff 1962; Kobayashi et al. 2009a) and EPS dissolution (Staats et al. 1999) as first purification steps. 1-3 ml of the xenic diatom cultures were harvested by centrifugation for 5 min at 3000 x g for *A. minutissimum* and *N. palea* and 5000 x g for *C. affiniformis*, respectively, and resuspended in 1 ml BM. Cell suspensions were treated with ultrasound (UP50H - Compact Homogenizer; Hielscher Ultrasound Technology, Teltow, Germany) for 30 s in the case of *A. minutissimum*, 60 s for *C. affiniformis* and 10-30 s for *N. palea*, with the instrument's settings at 40% amplitude and 0.5 s frequency. 500 µl of the cell suspensions were washed twice with BM, diluted 1:10 and

incubated with $98 \mu\text{g ml}^{-1}$ of the β -lactam antibiotic imipenem (Sigma-Aldrich, Germany) for 2-15 days at the same cultivation conditions as used for the xenic cultures.

In parallel approaches, loosely attached EPS were removed via incubation at 30°C for 2 h with shaking at 500 rpm directly after ultrasonic treatment. We suppose that the necessity of this step may depend on the biofilm structure and the respective species. According to our experience, the removal of EPS using the described methods is not harmful to a number of diatom species (Bruckner and Kroth 2009; this study). Cells were then washed three times, diluted 1:10 and incubated with $75 \mu\text{g ml}^{-1}$ of imipenem for 2-15 days. All approaches were pre-screened continuously at 400 x magnification using an inverted optical microscope (Axiovert 40 C; Carl Zeiss MicroImaging GmbH, Göttingen, Germany); cultures without bacteria were further cultivated. This alternative step was especially helpful when purifying *A. minutissimum* and *N. palea*. We chose the antibiotic imipenem, because β -lactam antibiotics inhibit the cell wall synthesis of bacteria and we therefore expected less toxic effects on chloroplasts as reported for other antibiotics (Shishlyannikov et al. 2011). Imipenem has a broad antimicrobial spectrum (Kropp et al. 1985) and was already successfully used to generate axenic cyanobacterial cultures from environmental isolates (Ferris and Hirsch 1991; Hong et al. 2010). In the first days after antibiotic treatment, the viability of the diatom cells was observed daily via inverse light microscopy, later on in irregular time intervals. *Nitzschia palea* and *A. minutissimum* did not show any microscopically observable morphological changes after treatment with imipenem and could be stably cultivated afterwards, suggesting that the diatom cells remained viable even after a long time of incubation (15 days) with this antibiotic. *C. affinis* isolate B-16, however, seems to be more sensitive against imipenem; here we observed many dead cells and empty frustules after an incubation period of 2 days. However, this culture was then able to regenerate in antibiotic-free medium and residual contaminations could be removed by an addition of $150 \mu\text{g ml}^{-1}$ of the β -lactam antibiotic carbenicillin (Duchefa Biochemie, Netherlands). After imipenem treatments we therefore recommend a post-treatment with other β -lactam antibiotics like for example carbenicillin, ampicillin, ticarcillin and cefotaxim, or a mixture of these drugs in case that bacterial contaminations should persist after treatment with imipenem. A repetition of the procedure with *A. minutissimum* from two separately cultivated xenic cultures confirmed our data although in one of these cultures bacterial cells remained visible when screened with inverse light microscopy. We speculate that in this culture the bacterial composition may have changed during separate cultivation for several months. Although the effectiveness of a purification method mainly depends on the individual diatom

species itself, the composition of the associated bacterial community is apparently also relevant. For example, the EPS formation of diatoms is influenced by the bacterial composition (Bruckner et al. 2008) and thus may change the effectiveness of the following treatment with antibiotics.

The axenic cultures were further cultivated in antibiotic free medium at the same standard cultivation conditions as the xenic cultures.

2.3.3 Microscopic surveillance of axenity

All approaches were microscopically pre-screened as described above and cultures without visible bacteria were selected for further checks. The respective cells were stained with SYBR Green I (Cambrex, Rockland, ME, USA) and screened for bacterial contaminants at 800 x magnification with an Olympus BX51 epifluorescence microscope (Olympus Europe, Hamburg, Germany) equipped with the filter set 41020 (Chroma Technology Corp, Rockingham, VT, USA). We could demonstrate that bacterial cells as well as algal nuclei are labelled in the xenic cultures, while in the axenic cultures only the fluorescence of the nuclear DNA of the diatom cells is visible (Figure 2.1).

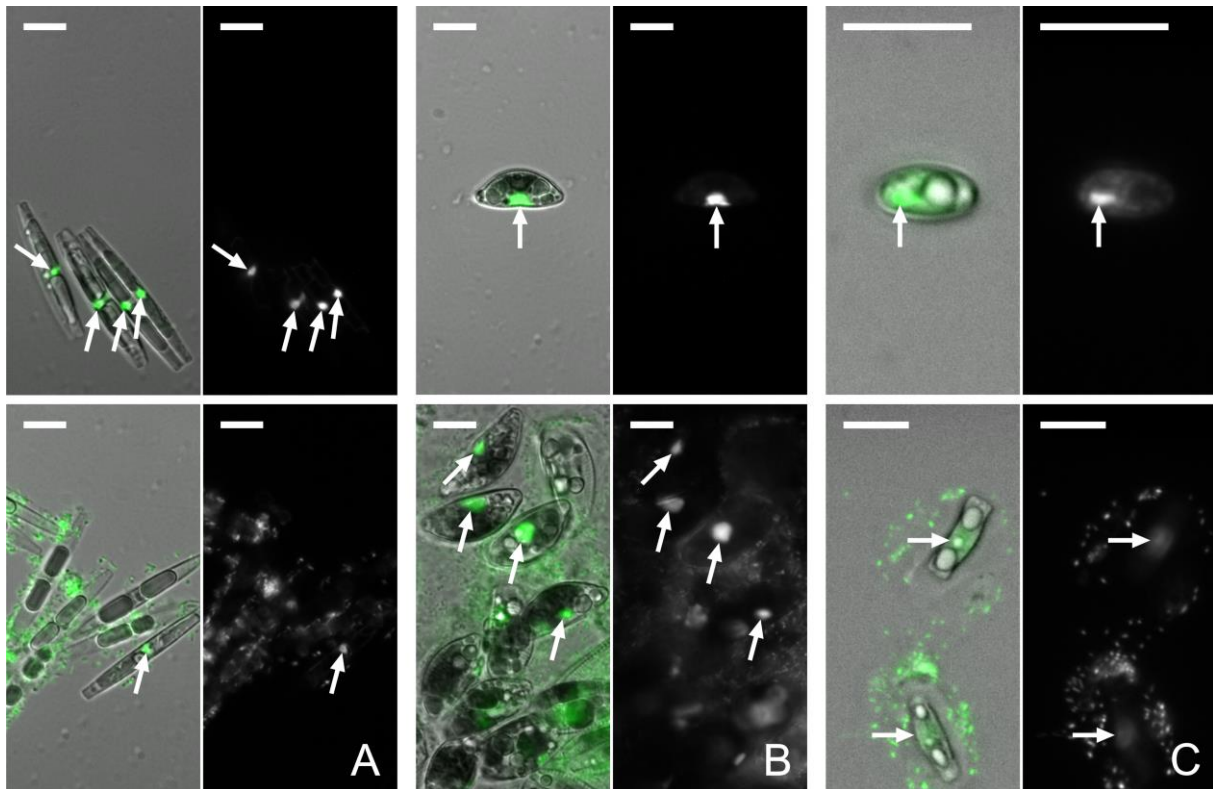


Figure 2.1: DNA staining of the xenic and axenic diatom cultures. A) *N. palea*, B) *C. affinis*, C) *A. minutissimum*. Upper row shows the axenic diatom cultures, lower row the corresponding xenic cultures. Left images show the merged transmitted light (grey-scale) and SYBR Green fluorescence (green), right images show SYBR Green fluorescence (grey-scale). Arrows mark the SYBR Green fluorescence of nuclei of diatoms. Scale bars denote 10 µm.

2.3.4 Surveillance of axenity via growth tests

The axenic state of the purified *A. minutissimum* culture was additionally verified by two different methods: The culture was transferred to different bacterial media like solid media with 1.5% Agar-Agar Kobe I (Roth, Germany) containing either diluted LB medium (25 or 50% (v/v), (Miller 1972)) or medium B (Jagmann et al. 2010) supplemented with 0.05% Trypton, 0.0005% yeast extract and 10 mM HEPES pH 6.8. After three days of cultivation at 16°C the plates were screened for bacterial growth. Additionally, 100 µl of the cultures were cultivated in liquid 50% (v/v) LB for five days at 20°C on a shaker (135 rpm). The xenic cultures quickly formed a bacterial lawn when spread on agar plates with bacterial media and dense bacterial growth could also be observed in the liquid medium. The axenic cultures did not show any bacterial growth on solid or liquid media.

2.3.5 Molecular biological surveillance of axenity

In axenic cultures, the only 16S rDNAs present should originate from the plastids and the mitochondria of the diatom species, whereas in xenic cultures also other 16S rDNAs may be present. We therefore extracted genomic DNA from xenic and axenic strains using a modified protocol from Murray and Thompson (1980). Cell pellets of 7-8 ml culture aliquots were pestled in 2 x cetyltrimethylammonium bromide (CTAB) extraction buffer with 1% 2-mercaptoethanol and processed as described in Bruckner et al. (2008). Precipitation was increased by adding one additional volume of isopropanol. Subsequently, we generated 16S rDNA clone libraries as described in Bruckner et al. (2008). From nine randomly selected colonies, the plasmids containing the 16S rDNA fragments were prepared (QIAprep Spin Miniprep Kit; Qiagen GmbH, Hilden, Germany), amplified and analysed by Restriction Fragment Length Polymorphism (RFLP) using the restriction enzymes MspI and AluI (Fermentas, Germany). rDNA fragments were separated by electrophoresis with 4% Sieve Agarose 3:1 (Biozyme Scientific GmbH, Germany). 16S rDNA fragments of the xenic culture, which showed different restriction patterns in RFLP analysis, were sequenced using the T7 promoter primer (GATC, Germany). We also sequenced five fragments cloned from the axenic *A. minutissimum* culture in the same way. BLAST searches were performed against the NCBI database (<http://www.ncbi.nlm.nih.gov/>). In the xenic cultures, we identified 16S rDNA fragments of *Bacteroidetes* and of β -*Proteobacteria*, while in the axenic cultures only 16S rDNA fragments of diatom plastid DNA were detected. This way we were able to demonstrate the absence of bacterial contaminations in the purified culture.

2.4 Conclusions

With this report we demonstrate the usability of the β -lactam antibiotic imipenem as a new tool for the repeated purification of the diatoms *Achnantheidium minutissimum*, *Cymbella affinis* and *Nitzschia palea* from xenic cultures. Generally, treatment with β -lactam antibiotics such as imipenem in combination with preceding EPS removal might also prove effective for the purification of other diatom species. Our protocol is particularly useful to re-establish axenic cultures from the long-term stable xenic cultures of the strains whenever axenic cultures are needed.

BOX 1 – Axenification protocol

All steps were performed under sterile conditions using a laminar flow and sterile consumables. For the detailed individual experimental procedures see text.

- Step 1** Transfer the diatom cells into new medium
- Step 2** *Sonication* – Treat cell suspension for 10, 30 or 60 sec with ultrasound
- (Step 3)** *Dissolution of EPS* – Alternative step
Incubate cell suspension at 30°C and 500 rpm for 2 h
- Step 4** *Washing step* – Wash 2-3 times with BM
- Step 5** *Antibiotic treatment* – Dilute the culture 1:10 and incubate the diluted suspension with 75-98 $\mu\text{g ml}^{-1}$ imipenem for 2-15 days, add optional 150 $\mu\text{g ml}^{-1}$ carbenicillin for after-treatment
Check the vitality of the diatom cells daily by microscopy
- Step 6** Cultivate cells in antibiotic free medium at appropriate cultivation conditions

2.5 Acknowledgements

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Chapter 3

Influence of bacteria on cell size development and morphology of cultivated diatoms

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Chapter 3: Influence of bacteria on cell size development and morphology of cultivated diatoms

3.1 Abstract

Vegetative cell division in diatoms often results in a decreased cell size of one of the daughter cells, which during long-term cultivation may lead to a gradual decrease of the mean cell size of the culture. To restore the initial cell size, sexual reproduction is required, however, in many diatom cultures sexual reproduction does not occur. Such diatom cultures may lose their viability once the average size of the cells falls below a critical size. Cell size reduction therefore seriously restrains the long-term stability of many diatom cultures. In order to study the bacterial influence on the size diminution process, we observed cell morphology and size distribution of the diatoms *Achnantheidium minutissimum* (Kützing) Czarnecki, *Cymbella affiniformis* Krammer and *Nitzschia palea* (Kützing) W. Smith for more than two years in bacteria-free conditions (axenic cultures) and in cultures that contain bacteria (xenic cultures). We found considerable morphological aberrations of frustule microstructures in *A. minutissimum* and *C. affiniformis* when cultivated under axenic conditions compared to the xenic cultures. These variations comprise significant cell length reduction, simplification and rounding of the frustule contour and deformation of the siliceous cell walls, features that are normally found in older cultures shortly before they die off. In contrast, the xenic cultures were well preserved and showed less cell length diminution. Our results show that bacteria may have a fundamental influence on the stability of long-term cultures of diatoms.

Keywords: axenic · cultivation · diatom · interaction · size reduction · xenic

3.2 Introduction

Long-term cultivation of diatoms is often challenging, one reason for this is their characteristic cell cycle. The intracellular generation of new valves during vegetative cell division results in disparately sized daughter cells, of which only one retains the initial cell size, while the other daughter cell is smaller (Chepurnov et al. 2004). This effect, known as the MacDonald–Pfitzer rule (MacDonald 1869; Pfitzer 1871) causes a gradual reduction of the median cell size of a culture (with a few known exceptions: *Eunotia pectinalis* var. minor (Geitler 1932), *Navicula muralis* (Locker 1950), *Nitzschia subtilis* var. *paleacea*, *N. palea* var. *debilis* (Wiedling 1948) and *Phaeodactylum tricorutum* (Lewin et al. 1958; Martino et al. 2007)). Recovery of the maximal (or initial) cell size takes place via auxospore formation, a process that mainly occurs after sexual reproduction (Chepurnov et al. 2004; Mann 2011). Once the cells reach a minimal size threshold, sexual reproduction can be triggered by external conditions such as light, temperature, presence of osmolytes and nutrient availability (Drebes 1977; Chepurnov et al. 2004). Many diatoms are not able to reproduce sexually in culture, for example if the gametes produced within a clonal line are self-incompatible or if external triggers for sexual reproduction are missing. In such cultures, cell size diminution often continues and finally the cultures die (Geitler 1932; Chepurnov et al. 2004). For the long-term maintenance of diatom cultures it is therefore necessary to detect and optimize cultivation conditions, which retard or even prevent the cell size diminution. In this study we investigate the bacterial influence on the long-term stability of three different pennate diatoms, isolated from freshwater biofilms. In their natural habitats diatoms are usually associated with bacteria and the presence as well as the composition of the accompanying bacterial community may considerably impact the physiology of the algae. Bacteria are known to influence the formation of marine snow (Grossart et al. 2006), growth and production of extracellular polymeric substances (Bruckner et al. 2008), reduce toxic compounds for example hydrogen peroxide (Hünken et al. 2008), supply essential organic compounds like vitamins (Croft et al. 2005), stimulate secondary metabolite production like domoic acid in case of *Pseudo-nitzschia multiseriis* (Bates et al. 1995; Kobayashi et al. 2009b) or even act algicidal (Mitsutani et al. 2001; Kang et al. 2011). Analyses of bacterial assemblages in diatom cultures revealed dominance of α -, β -, and γ -*Proteobacteria* and bacteria of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum (Schäfer et al. 2002; Kaczmarek et al. 2005; Sapp et al. 2007b; Bruckner et al. 2008). In such cultures, the composition of the co-cultivated bacterial community depends on the composition of the

bacterial community at the time of isolation, the diatom species itself and the cultivation conditions. It has been demonstrated that the bacterial composition in freshly isolated cultures differs from the associated bacterial community *in situ* and may change during extended cultivation (Sapp et al. 2007b). In contrast, Schäfer et al. (2002) found the diatom-bacteria associations in several investigated cultures to be stable. Furthermore, they showed that bacterial communities of different diatom cultures are distinct in composition (Schäfer et al. 2002). Even the same diatom species may be associated with different bacterial communities, depending on the cultivation conditions (Kaczmarska et al. 2005).

In this study, we followed the cell size distribution and morphological changes of the benthic diatoms *Achnantheidium minutissimum* (Kützing) Czarnecki, *Cymbella affiniformis* Krammer and *Nitzschia palea* (Kützing) W. Smith in presence and absence of their associated bacterial community for more than two years. We found that the average cell length and width as well as the frustule shape of two of the observed diatoms depend on the presence of bacteria. We further found evidence that the presence of bacteria in the culture slows down the overall size reduction. This study shows evidence that associated bacteria may be important for the long-term maintenance of clonal diatom cultures.

3.3 Materials and Methods

3.3.1 Isolation of diatoms

Achnantheidium minutissimum (Kützing) Czarnecki isolate B-13, *Cymbella affiniformis* Krammer isolate B-16 and *Nitzschia palea* (Kützing) W. Smith isolate B-01 were isolated in January 2009 from phototrophic, epilithic biofilms from the littoral zone of Lake Constance (47° 41' N; 9° 11' E, Germany). Detailed descriptions of isolation and purification from associated bacteria are described elsewhere (Windler et al. 2012). For isolation, the biofilm suspensions were streaked on agar plates containing a modified liquid Bacillariophycean Medium (BM) (Windler et al. 2012) and cultivated as described below. Single colonies were picked and transferred to liquid medium.

3.3.2 Cultivation conditions

Xenic and axenic diatom isolates were cultivated in liquid BM at standard cultivation conditions at 16°C in a 12:12 h light:dark cycle and at a photon flux density of 20-50 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Cells were cultivated under non-shaking conditions in tissue culture flasks (Sarstedt, Newton, USA). The strains were transferred to fresh media monthly, during the stationary

phase. The cells were scraped from the bottom of the culture flask, and 15-20 μl culture medium containing approximately 15 000-20 000 cells (in case of *A. minutissimum* and *N. palea*) were transferred into 10 ml fresh BM. Backup cultures of xenic and axenic *A. minutissimum* cells were stored on agar plates with solid BM at 16°C with a photon flux density of 20-40 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and transferred to fresh plates every two months, additional backup cultures were stored at 8°C with a photon flux density of 0.5-2 $\mu\text{mol m}^{-2}\text{s}^{-1}$; these backup cultures were transferred to fresh plates every three to six months.

3.3.3 Frustule preparation and identification of diatom isolates

For morphological identification and cell size determination of the diatom isolates, frustules were treated with 35 % hydrogen peroxide at 95°C for 4 h and again with 10 % hydrochloric acid at room temperature for 4 h, and mounted in Naphrax (index nD 1.710; Euromex, Arnhem, Netherlands) roughly following a procedure described by the European Committee for Standardization (2003). For morphological identification, the purified frustules were observed using an Olympus BX51 microscope (Olympus Europe, Hamburg, Germany) at 1000 x magnification and identified according to Krammer and Lange-Bertalot (1986, 1988, 1991b) and Krammer (2002).

For analysis of the 18S rDNA region, genomic DNA was extracted using a protocol from Murray and Thompson (1980) with slight modifications. Cell pellets of 7-8 ml cultures were pestled in 2 x cetyltrimethylammonium bromide (CTAB) extraction buffer with 1 % 2-mercaptoethanol and processed as described in Bruckner et al. (2008). Precipitation was increased using 1 volume of isopropanol. 18 S rDNA fragments were amplified using the primers 5`-AAC CTG GTT GAT CCT GCC AGT-3` and 5`-TTG ATC CTT CTG CAG GTT CAG CTA-3` modified from Medlin et al. (1988). 18 S rDNA clone libraries were established as described for 16 S rDNA libraries in Bruckner et al. (2008). 18 S rDNA fragments were sequenced (GATC, Konstanz, Germany) and BLAST searches were performed against the NCBI database.

3.3.4 Determination of cell length and width of diatom frustules

The first frustule preparation of xenic diatoms was performed in April 2009, after a cultivation period of three months and the next preparation in August 2011 after an additional cultivation period of 28 months. Axenic diatoms were purified from xenic cultures in June 2009 and the respective frustules were also prepared in August 2011 after a cultivation period of 26.5 months. The diatom frustules were observed at 400-1000 x magnification as described

above. Images were taken with a Zeiss AxioCam MRm digital camera system (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Frustule sizes were determined with the digital image processing software AxioVision LE (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Cell lengths were measured from pole to pole and cell widths at the widest part of the central area, 25 cells were measured from each sample.

3.3.5 Temporal progression of cell length of *Achnantheidium minutissimum*

Cell lengths of xenic and axenic *A. minutissimum* cultures were examined from June 2009 to November 2011 at irregular intervals from images of live cells. Length measurements were conducted either by direct observation in the cultivation vessels with an inverted optical microscope (Axiovert 40 C; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) or by observation of culture aliquots with an upright microscope (Olympus BX51; Olympus Europe, Hamburg, Germany). Length scales were determined individually for each of the used microscopes. Photographs were taken with Zeiss AxioCam MRc or MRm digital camera systems (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and analyzed with the digital image processing software AxioVision LE (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images of cultures taken after 150 (xenic and axenic), 305/306 (axenic) and 325 days (xenic) originated from cultures which were stained with crystal violet or DiOC₆ (Molecular Probes; Life Technologies, Darmstadt, Germany), respectively, directly before the pictures were taken. Whenever possible, 25 cells per time point were used for measurements (exceptions: 14 axenic cells on day 131, 16 axenic cells on day 150, 15 axenic cells on day 306 and 22 xenic cells on day 325). In order to test the influence of cultivation on solid medium on cell size reduction (compared to liquid cultivation), *A. minutissimum* cells were scraped from the backup cultures and transferred into liquid BM according to the test cultures. After one month cultivation under standard conditions, cell length was measured as described above. In the following we use the terms “frustule length” for measurements of frustule material and “cell length” for measurements of live cells.

3.3.6 Growth of *Achnantheidium minutissimum* and chlorophyll extraction

To observe growth of xenic and axenic *A. minutissimum*, the progression of the chlorophyll concentration was measured in triplicates. 150 ml of BM was inoculated with $1 \cdot 10^3$ cells ml⁻¹ and incubated at 16°C and 100 rpm with a photon flux density of 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and a light:dark cycle of 12:12 h. For each time point chlorophyll was extracted from an aliquot of 1 ml diatom culture by addition of a mixture of 5 % methanol and 95 % acetone to

the cell pellet. Chlorophyll concentrations were determined using the equation for diatoms, chrysomonads and brown algae according to Jeffrey and Humphrey (1975). The growth rates (μ) of the cultures were calculated during the exponential growth phase of the two cultures as:

$$\mu = (\ln(\text{Chl}_{t_2}) - \ln(\text{Chl}_{t_1})) / (t_2 - t_1)$$

with the chlorophyll concentrations Chl_{t_1} and Chl_{t_2} at the beginning (t_1) and at the end (t_2) of the exponential phase. The exponential growth phase lasted from day 11 to 22 in the axenic, and from day 4 to 18 in the xenic culture.

3.3.7 Statistical analysis

We compared the cell dimensions (length, width and length-to-width ratio) of diatom frustules of the xenic cultures in 2009, 2011 and of the axenic cultures in 2011 using analysis of variance (ANOVA) and Tukey's HSD posthoc tests. For *A. minutissimum* the temporal progression of mean cell length was analyzed for the xenic and axenic cultures with regression models using time since the start of the experiment as an independent variable. We further used a t-test to analyze differences in cell size of live cells of *A. minutissimum*. In addition, we used ANOVA and Tukey's HSD to test for differences in cell length between all liquid and backup cultures of *A. minutissimum* in 2012. Statistical analyses were performed in R (R Development Core Team 2011).

3.4 Results

3.4.1 Identification of diatom isolates

At the beginning of the experiments in 2009 the diatom isolates were identified using morphological criteria and 18S rDNA sequence analyses. Isolate B-13 exhibited typical features of *Achnantheidium minutissimum* (Kützing) Czarnecki (Krammer and Lange-Bertalot 1991b). The valves were linear-lanceolate with broadly rostrate ends. The axial area was narrow, linear or slightly lanceolate. The central area was formed by one or two shortened and more broadly spaced striae. The central area was variable and present on both sides or only on the one side of the valve and never formed a stauros (Figure 3.1). The BLAST results of the 18S rDNA fragment of isolate B-13 showed high similarities of 99 % with the database entry of *A. minutissimum* (GenBank AM502032).

Achnantheidium minutissimum

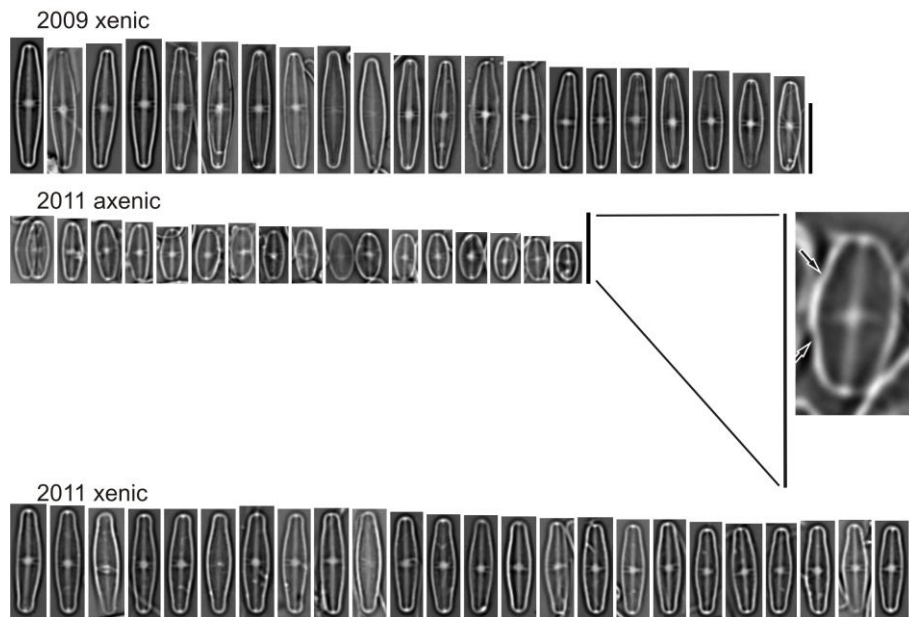


Figure 3.1: Frustules of *Achnantheidium minutissimum* in April 2009 and after cultivation in axenic and xenic state in August 2011. Arrows mark parts of the frustule deformation. Scale bars represent 10 μ m.

Cymbella affiniformis

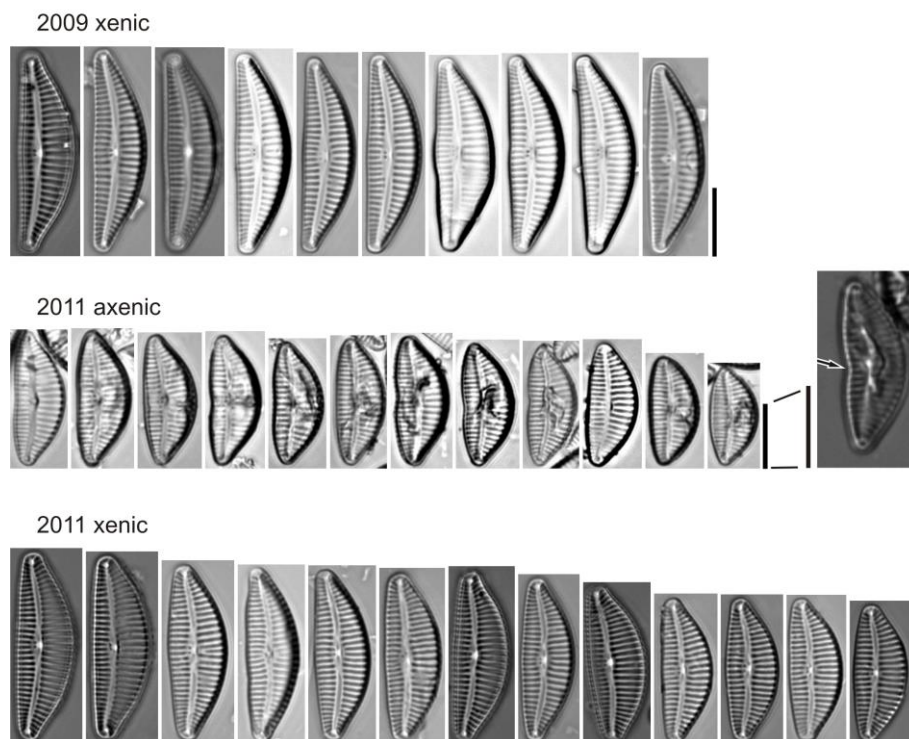


Figure 3.2: Frustules of *Cymbella affiniformis* in April 2009 and after cultivation in axenic and xenic state in August 2011. Arrow marks the concave ventral margin. Scale bars represent 10 μ m.

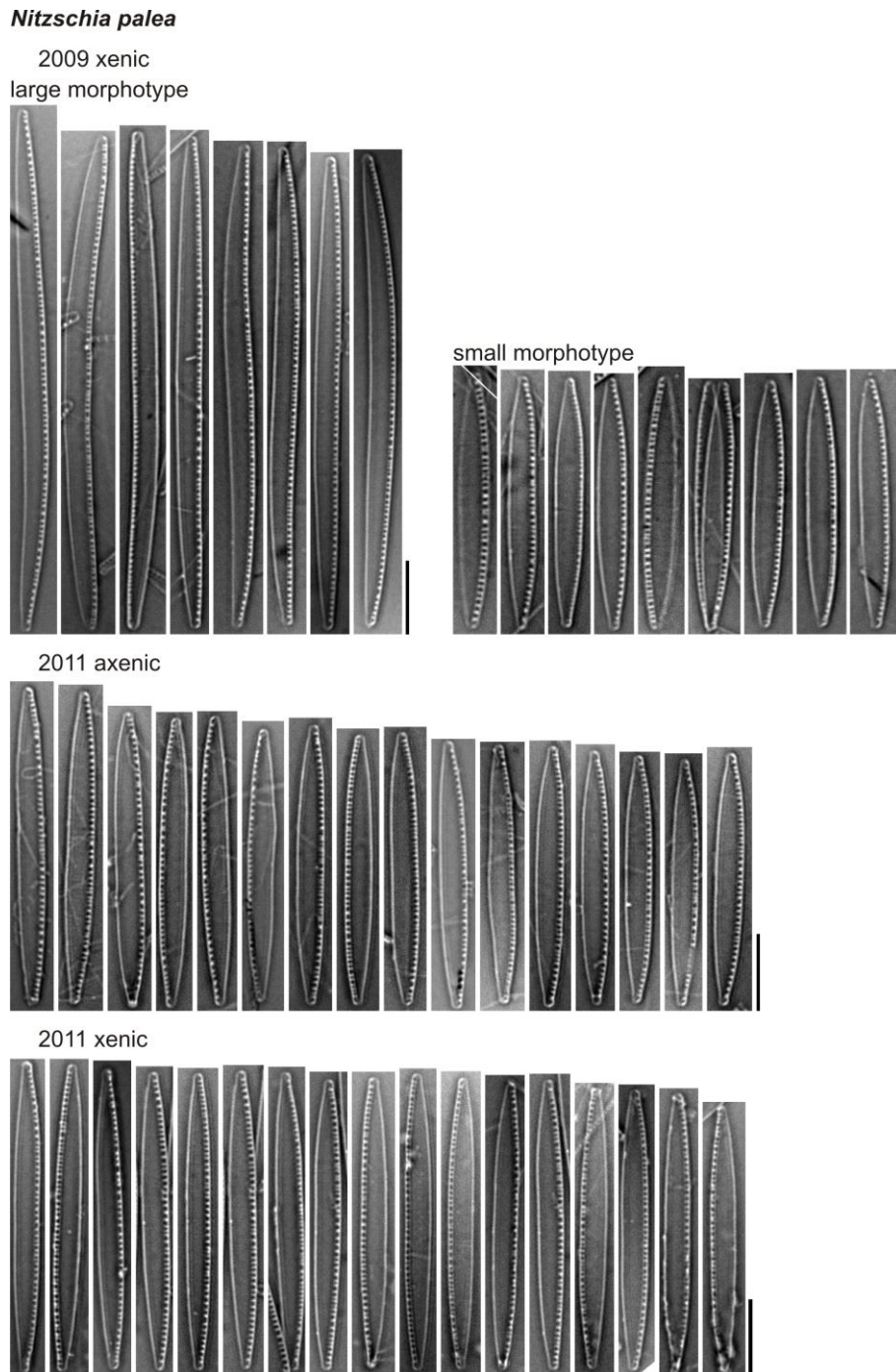


Figure 3.3: Frustules of *Nitzschia palea* in April 2009 and after cultivation in axenic and xenic state in August 2011. The culture consisted of *N. palea* f. *major* with longer cells (large morphotype) and *N. palea* with shorter cells (small morphotype) at the beginning of the experiment in 2009. In 2011 the xenic and axenic cultures contained only cells of the smaller morphotype. Scale bars represent 10 μm .

Based on the morphological characters we consider the isolate B-16 to represent *Cymbella affiniformis* Krammer (Krammer 2002). The valves were moderately dorsiventral, lanceolate, the dorsal margin was strongly convex and the ventral margin was slightly convex. The ends were subrostrate to rostrate and narrowly rounded (Figure 3.2). Sequence analysis of B-16 showed high similarities of 99% with *Cymbella affinis* (GenBank AM502018/AM502009) and *Cymbella excisa* (GenBank JN790273), however, there is no database entry for *C. affiniformis*.

Isolate B-01 showed all characters of *Nitzschia palea* (Kützing) W. Smith as described in Krammer and Lange-Bertalot (1988). Two forms were present in this culture, *N. palea* f. *major* Rabenhorst, which has considerably longer cells, and *N. palea* with shorter cells. The valves were linear-lanceolate (smaller morphotype) to linear (larger morphotype) and the valve margins at the centre were linear (larger morphotype) or slightly convex (smaller morphotype). The poles were rostrate, subrostrate or subcapitate. Striae were not visible under the light microscope. Fibulae were more or less square-like. The distance between the fibulae was irregular, but the central pair of fibulae was more widely separated than the others (Figure 3.3). The BLAST results of the 18S rDNA fragments of isolate B-01 coincide with the morphological identification, exhibiting 100% similarity with *N. palea* (GenBank AJ867008/AJ867006/ AJ867001).

3.4.2 Cell size and frustule morphology of axenic versus xenic diatom cultures

Figure 3.4 gives an overview over the dates of diatom isolation, purification from associated bacteria and sampling for length measurements. *A. minutissimum* exhibited considerable differences in frustule morphology when cultivated with or without bacteria in liquid medium (Figure 3.1). Frustules of xenic cells retained the typical features of *A. minutissimum* (Kützing) Czarnecki even after a long period of cultivation (Krammer and Lange-Bertalot 1991b). However, in our axenic *A. minutissimum* culture, the valve outline of the cells was rhombic to elliptic, with the ends broadly rounded and sometimes slightly protracted. The valves seemed partly to be thinner and deformed compared to valves of the original xenic culture. After more than two years in culture, the length and width of the xenic diatom cells were slightly shorter compared to the original length (Table 3.1, Figure 3.5). In contrast, the average frustule length and width of the axenic diatom culture were considerably more reduced after a comparable cultivation period. The length-to-width ratio in the axenic culture was substantially lower compared to the xenic *A. minutissimum* cultures (Table 3.1, Figure 3.5). As a result, we observed a slight reduction of frustule size of *A. minutissimum* of 16.2% in length and 5.5% in width in the presence of bacteria but an extensive reduction of 53.8% in length and 13% in width in the axenic culture after more than two years of cultivation. All observed differences in frustule length and width between xenic and axenic cultures at different time points are statistically significant (Figure 3.5).

No significant differences of cell length of live cells between xenic and axenic cultures of *A. minutissimum* were observed at the beginning of the experiment in June 2009 (Figure 3.6, $P > 0.05$, see Suppl. Data S3.1 for example measurements). The xenic culture thereby

exhibited a mean cell length of $13.65 \pm 1.22 \mu\text{m}$ and the axenic culture immediately after purification had a mean cell length of $13.06 \pm 1.21 \mu\text{m}$. This demonstrates that the applied purification methods had no direct influence on the cell length of *A. minutissimum*. The cell length of the axenic culture decreased ($n = 10$, $t = -4.8$, $P < 0.01$) to $9.68 \pm 0.97 \mu\text{m}$, whereas the cell length of the xenic culture did not decline ($n = 10$, $t = -1.0$, $P = 0.32$) and remained at $13.8 \pm 1.05 \mu\text{m}$. Interestingly, the xenic culture abruptly developed larger cells with a mean length of $15.9 \pm 0.82 \mu\text{m}$ after 325 days with a significant difference to cell length of the previous measurement after 150 days ($P < 0.05$).

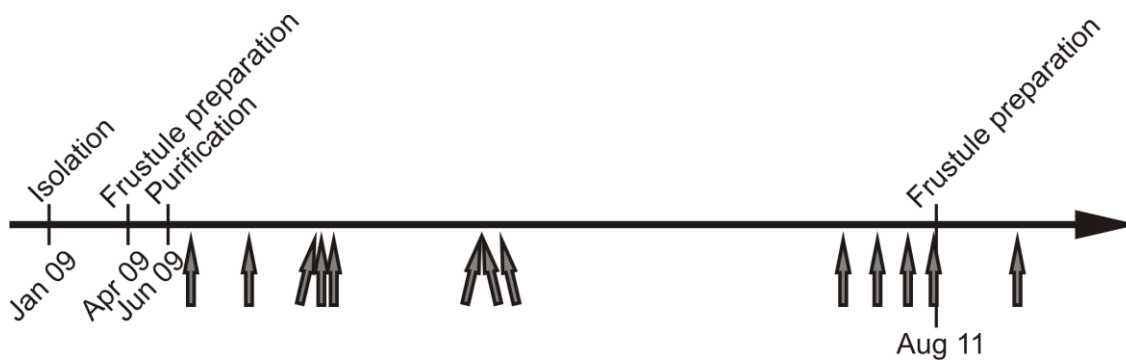


Figure 3.4: Timescale of diatom isolation, purification and frustule preparation. Small arrows mark points of length measurements of *A. minutissimum* live cells.

Table 3.1: Mean values of frustule length, width and length-to-width ratio of *Achnantheidium minutissimum*, *Cymbella affiniformis* and *Nitzschia palea* in April 2009 and after long-term cultivation under xenic and axenic conditions in August 2011.

		xenic 2009	xenic 2011	Ratio [%]†	axenic 2011	Ratio [%]†
<i>A. minutissimum</i>	length [μm]	16.52 (± 1.59)	13.84 (± 0.6)	-16.2	7.64 (± 0.76)	-53.8
	width [μm]	4.16 (± 0.24)	3.93 (± 0.2)	-5.5	3.62 (± 0.18)	-13
	length:width	3.98	3.53		2.11	
<i>C. affiniformis</i>	length [μm]	30.02 (± 0.8)	26.6 (± 3)	-11.4	20.14 (± 1.51)	-32.9
	width [μm]	8.6 (± 0.39)	9.42 (± 0.54)	+9.5	8.67 (± 0.4)	n.s.
	length:width	3.5	2.83		2.33	
<i>N. palea</i>	length [μm]	68.59 (± 2.46) ^a	40.06 (± 1.89)	-41.6	36.23 (± 2.13)	-47.2
		31.74 (± 0.54) ^b				
	width [μm]	4.35 (± 0.68) ^a	3.78 (± 0.1)	-13.1	3.68 (± 0.17)	-15.4
		4.04 (± 0.31) ^b				
	length:width	16.08 ^a	10.6		9.87	
		7.9 ^b				

† describes the percentage differences in frustule length and width compared to the original sizes in 2009. Cell lengths and widths of *N. palea* measured in 2011 were thereby compared to those of *N. palea* f. *major* (large morphotype). n.s. reveals no significant differences. ^a *N. palea* f. *major*, ^b *N. palea* small morphotype. Standard deviation is given in parentheses.

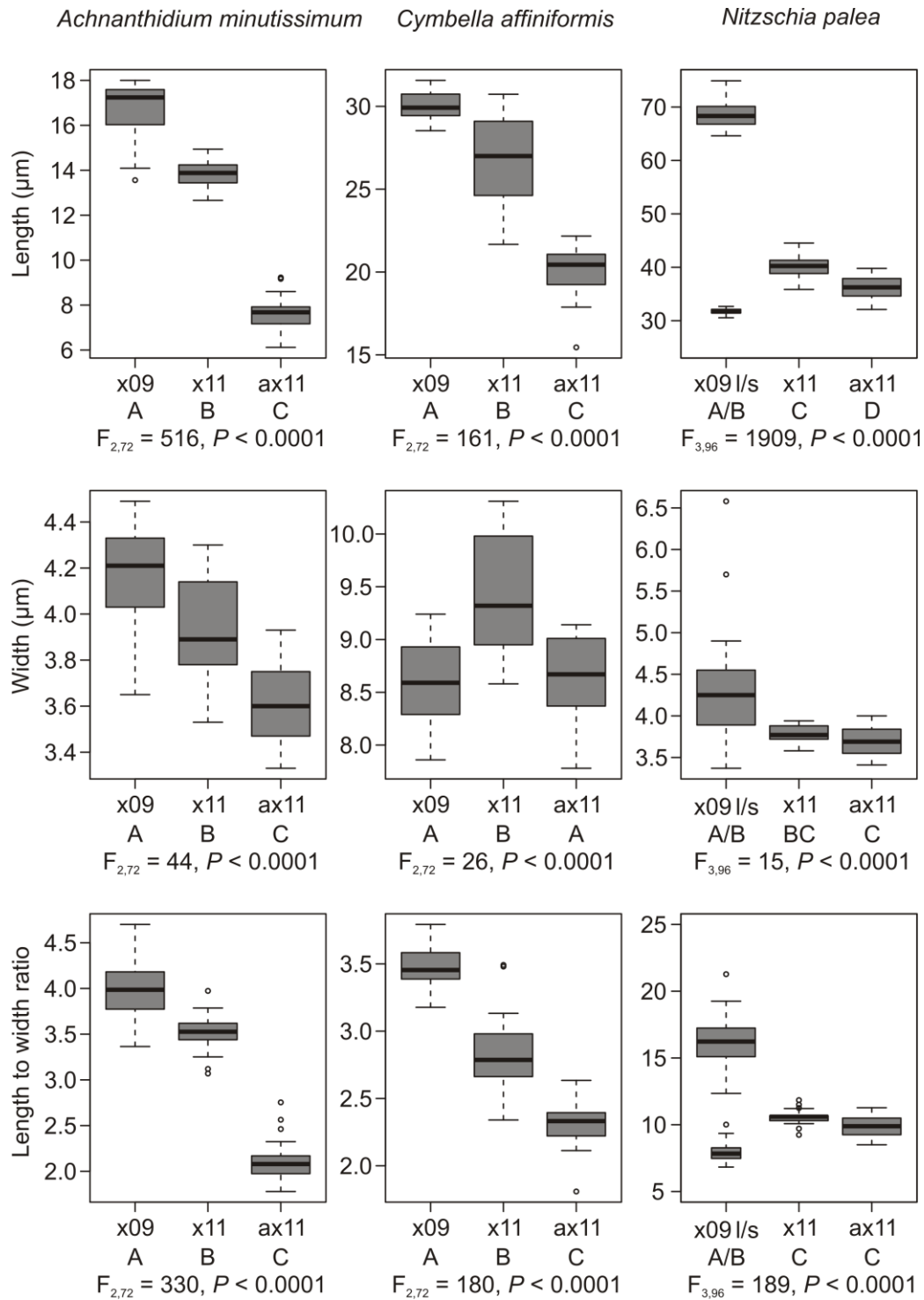


Figure 3.5: Frustule length, width and length-to-width ratio of *Achnantheidium minutissimum*, *Cymbella affinisformis* and *Nitzschia palea* in the xenic cultures in April 2009 (x09) and August 2011 (x11) and in the axenic cultures in August 2011 (ax11). For *N. palea* dimensions of the large (l) and small (s) morphotypes observed in the xenic culture in April 2009 (x09l/s) are shown. Widths of the large and small morphotypes completely overlap. Results of ANOVA and Tukey HSD testing are shown below each chart. The first line points to significant differences ($P < 0.05$) between the cultures based on post-hoc tests (Tukey HSD, means not differing share the same letter). The second line gives the F-value, degrees of freedom and significance level.

During the long-term cultivation of xenic and axenic *A. minutissimum* cultures in liquid BM (three parallel axenic cultures and two parallel xenic cultures), it was occasionally necessary to re-inoculate the axenic liquid medium cultures from backup cultures kept on solid BM at 16°C and at 8°C. To exclude the possibility that the cell size reduction of the axenic diatom culture was induced by such changes between cultivation on solid medium versus liquid medium or by the lower cultivation temperature of the backup cultures, we measured and compared cell lengths of all existing xenic and axenic *A. minutissimum* cultures at the end of the experiment in February 2012. Interestingly, cells of *A. minutissimum*, when cultivated at 16°C on solid diatom medium, in our hands became considerably smaller compared to those which were cultivated in liquid medium (Figure 3.7). Nevertheless, in each observed case the xenic cells were significantly longer than the axenic ones, independent from the type of medium used for cultivation. This proves that cultivation on solid medium alone does not explain the differences in length between xenic and axenic *A. minutissimum* and thus the observed differences have to be attributed to the presence or absence of bacteria.

To evaluate possible differences in the numbers of cell divisions between xenic and axenic cultures, the temporal progression of the chlorophyll concentration of these cultures was measured (Figure 3.8). The xenic culture had a high potential to form cell aggregates, whereas the axenic culture grew completely suspended. This condition made it impractical to determine the growth rate by cell counting methods and we therefore chose chlorophyll concentration as a measure for growth. Logarithmic scale of the growth curves (not shown) showed a prolonged lag-phase of 11 days for the axenic culture, while the xenic culture entered the exponential phase earlier after four days of cultivation. *A. minutissimum* exhibited a slightly higher growth rate of 0.4 day⁻¹ in the axenic state compared to 0.31 day⁻¹ in the xenic state. This corresponds to a doubling time of 1.73 days in the axenic and 2.21 days in the xenic diatom culture. Both cultures reached similar chlorophyll concentrations in the stationary phase (1.25 ± 0.39 µg ml⁻¹ in the xenic, 1.28 ± 0.1 µg ml⁻¹ in the axenic culture after 22 days of cultivation). In the late stationary phase, after 25 days of observation, the chlorophyll concentration of the axenic culture declined, whereas that of the xenic culture remained stable for a longer time. The xenic culture formed cell aggregates in the course of the stationary phase resulting in high standard deviations of chlorophyll measurements.

In all studied xenic *C. affiniformis* cultures the frustules exhibited typical morphological characteristics as described in Krammer (2002) (Figure 3.2). Apart from a decreased length-to-width ratio, frustules of cells cultivated in xenic conditions exhibited no major differences to frustules of cells harvested at the beginning of the experiment. In the

axenic culture however, we frequently found cells with a concave ventral margin. Valve ends were slightly protracted and acutely rounded. After the cultivation period of about 28 months the frustule length of the xenic diatom culture showed a relatively broad distribution but a significant shift of 11.4% to smaller lengths compared to the original value in April 2009 (Table 3.1, Figure 3.5). An extensive reduction of frustule length of about 33% was also observed in the axenic diatom culture. Frustule width persisted in case of the axenic culture, but increased significantly for the xenic diatom culture. In both cases a significant decrease of the length-to-width ratio was observed compared to the original value in April 2009.

At the beginning of the experiment *N. palea* showed all characters described in Krammer and Lange-Bertalot (1988) (Figure 3.3). The ratio between the large and small morphotypes in the cultures was 39:61. After more than two years in culture, frustules which originated from xenic or axenic cultures exhibited no visible differences in microstructure. All cells were uniform and corresponded to the *N. palea* smaller morphotype description. The frustules of xenic and axenic cultures were significantly shorter compared to those of *N. palea* f. major in 2009, but significantly longer than *N. palea* smaller morphotype in 2009 (Table 3.1, Figure 3.5). Therefore we consider *N. palea* f. major as parental for the cultures observed in 2011. *N. palea* exhibited a significantly shorter length when cultivated without bacteria compared to the xenic culture. Frustule width of xenic and axenic cultures also decreased during the experiment but did not differ significantly from each other in 2011. Length-to-width ratio decreased remarkable in both cultures in 2011 when compared to *N. palea* f. major in 2009.

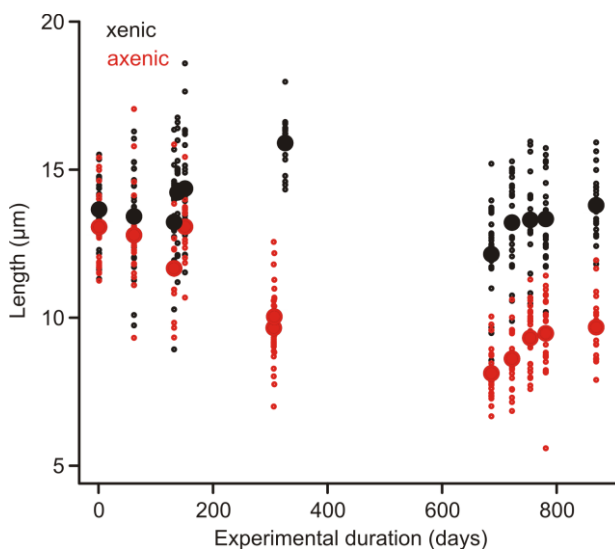


Figure 3.6: Temporal progression of cell length of xenic and axenic *Achnantheidium minutissimum*. Cell length was determined for live cells from June 2009 till November 2011. Red, axenic culture; black, xenic culture; small dots, individual measurements; large dots, arithmetic means.

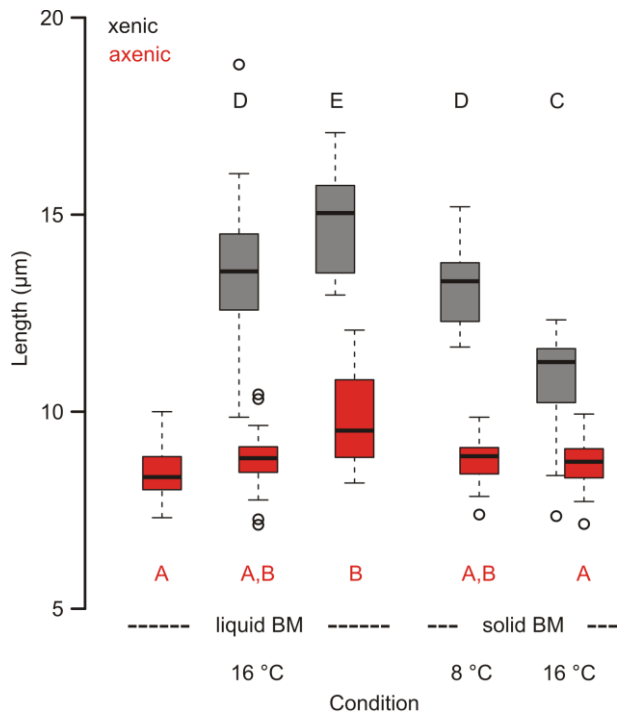


Figure 3.7: Cell lengths of liquid and backup cultures of *Achnantheidium minutissimum* cultivated under different conditions in February 2012. Grey, xenic cultures; red, axenic cultures. Significant differences were denoted with different letters. P -values > 0.05 were determined as the cut-off for significance.

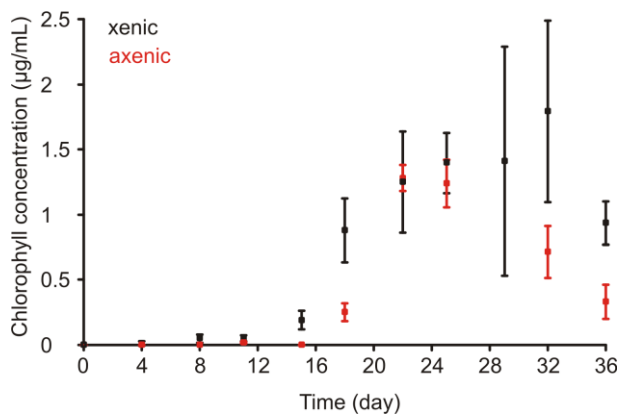


Figure 3.8: Growth of *Achnantheidium minutissimum* in xenic (black) and axenic state (red). Both cultures reached similar chlorophyll concentrations in the stationary phase ($1.25 \pm 0.39 \mu\text{g ml}^{-1}$ in the xenic culture, $1.28 \pm 0.1 \mu\text{g ml}^{-1}$ in the axenic culture after 22 days of cultivation). High error bars in the late stationary phase are attributed to the formation of cell aggregates.

3.5 Discussion

Changes of cell size and morphology of diatoms during their life cycle have been known for a very long time (MacDonald 1869; Pfitzer 1871; Geitler 1932). These changes are crucial for the long-term stability of many diatom cultures because small cells, if auxosporeulation is omitted, often become continuously smaller resulting in a loss of the cultures. Furthermore, as we know from our own experience, very small cells may show a different physiological behavior and thus compromise the reproducibility of experiments conducted with the strain. For example, stalk formation of *Achnanthes longipes* is strongly

reduced in small cells compared to large cells (von Stosch 1965). It is thus very important to develop methods that avoid or reduce cell size diminution of diatoms. One strategy is, if feasible, the maintenance of clones of the opposite mating type to induce sexual reproduction (Chepurnov et al. 2004). However, sexual reproduction will change the gene pool of the culture and for many genetic studies clonal cultures are essential. Long-term maintenance of diatoms via standardized cryopreservation therefore might represent an ideal solution but only few protocols for individual freshwater species are already available (McLellan 1989; Buhmann et al. 2013). In this study we observed the influence of associated bacteria on the long-term maintenance of three pennate diatom cultures and our results show that in two of the three investigated diatom species the axenic culture exhibited considerably shorter cells and aberrations in frustule morphology, whereas the cell length and frustule shapes were quite preserved in the xenic cultures.

The strongest effect of bacteria on cell size preservation was observed for *A. minutissimum* where cell length of the axenic culture decreased by more than half, whereas in the xenic culture just a slight reduction was observed (Table 3.1, Figure 3.5). Comparing the original length of frustules and live cells in April and June, the major length decrease of the xenic *A. minutissimum* culture happened at the beginning of the experiment, soon after isolation of the diatom. Afterwards the cell length of the xenic culture remained around 13.8 μm , both when measuring frustules or live cells. However, it has to be taken into account that there may be slight differences between the cell lengths that were determined either by frustule or live cell measurements. For example, the axenic *A. minutissimum* culture showed a mean frustule length in August 2011 of $7.64 \pm 0.76 \mu\text{m}$ (frustule measurement), compared to $9.63 \pm 1.00 \mu\text{m}$ (live cell measurement). The measurement of frustules is more accurate than the measurement of live cells. One reason could be the organic layer of live cells, which makes the exact outline of the cells more difficult to distinguish. The xenic *A. minutissimum* cells form capsules of extracellular polymeric substances (EPS) which make it even harder to define the correct cell border and due to the EPS matrix the cells were not always oriented completely planar to the microscope slide. Measurements of live cells can therefore indicate a trend; the absolute cell length values must be determined by means of frustules. According to Figure 3.6, the cell lengths of the xenic and axenic *A. minutissimum* cells may be slightly increased at the end of the experiment. Thus, the alga exhibited mean cell lengths of $12.15 \pm 1.69 \mu\text{m}$ after 685 days and $13.8 \pm 1.05 \mu\text{m}$ after 868 days in the xenic and $8.12 \pm 0.90 \mu\text{m}$ after 685 days and $9.68 \pm 0.97 \mu\text{m}$ after 868 days in the axenic culture. However, cell lengths of the parallel cultures of *A. minutissimum* slightly differ among themselves (Figure 3.7). The

axenic cultures thereby range from $8.49 \pm 0.72 \mu\text{m}$ to $9.69 \pm 1.15 \mu\text{m}$ and the xenic cultures from $13.61 \pm 1.85 \mu\text{m}$ to $14.83 \pm 1.30 \mu\text{m}$. Thus, we attribute those observed cell length differences to variances within the single cultures rather than to an actual increase. The same pattern of a differential cell length decrease between the xenic and axenic cultures was also observed for *C. affiniformis*. It must be noted here, that the identification results for this isolate were controversial. According to Krammer and Lange-Bertalot (1986), this strain should be identified as *Cymbella affinis*. However, according to Krammer (2002) the strain represents neither *C. affinis* due to the absence of a central area, nor *Cymbella excisa* due to the presence of two stigmas; instead, the characters of the strain are in agreement with the description for *Cymbella affiniformis*. Interestingly, although the cell length reduction was not prominent in the xenic *C. affiniformis* culture, the cells increased significantly in width. The ability to increase cell width to counteract a decrease of the cell volume was already mentioned by Geitler (1932), but only in very small cells. It seems that *C. affiniformis* attempts to regulate the decrease of volume by broadening the cell width in early stages of the length diminution process. The cell width increase was not observed in the axenic cultures. However, it was possibly falsified by a concave ventral margin which was found in some cells of this culture. Axenic *A. minutissimum* and *C. affiniformis* showed aberrations in frustule morphologies which was not observed in the xenic cultures. Changes in frustule shape of pennate diatoms during size reduction have been known for a long time (Geitler 1932) and correlate with our observation of decreasing length-to-width ratio, simplification and rounding of the frustule contour and weakening of the frustules. Atypical changes of the morphology in axenic state were also reported for the green algae *Ulva linza*, here the normal morphology could be restored by adding specific bacteria to the alga (Marshall et al. 2006). The authors speculate that nitrogen supply, hormone production and production of secondary metabolites by the bacteria might explain the bacterial influence on the morphology of the alga. To which extent the observed aberrations in our study are directly contributed to the absence of bacteria or to the normal shape changes, which usually occur during the diminution process, remained uncertain. The *N. palea* culture consisted of two cell types in 2009, a large and a small morphotype. The cultures are clonal as we isolated the cells by picking single colonies on agar plates, thus the two cell types must have developed from each other, either by auxosporulation or by abrupt size reduction. The large cells show irregular shapes or bends along the apical axis, which are also known for initial cells of other *Nitzschia* species (Geitler 1928; Trobajo et al. 2006). It is therefore conceivable that the long cells developed from the small morphotype by auxosporulation. Compared to the other two diatom species, the

differences in cell length between xenic and axenic in 2011 are not that remarkable in the *N. palea* culture.

We can currently only speculate about the underlying mechanisms bacteria use to be able to influence the cell size and morphology of *A. minutissimum* and *C. affiniformis*, but the following scenarios are theoretically conceivable:

- a) Bacteria may influence the frequency of mitotic cell division
- b) Bacteria may influence sexual reproduction
- c) Bacteria may influence other processes like vegetative cell enlargement or abrupt cell size reduction
- d) Bacteria may improve cell wall synthesis of the diatoms

Bacteria could possibly influence the frequency of mitotic cell division and, if the culture is able to reproduce sexually, consequently the frequency of auxosporulation. An increase of vegetative cell divisions in this case might enhance the frequency of auxosporulation, because the critical size threshold for auxosporulation is attained faster, resulting in a culture of mostly large cells. If the culture is not capable of sexual reproduction, a decrease of the division rate in the xenic culture could be another possible explanation. In our growth experiments with *A. minutissimum*, the axenic culture exhibited a longer lag phase and a shorter cell doubling time compared to the xenic culture; both cultures had similar chlorophyll concentrations in the stationary phase. This suggests similar cell densities of both cultures in the stationary phase and consequently similar cell division numbers. The shorter cell lengths and altered morphology observed in axenic cultures can therefore not simply be attributed to different numbers of mitotic cell divisions during vegetative growth.

Direct stimulation of sexual reproduction of diatoms by bacteria was already reported by Nagai et al. (1994; 1999) and Nagai and Imai (1998). They found that spermatogenesis of the centric diatom *Coscinodiscus wailesii* is induced by bacteria, a hint that bacteria indeed might externally trigger sexual reproduction and auxosporulation of diatoms. Sexual reproduction and accompanied auxosporulation of *A. minutissimum* has been observed and described by Geitler (1932) (using the synonymous species name *Achnanthes minutissima*). Auxosporulation was not directly observed in our cultures. Furthermore, the cultures can be considered as clonal, making sexual reproduction only possible if the diatoms are homothallic or capable for automixis. Both processes were found in pennate diatoms but represent rather exceptional processes in this group (Chepurnov et al. 2004). However, if auxosporulation

would have occurred, conspicuously larger cells should have been observed at least occasionally in *A. minutissimum*, since cell length distribution of the culture was observed throughout the experimental duration. After 325 days of cultivation, the average length of xenic *A. minutissimum* indeed increased to $15.9 \pm 0.82 \mu\text{m}$. According to Geitler (1932), 25 μm is the maximal cell length of *A. minutissima*, while initial cells of this diatom in culture were reported to be smaller with 16,5 μm (Locker 1950). This could be a hint that the length of the initial cell observed in environmental samples is not achieved under artificial conditions. However, *A. minutissimum* represents a species complex (Potapova and Hamilton 2007) and it is nearly impossible to clarify whether Geitler and Locker used the same species. Locker (1950) observed that auxosporulation of *A. minutissima* Kützing occurred again after 15 months, while Byllaardt and Cyr (2011) noticed that cell reproduction of *A. minutissimum* *in situ* takes place with incidental auxosporulation occurring in spring. We did not observe periodic cell enlargement even after further cultivation for nearly one and a half year, possibly due to a lack of additional stimulating triggers in our comparatively constant cultivation conditions without annual changes in light and temperature compared to the study of Locker (1950). We therefore cannot exclude that bacteria might be involved in sexual reproduction and thus preserve cell lengths in case of *A. minutissimum* and *C. affiniformis* even if we do not consider this to be likely.

Other mechanisms which affect cell size of diatoms like vegetative cell enlargement (von Stosch 1965), vegetative auxosporulation (Nagai et al. 1995; Nagai and Imai 1997; Sabbe et al. 2004), apomixis (Chepurnov et al. 2004) or abrupt cell size reduction (Locker 1950; von Stosch 1965; Chepurnov and Mann 1997; Chepurnov et al. 2004) could also not be ruled out as possible explanation for the cell size differences observed in this study as bacteria may possibly serve as an additional external trigger for one of these mechanisms.

Another possible explanation for the differences in cell length between xenic and axenic diatom cultures could be the complex cell wall synthesis of diatoms. Silica biomineralization in diatoms strongly depends on long-chain polyamines (LCPA) (Kröger and Poulsen 2008). Synthesis of a single molecule of LCPA with 20 methylated aminopropyl units consumes 40 molecules of *S*-adenosylmethionine, which is a derivate of the amino acid methionine (Michael 2011). The vitamin B₁₂ dependent methionine synthase may play an important role in the synthesis of the LCPA precursor methionine. This suggests that species with siliceous cell walls may have higher methionine requirements compared to the species which do not build siliceous cell walls. It was shown for algae from various groups that bacteria supplement vitamin B₁₂ to the algae (Croft et al. 2005). Possibly, the addition of

vitamin B₁₂ into the medium was not enough for the synthesis of a sufficient amount of methionine during the cell wall synthesis in the axenic culture, while in the xenic culture bacteria might supply additional amounts of vitamin B₁₂.

In this study we showed that the presence of bacteria is important for morphology and size preservation of some diatoms and hence for healthy and stable long-term cultures. The extent of this effect apparently differs from species to species, as we found largest length differences between xenic and axenic states in *A. minutissimum* and *C. affinis* cultures and smaller differences in case of *N. palea*. Our findings can be exploited for the long-term maintenance of diatom cultures and we recommend the storage of xenic diatom cultures for backup, from which axenic cultures can be repeatedly purified. Furthermore, we found that cultivation on solid medium may enhance the size diminution process of *A. minutissimum* cells. Apart from fundamental insights into the influence of bacteria on diatoms, our results may also help to optimize conditions for the stable long-term cultivation of diatoms. On the long-term, the identification of putative bacterial molecules which facilitate cell size maintenance of diatoms will be extremely valuable for keeping axenic diatom cultures stable even in the absence of bacteria.

3.6 Acknowledgements

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Supplementary Data

S3.1 Example measurements of cell length of *A. minutissimum* live cells

Chapter 4

Biofilm and capsule formation of the diatom *Achnantheidium minutissimum* are strongly affected by a bacterium

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Chapter 4: Biofilm and capsule formation of the diatom *Achnantheidium minutissimum* are strongly affected by a bacterium

4.1 Abstract

Photoautotrophic biofilms play an important role in various aquatic habitats and are composed of prokaryotic and/or eukaryotic organisms embedded in extracellular polymeric substances (EPS). We have isolated diatoms as well as bacteria from freshwater biofilms in order to study organismal interactions between representative isolates. We found that bacteria may have a strong impact on the biofilm formation of the pennate diatom *Achnantheidium minutissimum* (Kützing) Czarnecki. This alga produced extracellular capsules of insoluble EPS, mostly carbohydrates, only in the presence of bacteria (xenic culture). The EPS itself also had a strong impact on the aggregation and attachment of the diatom. In the absence of bacteria (axenic culture), *A. minutissimum* did not form capsules and the cells grew completely suspended. For investigation of biofilm formation by *A. minutissimum*, a bioassay was established using a diatom satellite *Bacteroidetes* bacterium which had been shown to induce capsule formation of *A. minutissimum*. Interestingly, capsule and biofilm induction could be achieved by addition of bacterial spent medium, indicating that soluble hydrophobic molecules produced by the bacterium may mediate the diatom/bacterium interaction. Fractionation and quantification of carbohydrates revealed that the diatom in axenic culture produced large amounts of soluble carbohydrates, whereas in the xenic culture mainly insoluble carbohydrates were detected.

Keywords: *Achnantheidium* · biofilm · capsule · diatom · EPS

4.2 Introduction

Photoautotrophic biofilms are a typical feature in the littoral zones of lakes, streams and oceans. Stones or any other substrata can be covered by a brownish or greenish mucous layer, whenever sufficient light and water is available. These biofilms are a habitat of high primary production (Wetzel 1964) and may be responsible for sediment stabilization (Wigglesworth-Cooksey et al. 2001). However, biofilms also have negative effects, for instance biofouling on human made surfaces like ship hulls or pipes causes high costs in shipping and water management (Gaylarde and Morton 1999; Schultz et al. 2011; Wingender and Flemming 2011). Besides exogenous factors like light, wave disturbance, temperature, water level fluctuations as well as grazing pressure (Hoagland and Peterson 1990; Schmieder et al. 2004; Rao 2010), the formation of photoautotrophic biofilms is also strongly influenced by the physiology of the inhabitants of the biofilms and their interactions. Diatoms are common members and early colonizers of photoautotrophic biofilms (Cooksey and Wigglesworth-Cooksey 1995; Wetherbee et al. 1998) and their productivity may have a strong influence on the whole biofilm. They can produce copious amounts of extracellular polymeric substances (Myklestad et al. 1989) which are classified as cell bound EPS like stalks, tubes and capsules (Hoagland et al. 1993) or soluble EPS. Diatoms are generally associated with bacteria belonging mostly to α -, β - and γ -*Proteobacteria*, to *Bacteroidetes* and to *Actinobacteria* (Knoll et al. 2001; Sapp et al. 2007c; Stanish et al. 2012). Interactions between diatoms and bacteria may occur on different levels and can span from synergy via competition to parasitism or defence reactions (Amin et al. 2012). Algae as primary producers provide organic substrates which serve as energy and carbon source for heterotrophic bacteria (Cole 1982). Bell and Mitchell (1972) introduced the “phycosphere” concept, describing the zone around the algal cell “in which bacterial growth is stimulated by extracellular products of the alga”. The diatoms may in turn require essential compounds from the bacteria, e.g. vitamins (Croft et al. 2005). Little is known about the molecular processes underlying diatom/bacteria interactions within these biofilm communities. Biofilm inhabitants may affect the physiology of other organisms by soluble molecules, indicating that many of these interspecific interactions are based on chemical signals released by diatoms and by bacteria. Thomas and Robinson (1987) observed that the exudates of the xenic diatom *Amphora coffeaeformis* led to enhanced tolerance of the diatom against copper and tributyltin fluoride (TBTF). This suggests that either bacterial substances themselves or algal exudates induced by bacteria may trigger the stress response of *A. coffeaeformis*. Such unknown substances

may act as signals which mediate recognition and communication between the interaction partners or directly cause a specific effect as e.g. toxic compounds. Amin et al. (2012) suggested that substances which are used for intraspecies communication, like autoinducers in bacterial quorum sensing or pheromones in the case of diatoms, might also be used for interspecific interactions between diatoms and bacteria. Such interkingdom signalling was already described to play a role for the seaweed *Ulva*, where zoospores are attracted by bacterial biofilms via released *N*-acylhomoserine lactones (AHLs) (Joint et al. 2007). These AHLs are common autoinducers of Gram-negative bacteria (Chhabra et al. 2005). However, as Amin et al. (2012) stated, a reliable bioassay comprising a diatom/bacterium pair with a stable interdependency is needed to elucidate the molecular and chemical basics of these interactions.

The goal of this study was to establish a model system for studying the interaction of benthic diatoms and bacteria during biofilm formation. The model organisms, *Achnantheidium minutissimum* (Kützing) Czarnecki and *Bacteroidetes* strain 32, were isolated from photoautotrophic, epilithic biofilms taken from the littoral zone of Lake Constance. *A. minutissimum* (renamed from *Achnanthes minutissima* (Kützing) (Czarnecki 1994)) is one of the most abundant freshwater diatoms (Patrick and Reimer 1966; Krammer and Lange-Bertalot 1991b). It is frequently found in epilithic biofilms of Lake Constance and represents an eukaryotic pioneer during the initial processes of biofilm formation (Sekar et al. 2004). Bacteria of the *Bacteroidetes* phylum are frequently associated with diatoms (Amin et al. 2012) and were also found to be prominent in xenic diatom isolates from photoautotrophic, epilithic biofilms of Lake Constance (Bruckner et al. 2008). *Bacteroidetes* strain 32 belongs to the *Dyadobacter* genus. Bacteria of this group were found in freshwater, soil samples or to be associated with maize (Chelius and Triplett 2000; Baik et al. 2007; Zhang et al. 2010).

4.3 Materials and Methods

4.3.1 Organisms and cultivation conditions

Achnantheidium minutissimum (Kützing) Czarnecki was isolated from photoautotrophic, epilithic biofilms of Lake Constance (Windler et al. 2012). The diatom was either cultivated with co-isolated bacteria as “xenic culture” or additionally as “axenic culture” after removal of associated bacteria as described earlier (Windler et al. 2012). The diatom stock cultures were cultivated in a modified liquid Bacillariophycean Medium (BM) (Windler et al. 2012) in cell culture flasks with ventilation caps (Sarstedt, Newton USA), in

which the cells could form a biofilm on the vessel surface. Monthly, these cultures were scraped off and sub-cultured into new BM. For aggregation, the xenic and axenic diatom cultures were cultivated at 100 rpm on an orbital shaker (type 3019; GFL, Burgwedel, Germany). The diatom cultures were exposed to a 12:12 h light:dark cycle with light intensities of 20-50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 16°C (in this study denoted as standard cultivation conditions for diatoms).

Bacteroidetes strain 32 was isolated by Bruckner et al. (2008). This bacterium was enriched from a xenic *Cymbella microcephala* Grunow strain D-32 culture, which originated from photoautotrophic, epilithic biofilms from the same sampling site as *A. minutissimum* (47° 41' N; 9° 11' E, Germany) (Bahulikar 2006). The bacterium was cultivated at 22°C on agar plates containing 50% (v/v) Luria Broth medium (diluted LB (Miller 1972)), sub-cultivated monthly and stored at 4-8°C.

4.3.2 Staining procedures and microscopy

Carbohydrates associated with cells were stained either with alcian blue or crystal violet. For crystal violet staining, we used a 1:100 dilution of the Gram-staining reagent described by Kaplan and Fine (2002). Supernatants of the diatom cultures were gently removed, the dye was added to the cultures and incubated for 1 min. Excessive dye was removed and the cultures were rinsed with water to remove non-attached cells (Izano et al. 2007). To quantify biofilm formation, crystal violet was extracted from the cultures with 1 ml ethanol and the absorption of the dye was determined photometrically at 580 nm. The alcian blue dye solution was modified according to Staats et al. (1999), here alcian blue (1% (w/v); Roth, Germany) in 3% acetic acid was added and the cultures were washed once with water.

Cells were observed with an upright light microscope (Olympus BX51; Olympus Europe, Hamburg, Germany), equipped with the filter set 41020 (Chroma Technology Corp, Rockingham, VT, USA) or by using an inverted light microscope (Axiovert 40 C; Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were taken with the Zeiss AxioCam MRm or MRc digital camera systems (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

4.3.3 Co-cultivation experiments and bioassay for biofilm formation

A. minutissimum was cultivated either with bacteria, the sterile supernatant of *Bacteroidetes* strain 32, or single fractions of the bacterial supernatant. The biofilms were stained as described above. In the following, *A. minutissimum* together with all co-isolated

bacteria is termed a “xenic culture”, whereas the purified diatom in co-culture with strain 32 is termed a “co-culture”. Cultivation was performed in BM at standard cultivation conditions for diatoms (see above) in well plates and was stopped by gently removing the culture supernatant.

Comparison of diatom growth in xenic and axenic cultures

Axenic *A. minutissimum* cells were washed with fresh BM and 10^5 cells ml⁻¹ were cultivated in 500 µl BM at standard cultivation conditions for diatoms (see above). For the co-cultivation experiments, *Bacteroidetes* strain 32 was grown in diluted LB (50% (v/v)), washed three times with BM to remove the bacterial medium and the strain 32 suspension was adjusted to an optical density at 600 nm (OD₆₀₀) of 0.1, of which 5 µl were used to inoculate axenic *A. minutissimum* cultures. The xenic *A. minutissimum* cultures were inoculated with the same chlorophyll (chl) concentration as the axenic cultures and the co-cultures. The chl concentrations of suspended cells in the supernatants (non-adherent cells) and of cells embedded in the biofilm (adherent cells) were determined as described below. Each experiment was conducted in triplicates.

*Induction of capsule and biofilm formation in the axenic *A. minutissimum* culture by co-cultivation with *Bacteroidetes* strain 32 or its sterile supernatant*

Xenic cultures, axenic cultures and co-cultures with *Bacteroidetes* strain 32 were performed as described above and biofilms were stained with crystal violet solution or alcian blue.

For those experiments requiring the sterile supernatant of the bacterial culture, strain 32 was cultivated in liquid BM supplemented with 10 mM glucose (glcBM) at 20°C and 135 rpm. The supernatant of the bacterial culture was harvested when the culture reached an OD₆₀₀ of 0.235. The culture was centrifuged at 5525 x g (Allegra™ 25R centrifuge with TS-5.1-500 rotor and swinging buckets, 10 or 50 ml tubes, depending on the volume of the supernatant; Beckman Coulter, Germany) and the supernatant was filtered using a 0.2 µm filter (polyethersulfone, Filtropur S; Sarstedt, Germany). The sterility of the filtrate was randomly tested by plating an aliquot on agar plates containing diluted LB medium (50% (v/v)) and incubated as described for *Bacteroidetes* strain 32. $2.8 \cdot 10^6$ cells ml⁻¹ of the axenic diatom culture, previously washed and resuspended in fresh BM, were incubated with different volumes of the sterile bacterial supernatant and complemented with BM to a total volume of 500 µl. Equivalent volumes of glcBM were added to axenic diatom cultures for

negative controls. Each experiment was conducted in triplicates. After a cultivation period of 30 days at diatom standard cultivation conditions, the cultures were stained with crystal violet and the biofilms were quantified as described above.

For fractionation, 10 ml of the bacterial supernatant harvested at an OD₆₀₀ of 0.239, were separated via solid-phase extraction (SPE) using C18-SPE endcapped cartridges (530 mg; Macherey-Nagel, Germany) according to Von Elert and Pohnert (2000). The bacterial supernatant and medium control were adjusted to pH 7 with citric acid or sodium hydroxide. Flow-through, wash fraction and eluate were collected separately. Flow-through and wash fraction were evaporated to near dryness and diluted in 1 ml BM. The eluate was evaporated to dryness to remove methanol and resuspended in 2 ml BM. Aliquots of this fraction were incubated for 1 h at 30 and 80°C, respectively, for further dissolution of solid material. The bacterial growth medium (glcBM) was treated the same way and was used as a negative control. The fractions were sterile filtered as described above and aliquots were diluted with BM to the original concentration (1:5 for the eluate, 1:10 for flow-through and wash fraction). The fractions were tested for bioactivity and applied to the bioassay. $7 \cdot 10^5$ cells ml⁻¹ of the axenic diatom culture were incubated with 250 µl of the concentrated and diluted fractions in a total volume of 500 µl. The unseparated bacterial supernatant was used as positive control. The cultures were incubated at standard cultivation conditions for diatoms for 11 days.

For further fractionation, 910 ml of the sterile bacterial supernatant of the *Bacteroidetes* strain 32 culture (OD₆₀₀ of 0.18) were harvested by centrifugation and successive filtration through 3.0 µm membrane filter (mixed cellulose esters; Millipore, Ireland), 0.2 µm filter (mixed cellulose esters; Whatman, Germany) and 0.2 µm filter (Filtropur S; Sarstedt, Germany). The supernatant was loaded on an endcapped C18-SPE column (10 g; Macherey-Nagel, Germany) and was eluted stepwise with methanol at increasing concentrations (20%, 40%, 60%, 80% and 100% methanol in ultrapure water). Each fraction was eluted with 20 ml of the solvent, except the 100% methanol fraction. This fraction was eluted with 25 ml. After evaporation, the residues were resuspended in 1 ml of ultrapure water, sterile filtered and diluted in BM. The fractions were tested in duplicate for bioactivity. $1.2 \cdot 10^6$ cells ml⁻¹ of the axenic diatom culture were incubated with 250 µl of the diluted fractions in a total volume of 500 µl. Quantity of the biofilm was determined after 12 days of incubation as described above.

The sterile bacterial supernatant was harvested at different growth phases of *Bacteroidetes* strain 32, which was cultivated in 100 ml glcBM in triplicates. 1.2 ml were removed at each time point. 100 µl were used to measure OD₆₀₀ of the bacterium and the

supernatant of the remaining volume was sterile filtered as described above. Half of the volume was frozen for determination of the glucose concentration via HPLC as described in Jagmann et al. (2010). The other half volume was stored at 4°C until it was tested for bioactivity. $1.2 \cdot 10^6$ cells ml⁻¹ of the axenic diatom culture were treated with 50% (v/v) of the bacterial supernatant in a volume of 500 µl. Each approach was performed in triplicates. Biofilms were stained and quantified after 11 days of cultivation.

4.3.4 Quantification of soluble and bound carbohydrates

Triplicates of xenic as well as axenic cultures were cultivated in 100 ml BM in cell culture flasks at standard diatom cultivation conditions. The axenic cultures were inoculated at a cell density of 10^3 cells ml⁻¹. The chl concentrations were determined as described below and the xenic cultures were inoculated at the same chl concentrations as the axenic cultures. Soluble and bound EPS of xenic and axenic *A. minutissimum* were stepwise extracted according to the protocol established for isolation of “soluble EPS (SE)”-, “warm water soluble EPS (WW)”-, “hot water soluble EPS (HW)”-, “hot bicarbonate soluble EPS (HB)”- and “hot alkali soluble EPS (HA)”-fractions according to Bahulikar and Kroth (2008) with modifications: The cells were scraped from the bottom of the tissue culture flasks and the suspensions were centrifuged for 20 min at $5525 \times g$ and 20°C (Allegra™ 25R centrifuge with TS-5.1-500 rotor and 50 ml swinging buckets; Beckman Coulter, Germany). The supernatants containing the soluble carbohydrates were carefully separated by decanting from the cell pellets containing the bound carbohydrates. The supernatants were concentrated to 5 ml using a rotary evaporator. Polymers were precipitated overnight in 5 volumes of 96% ethanol at -20°C. The precipitates were centrifuged for 20 min and 4°C at $4300 \times g$ without active deceleration (Megafuge 1.0R with swing-out rotor #2705; Heraeus Instruments, UK), the pellets were dried under nitrogen gas and subsequently dissolved in 1 ml ultrapure water. To increase solubility, the suspensions were acidified with 2-10 µl concentrated H₂SO₄ and heated to 60-80°C when required to dissolve the pellets. The ethanol fractions containing the oligo- and monomers were evaporated and the pellets were dried and processed as described for the polymers. The bound carbohydrates were extracted stepwise. First, the cell pellets were resuspended in 1 ml sterile-filtered tap water to keep the osmolarity in the physiological range and incubated for 1 h at 30°C and 300 rpm. Cells were centrifuged ($5000 \times g$ for 10 min at 20°C, 5417R microcentrifuge; Eppendorf, Germany) and the supernatants containing the “warm water soluble EPS” (WW) were separated from the pellets. The following centrifugation steps were conducted at $5000 \times g$ for 7 min at 4°C (5417R microcentrifuge;

Eppendorf, Germany). Pellets were defatted by incubation in 1 ml 90% ethanol at 600 rpm for 15 min and subsequently centrifuged (Wustman et al. 1997; Bahulikar and Kroth 2008). This procedure was repeated 5-7 times until the pellets were colourless or slightly green. Cells were further washed twice with distilled water, then incubated in 1 ml distilled water at 90°C for 1 h and centrifuged. The supernatants were used for determination of the “hot water soluble EPS” (HW) and the cell pellets were incubated for 1 h in 1 ml 0.5 M NaHCO₃ and 0.1 M EDTA at 95°C (Chiovitti et al. 2003) to harvest the “hot bicarbonate soluble EPS” (HB). The remaining portion of the bound EPS was extracted by resuspending the pellets in 1 ml 1 M NaOH and 0.2 M NaBH₄ at 95°C for 1 h and subsequent centrifugation of the cell fragments, resulted in “hot alkali soluble EPS” (HA). Mono-/oligo- and polysaccharides of the soluble carbohydrate fractions as well as the total carbohydrate content of the WW-, HW-, HB- and HA-fractions were measured using the phenol-sulphuric acid assay (Dubois et al. 1956) according to De Brouwer et al. (2002) and were specified as µg carbohydrate per µg chl (µg CHO·(µg chl)⁻¹).

4.3.5 Determination of chlorophyll concentration

Chlorophyll was extracted by addition of a mixture of 5 % methanol and 95 % acetone to the diatom cell pellet. The chl of surface-adherent cells was extracted by flushing the biofilm with the solvent (5% methanol diluted in acetone) for several times directly in the cultivation well. Chl concentrations were determined using the equation and extinction coefficients for diatoms, chrysomonads and brown algae according to Jeffrey and Humphrey (1975), and specified as total chl concentration (a sum of chl *a* and *c*).

4.4 Results

4.4.1 Differences in biofilm formation of xenic and axenic *Achnantheidium minutissimum*

A. minutissimum is a benthic diatom growing attached to surfaces. Interestingly we observed that xenic and axenic *A. minutissimum* cells showed very different aggregation behaviour. When cultivated on a shaker the xenic *A. minutissimum* cells formed macroscopically visible aggregates, while the axenic diatom culture grew completely suspended and aggregate formation was not visible (Figure 4.1A). Under non-shaking conditions, the xenic diatom cells were attached to the surface of the cultivation vessel forming a biofilm. Microscopic analyses in combination with alcian blue staining revealed that the cells in the late stationary phase were surrounded by large capsules of bound EPS

which were formed in the early stationary phase (Figures 4.1B and C). The respective diatom cells were attached to each other via the capsules partially resulting in macroscopically visible aggregates. Cells of the axenic culture did not show any capsules, remaining freely dispersed, forming, if at all, only small aggregates of a few cells. In addition to the capsules, the xenic cultures showed diffuse EPS structures that were stainable by alcian blue (Figure 4.1 B). This diffuse, unstructured form of EPS could also be found in the axenic culture.

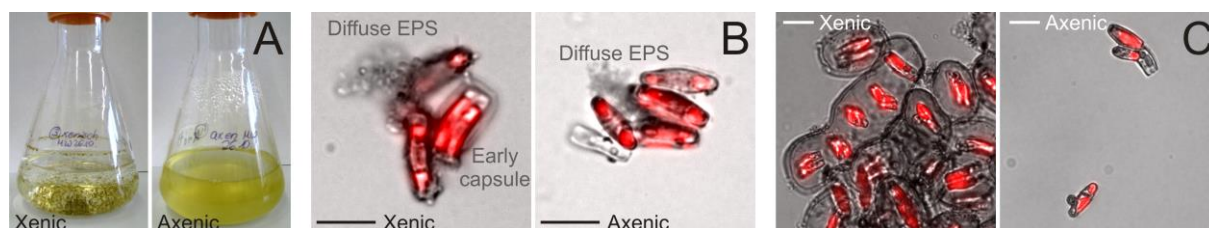


Figure 4.1: Xenic and axenic cultures of the diatom *Achnantheidium minutissimum*. A) The xenic culture with co-isolated bacteria from the original habitat and the axenic culture after cultivation on a shaker. B) Alcian blue stained cells with diffuse EPS in the xenic and axenic cultures and early capsule formation in the xenic culture after 20 days of cultivation (early stationary phase). C) Xenic culture with fully developed capsules and the axenic culture without such structures after 33 days of cultivation (late stationary phase). Autofluorescence of chl appears in red. Scale bars denote 10 μm .

4.4.2 Diatom growth in xenic and axenic cultures and in co-culture with *Bacteroidetes* strain 32

Growth of the cultures was determined by measuring the chl concentration (Figure 4.2). This was important, because of the strong tendency of the co-culture as well as of the xenic culture to form biofilms, making it difficult to determine cell growth by counting methods. We therefore chose the chl concentration to represent a surrogate parameter for diatom growth. The axenic cultures reached a similar maximum of chl content as the xenic culture and the co-culture ($0.78 \pm 0.13 \mu\text{g ml}^{-1}$ after 10 days for the axenic culture, $0.86 \pm 0.05 \mu\text{g ml}^{-1}$ and $0.81 \pm 0.06 \mu\text{g ml}^{-1}$ after 7 days for the xenic culture and co-culture, respectively; Figure 4.2A). Interestingly, the chl content of the xenic culture and co-culture showed a plateau in the late stationary phase after 17 days of cultivation, whereas the chl concentration of the axenic culture decreased continuously. In the xenic culture and in the co-culture the main chl concentrations were found in the biofilm fraction, indicating that these cultures consisted mostly of adherent cells (Figures 4.2B and C). Nearly no chl was detected in the supernatant after 10 and 14 days of cultivation, respectively, at a time when the cells exhibited capsules. In contrast, most of the chl of the axenic culture was found in the non-adherent fraction (Figure 4.2D).

The bacterium was not able to grow in BM in the absence of the diatom (Figure S4.1 Suppl. Data S4.1). Determination of bacterial growth within the biofilm was not performable due to technical constraints.

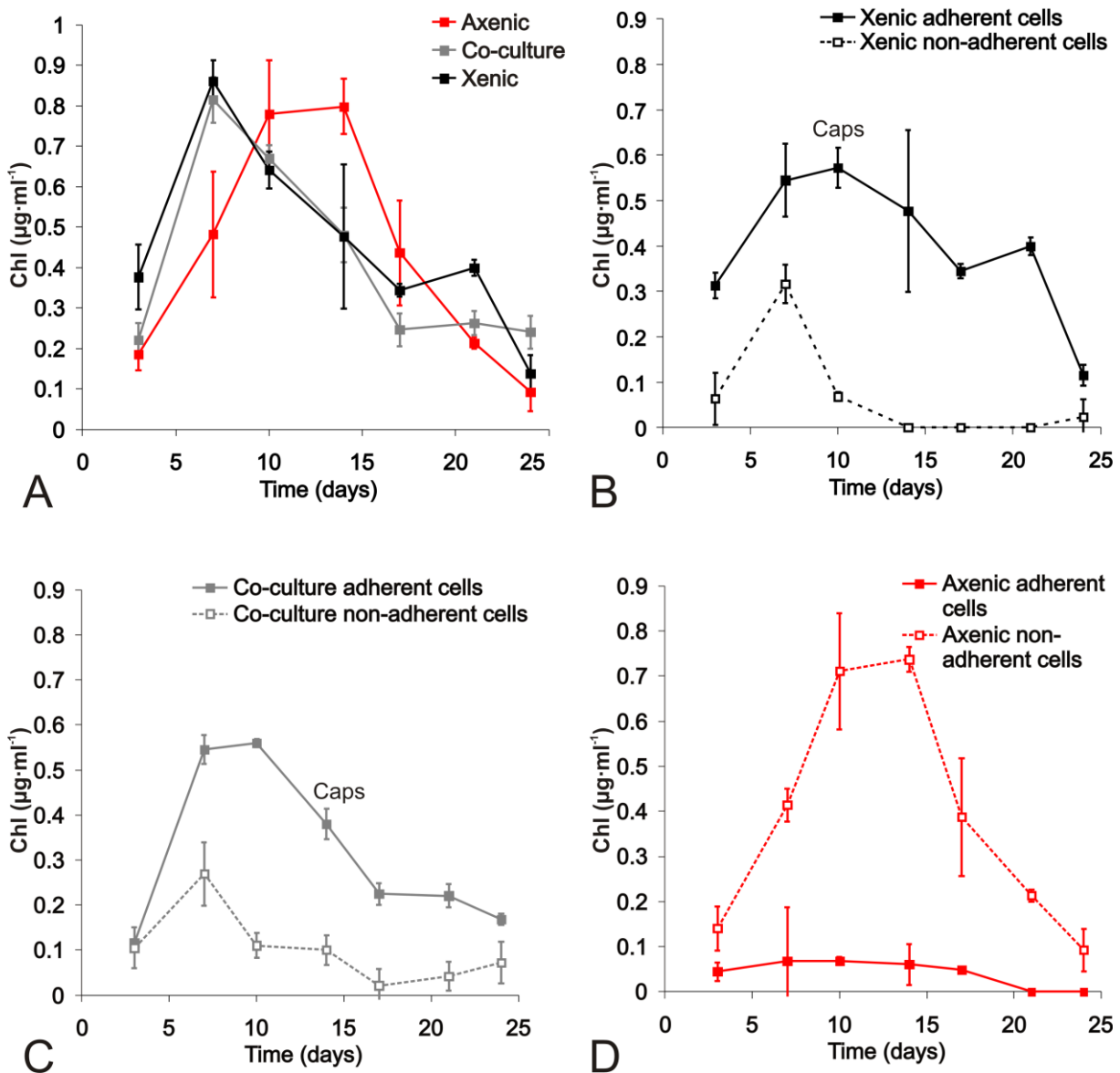


Figure 4.2: Chlorophyll concentrations of *Achnanthisidium minutissimum* in axenic and xenic conditions and in the co-culture with *Bacteroidetes* strain 32. A) Sum of the chl concentrations of adherent and non-adherent cells of the cultures. B) Chl concentrations of adherent and non-adherent cells of the xenic culture, C) of the co-culture with strain 32 and D) of the axenic culture. Capsules (Caps) in the xenic and co-culture are formed after 10 and 14 days of cultivation, respectively (n=3; error bars indicate standard deviation).

4.4.3 Induction of capsule and biofilm formation in the axenic *Achnanthydium minutissimum* culture

For further analysis of biofilm formation of *A. minutissimum*, a bioassay was established according to Izano et al. (2007), including fast quantification of biofilm by staining of bound carbohydrates with crystal violet. The dye stains the cells as well as the bound EPS and its intensity depends on the amount of attached cells and insoluble EPS and therefore is proportional to the biomass.

We found that the biofilm formation of the xenic culture and of the co-culture with *Bacteroidetes* strain 32 is reflected by much higher crystal violet adsorption as compared to the axenic diatom culture (Figures 4.3A and B). The stable biofilm of the xenic culture allowed rough washing steps because the cells adhered strongly to the surface of the wells and resulted in high crystal violet absorption already after three days of cultivation (Figure 4.3B). At that time, the chl concentration was still relatively low in the xenic culture ($0.38 \mu\text{g ml}^{-1}$; Figure 4.2A). The co-culture also exhibited considerably strong biofilm formation after three days and reached the highest values of crystal violet absorption after 10 days (Figure 4.3B). After three days of cultivation the diatom cells in the xenic culture and in the co-culture exhibited stalks that apparently mediate adherence in early stages of biofilm formation. Such stalks are representatively shown in Figure 4.3C for the co-culture. The cells of the xenic culture started with capsule formation after 10 days, those of the co-culture after 14 days of cultivation when cells were in the stationary phase. However, in the axenic culture, there was no biofilm and capsule formation observable even after 24 days. After staining only a few cells were left in this culture, indicating that the cells did not adhere to the well surface and were removed during the wash steps of the staining procedure. In some cases crystal violet did not stain the capsules properly, especially when the biofilm was fully developed. In these cases the cultures were additionally stained with alcian blue for microscopic observation and visualisation of capsules in the xenic culture and co-culture (Figure 4.3C). Taken together our results indicate that biofilm and capsule formation of the axenic *A. minutissimum* can be induced by co-cultivation with *Bacteroidetes* strain 32.

Capsule and biofilm induction by the sterile supernatant of Bacteroidetes strain 32

Bacteroidetes strain 32 was cultivated in BM supplemented with 10 mM glucose (glcBM) before harvesting of the spent medium. This medium allowed bacterial growth but did not have inhibiting effects on diatom growth as we observed for diluted LB. Capsule and biofilm formation of *A. minutissimum* can be induced by incubation of the axenic culture with

the sterile filtered supernatant of *Bacteroidetes* strain 32 (Figures 4.3A and 4.4). The intensities of crystal violet adsorption of *A. minutissimum* cultures showed a dose depended pattern when treated with different volumes of the sterile bacterial supernatant and reached a maximal absorption after addition of 25% and 50% (v/v) of the bacterial supernatant (Figure 4.4A). Neither the control cultures, which had been treated with glcBM only, nor the axenic culture did induce a comparable biofilm formation. Diatom capsules were found in cultures which had been supplemented with 12.5%, 25% and 50% (v/v) of the bacterial supernatant. The respective control cultures did not show any capsule formations. This indicates that biofilm and capsule formation of *A. minutissimum* are inducible by soluble molecules released by the bacterium.

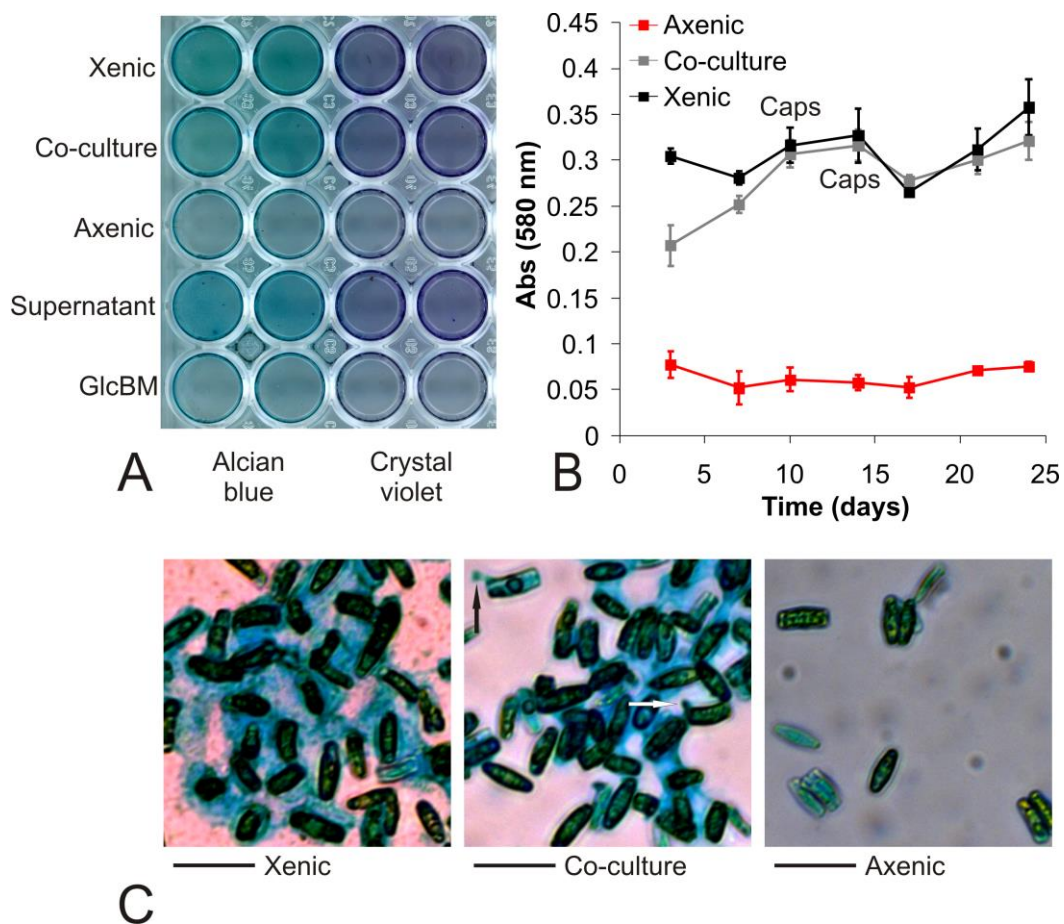


Figure 4.3: Induction of capsule and biofilm formation in the axenic *Achnantheidium minutissimum* culture. A) Macroscopic image of the alcian blue or crystal violet stained cultures of the diatom cultivated in a 48-well plate. Stronger staining of the xenic cultures, the co-cultures and of the cultures which were treated with the bacterial supernatant compared to the controls (Axenic, GlcBM) indicates biofilm formation. B) Absorption of crystal violet extracted from the axenic culture, the co-culture with *Bacteroidetes* strain 32 and the xenic diatom culture. Increased biofilm formation of the xenic and co-culture is visualized by a stronger crystal violet adsorption compared to the axenic diatom culture. The xenic culture started to form capsules (Caps) after 10 days, the co-culture after 14 days of cultivation (n=3; error bars indicate standard deviation). C) Microscopic images: Cells of the xenic culture, the co-culture with strain 32 and axenic *A. minutissimum* culture stained with alcian blue after 21 days of cultivation. Arrows mark stalks of the diatom cells. Scale bars denote 20 μ m.

For enrichment of the biofilm-inducing substance(s), we further separated the bacterial supernatant by solid phase extraction (SPE) according to Von Elert and Pohnert (2000). The fractions were diluted to the original concentration previous to application and Figure 4.4B shows the treatment with the diluted fractions. The eluate fraction (Elu) showed a similar induction of biofilm formation as the unseparated bacterial spent medium (positive control) and capsule formation was only found to be induced by this fraction. The methanol used as eluent was completely evaporated, therefore we can exclude an induction of capsule and biofilm formation by the alcohol itself. As a further control, the solid phase extraction was performed with bacterial growth medium in an identical way. Here capsule or biofilm formation was not observed in any of the control fractions including the eluate. Flow-through (Ft) and wash fraction (Wf) of the extracted bacterial spent medium did not induce a considerably stronger biofilm formation as the fractions of the bacterial growth medium, indicating that the biological activity was completely absorbed by the nonpolar C18-sorbent and fully extracted by elution with methanol. Incubation of the bioactive fraction at 30°C and 80°C as well as evaporation to dryness did not diminish the activity of the fraction compared to the positive control. The undiluted fractions did not differ remarkably in biofilm formation (data not shown). The results suggest a nonpolar character of the bioactive molecule which also is resistant to heat and dryness.

The bacterial supernatant was also eluted from the C18-SPE column with increasing methanol concentrations. The bioactivity was found in fractions which were eluted with high methanol concentrations, thus increased biofilm formation was mainly found in *A. minutissimum* cultures which had been treated with the 60-100% methanol fractions and capsules were only found in cultures treated with the 80-100% methanol fractions (Figure 4.4C).

To investigate the optimal growth phase of the bacterium for sufficient production of the bioactive substance(s), the bacterial supernatant was harvested at different time points and each supernatant was tested for bioactivity. Figure 4.5A shows the growth of *Bacteroidetes* strain 32 in glcBM. The bacterium reached an OD₆₀₀ of 0.24 in the stationary phase. Glucose consumption resulted in a decrease of the glucose concentration from 10.3 to 8.5 mM during this time. Biofilm formation of *A. minutissimum*, as based on an increase of extractable crystal violet adsorption from 0.16 to 0.35, was already induced by the bacterial supernatant taken in the early exponential growth phase of the bacterium at an OD₆₀₀ of 0.016 (Figure 4.5B). Accordingly, capsule formation was only induced by supernatants taken after the bacterium reached the mid-exponential phase at an OD₆₀₀ of 0.03.

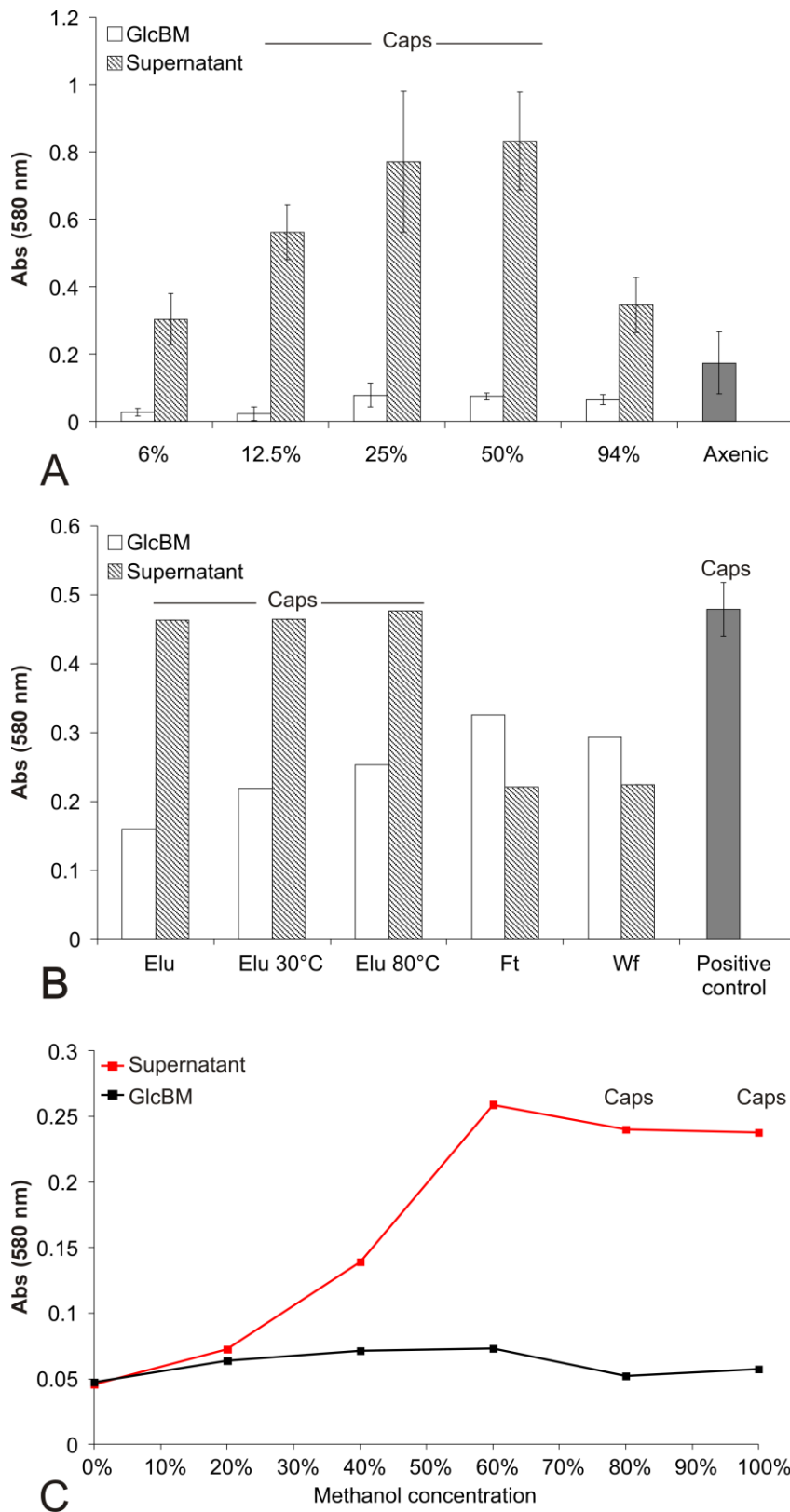


Figure 4.4: Induction of capsule and biofilm formation of *Achnanthyidium minutissimum* with the sterile bacterial supernatant of *Bacteroidetes* strain 32. Biofilms were stained with crystal violet and the absorption of the dye was determined. Cultures exhibiting capsules are marked with (Caps). A) Incubation with 6%, 12.5%, 25%, 50% and 94% (v/v) of the sterile supernatant. The control assays were conducted either by addition of equivalent volumes of the bacterial medium (glcBM) or with the pure axenic diatom culture (n=3). B) *A. minutissimum* incubated with different SPE-fractions of the bacterial supernatant: eluate (Elu), flow through (Ft) and wash fraction (Wf). The graphic shows cultures treated with fractions which were diluted to the original concentration. Eluate fractions were additionally heated to 30 and 80°C. Fractions of the bacterial medium glcBM and the unseparated bacterial supernatant were used for negative and positive control (n=1 for the fractions, n=3 for the control). C) *A. minutissimum* incubated with different fractions of the separated bacterial supernatant, which were eluted with increasing methanol concentrations. Fractions of the bacterial growth medium glcBM were used for negative controls (n=2). Error bars indicate standard deviation.

4.4.4 Analysis of carbohydrates

We analysed the carbohydrate content and quality by stepwise extraction of soluble and insoluble EPS from xenic and axenic *A. minutissimum* cultures as described in the Materials and Methods section. Determination of carbohydrate amounts in each fraction showed that in the late exponential growth phase, after 12 days of cultivation, the axenic and xenic cultures exhibited similar amounts of bound carbohydrates (Figure 4.6A). At that time, the xenic diatoms did not show capsules and the axenic cells secreted about five-fold more soluble carbohydrates as those of the xenic culture. In the early stationary phase, after 20 days of cultivation, the amount of bound carbohydrates in the HB fraction of the xenic culture increased (Figure 4.6B) and light microscopy indicated that the xenic cells started to form capsules (Figure 4.1B). The capsules were fully developed in the late stationary phase, after 33 days, when the HW and HB fractions exhibited large amounts of insoluble carbohydrates (Figures 4.1C and 4.6C). Thus, the capsular material of the xenic culture can be dissolved with the hot water and hot bicarbonate treatments, leaving only very little carbohydrate material in the hot alkali fraction. During the whole cultivation period, the amount of soluble carbohydrates remained relatively low in the xenic culture, especially in the monomer fractions. The axenic *A. minutissimum*, on the other hand, showed less carbohydrates in the insoluble fractions but produced large amounts of soluble carbohydrates.

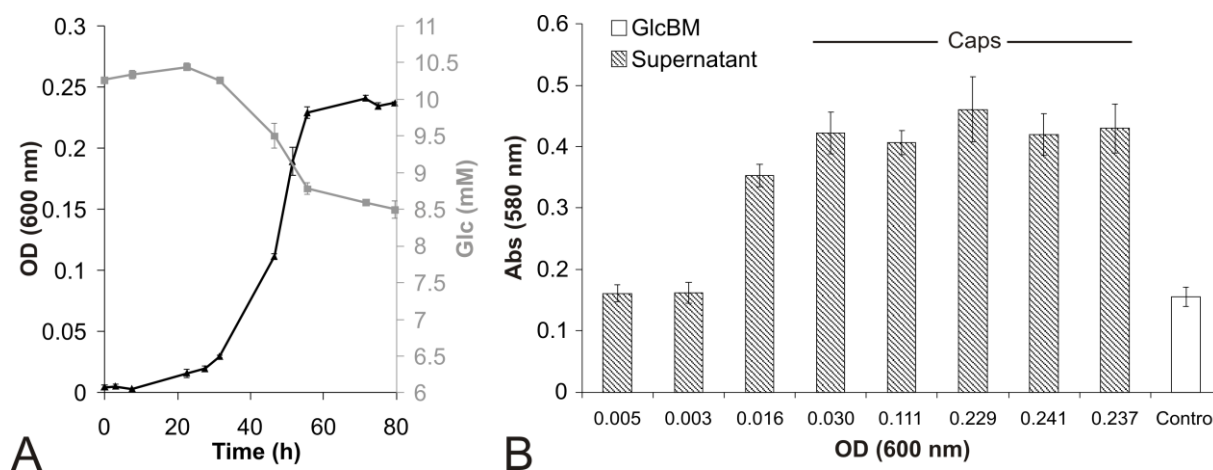


Figure 4.5: Biofilm induction according to different growth phases of *Bacteroidetes* strain 32. A) Optical density (OD₆₀₀) of strain 32 in glcBM (black) and simultaneous decrease of the glucose concentration (grey). B) Intensity of crystal violet extracted from *A. minutissimum* cultures treated with the sterile supernatant of strain 32. The bacterial supernatant was harvested at different growth phases of the bacterium (measured by means of OD₆₀₀). Capsules (Caps) were induced when treated with supernatant harvested at a bacterial OD₆₀₀ of 0.03 and above. The diatom culture was treated with glcBM for control. (n=3; error bars indicate standard deviation).

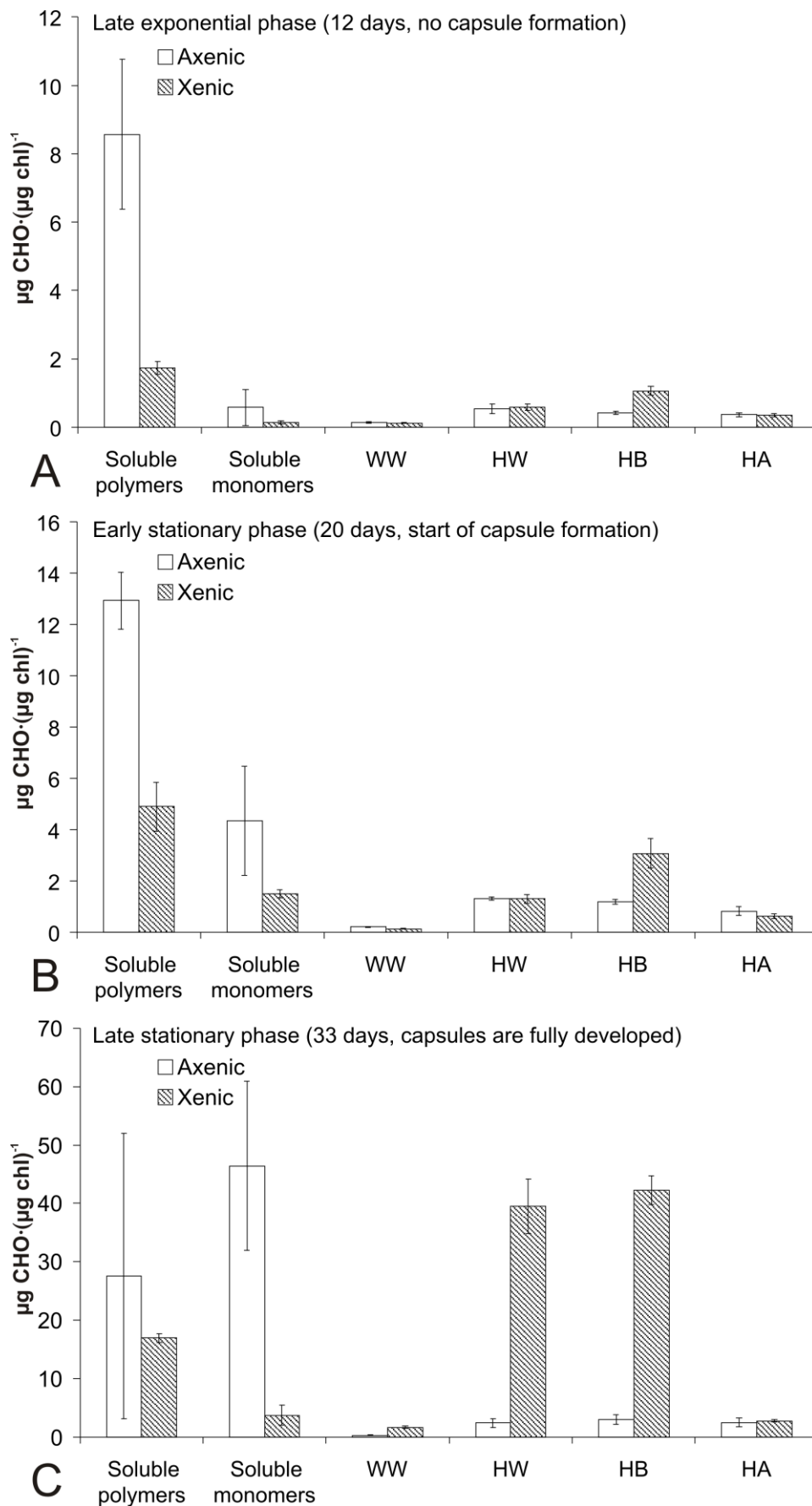


Figure 4.6: Quantification of soluble and bound carbohydrates of axenic and xenic *Achnanthes minutissimum* cultures. Carbohydrate quantity is specified in $\mu\text{g CHO}\cdot(\mu\text{g chl})^{-1}$. Carbohydrates were extracted at different growth phases of the diatom: late exponential growth phase (12 days of cultivation), early stationary phase (20 days of cultivation) and late stationary phase (33 days of cultivation). Soluble carbohydrates were measured as soluble polymers and monomers. Bound carbohydrates were stepwise extracted with warm water (WW), hot water (HW), hot bicarbonate (HB) and hot alkali (HA). (n=3; error bars indicate standard deviation).

4.5 Discussion

When cultivated in presence or absence of distinct bacteria, the diatom *A. minutissimum* showed a considerably different behaviour regarding cell aggregation, cell adhesion and biofilm formation, which is most likely due to a changed generation of soluble and insoluble EPS by the diatom. Positive as well as negative bacterial influences on aggregation formation have been described in the context of marine snow, aggregated material of cells and organic matter in the oceans (Grossart et al. 2006; Gärdes et al. 2010) and several possible mechanisms have been discussed. Bacteria may increase the amount of matrix material either by contribution of bacterial EPS (Decho 1990) or by stimulation of EPS secretion by the algae. Also bacterial modification of organic material and subsequent changes of the adhesive properties were suggested (Grossart et al. 2006). Cellular aggregation is of ecological relevance as it increases the sinking velocity and thus plays an important role in the flux of organic matter into deeper parts of the water column (Fowler and Knauer 1986; Gärdes et al. 2010). It also may alter the consumption by grazers and thereby the transfer of organic matter to upper trophic levels (Decho 1990).

Under non-shaking conditions, we found that the xenic *A. minutissimum* cells stick to the well surface and the microstructures of the biofilm exhibit capsules of extracellular organic matter. The axenic culture revealed no such capsules and the cells were completely detached. The missing capsule and biofilm formation of axenic *A. minutissimum* could also be re-induced by addition of *Bacteroidetes* strain 32. From eight other tested bacterial isolates in our hands also some led to stronger biofilm formation, but none of them was able to induce capsule formation (Figure S4.2 Suppl. Data S4.2). We made similar observations in earlier experiments showing that capsulation and altered EPS structures of the freshwater biofilm diatom *Cymbella microcephala* occur in co-culture with strain 32, indicating that this might be a specific property of the bacterium (Bruckner et al. 2008).

Interestingly, *A. minutissimum* reacted with biofilm and capsule formation also when treated with the sterile supernatant of strain 32. Apparently, the diatom is able to recognize one or more soluble molecules produced by the bacterium, a direct cell-to-cell contact between the interaction partners obviously is not necessary as it was shown previously for *Pseudo-nitzschia multiseriis* (Kobayashi et al. 2009b). Interestingly, *Bacteroidetes* strain 32 produced the putative infochemicals even when cultivated separately, which indicates a constitutive production and/or secretion. As the biotic activity of this or these substance(s) could be demonstrated already in the early exponential growth phase of strain 32, they

apparently are produced by intact bacterial cells and do not constitute substances that are released after cell damage. As shown in Figure 4.5B, *A. minutissimum* reacted with a sharp increase of biofilm formation even at relative low bacterial abundance, thus either the diatom is very sensitive for the substance(s) or the production of the substance(s) is strongly increased once the bacterial density exceeds a certain threshold. The bioactive molecule(s) was extractable via reversed phase cartridges indicating a hydrophobic character, similarly to AHLs, a major class of autoinducers produced by Gram-negative bacteria (Chhabra et al. 2005). The inducibility of capsule formation using the spent medium of the bacterium shows that the diatom itself is the active producer of biofilm material and the active contribution of bacterial EPS can therefore be excluded.

Carbohydrate quantification revealed that the axenic diatom culture contained large amounts of soluble carbohydrates. In the late stationary phase, we found variable values of soluble polymers within the triplicates of this culture (15.9, 11.1 and 55.6 $\mu\text{g CHO}\cdot(\mu\text{g chl})^{-1}$; Figure 4.6C), resulting in a high standard deviation. From former experiments we know that the axenic cultures usually secrete large amounts of soluble polymeric carbohydrates in the stationary phase (up to 105 $\mu\text{g CHO}\cdot(\mu\text{g chl})^{-1}$; data not shown). However, in the xenic cultures insoluble carbohydrates were dominant, especially in the late stationary phase when the capsules were fully developed. In this growth phase, carbohydrates of the xenic culture were primarily located in the HW and HB fractions, thus the capsule material can be assigned mainly to this fraction. As bacteria in the xenic culture potentially may consume the soluble carbohydrates (probably especially the easily accessible monomers, whereas the bound carbohydrates may be more resistant to bacterial degradation), we cannot estimate whether the total amount of carbohydrates in xenic and axenic culture is comparable. However, it might be possible that axenic and xenic cultures produced similar amounts of carbohydrates, but the condition of the secreted carbohydrates changed from a dissolved to an insoluble state in the presence of the bacteria. Nonetheless, in both cases it is evident that *A. minutissimum* strongly secreted carbohydrates even when no structured EPS were visible. When nutrients become limited - thus within the stationary phase - increased EPS production and capsule formation is a known phenomenon (Lewin 1955; Bhosle et al. 1995; Staats et al. 2000) and is assumed to serve also as an overflow mechanism (Staats et al. 2000).

The exact function of capsules produced by *A. minutissimum* is still unclear. Geitler (1977) proposed that capsule formation of this alga is involved in sexual reproduction, whereas pads and stalks are regarded as the common structures of vegetative cells. This

proposal was based on the observation that the capsule may engulf both mating partners, however, we also found single cells to be surrounded by a capsule.

According to Lewin (1955) the production of cell bound gelatinous matter may provide attachment. This would agree with our observation that the cells were attached to each other or to artificial surfaces when exhibiting capsules. Furthermore, the capsules can be stained with a solution of alcian blue dissolved in acetic acid, which stains anionic polysaccharides (Staats et al. 1999) that are thought to facilitate adhesion (Dade et al. 1990). However, in the xenic culture and in the co-culture with strain 32, the diatom cells within a few days adhered to the surface of the well even when capsules were not yet visible, obviously mediated by stalks. We therefore conclude that bacterial induced capsulation may not be a prerequisite for surface-attachment, but possibly for strengthening the attachment and irreversible binding. Diffuse EPS, also known as transparent exopolymer particles (TEP) (Passow 2002), which were observed in both the axenic and xenic cultures, obviously did not facilitate surface adhesion, as axenic cells can be easily removed during the washing procedures. Surface adhesion of diatoms could be relevant once the cells found a convenient environment for example when they recognize the presence of an interaction partner. This would implicate a mutualistic character of the interaction with *Bacteroidetes* strain 32. The bacterium was not able to grow in BM alone, indicating that it might receive organic substances from the alga in co-culture. The finding of a clearly higher abundance of bacteria in co-culture with diatoms compared to the bacterial growth in the respective diatom medium, supports that this is the case for exudates from other diatom species (Grossart et al. 2006; Gärdes et al. 2010). Thus, a further explanation for capsulation in the presence of bacteria could be that the capsules themselves may serve as feeding grounds for the bacteria and may help to keep them in close proximity to the diatom cells. It is tempting to speculate that bacteria may induce cell aggregation and biofilm formation of the diatom via soluble molecules in order to keep the alga in spatial proximity and to ensure an adequate nutrient supply by the primary producer. The diatom in turn may benefit from the general properties of the biofilm, for example protection from toxic compounds (Ceri et al. 1999), UV radiation (Ehling-Schulz et al. 1997) and grazer protection, but may also directly benefit from the bacterium, for example by bacterial supply of essential nutrients (e.g. vitamins). The used medium in this study indeed contains sufficient basic nutrients for unlimited diatom growth. However, once a biofilm is established, local nutrient deficiency may occur which may be compensated by bacteria. Hassler et al. (2011) showed that mono- and polysaccharides, such as glucuronic acid and dextran, enhance iron accessibility to phytoplankton, possibly by

acting like ligands. In this context it would be conceivable that the capsules of *A. minutissimum* could be a mechanism to create a reservoir for micronutrients. This would explain why the capsules are induced in the stationary phase under nutrient limited conditions. Amin et al. (2012) speculated about a synergistic interaction in which the satellite-bacteria also receive iron from the pool of the phytoplankton, but it could also represent a competition mechanism of the diatom to increase iron availability.

Microscopic analyses revealed that the bacterial cells of the xenic culture most likely cannot penetrate the capsules, visible as bacteria-free areas around the diatom cells (data not shown), and thus the diatom may prevent the cells from being overgrown or parasitized by bacteria. This may be much more evident when the diatom cells are stressed and thus much more vulnerable. In this context capsulation could also represent a defense mechanism. The capsules could further prevent the diatom cells from toxic bacterial compounds as EPS may reduce the susceptibility of biofilm organisms to some substances (Stewart and William Costerton 2001).

The identification of the bioactive compound(s) of strain 32 and further physiological studies will be important to show whether the interaction between *A. minutissimum* and *Bacteroidetes* strain 32 has a mutualistic or an antibiotic character. The bioassay for analysis of the bacterial influence on biofilm structure and quantity of *A. minutissimum* proved to be very reliable. The enhanced biofilm formation when stained with crystal violet is visible by eye which allows a fast screening of a large number of substances as shown here for fractions of the separated bacterial supernatant. The assay could possibly be extended to test impacts of other chemicals on the biofilm formation of this or even other diatoms. *A. minutissimum* turned out to be an excellent model organism for the investigation of biofilm formation of diatoms as it produces large amounts of soluble and bound EPS and it can be cryopreserved by using a modified protocol according to Buhmann et al. (2013) (see Chapter 6).

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Supplementary Data

S4.1 Growth of *Bacteroidetes* strain 32 in diatom full medium

S4.2 Co-cultivation of *Achnanthydium minutissimum* with different bacterial isolates

Chapter 5

Genetic characterization of a diatom growth-promoting bacterium

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Manuscript in preparation

Chapter 5: Genetic characterization of a diatom growth-promoting bacterium

5.1 Abstract

Photoautotrophic biofilms can be found on most submerged, irradiated surfaces. Here, diatoms and bacteria are often primary colonizers. Various interactions between these two types of organisms have been described, ranging from mutualism to parasitism and competition, however, the nature and mechanisms of these interactions are largely unknown. In this study we showed that the bacterium *Bacteroidetes* strain 32, isolated from photoautotrophic, littoral biofilms, had a strong growth-promoting effect on the benthic diatom *Fragilaria brevistriata* Grunow when co-cultivated on solid medium. Heat treated bacterial cells as well as the sterile bacterial supernatant had no effects on growth of the diatom. To further investigate this interaction, transposon tagged mutants of strain 32 were prepared and screened in a newly developed biotest utilising *F. brevistriata*. Genetic characterization of bacterial mutants that had no influence on diatom growth revealed that two genes, whose products are homologous to transcriptional regulators of the MarR family and to a calcium transporter, respectively, may be involved in the growth stimulating effect of the bacterium.

Keywords: Growth-stimulation · MarR · Ca²⁺-ATPase · bacterium · diatom

5.2 Introduction

Diatoms are commonly associated with bacteria (Weiss et al. 1996; Knoll et al. 2001; Schäfer et al. 2002; Makk et al. 2003; Kaczmarska et al. 2005; Sapp et al. 2007a, b; Bruckner et al. 2008), promoting various interactions which may exceed the trophic interactions of nutrient supply and competition for limited substances (Amin et al. 2012). Stimulating effects of bacteria on algal growth have been reported (Ukeles and Bishop 1975; Delucca and McCracken 1977; Fukami et al. 1991; Watanabe et al. 2005; Marshall et al. 2006; Bruckner et al. 2008; Park et al. 2008; Bruckner et al. 2011; Le Chevanton et al. 2013) and several possible mechanisms of these interactions are currently under discussion. For example, bacteria may supply essential nutrients for auxotrophic algae. Croft et al. (2005) surveyed that a large number of algae species are vitamin B₁₂ auxotrophs and have to take up this vitamin from the surrounding medium to cover their demand. It is most likely that in nature bacteria are a source of this vitamin (Haines and Guillard 1974). Furthermore, bacteria may supply inorganic nutrients like nitrogen and phosphate by remineralisation of dead cells and allochthonous material as reviewed in Cole (1982). They may enhance nutrient availability for the algae as it was shown for iron uptake mediated by a bacterial siderophore (Amin et al. 2009). It was further suggested that bacteria may create a favourable microenvironment around the algal cells for example by consumption of oxygen and/or supply of carbon dioxide (Mouget et al. 1995). Furthermore, bacterial decomposition of some algal waste products may also enhance viability of the algae. Some studies reported that an observed supportive effect on algal growth was even inducible by the spent medium of the bacterial culture or of the algal/bacterial co-culture (Riquelm et al. 1988; Bruckner et al. 2011), indicating that soluble bacterial molecules act as growth factors, while other spent media had no effects (Ukeles and Bishop 1975; Delucca and McCracken 1977; Mouget et al. 1995). Maruyama et al. (1986) discovered cytokinin-like substances in cultures of aquatic bacteria, leading to the assumption, that these substances are putative stimulators of algal growth. The bacteria in turn may profit from a mutualistic interaction with the algae as the primary producers may provide carbon sources for heterotrophic bacteria (Cole 1982). All these mechanisms may have additional importance in biofilms where the inhabitants are in close spatial proximity and material exchange with the surrounding medium is limited by diffusion through the matrix or by microcurrents through the biofilm matrix pores. Thus, a division of work within symbiotic relationships of interaction partners may be one strategy which makes living in a biofilm such

a successful life style. However, the mechanisms of how bacteria influence algal growth remained largely unclear.

One essential goal of this study was the development of a bioassay to investigate the mechanisms of growth-promoting bacteria on diatom growth in photoautotrophic biofilms. *Bacteroidetes* strain 32 and the diatom *Fragilaria brevistriata* Grunow, both isolates of photoautotrophic, epilithic biofilms, were used as model organisms and transposon tagged mutants of strain 32 were generated and used to explore the genetic background of the growth-promoting mechanism of this bacterium.

5.3 Materials and Methods

5.3.1 Organisms and cultivation conditions

The axenic diatom *Fragilaria brevistriata* Grunow (isolate F-02) was obtained from Dr. Rahul Bahulikar (The Samuel Roberts Noble Foundation, Ardmore, USA). Frustules were prepared as described in Chapter 3 and morphologically identified according to Krammer and Lange-Bertalot (1991a) by Anastasiia Kryvenda (University of Göttingen). The diatom stock cultures were cultivated in liquid Bacillariophycean Medium (BM) (Schlösser 1994), which was modified according to Windler et al. (2012). Cultures were exposed to a 12:12 h light:dark cycle with light intensities of 20-50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 16°C (denoted in this study as standard cultivation conditions for diatoms). Monthly, an aliquot of the diatom culture was sub-cultured in fresh BM.

Bacteroidetes strain 32 was isolated by Bruckner et al. (2008). This bacterium was enriched from a xenic *Cymbella microcephala* Grunow culture which originated from photoautotrophic, epilithic biofilms from the same sampling site as *F. brevistriata* (47° 41' N; 9° 11' E, Germany) (Bahulikar 2006). According to 16S rDNA sequence comparison, strain 32 is classified to the genus of *Dyadobacter* (Bruckner et al. 2008). The bacterium was cultivated at 22°C on agar plates containing diluted Luria Broth (LB) medium (50% (v/v) (Miller 1972)), sub-cultivated monthly and stored at 4-8°C. The *Bacteroidetes* mutants were cultivated on diluted LB agar plates containing 150 $\mu\text{g ml}^{-1}$ erythromycin (Sigma Aldrich Chemie GmbH, Steinheim, Germany).

5.3.2 Bacterial conjugation

The *Escherichia coli* strain BW19851 containing the plasmid pEP4351 was used as donor for conjugation (Metcalf et al. 1994; Cooper et al. 1997). Conjugation was performed

according to McBride and Kempf (1996) with following modifications: The donor strain BW19851 was grown in 5 ml LB with 30 $\mu\text{g ml}^{-1}$ chloramphenicol over night at 37°C and 200 rpm. The culture was diluted in 25 ml LB containing chloramphenicol and cultivated at room temperature and 200 rpm to an optical density at 600 nm (OD_{600}) of 0.5-0.6. *Bacteroidetes* strain 32 was cultivated in 50 ml diluted LB at 20°C and 85 rpm up to an OD_{600} of 0.8-0.9. OD_{600} of the cell suspension of strain 32 was adjusted in diluted LB to a ratio of recipient:donor of 1:1 and 1:2. The cultures of donor and recipient were centrifuged and the pellets were washed twice with 20 ml diluted LB and afterwards with 1-2 ml diluted LB. Cells of donor and recipient were resuspended in 100 μl diluted LB and 50 μl of each culture were mixed together. An agar plate containing diluted LB was prepared with 8 mM CaCl_2 (100 μl per plate) one hour before the mixed cell suspension was spotted. For conjugation the cells were incubated over night at 30°C. Cells were scraped from the plates, resuspended in 2 ml diluted LB and Tn4351-mutants were selected on agar plates containing diluted LB with 100 and 200 $\mu\text{g ml}^{-1}$ erythromycin, respectively. The plates were incubated at 22°C until bacterial colonies appeared.

5.3.3 Co-cultivation of *Fragilaria brevistriata* and *Bacteroidetes* strain 32 – Bioassay for growth-promoting influence

Co-cultivation of F. brevistriata with Bacteroidetes strain 32 wild type and Tn4351-mutants

F. brevistriata and *Bacteroidetes* strain 32 were co-cultivated in 48-well plates on 700 μl solid BM containing 1.5% Bacto™ Agar per well (Becton, Dickinson and Company, USA). *F. brevistriata* cultures were inoculated with 1070, 3100 or 8350 cells per well which were dropped in the centre of each well. Bacterial cells were added either with undefined cell densities by transferring of single colonies using a sterile toothpick or with defined cell densities by adding of 5 μl of the diluted or undiluted bacterial culture with an OD_{600} of 0.8. For this, the bacterium was cultivated in liquid diluted LB, washed three times with BM to remove the bacterial medium, and the optical density was adjusted. The axenic diatom culture was used for negative control. For negative controls of experiments in which bacterial colonies were transferred, the agar medium of the axenic diatom culture was gently penetrated with a sterile toothpick without transferring bacterial cells. Each approach was conducted in triplicates. To test the bacterial growth in the absence of the diatom on solid BM, the bacterium was transferred with a sterile tooth pick to the medium. The cultures were incubated at standard cultivation conditions for diatoms.

The bacterial mutants were pre-screened in co-cultures of 5 μ l per well of the axenic *F. brevistriata* culture with an undefined cell density and bacterial mutants were added by transferring of single colonies. Mutants with the desired phenotype were screened again in triplicates. Mutants B-16, 21.20 and 31.90 were further screened with defined diatom cell densities in triplicates as described above. For mutant B-16 also the influence of the bacterial cell density was tested as described above. The co-culture of diatom and bacterial wild type cells as well as the axenic diatom culture were used as positive and negative controls. Microscopy was performed by using an inverse microscope (Axiovert 40 C; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and photographs were taken with a Zeiss AxioCam MRc digital camera system (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Influence of vitamins, soluble bacterial substances and heat treated bacterial cells on diatom growth

The influences of vitamins, sterile bacterial supernatant and heat treated bacterial cells were further tested on solid BM. 24-well plates were filled with 1.5 ml per well solid BM and 10 μ l of the diatom culture were added into each well. For the experiments with vitamin deficient medium, BM without vitamin B₁₂, thiamine and biotin was used. Here, single colonies of *Bacteroidetes* strain 32 were transferred with a sterile toothpick. Axenic *F. brevistriata* was cultivated on the vitamin deficient BM for negative control. Each approach was conducted four times. To test the influence of the sterile bacterial supernatant, the bacterium was cultivated in liquid diluted LB at 20°C and 135 rpm and harvested when the culture reached an OD₆₀₀ of 1.25. The culture was centrifuged for 5 min at 16100 x g and filter-sterilized with a 0.2 μ m filter (Filtropur S; Sarstedt, Germany). Different volumes (2, 5, 10, 20 and 50 μ l) of the filter-sterilized supernatant were added to axenic diatom cultures. The same volumes of the bacterial growth medium (diluted LB) were added to axenic diatom cultures for negative control. Each experiment was conducted in duplicates (except with 5 μ l (n=6) and 10 μ l (n=1) of the bacterial supernatant). *F. brevistriata* was further cultivated with 5 μ l of the heat-treated bacterial culture. The bacterial cells were therefore autoclaved. 5 μ l of the untreated bacterial culture, grown in diluted LB, was added to the axenic culture for positive control. Each experiment was conducted 4 times. The cultures were incubated at standard cultivation conditions for diatoms for 33 days.

Comparison of growth of Bacteroidetes strain 32 wild type and mutants

Bacteroidetes strain 32 wild type and Tn4351-mutants were inoculated in diluted LB to an OD₆₀₀ of 0.01. The cultures were incubated at 20°C and 200 rpm. The OD₆₀₀ of the cultures were determined by using the M107 Spectrophotometer (Camspec, Cambridge United Kingdom). Each approach was conducted four times.

Co-cultivation of F. brevistriata and Bacteroidetes strain 32 wild type and mutant B-16 in liquid medium

For co-cultivation in liquid medium, 60 ml BM were inoculated with *F. brevistriata* to a cell density of $2.45 \cdot 10^4$ cells ml⁻¹. The co-cultures with the wild type and mutant B-16 were inoculated with 1 ml of the bacterial cultures with an OD₆₀₀ of 0.13-0.14. The cultures were cultivated at standard cultivation conditions for diatoms at 100 rpm. Chlorophyll was extracted from 1 ml per sampling by resuspension of the cell pellet with 50 µl methanol and subsequent incubation for 10 min at 1400 rpm. After addition of 950 µl acetone, cell fragments were centrifuged and the chlorophyll concentration was measured in the supernatant by using the equation for diatoms, chrysomonads and brown algae according to Jeffrey and Humphrey (1975). Specified is the total chlorophyll concentration which is the sum of chlorophyll *a* and *c*. CFUs (colony forming units) were determined by spotting of 5 µl of the diluted co-cultures on agar plates containing diluted LB. The plates were incubated as described above for *Bacteroidetes* strain 32.

5.3.4 Genetic characterization of transposon mutants

DNA sequence of the Tn4351-transposon was obtained from Prof. Dr. M.J. McBride (University of Wisconsin-Milwaukee, USA). Genomic DNA of the mutants B-16, 21.20 and 31.90 and of the wild type was extracted by using the DNA purification protocol for Gram-negative bacteria of the Genra Puregene Yeast/Bact. Kit (Qiagen, Maryland, USA). A 450 bp fragment of the Tn4351 transposon was amplified with the Tn4351_Tet_fw and Tn4351_Tet_rev primers (Table 5.1) using a Taq-polymerase (F-100; Biozym Scientific, Oldendorf, Germany) according to the manufacturer's protocol. Genomic DNA was digested each with the restriction enzymes BsoBI, BstNI (New England BioLabs, Ipswich, USA) and HindIII (Fermentas, Thermo Scientific, Schwerte, Germany) modified after the manufacturer's protocol: 4 µg DNA was digested with 30 units of the respective restriction enzyme in a total volume of 200 µl and the reactions were incubated for 1 h. The same amounts of enzymes were added afterwards and the reactions were incubated overnight.

Restriction sites for the enzymes were calculated with the NEBcutter V2.0 tool (New England BioLabs, Ipswich, USA, <http://tools.neb.com/NEBcutter2/index.php>). A DIG-labeled probe against the Tn4351-transposon was synthesized with the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) and the primers Tn4351_Tet_fw and Tn4351_Tet_rev (Table 5.1). The Tn4351-transposon was localized in the digested genomic DNA via Southern blot according to the DIG Application Manual for Filter Hybridization (Roche Diagnostics GmbH, Mannheim, Germany) with modifications in the following steps: The Tn4351-DIG-labeled probe was hybridized to the membrane bound samples in High SDS hybridization buffer at 42°C. Duration of denaturation and neutralization were reduced to 15 min and equilibration with 20 x SSC to 5 min. After blotting, the membrane was briefly washed two times for 5 min with 5 x SSC and again 2 times for 5 min with 2 x SSC. The blot was incubated in High Stringency buffer (0.1 x SSC and 0.1% SDS) at 68°C. The detection buffer contained 0.1 M Tris HCl and 0.1 M NaOH (pH 9.5). The DIG-labeled DNA Molecular Weight Markers II and III (Roche Diagnostics GmbH, Mannheim, Germany) were used for molecular weight determination. Sequence analysis of the insertion sites of the transposon were conducted partially by the Trenzyme company (Trenzyme GmbH, Konstanz, Germany). The procedure of this step is listed in the final report for “Localization of transposon insertion conducted by the Trenzyme company” in the Supplementary Data S5.1. Furthermore, fosmid libraries of randomly sheared genomic DNA of the mutants were constructed using the EpiFOS™ Fosmid Library Production Kit (Epicentre Biotechnologies, Madison, USA). Fosmids containing the transposon were selected on LB agar plates with 10 µg ml⁻¹ tetracycline and prepared with the Qiagen Large-Construct Kit (Qiagen GmbH, Hilden, Germany). Primer walking was performed by GATC Biotech (GATC Biotech AG, Konstanz, Germany) with the primers listed in Table 5.1 and sequence assembly, including the sequences of the Trenzyme report and the Tn4351-transposon sequence, was performed using the SeqMan software (Lasergene; DNASTAR, Madison, USA). Binding sites of the primer B16-FosmidprimerRev2 are located in the direct repeats of the transposon and sequencing with this primer resulted in two overlapping sequences (Figure M5.1). The known transposon sequence was subtracted from the overlapping sequences and thus, a putative sequence of the insertion site was obtained. On the basis of the predicted sequence the primers B16-FosmidprimerRev3, B16-FosmidprimerFW4 and B16-FosmidprimerFW5 were designed and amplification with these primers led to the actual sequence of the transposon insertion site. Binding sites of 21.20-FosmidprimerFW1 and 31.90-FosmidprimerFW1 were also located in the direct repeats of the transposon, but only one direct repeat was in each case

integrated in the fosmids, resulting in clear electropherograms. Up- and downstream nucleotide sequences were transcribed into amino acid sequences with the DNA to protein translation tool of insilico.ehu.es (<http://insilico.ehu.es/translate/> (Bikandi et al. 2004)) to detect open reading frames (ORFs). ORFs were defined by the first methionine after the previous stop codon up to the following stop codon. The ORFs were blasted against the protein database of NCBI using the blastp program (<http://www.ncbi.nlm.nih.gov/>). Blast hits exhibiting an e-value $>10^{-6}$ were excluded. The protein sequences were aligned with those of proteins with the highest blastp identities by using ClustalW2.1 (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>; EMBL-EBI, Hinxton, UK).

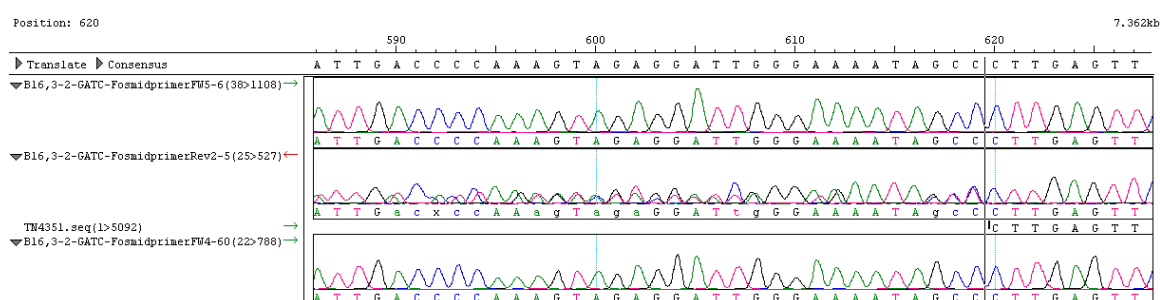


Figure M5.1: Primer walking for sequencing of the Tn4351-transposon insertion site of mutant B-16. Sequencing with the B16-FosmidprimerRev2 led to two overlapping sequences outside of the Tn4351-transposon sequence. One sequence belongs to the direct repeat of the transposon and the other to the desired sequence of the transposon insertion site. Sequencing with the primers B16-FosmidprimerFW4 and B16-FosmidprimerFW5 resulted in clear electropherograms.

Table 5.1: Primers used in this study.

Name	Primer sequence	Application
Tn4351_Tet_fw	CGC AAG CAG GGG GTT CGT GC	DIG labeled DNA probe synthesis; Tn4351-fragment amplification
Tn4351_Tet_rev	CTC TCG GTC GTT GTC TCT TTC GTA AAC	DIG labeled DNA probe synthesis; Tn4351-fragment amplification
B16-FosmidprimerFW2	GAC AGT ATG ACG TAA CAG GAG ACC	Primer walking mutant B-16
B16-FosmidprimerFW3	TAG ACG AAG ATA CGC ATC ACA GC	Primer walking mutant B-16
B16-FosmidprimerFW4	CGG AGC AGT ATA ATG TGC TCA GGA	Primer walking mutant B-16
B16-FosmidprimerFW5	TTG GAG AGT TAG AGG CAA GAA TCA G	Primer walking mutant B-16
B16-FosmidprimerRev2	TAT CTA CTC CGA TAG CTT CCG C	Primer walking mutant B-16
B16-FosmidprimerRev3	TCA CTA CAT CCA CTG CGC	Primer walking mutant B-16
B16-FosmidprimerRev5	GGT CTC CTG TTA CGT CAT ACT GTC	Primer walking mutant B-16
21.20-FosmidprimerFW1	GGT GCC ATT CTT ACC ATT AAC GAC AG	Primer walking mutant 21.20
21.20-FosmidprimerFW2	GCT TCA GAT CGA AGA AAT GCC	Primer walking mutant 21.20
21.20-FosmidprimerRev1	ACA CCA GTC ACC TCC GGT C	Primer walking mutant 21.20
21.20-FosmidprimerRev2	TTG CAT ACC AGT ACA CAC CC	Primer walking mutant 21.20
31.90-FosmidprimerFW1	GCA GTA TAT CCC AAA GGG TAA GGA C	Primer walking mutant 31.90

The primers are listed in 5'-3' direction. Primers for nested PCR and sequencing reactions used by the Trenzyme company are listed in the final report of localization of transposon insertion (Suppl. Data S5.1).

5.4 Results

5.4.1 Co-cultivation of *Fragilaria brevistriata* and *Bacteroidetes* strain 32 – Bioassay for growth-promoting influence

Growth of the diatom *F. brevistriata* was strongly enhanced when co-cultivated on solid medium with *Bacteroidetes* strain 32. In co-culture, the diatom formed macroscopically visible colonies, whereas in the axenic culture no or only small colonies were observed (Figure 5.1). The microscopic image shows that *F. brevistriata* formed large cell chains in the co-culture. Chain formation emerged from the inoculation site with the bacterium. The growth of the axenic diatom culture was decelerated and the appearance of macroscopically visible colonies was, if at all, strongly delayed compared to the xenic culture. Microscopical analysis of the axenic culture revealed only short diatom cell chains distributed on the agar surface.

Growth of the bacterium in the co-culture with the diatom was visible as the bacterium formed a smooth film starting from the inoculation site on the solid medium (Figure 5.1). A comparable bacterial film was also visible when cultivated without diatom cells (data not shown), suggesting that the bacterium was able to grow on solid BM even in the absence of the alga.

For genetic characterization of the bacterial influence on diatom growth, Tn4351-transposon mutants of *Bacteroidetes* strain 32 were generated and screened for a loss of growth enhancement on *F. brevistriata*. For this purpose we developed a bioassay on solid agar medium in 48-well plates, in which growth of *F. brevistriata* was observed by visual inspection after addition of individual bacterial mutant cell lines (see Materials and Methods section for a detailed description). At least 2858 Tn4351-mutants were pre-screened this way in co-cultures with *F. brevistriata* and three mutants (B-16, 31.90 and 21.20) with the desired phenotype were identified (Figure 5.1). These mutants were further characterized by inoculation with different diatom and bacterial cell densities and different incubation durations (Figure 5.2). When inoculated with bacterial colonies (undefined cell density), we found the strongest visible differences of diatom growth induction between wild type cells and Tn4351-mutants with 3100 diatom cells per well and after 25 days of cultivation. Under these conditions, the co-cultures with the mutants did not show diatom colony formation, while all three co-cultures with the wild type cells did. At lower diatom cell density (1070 cells well⁻¹) even the wild type cells did not induce diatom colony formation within 25 days. At higher diatom cell density (8350 cells well⁻¹) or longer cultivation duration (62 days) also the co-cultures with the mutants started to form colonies. No colony formation was observed

in the axenic *F. brevistriata* cultures under all tested conditions. Obviously, the mutants enhanced growth of the diatom compared to the axenic culture, but the potency of the wild type was not achieved by the mutants. We further tested the influence of the bacterial cell density on the reliability of the bioassay for the wild type cells and mutant B-16. We found the best correlation when bacteria with an OD₆₀₀ of 0.8 were incubated with about 3100 or 8350 diatom cells per well and cultivated for 25 days. At these conditions, the wild type cells induced diatom colony formation in all replicates, whereas the co-culture with mutant B-16 and the axenic diatom culture showed no colonies (Figures 5.1 and 5.2).

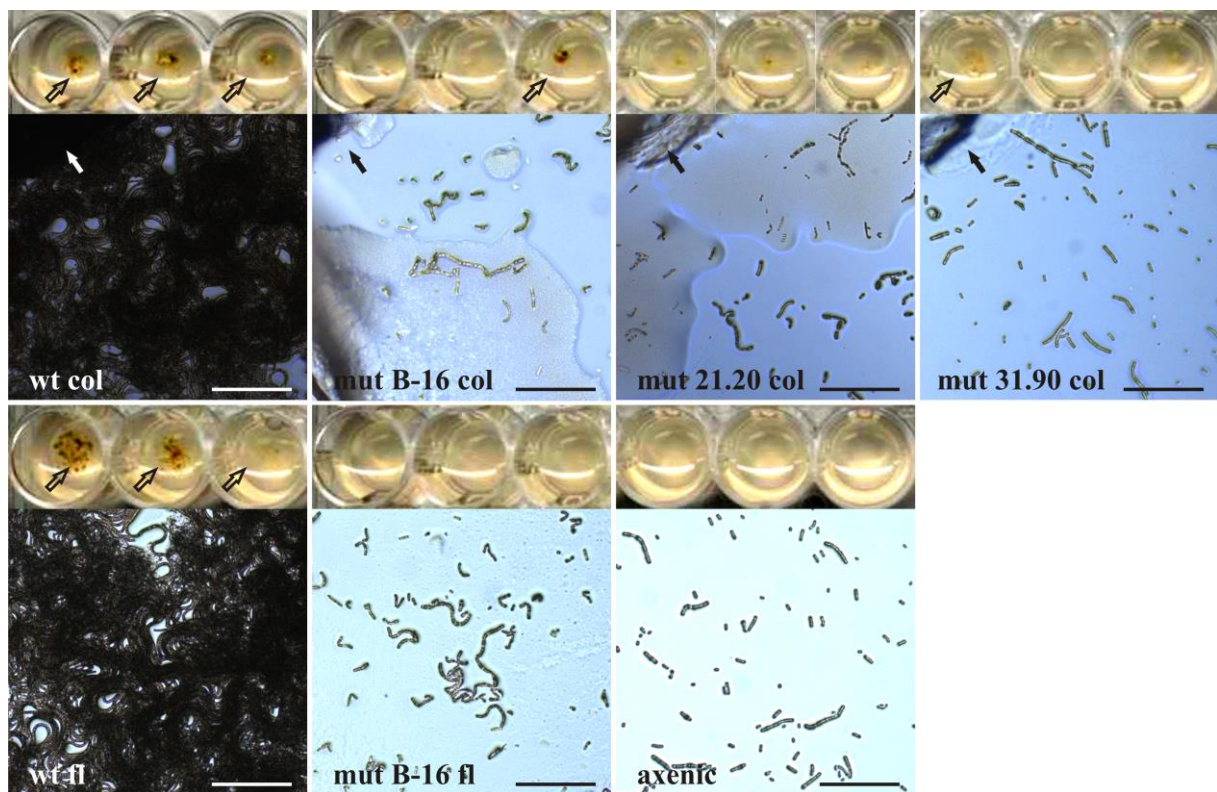


Figure 5.1: Growth of *Fragilaria brevistriata* in co-cultures with *Bacteroidetes* strain 32 wild type and mutants on solid medium. Each picture shows the macroscopic (top) and microscopic (below) images of the cultures. The upper row shows the co-cultures with the wild type (wt col), mutant B-16 (mut B-16 col), mutant 21.20 (mut 21.20 col) and mutant 31.90 (mut 31.90 col). In these cultures, bacterial cells were inoculated by transfer of bacterial colonies (undefined bacterial cell densities). Macroscopically visible colonies are marked with unfilled arrows. The microscopic images were taken near the inoculation sites of the bacteria (filled arrows). Bacterial growth is visible as the bacteria formed smooth films starting from the inoculation sites. The microscopic images of co-cultures with mutants B-16 and 31.90 show one of the triplicates without colony formation. The lower row shows the co-cultures with defined bacterial cell densities (OD₆₀₀ = 0.8) of the wild type (wt fl), mutant B-16 (mut B-16 fl) as well as the axenic diatom culture. Scale bars resemble 100 μ m. The cultures were inoculated with 8350 diatom cells per well and cultivated for 25 days. All cultures originated from the same well plate.

		25 days			62 days
diatom (cell per well)		1070	3100	8350	1070
bacteria	colony		XXX	XXXXX	XXXXXXX
	pure	XX	XXX	XXX	XXX
	0.8 OD		XX	XX	XXX
	1:10		XX	XX	XXX
	1:100		X	X	XXX
	1:1000				XXX

Figure 5.2: Colony formation of *Fragilaria brevistriata* in co-cultures with *Bacteroidetes* strain 32 wild type and mutants on solid medium. Co-cultures were inoculated with different diatom and bacterial cell densities and cultivated for 25 and 62 days, respectively. Bacterial cells were added either by transferring of single colonies (undefined cell densities) or by addition of the bacterial culture with an OD₆₀₀ of 0.8 and in several dilutions. X denotes diatom colony formation in the co-culture with the wild type (black), mutant B-16 (red), mutant 31.90 (green) and mutant 21.20 (blue) (n=3).

We further tested the influence of vitamins on the co-culture with the wild type and the axenic diatom culture and found that the bacterium enhanced diatom growth even on vitamin depleted growth medium (Table 5.2). Filter-sterilized bacterial supernatant or heat treated bacterial cells had no effects on growth of the diatom culture (Table 5.2), indicating that soluble bacterial molecules are not responsible for the observed effect and bacterial cells have to be viable to cause growth stimulation.

Table 5.2: Influence of vitamins, sterile bacterial supernatant and heat treated bacterial cells on growth of the diatom *Fragilaria brevistriata*. (+) Diatoms formed macroscopic visible colonies, (-) diatom growth was not observable. The number of +/- represents the number of replicates. -medium is BM without vitamin B₁₂, thiamine and biotin.

Experiment	Diatom growth
1. <i>F. brev</i> /bacterial colony on vitamin -medium	++++
2. axenic <i>F. brev</i> on vitamin -medium (control for exp. 1)	----
3. <i>F. brev</i> /filter-sterilized bacterial supernatant:	
2 µl	--
5 µl	-----
10 µl	-
20 µl	--
50 µl	--
4. <i>F. brev</i> /heat treated bacteria	----
5. <i>F. brev</i> /bacterial colony (pos. control for exp. 1, 2)	++++
6. <i>F. brev</i> /bacterium grown in liquid medium (pos. control for exp. 3, 4)	++++
7. axenic <i>F. brev</i> (neg. control)	--
8. <i>F. brev</i> /bacterial growth medium: (neg. control for exp. 3)	--
2 µl	-----
5 µl	--
10 µl	--
20 µl	--
50 µl	--

To exclude that the delayed growth of *F. brevistriata* in co-cultures with the mutants was due to lower cell densities of the mutants themselves, we measured the OD₆₀₀ of the mutants cultivated in liquid diluted LB medium. None of the mutants showed a considerably different growth behavior compared to the wild type (Figure 5.3).

Growth of *F. brevistriata* in co-cultures with mutant B-16 and with the wild type as well as of the axenic diatom culture was observed in liquid medium (BM) via estimation of the chlorophyll concentrations (Figure 5.4). In the late stationary phase, all cultures reached similar chlorophyll concentrations (e.g. $0.52 \pm 0.04 \mu\text{g ml}^{-1}$ in the axenic culture, $0.6 \pm 0.04 \mu\text{g ml}^{-1}$ in the co-culture with the wild type and $0.6 \pm 0.02 \mu\text{g ml}^{-1}$ with mutant B-16 after 21 days of cultivation). CFUs of the wild type and mutant B-16 revealed that the bacteria were not able to grow in the co-culture when cultivated in liquid BM (Figure 5.4). The values for CFUs varied in the range of $1.4 \cdot 10^6 \pm 5.9 \cdot 10^5 - 4.1 \cdot 10^6 \pm 2.3 \cdot 10^6$ for the wild type and $1.2 \cdot 10^6 \pm 3.9 \cdot 10^5 - 2.7 \cdot 10^6 \pm 4.8 \cdot 10^5$ for the mutant B-16 throughout the experiment.

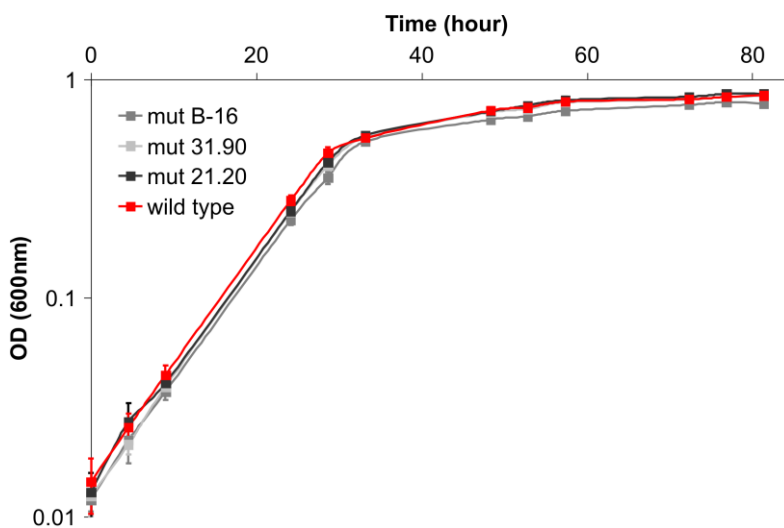


Figure 5.3: Growth of *Bacteroidetes* strain 32 wild type and of the mutants B-16, 31.90 and 21.20 in bacterial full medium. (n=4, error bars indicate standard deviation).

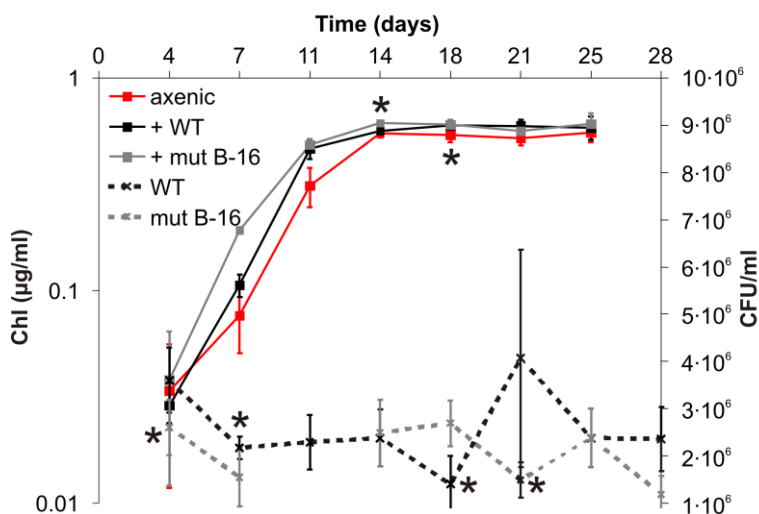


Figure 5.4: Growth of *Fragilaria brevistriata*, *Bacteroidetes* strain 32 wild type (WT) and mutant B-16 in co-culture in liquid medium. Diatom growth was measured by means of chlorophyll concentration (chl, solid lines; n=3, except for co-cultures with mutant B-16 at day 14 and with wild type at day 18 (n=2), marked with *). Growth of the bacterium was measured by means of CFUs (dashed lines; n=9 for spot tests, except for mutant B-16 at day 4 and day 21 (n=7) and for the wild type on day 7 (n=6) and day 18 (n=7), marked with *). Error bars indicate standard deviation.

5.4.2 Genetic characterization of transposon mutants

Figure 5.5 shows that the Tn4351-transposon was integrated into the genomes of the three mutants whereas the *Bacteroidetes* strain 32 wild type shows no PCR product band after amplification with Tn4351 specific primers (Tn4351_Tet_fw and _rev, Table 5.1). For Southern blot labeling of fragments containing the Tn4351 insertions, the genomic DNA of the mutants B-16, 21.20 and 31.90 were digested with the restriction enzymes BstNI, BsoBI and HindIII. Tn4351 exhibits four cleavage sites for BstNI and two for BsoBI (up- and downstream of the binding site of the DIG-labeled probe) (Figures S5.1-3 Suppl. Data S5.2). Cleavage of the genomic DNA with these restriction enzymes we obtained the desired fragments of 3937 bp for BsoBI and 1543 bp for BstNI (Figure 5.6). The transposon has one HindIII cleavage site outside of the binding site of the DIG-labeled probe (Figures S5.1-3 Suppl. Data S5.2).

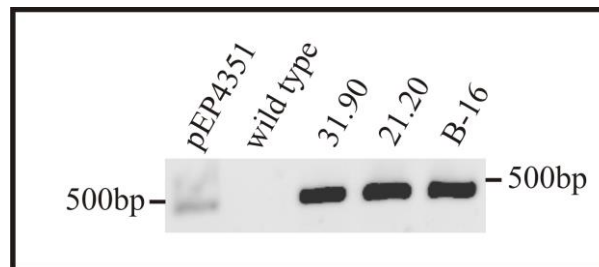


Figure 5.5: Amplification of a 450 bp fragment of the Tn4351-transposon. The vector pEP4351 containing the transposon and the genomic DNA of the wild type were used for positive and negative control, respectively.

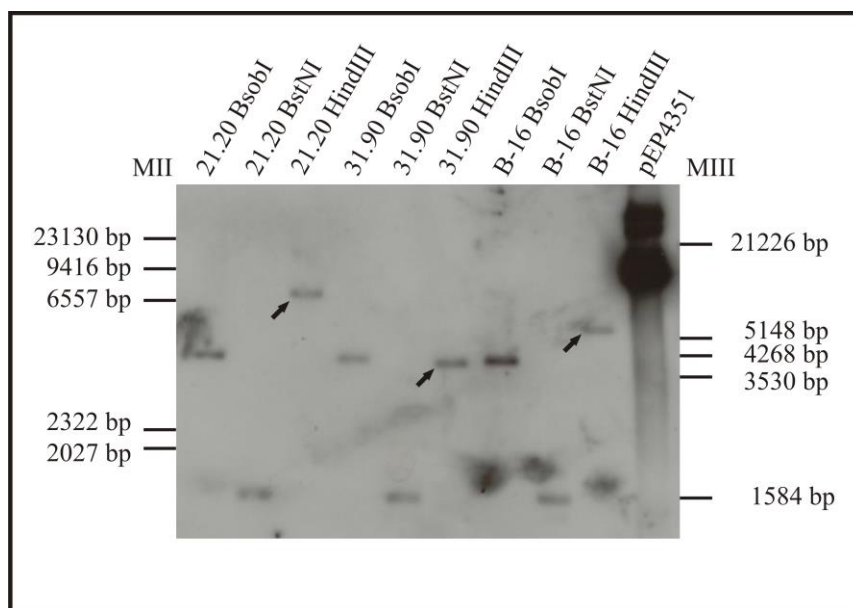


Figure 5.6: Detection of the Tn4351-transposon in the genomic DNA of the mutants 21.20, 31.90 and B-16 digested with the restriction enzymes BsoBI, BstNI and HindIII. The vector pEP4351, containing the Tn4351-transposon, was used to control the specificity of hybridization with the DNA probe. The markers MII and MIII were used for molecular weight determination. Arrows mark bands in HindIII digested DNAs.

We expected fragments with at least 1608 bp, depending on the second restriction site which is located next to the transposon insertion in the genomic DNA of the mutants and which should be different for each mutant. Accordingly, we obtained fragments of about 6.5 kbp for mutant 21.20, 4 kbp for mutant 31.90 and 5 kbp for mutant B-16 (Figure 5.6). Southern blot localization of the HindIII digested DNA showed one signal with different length for each mutant suggesting that the transposon was integrated once in each mutant genome at different sites. Nucleotide and protein sequences of the insertion sites and alignments of the protein sequences with similar sequences of other bacteria were attached in the Supplementary Data (Figures S5.1-12 Suppl. Data S5.2). ORF1 of mutant B-16 consists of 502 nucleotides and was disrupted 74 nucleotides next to the stop codon by the transposon (Figure 5.7; Figure S5.1 Suppl. Data S5.2). Blast searches in the NCBI database revealed that the protein sequence is similar to those of the MarR (multiple antibiotic resistance regulator) family of transcriptional regulator proteins (Figure S5.4 Suppl. Data S5.2). The sequenced ORFs in the region next to the disrupted gene were identified to encode for an YceI family protein (ORF2), a restriction endonuclease (ORF3) and an AMP nucleosidase (ORF4) (Figures S5.5-7 Suppl. Data S5.2). The gene region seems to be highly conserved as we found similar gene arrangements in two other *Dyadobacter* species and in the distantly related *Runella slithyformis* (Figure 5.7). ORF1 of the mutant 21.20 is disrupted by the transposon 69 nucleotides next to the stop codon (Figure 5.8; Figure S5.2 Suppl. Data S5.2). Interestingly, in the mutant 31.90 the transposon was integrated next to the same ORF as in mutant 21.20 (Figure 5.8; Figure S5.3 Suppl. Data S5.2). The best Blast hit for ORF1 and 2 of the two mutants was a Ca²⁺-ATPase of *Fibrisoma limi* (Figures S5.8 and 12 Suppl. Data S5.2). The regions next to the ATPase gene were sequenced in mutant 31.90 and 21.20, respectively. For ORF1 of mutant 31.90 we found weak similarities to a hypothetical protein in diverse bacteria (Figure S5.11 Suppl. Data S5.2). For ORF2 and ORF3 of mutant 21.20 we found similarities with a cupin protein or cupin-domain containing proteins and an alcohol dehydrogenase, respectively (Figures S5.9 and 10 Suppl. Data S5.2). The gene region next to the transposon insertion site of the two mutants seems to be less conserved as we found no or only low Blast hits within the genomes of *Dyadobacter fermentans* and *Dyadobacter beijingsensis*. Additionally, in *F. limi*, which showed high Blast hit values for the ATPase of the mutants, we found only low or no similarities with the adjacent ORFs and if so, the corresponding genes are located in other genome regions (Figure 5.8). The same is true for *Mucilaginibacter paludis*, where we found high Blast hits for ORF2 and 3 of mutant 21.20 (Figure 5.8).

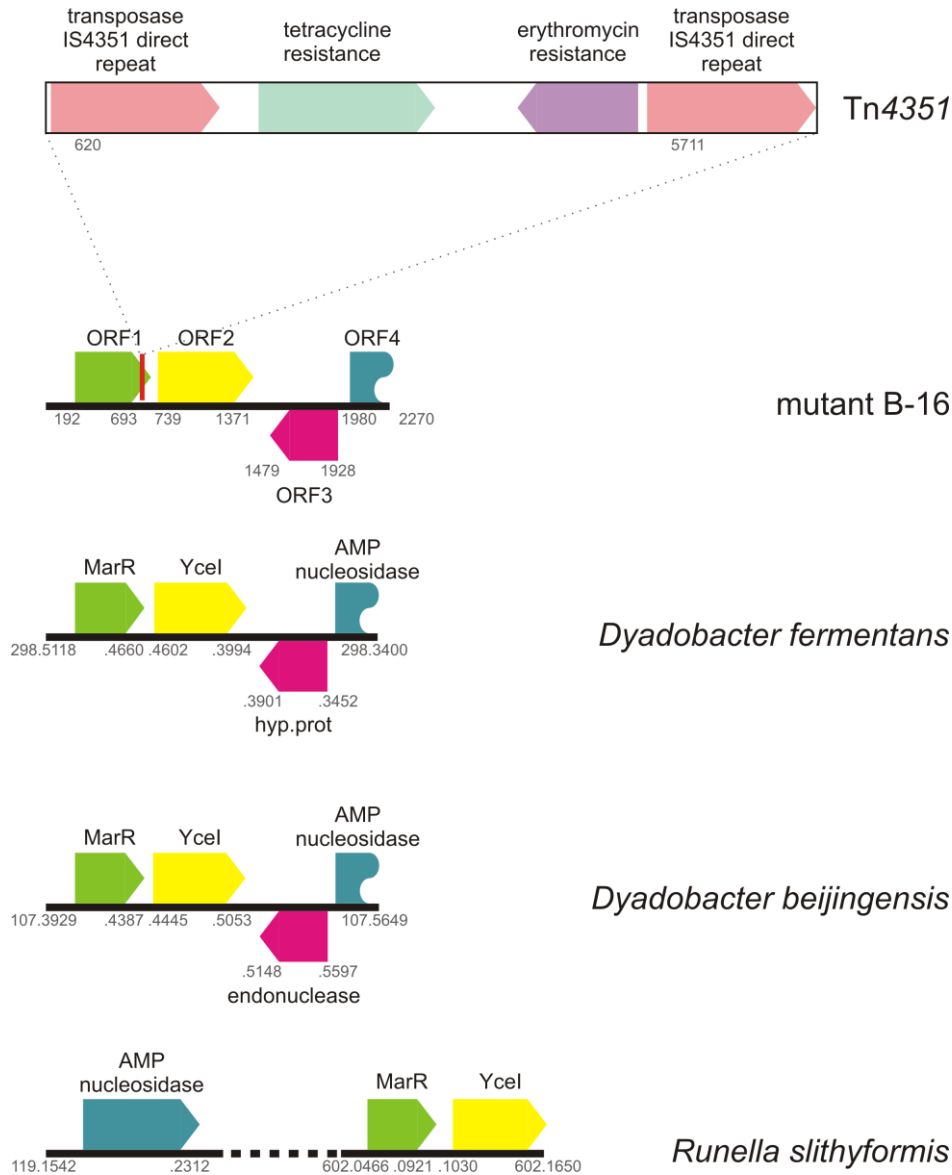


Figure 5.7: Insertion site of the Tn4351-transposon of the mutant B-16 and comparison with the respective genome regions of other bacteria. The transposon is integrated in the open reading frame 1 (ORF1), marked by a red bar. Genes whose protein sequences exhibited high similarities are stained with the same colour. Genome informations were obtained from the NCBI database. *D. fermentans*: MarR family transcriptional regulator, YP_003086806.1, max. ident. 72% (green); YceI family protein, YP_003086805.1, max. ident. 82% (yellow); hypothetical protein, YP_003086804.1, max. ident. 80% (hyp. prot., pink); AMP nucleosidase, YP_003086803.1, max. ident. 93% (blue). *D. beijingensis*: Transcriptional regulators MarR, WP_019940850.1, max. ident. 71% (green); lipid-binding protein, WP_019940851.1, max. ident. 81% (YceI, yellow); restriction endonuclease, WP_019940852.1, max. ident. 80% (endonuclease, pink); AMP nucleosidase, WP_019940853.1, max. ident. 92% (blue). *R. slithyformis*: AMP nucleosidase, YP_004654579.1, max. ident. 86% (blue); regulatory protein MarR, YP_004658609.1, max. ident. 60% (green); YceI family protein, YP_004658610.1, max. ident. 53% (yellow).

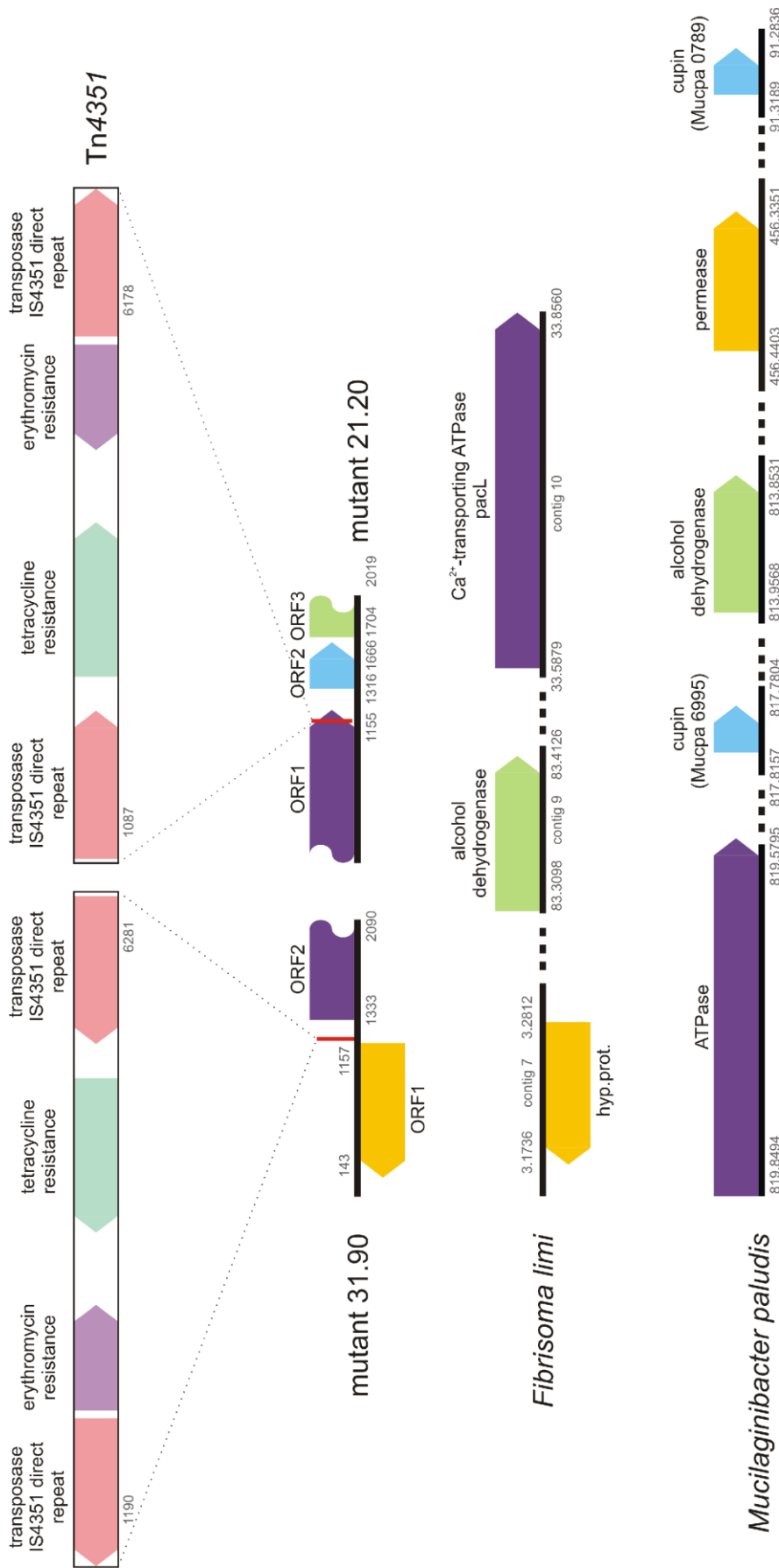


Figure 5.8: Insertion sites of the Tn4351-transposon of the mutants 21.20 and 31.90 and comparison with the respective genome regions of other bacteria. The transposon is integrated in the open reading frame 1 (ORF1) of the mutant 21.20 and between ORF1 and 2 in case of mutant 31.90, marked each with a red bar. Genes whose protein sequences exhibited high similarities are stained with the same colour. Genome informations were obtained from the NCBI database. *F. limi*: hypothetical protein, WP_009283022.1, max. ident. 31% (hyp. prot., yellow); alcohol dehydrogenase, WP_009284351.1, max. ident. 65% (green); Ca²⁺-transporting ATPase, WP_009285602.1, max. ident. 72% for mutant 21.20 and 65% for mutant 31.90 (violet). *M. paludis*: ATPase, WP_008513200.1 and WP_008504582.1, max. ident. 46% for mutant 21.20 and 47% for mutant 31.90; cupin, WP_008513200.1 and WP_008508719.1, max. ident. 65% and 58% (blue); alcohol dehydrogenase, WP_008513141.1; max. ident. 80% (green); permease, WP_008508719.1, max. ident. 34% (yellow).

5.5 Discussion

We found that growth of the benthic diatom *F. brevistriata* is strongly enhanced when co-cultivated with *Bacteroidetes* strain 32 on agar plates, while under axenic conditions growth is strongly retarded. This different growth behaviour can be easily observed in well plates by following diatom colony formation. The reason for this behaviour is yet unclear. According to Provasoli and Carlucci (1974), *F. brevistriata* shows vitamin B₁₂ auxotrophy. However, the used medium was supplemented with a number of vitamins and trace metals. Furthermore, Bruckner et al. (2008) have demonstrated that *Bacteroidetes* strain 32 also had a growth-enhancing effect on the diatom *Cymbella microcephala*. The authors conducted their experiments using the same medium as in this study (there denoted as diatom medium (DM)) and have ruled out nitrate limitation of the axenic culture. In order to identify transposon tagged mutants of strain 32 that do not promote growth of the diatom anymore, we have established a bioassay on solid medium in multi well plates, allowing a fast screening of a huge number of co-cultures while simulating the conditions of the biofilm matrix. Exchange of nutrients may here be limited by diffusion through the agar layer, in contrast to cultivation in shaking liquid medium. Thus, local nutrient limitations and accumulation of waste products may play a role on solid medium and bacterial nutrient supply or degradation of waste products is conceivable. Furthermore, growth-promotion was also visible on vitamin depleted medium, indicating that the bacterium may supply the alga with vitamins. This may explain, besides low bacterial cell density, why the observed growth differences of xenic and axenic diatom was not visible when cultivated in liquid medium. However, the filter-sterilized bacterial supernatant, which should contain possible secreted bacterial substances or nutrients, had no effect on algal growth, a hind, that nutrients alone are not responsible for the growth effect. This should be treated with caution, as we observed toxic effects of the bacterial growth medium (diluted LB) itself on the diatom *Achnantheidium minutissimum* (data not shown). Possible toxic effects of diluted LB on axenic *F. brevistriata* could not be determined, as diatom growth itself is strongly decelerated without bacteria, but the diatom grew well when co-cultivated with the bacterium which was previously cultivated in liquid diluted LB medium. When inoculated with the bacterium, some residues of the bacterial growth medium had obviously no toxic effects here.

Heat treated bacterial cells had also no effect on the diatom and showed, that the presence of viable bacteria is needed to induce growth enhancement. This is in broad agreement with the studies of growth influence of three bacteria on ten different

phytoplankton species on agar plates, including two diatom species (Ukeles and Bishop 1975). One *Vibrio anguillarum* strain enhanced growth of all tested algae and this effect depended also on the vitality of the bacterium. Heat treated suspensions of bacteria or vacuum-dried bacteria did not achieve the same effect as viable cells. Filter-sterilized supernatant of the bacterium as well as vitamin supply to the axenic cultures had no influence as well. The authors instead described some evidence that growth stimulatory factors released by bacterial degradation of the agar may have played a role. *Bacteroidetes* strain 32 is indeed able to grow on solid BM but not in liquid BM (Figure S4.1 Suppl. Data S4.1). Thus, the bacterium may be able to metabolize the agar of the medium, supporting the hypothesis of (Ukeles and Bishop 1975). Carbohydrates of the diatom *F. brevistriata* did not seem to play a role as carbon source for the bacterium as we observed no bacterial growth in the liquid co-culture. In nature, the bacterium may receive substrates from other sources, for example other diatoms. A biofilm is a highly heterogeneous entity, in which different organisms live together in spatial proximity. The bacterium may live together in a mutualistic interaction with a specific diatom species, but other diatoms like *F. brevistriata* might be beneficiary of this interaction.

In order to characterize the growth-promoting bacterial strain genetically, we conducted transposon mutagenesis of this strain using the transposon Tn4351. This transposon was already successfully used to study several functions of *Bacteroides thetaiotaomicron* and *Cytophaga johnsonae* (Anderson and Salyers 1989; Cheng and Salyers 1995; McBride and Kempf 1996). The transposon was isolated from a *Bacteroides* plasmid and conveys erythromycin resistance when expressed in *Bacteroides* spp., but not in *E. coli*, allowing selection of the mutants (Shoemaker et al. 1986). Furthermore, the transposon carries a tetracycline resistance gene which is only expressed in *E. coli* (McBride and Kempf 1996). This was utilized for the selection of *E. coli* which carried Tn4351 in their fosmids. At least 2858 Tn4351-mutants were screened for a loss of influence on growth of *F. brevistriata* and three of them showed the desired phenotype. Genetic characterization of these mutants revealed two independent gene loci, a gene whose product is related to the MarR family of transcriptional regulators and a gene encoding a Ca²⁺-ATPase homolog. Two of the mutants were affected in the same gene region: in mutant 21.20 the ATPase gene was disrupted directly, while in mutant 31.90 the transposon was integrated 143 nucleotides next to the ATPase gene, which may also here influence the expression of the transporter.

The *mar* locus of *E. coli* is known to confer resistance to multiple antibiotics, organic solvents, oxidative stress agents and household disinfectants and is regulated by its regulator

MarR (Aleksun and Levy 1999). MarR of *E. coli* in its activated state represses the transcription of the *marRAB* operon. Inactivation of MarR leads to the expression of the *marRAB* operon and its products in turn regulate the expression of other genes for example those for antioxidant defence or antibiotic resistance (Ariza et al. 1994; Aleksun and Levy 1999). Besides other substances, MarR of *E. coli* binds to salicylate, which is a derivate of the plant hormone salicylic acid, and thus the ability of the regulator to complex with the *mar* promoter is reduced (Martin and Rosner 1995), which in turn increases the transcription of the *marRAB* operon (Cohen et al. 1993). Transposon insertion in a putative *marR* of *Xanthomonas campestris*, which is responsible for the plant black rot disease, led to a loss of pathogenicity of the bacterium (Qian et al. 2005; Wei et al. 2007), which supports the idea, that the *mar* operon may indeed play a role in interkingdom signalling between bacteria and plants or algae. In general, MarR homologs in bacteria are thought to be regulatory factors whose activities are regulated by phenolic compounds, which are often of plant origin (Sulavik et al. 1995). We thus speculate that *Bacteroidetes* strain 32 may recognize similar substances released by diatoms via binding to MarR, which in turn may mediate acclimatisation and response mechanisms of the bacterium resulting in the observed growth effect. For the mutant B-16 this would imply that the bacterium might not respond to the presence of the diatom and may not induce growth enhancement. In this case we would have the interesting situation that the bacterium might perceive signals from diatoms before intracellular mechanisms lead to phenotypic changes which in turn induce diatom growth. This could be a further explanation why the bacterial supernatant, harvested from bacterial cells which were previously cultivated separately from the alga, showed no effects. Such well-established interplay would indicate a close and specific interaction of the bacterium with diatoms.

The potential role of the Ca²⁺-ATPase for the diatom/bacterium interaction is also almost unclear. Ca²⁺ is a ubiquitous intracellular second messenger in eukaryotes (Alberts et al. 1995). The eukaryotic Ca²⁺-ATPase restores the low cytosolic Ca²⁺ concentration to the level of the non-stimulated state by active efflux of this ion. The role of calcium in prokaryotes is comparatively less investigated but is implicated in several functions as in chemotaxis, heat shock, differentiation, cell cycle and pathogenicity or symbiosis (Norris et al. 1996). Naseem et al. (2009) showed that Ca²⁺ regulates at least 110 genes in *E. coli* and a set of virulence genes of the human pathogen *Yersinia pestis* is also Ca²⁺ regulated (Straley et al. 1993). A putative role of this cation as a second messenger in prokaryotes is under discussion (Dominguez 2004). It is thus conceivable that the Ca²⁺-ATPase of *Bacteroidetes*

strain 32 may play a role in the regulation of the bacterial calcium homeostasis and a deletion of its function may disrupt intracellular regulation processes which are possibly involved in the growth enhancement of the diatom.

Taken together, we showed that the *Bacteroidetes* strain 32 enhances growth of the diatom *F. brevistriata* when co-cultivated on solid medium and that a MarR homolog and a calcium pump may be involved in the growth-promoting potency of this bacterium.

5.6 Acknowledgements

The authors thank Annette Ramsperger and Kirstin Meyer for their assistance in screening of the transposon mutants. We further gratefully acknowledge the financial support by the Konstanz Research School Chemical Biology (GSC 218) and the University of Konstanz.

Supplementary Data

S5.1 Localization of transposon insertion conducted by the Trenzyme company

S5.2 Nucleotide and protein sequences of the up- and downstream regions of the transposon insertion sites

S5.3 Alignments of the open reading frames with similar protein sequences from other bacteria

Chapter 6

Characterization of the model organisms *Achnanthydium minutissimum*, *Fragilaria brevistriata* and *Bacteroidetes* strain 32 and their cultivation conditions

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Chapter 6: Characterization of the model organisms *Achnantheidium minutissimum*, *Fragilaria brevistriata* and *Bacteroidetes* strain 32 and their cultivation conditions

6.1 Abstract

The diatoms *Achnantheidium minutissimum* (Kützing) Czarnecki and *Fragilaria brevistriata* Grunow have recently been established as model organisms in combination with a *Bacteroidetes* isolate to study diatom/bacteria interactions in freshwater biofilms. Both diatom strains showed strong physiological changes when cultivated in the presence of the bacterium like enhanced growth and biofilm formation. We here improved cultivation conditions and characterized the physiology of these organisms with regard to vitamin B₁₂ requirement of *A. minutissimum* and growth behaviour of the bacterium in the co-culture with *A. minutissimum*. We further describe in this chapter a protocol for the cryopreservation of both diatom strains.

Keywords: Diatom medium · vitamin requirement · cryopreservation

6.2 Introduction

A. minutissimum (renamed from *Achnanthes minutissima* (Kützing) (Czarnecki 1994)) is one of the most abundant freshwater diatoms (Patrick and Reimer 1966; Krammer and Lange-Bertalot 1991b) and is an eukaryotic pioneer during the initial processes of biofilm formation (Sekar et al. 2004). Bahulikar (2006) demonstrated that this diatom is also abundant in photoautotrophic, epilithic biofilms of Lake Constance. Similarly, *Fragilaria brevistriata* Grunow is also a very wide spread diatom species (<http://www.algaebase.org/>). Bacteria of the *Bacteroidetes* phylum are common satellite bacteria of diatoms (Amin et al. 2012). *Bacteroidetes* strain 32 belongs to the *Dyadobacter* genus (Bruckner et al. 2008) and bacteria of this group were found in freshwater and soil samples or to be associated with maize (Chelius and Triplett 2000; Baik et al. 2007; Zhang et al. 2010). Both, *A. minutissimum* and *F. brevistriata* showed strong physiological changes when co-cultivated with *Bacteroidetes* strain 32: The bacterium induced capsulation, aggregation and biofilm formation of *A. minutissimum* (Chapter 4), while it further enhanced growth of the diatom *F. brevistriata* (Chapter 5).

6.3 Materials and Methods

6.3.1 Organisms and cultivation conditions

Achnantheidium minutissimum (Kützing) Czarnecki was isolated from photoautotrophic, epilithic biofilms of Lake Constance and purified from associated bacteria (Windler et al. 2012). The axenic culture of *Fragilaria brevistriata* Grunow (isolate F-02) was obtained from Dr. Rahul Bahulikar (The Samuel Roberts Noble Foundation, Ardmore, USA). Both diatoms were morphologically identified according to Krammer and Lange-Bertalot (1991a, b) by Anastasiia Kryvenda (University of Göttingen). The diatom stock cultures were cultivated in liquid Bacillariophycean Medium (BM) (Schlösser 1994), which was modified according to Windler et al. (2012). Cultures were exposed to a 12:12 h light:dark cycle with light intensities of 20-50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 16°C (denoted in this study as standard cultivation conditions for diatoms). The diatom cultures were sub-cultured monthly into fresh medium.

Bacteroidetes strain 32 was isolated by Bruckner et al. (2008). The bacterium was cultivated at 22°C on agar plates containing diluted Luria Broth (LB) medium (50% (v/v) (Miller 1972)), sub-cultivated monthly and stored at 4-8°C.

6.3.2 A new diatom medium without soil extract for *Achnanthydium minutissimum*

The basal medium for the Achnanthydium Medium (AM) is BM (Windler et al. 2012) without soil extract. The basal medium was supplemented with 2 x f/2 vitamin solution and 0.5 x f/2 Trace Metals solution described by Guillard (1975), 50 mg l⁻¹ Na₂SiO₃·5H₂O and 0.75 µg l⁻¹ H₂SeO₃. To test the diatom growth, axenic *A. minutissimum* was inoculated in 250 ml AM and BM and cultivated with a 8:16 h light:dark cycle and a light intensity of 30 µmol photons m⁻² s⁻¹ at 20°C and about 100 rpm. Cells were counted with a hemocytometer.

6.3.3 Vitamin B₁₂ requirement

Xenic and axenic *A. minutissimum* cells were cultivated on agar plates containing AM with and without vitamin B₁₂ and cultivated at 18°C with a light intensity of 35 µmol photons m⁻² s⁻¹.

6.3.4 Cryopreservation of *Achnanthydium minutissimum* and *Fragilaria brevistriata*

Axenic cultures of *F. brevistriata* and *A. minutissimum* and the xenic culture of *A. minutissimum* were treated with 4, 6 or 8% (v/v) methanol and incubated for 3, 5 or 8 min at room temperature before freezing. Cryopreservation was performed in a Cryo Freezing Container (#115650; Qualilab, Olivet, France) for 1.5 h at -80°C which allowed a controlled decrease of the temperature for 1°C per min. After storage of several days in liquid nitrogen the cells were thawed at 35°C, transferred into 20 ml AM and incubated at standard cultivation conditions for diatoms for recovery. To test the viability of the cryopreserved cells, aliquots of the thawed cell suspensions were either spotted on agar plates containing BM to determine the colony forming units (CFUs) or 100 µl were transferred into 5 ml AM and incubated at standard cultivation conditions for diatoms.

6.3.5 Organic substrates for *Bacteroidetes* strain 32

The bacterium was cultivated in liquid BM with different organic substrates: Tryptone or yeast extract, with the same concentration as in diluted LB, or with 10 mM glucose (glcBM). The cultures were cultivated for 7 days at 20°C and about 100 rpm.

6.3.6 Growth of *Bacteroidetes* strain 32 in co-culture with *Achnanthydium minutissimum*

Growth of *Bacteroidetes* strain 32 was measured in the same experiment as described for diatom growth in Chapter 4. 10⁵ cells ml⁻¹ of the axenic *A. minutissimum* culture were

inoculated with 5 μl of the bacterial culture with an optical density at 600 nm (OD_{600}) of 0.1 in a total volume of 500 μl BM. The co-cultures were cultivated at standard cultivation conditions for diatoms without shaking to allow biofilm formation. CFUs of the bacterium were measured in the supernatant of the co-cultures (non-adherent cells). 5 μl of the diluted supernatant were spotted on agar plates containing diluted LB and cultivated as described above for the bacterium. Each experiment was conducted in triplicates.

6.4 Results and discussion

6.4.1 A new diatom medium without soil extract for *Achnantheidium minutissimum*

BM is a diatom full medium which was frequently used in former studies, there partially denoted as Diatom Medium (DM) (Bahulikar and Kroth 2007; Bahulikar and Kroth 2008; Bruckner et al. 2008; Bruckner and Kroth 2009; Bruckner et al. 2011; Windler et al. 2012). This medium contains soil extract, which is an extract of garden mould, whose chemical composition is rather unknown. However, especially for studies of chemical interactions between diatoms and bacteria, a medium with an exact composition may be quite necessary. The soil extract itself may contain bacteria and bacterial products which possibly may impact the interaction studies. Further, soil extract is quite elaborate. It takes multiple steps of autoclaving, disrupted by several overnight incubations at room temperature and filter sterilisation steps to remove potential dormant bacteria, but still remains a potential source of contamination. We therefore developed a new medium on the basis of the BM for diatoms without soil extract (see Materials and Methods section). *A. minutissimum* reached about 50-60% higher chlorophyll yields in the stationary phase when cultivated in the Achnantheidium Medium compared to BM (Figure 6.1). The medium hence represents a good alternative for cultivation of the diatom without soil extract.

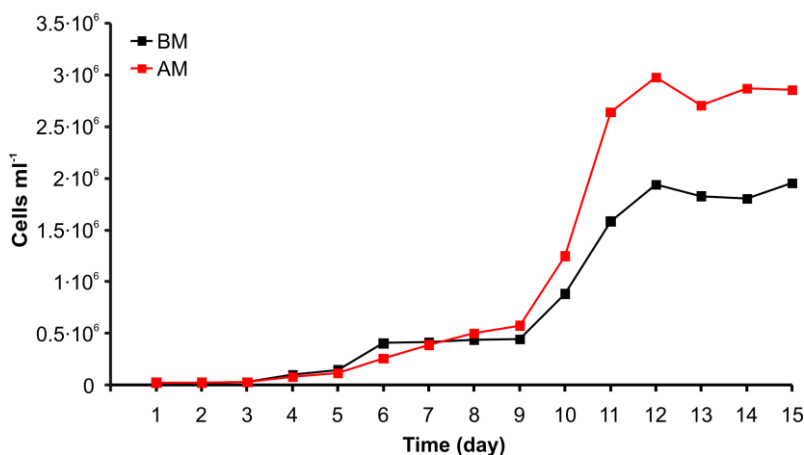


Figure 6.1: Growth of *Achnantheidium minutissimum* in different media. The diatom was cultivated in Bacillariophycean Medium (BM) and in Achnantheidium Medium (AM) (n=1).

6.4.2 Vitamin B₁₂ requirement

Croft et al. (2005) surveyed several algae species and found a large number of vitamin B₁₂ auxotrophs for example *F. brevistriata* (Provasoli and Carlucci 1974). These algae have to take up this vitamin from the surrounding medium to cover their demand and it is most likely that in nature this vitamin is of bacterial origin (Haines and Guillard 1974). However, in case of the diatom *A. minutissimum*, no growth differences were observable when cultivated on BM agar plates which were enriched with vitamin B₁₂ compared to the vitamin B₁₂ deficient medium. We hence classify *A. minutissimum* to be autotroph for this vitamin.

6.4.3 Cryopreservation diatom strains

A. minutissimum and *F. brevistriata* have been cryopreserved in different volumes of methanol with various incubation times (see Materials and Methods section). Spot tests of the axenic *A. minutissimum* culture showed no cell colonies after thawing and revealed that the diatom could not be recovered after cryopreservation. However, the xenic culture showed colony formation when incubated for 5 min with methanol before freezing. Incubation with 6% (v/v) methanol resulted in the highest CFU values. However, the best condition for the cryopreservation of *F. brevistriata* was not as clear as for *A. minutissimum*. Inoculation of liquid AM with an aliquot of the thawed *F. brevistriata* culture revealed that cells were still viable after cryopreservation with variable methanol concentrations and incubation times as shown in Table 6.1.

Table 6.1: Cryo-solutions and incubation times recommended for the cryopreservation of *Achnantheidium minutissimum* and *Fragilaria brevistriata*.

Diatom	Cryo-solution methanol (v/v)	Incubation time (min)
<i>A. minutissimum</i>	6%	5
<i>F. brevistriata</i>	4%	8
	6%	5
	8%	3
	8%	8

6.4.4 Organic substrates for *Bacteroidetes* strain 32

Bacteroidetes strain 32 was able to grow in BM complemented either with glucose (glcBM), tryptone or yeast extract. Interestingly, the bacterial culture lost its characteristicly

yellowish colour when cultivated in glcBM (Figure 6.2). 16S rDNA analysis excluded any potential contaminations with other bacteria (data not shown).

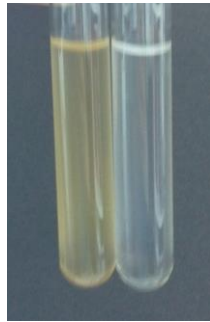


Figure 6.2: Growth of *Bacteroidetes* strain 32 in diluted LB and glcBM. The bacterium exhibited the typical yellowish colour when cultivated in diluted LB (left). In glcBM the bacterium grew colourless (right). The picture was obtained from Katrin Leinweber (University of Konstanz).

6.4.5 Growth of *Bacteroidetes* strain 32 in co-culture with *Achnanthisidium minutissimum*

The presence of *Bacteroidetes* strain 32 or its sterile supernatant induced capsulation and biofilm formation of the diatom *A. minutissimum* (Chapter 4). We here observed the growth behaviour of the bacterium in the co-culture with the diatom. Under this condition, the diatom formed a strong biofilm and cells stuck together and to the vessel surface making it impossible to measure CFUs within the biofilm (attached fraction). We hence measured the CFUs of the bacterium in the supernatant of the co-culture and observed relatively low values during the exponential phase and in the early stationary phase of the diatom (Figure 6.3). However, the CFUs strongly increased, when the diatom reached a plateau in the late stationary phase.

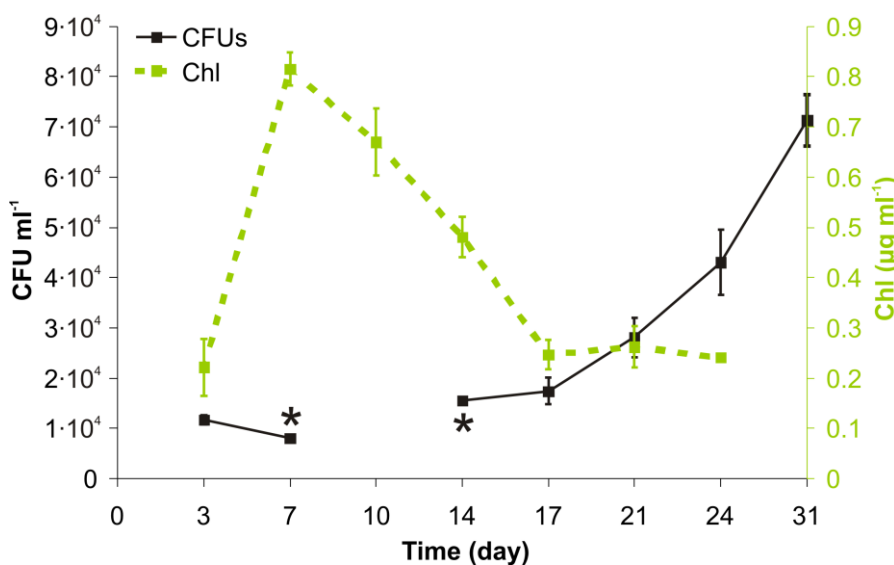


Figure 6.3: Growth of *Bacteroidetes* strain 32 in co-culture with *Achnanthisidium minutissimum*. Growth of the bacterium was measured in three parallel cultures (n=3; except for day 7 (n=2) and day 14 (n=1), marked by asterisks). Data of the chlorophyll measurement were collected from Chapter 4. Error bars indicate standard deviation.

Several explanations regarding the course of the CFU values are possible: The bacteria may either be strongly attached to the diatom and thus located in the adherent fraction until

the late stationary phase. Afterwards, the biofilm may be disintegrated and the bacterial cells may be released into the supernatant. This would suggest that the bacterium may live together with the diatom in a mutualistic interaction, possibly as it metabolizes extracellular polymeric substances released by the alga. However, another possible scenario could be that the number of CFUs in the supernatant may resemble the total number of bacteria (attached and non-attached bacteria). This would mean that the bacterial density within the biofilm showed also the same pattern of low values during the exponential phase and early stationary phase of the diatom and that bacterial growth was boosted in the late stationary phase. This could be a hint that the vital diatoms may have some defence mechanisms during the exponential phase and early stationary phase, for example capsule formation, to protect themselves from being overgrown and decomposed by the bacteria. The putative defence mechanisms may eventually collapse in old diatom cultures. This scenario would indicate that the bacterium might utilize organic substances from dead algae cells rather than extracellular compounds produced by viable diatom cultures.

6.5 Conclusion

The diatoms *A. minutissimum* and *F. brevistriata* showed strong physiological changes when co-cultivated with *Bacteroidetes* strain 32 (Chapters 4 and 5). Based on these observations, both diatom strains were recommended, together with the bacterium, to represent good model organisms to study diatom/bacteria interactions. *F. brevistriata* turned out to be a highly stable diatom as it is kept in our culture collection since several years (Bahulikar 2006). *A. minutissimum* showed indeed the typical cell size reduction and morphological aberrations of diatoms during long-term cultivation; however this diatom could now be cryopreserved and stored for a long time. Furthermore, transcriptome data and gene annotations of *A. minutissimum* are now available in our lab (Rottberger 2013).

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Chapter 7

General Discussion

Chapter 7: General Discussion

7.1 Cellular mechanisms involved in the interactions between *Bacteroidetes* strain 32 and diatoms

In this thesis it was observed that bacteria may considerably influence different physiological characteristics of diatoms:

- 1) Cell size diminution and structure of the silica frustule during long-term cultivation (Chapter 3)
- 2) Capsulation and biofilm formation (Chapter 4)
- 3) Culture growth (Chapter 5)

Up to now little is known about the cellular mechanisms which are involved in the diatom/bacteria interactions, not at least because of the lack of convenient and reliable model systems to study such interdependencies especially with regard to biofilm formation. Two bioassays were therefore established in the course of this work. Both assays have proved to be suitable for the investigation of cellular mechanisms of diatom/bacteria interactions which lead to enhanced diatom growth and matrix material production, important factors of biofilm formation as both groups of organisms are pioneers in the colonization of wet and irradiated surfaces (Cooksey and Wigglesworth-Cooksey 1995; Wetherbee et al. 1998; Sekar et al. 2004). With the help of these bioassays we obtained important insights in the complex genetic and chemical mechanisms which underly the interactions between representatives of benthic diatoms and bacteria.

In Chapter 4 we showed that capsule and biofilm formation of the diatom *A. minutissimum* considerably depends on the presence of bacteria. During the development of the bioassay, we screened three further diatom species (*Nitzschia palea*, *F. brevistriata* and *Fragilaria capucina*), in addition to *A. minutissimum*, and found that biofilm formation in most cases depended on the presence of bacteria. Further investigations revealed that capsulation and biofilm formation were induced by one or more soluble molecule(s) of a bacterium belonging to the *Bacteroidetes* phylum. This molecule(s) apparently possesses hydrophobic properties and is quite thermostable. Known bacterial signal molecules with hydrophobic characters are for example *N*-acylhomoserine lactones (AHLs), common autoinducers of Gram-negative bacteria which mediate quorum sensing (Chhabra et al. 2005). Amin et al. (2012) speculated that such intraspecific signal molecules may also be exploited

in interkingdom signalling between diatoms and bacteria which might be also the case for *A. minutissimum* and *Bacteroidetes* strain 32. We further found evidence that two genes of *Bacteroidetes* strain 32, whose products are related to the transcriptional regulators of the MarR family and a Ca^{2+} -ATPase, respectively, are involved in the cellular mechanisms which lead to growth enhancement of *F. brevistriata* (Chapter 5). Figure 7.1 shows a hypothetical scheme of the cellular mechanisms of *Bacteroidetes* strain 32 which may be involved in the bacterial influence on diatom growth and biofilm formation. In case of capsulation/biofilm formation of *A. minutissimum*, the bacterium produced the substance(s) even when cultivated without the diatom, indicating a constitutive production which is independent of the presence of the diatom. In contrast, members of the MarR family are thought to be regulators which are in turn regulated by environmental signals like phenolic compounds which are often of plant origin (Sulavik et al. 1995). The involvement of a Ca^{2+} -ATPase might further indicate intracellular regulation processes with Ca^{2+} as a putative intracellular second messenger (Straley et al. 1993; Naseem et al. 2009; Dominguez 2004). In case of growth stimulation of *F. brevistriata* we hence suggest that the bacterium may at first recognize the presence of the diatom, possibly by secondary metabolites of the alga via the MarR regulator, which in turn may alter intracellular processes in which calcium might also be involved and which finally leads to a response mechanism of the bacterium. This suggests that the sense- and regulatory mechanisms of the bacterium to induce capsulation/biofilm formation of *A. minutissimum* and growth of *F. brevistriata* may be quite diverse and reveals the complexity of the underlying processes which mediate diatom/bacteria interactions.

7.2 *Bacteroidetes* strain 32 and diatoms – Is it love?

Members of the *Bacteroidetes* phylum are often associated with diatoms (Knoll et al. 2001; Schäfer et al. 2002; Makk et al. 2003; Kaczmarska et al. 2005; Sapp et al. 2007a, b; Bruckner et al. 2008). *Bacteroidetes* strain 32 possesses several impacts on the physiology of different diatom species: It changes the formation of EPS of *A. minutissimum* (Chapter 4) and *Cymbella microcephala* (Bruckner et al. 2008) and thus induces capsule and biofilm formation of both diatoms. It further induces enhanced growth of *F. brevistriata* (Chapter 5) and *C. microcephala* (Bruckner et al. 2008) (Table 7.1). This leads to questions on the nature of the interactions between the bacterium and diatoms that trigger the observed physiological changes. Do these interactions represent a mutualistic or a commensalistic relationship or in case of capsule/biofilm formation of *A. minutissimum* rather a parasitic relationship? Does capsules/biofilm formation offers protection against the bacteria, or do the bacteria implement

a convenient environment for the diatom to settle and to attach irreversibly by forming a biofilm?

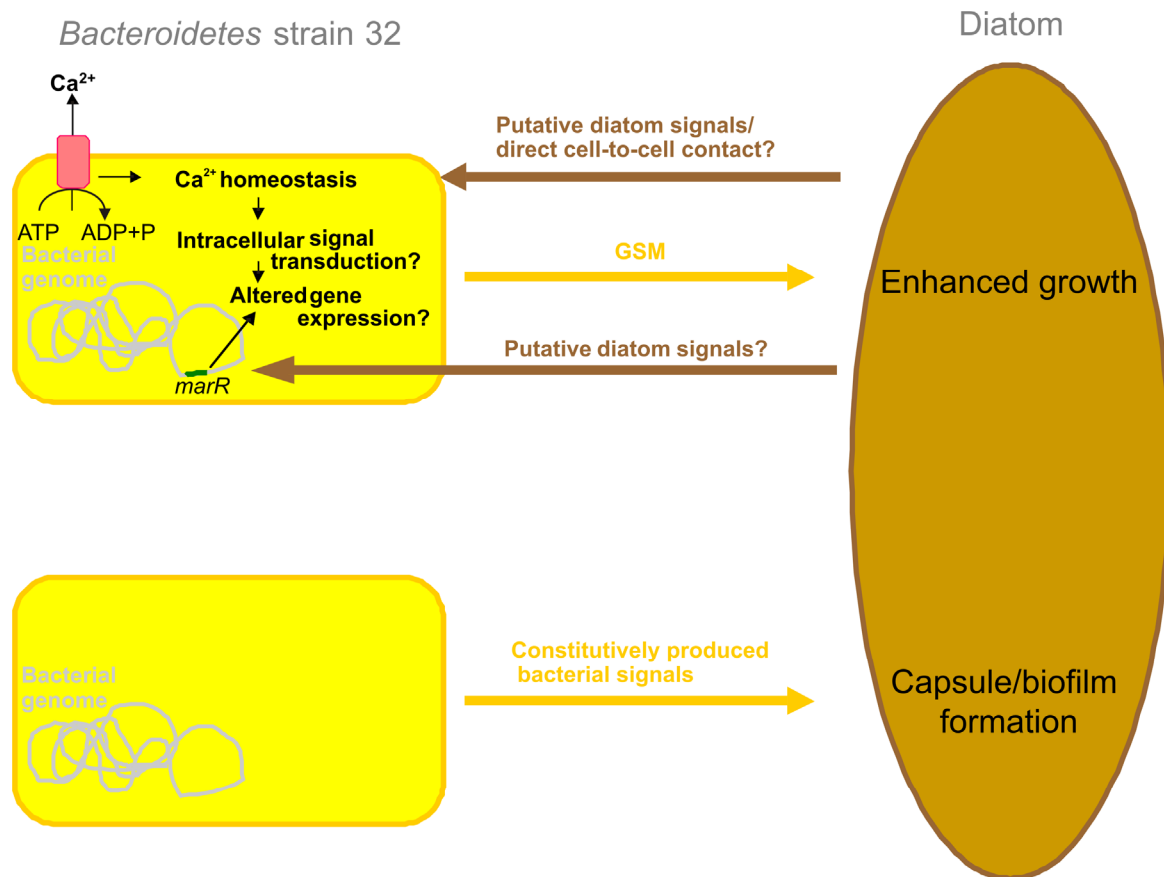


Figure 7.1: Hypothetical scheme of the cellular mechanisms involved in the interactions of *Bacteroidetes* strain 32 and diatoms based on the results of this thesis. The cellular bacterial mechanisms which lead to the observed physiological changes of the diatoms may be quite different: In case of growth stimulation of *F. brevistriata* the bacterium may sense the presence of the diatom, possibly via algal compounds recognized by a MarR homolog, which may result in an altered gene expression. Calcium may also play a role in the regulation of the growth stimulating mechanism (GSM) of the bacterium. The bacterial induction of capsulation and biofilm formation of *A. minutissimum* may be rather a constitutive mechanism of the bacterium mediated by soluble bacterial compounds.

Table 7.1: Overview of the influence of *Bacteroidetes* strain 32 on growth and capsulation/biofilm formation of the diatoms investigated in this thesis compared to the influence on the original host diatom *Cymbella microcephala*.

Diatom	Growth	Capsulation/Biofilm	Reference
<i>A. minutissimum</i>	-	+	Chapter 4
<i>F. brevistriata</i>	+	-	Chapter 5
<i>C. microcephala</i>	+	+	Bruckner et al. (2008)

Bacteroidetes strain 32 was originally isolated from a xenic culture of *C. microcephala* that previously had been cultivated in xenic condition for more than two years and can be considered as a satellite bacterium of this alga (isolation and cultivation of *C. microcephala* was described in (Bahulikar 2006); isolation of the bacterium was performed by Bruckner et al. (2008)). Schäfer et al. (2002) investigated xenic cultures of six different diatom species and found that the so called “satellite community” (“accompanying bacterial community”) of each diatom was unique and they proposed that “algal cells constitute niches for specific bacterial species”. Gärdes et al. (2010) distinguished between free-living and diatom-attaching bacteria showing that attached bacteria may influence aggregation whereas free-living bacteria do not. It is thus conceivable that the interactions between the host diatoms and their satellite bacteria are specific and particularly tight. Application of *Bacteroidetes* strain 32 to *F. brevistriata* and *A. minutissimum* may of course represent artificial situations as it has not been proven that this bacterium is also a natural part of the satellite communities of these diatoms. However, both diatom strains were isolated from biofilms from the same sampling site as *C. microcephala* and *Bacteroidetes* strain 32, indicating that all organisms used in this thesis are inhabitants of the same biofilm community. Furthermore, *A. minutissimum* showed the same physiological changes when cultivated with its co-isolated satellite community as with *Bacteroidetes* strain 32: both the co-culture with strain 32 as well as the xenic *A. minutissimum* with its satellite bacteria showed capsule and biofilm formation, suggesting that these effects may indeed be of relevance in the interaction of this diatom with its satellite community.

The presence of the bacterium could be of advantage for *F. brevistriata*, as diatom growth was enhanced in the co-culture. The bacterium in turn seems not to be able to grow with organic substrates secreted by *F. brevistriata*, algal organic substrates from the diatom might at the most help to survive the observed cultivation period of about one month (Chapter 5). Utilization of organic substrates from *F. brevistriata* by strain 32 is hence not proved and we therefore classify this interaction to be rather commensalistic with a benefit for the diatom than being mutualistic. As mentioned in Chapter 5, the heterotrophic bacterium must obtain organic substrates from other sources, under artificial culture conditions this could be from the agar, in nature possibly in the form of organic material from other diatoms like for example its putative original host *C. microcephala*. We thus assume that the growth stimulating mechanisms of the bacterium may be focused on its original host, the diatom from which the bacterium receives substrates, while other diatoms like *F. brevistriata* may also be beneficiaries of this interaction. This may not only play a role under artificial conditions as in

the co-culture of *F. brevistriata* and *Bacteroidetes* strain 32, but also in natural habitats, where different organisms in a biofilm live together in spatial proximity.

For the interpretation of the interaction between *Bacteroidetes* strain 32 and *A. minutissimum* we suggest two contradictory scenarios: the interaction can either be mutualistic or commensalistic, but also a parasitic relationship might be conceivable. Axenic and xenic *A. minutissimum* achieved similar cell densities, thus an advantage or disadvantage of the presence of the bacterium for the alga is not directly obvious as shown for *F. brevistriata*. However, within three days of co-cultivation, the diatom was attached to a surface via stalks and started with biofilm formation (Chapter 4). The presence of the bacterium might constitute a friendly environment and a favourable condition to start biofilm formation. As the axenic *A. minutissimum* cells did neither produce capsules nor attach to surfaces, this could mean that diatoms without suitable bacteria might, under natural conditions, be easily dispersed by water currents helping them to find new niches. The same changes of capsulation and biofilm formation were previously found in *C. microcephala*, where growth was enhanced in the presence of the bacterium (Bruckner et al. 2008), indicating that capsulation/biofilm formation may indeed be the reaction of a mutualistic or at least commensalistic interaction.

A. minutissimum might also react with capsulation as a defence or protection mechanism against bacteria. Without staining, capsules were indirectly visible as bacteria free zones around the diatom cells indicating that the capsules indeed keep bacteria away from the diatom cells. Furthermore, capsulation of *A. minutissimum* usually started in the stationary growth phase of the diatom (Chapter 4) when cells may be stressed due to nutrient limitation and much more vulnerable to a bacterial attack. The diatom produced copious amounts of EPS anyway, in axenic conditions in a soluble form and in xenic conditions as capsular material. Thus, capsulation might be associated with relative low energetic cost differences for the xenic diatom compared to the axenic culture, as polymers apparently are converted from a soluble to a bound form. This might constitute a convenient and rather “cheap” defence mechanism.

The impact of *A. minutissimum* on growth of the bacterium could not be determined as the bacterial cell density within the biofilm was not measurable due to technical constraints. We thus investigated the bacterial cell density in the supernatant (non-attached fraction) (Chapter 6). As described in this chapter, the bacterial cell density in the supernatant increased in the late-stationary phase of the alga. On the one hand, this could mean that the bacterium was tightly attached to the diatom within the biofilm and thus bacterial growth was

not detectable in the supernatant until the biofilm disintegrated. This would support the hypothesis of a mutualistic/commensalistic interaction. In this context the capsule of the algal cell might serve as a feeding ground for the bacterium to keep it close to the algal cell. On the other hand, the bacterium might decompose dead algal cells rather than extracellular compounds released by vital diatom cells, which would represent a parasitic interaction.

If we consider bacteria to be mutual partners of their host diatom but parasites for other diatom species, this could represent a mechanism that allows the diatom host to weaken other diatom species for example when competing for limiting nutrients (Grover 1988). Such a possibility may give a glimpse on the complex interactions in multispecies photoautotrophic biofilms *in natura* (Figure 7.2). In contrast to the highly simplified and artificial situation within a bioassay, which is reduced to investigate the interaction between one pair of organisms, the organisms in nature are faced with several different interactions at the same time. In case of photoautotrophic epilithic biofilms, this may comprise, besides the interactions between the host diatom and its satellite bacteria, further interactions within the microbial community for example interactions within the bacterial satellite community, with neighbouring diatoms, with free-living bacteria outside of the phycosphere and with other microbes like protozoa or fungi, up to animals like insect larvae and snails (Hunter 1980; Parry 2004; Ceola et al. 2013; Gao et al. 2013). These interactions may comprise all facets of interspecies up to interkingdom interactions and may range from mutualism to competition and even to parasitism. Together with influences of permanently altered external abiotic factors like light, wave disturbance, temperature and water level fluctuations (Hoagland and Peterson 1990; Schmieder et al. 2004; Rao 2010), a photoautotrophic, epilithic biofilm is a highly dynamic entity which is influenced by manifold parameters.

- GSM - growth stimulating mechanism
- CH - carbohydrates
- - *Bacteroidetes* strain 32

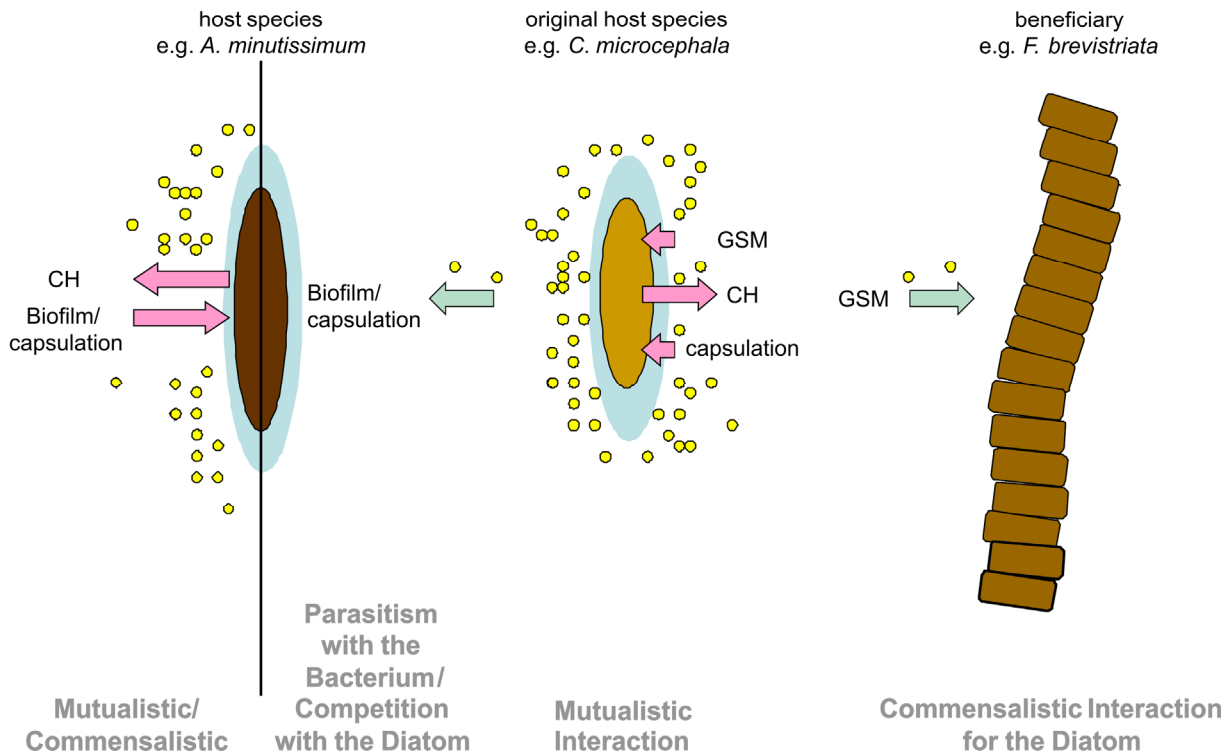


Figure 7.2: Hypothetical scenario of the interactions between *Bacteroidetes* strain 32 and diatoms within a biofilm. It is assumed that the satellite bacterium may live in a close and mutualistic relationship with its original host diatom e.g. *C. microcephala*. This diatom may provide organic substrates like carbohydrates (CH) for the bacterium which in turn possesses some growth stimulating mechanisms (GSM) to support algal growth and induces capsulation and biofilm formation of its host. The GSM might affect other diatoms which live in spatial proximity within the biofilm. These diatoms might benefit from the interaction between the bacterium and its host, but in turn exert no effects on the bacterium (commensalistic interaction with a benefit for the diatom). Capsulation and biofilm formation of *A. minutissimum* may either be the reaction of the diatom in a mutualistic/commensalistic interaction with the bacterium comparable to the relationship of the bacterium with its original host or a defence mechanism to save cells from parasitism by the bacterium. Such a hypothetical dual character of a bacterium, mutualistic partner for its original host diatom and parasite for other diatoms, might be exploited by the original host to weaken competitors.

Supplementary Data

Supplementary Data Chapter 3

S3.1 Example measurements of cell lengths of *Achnanthydium minutissimum* live cells

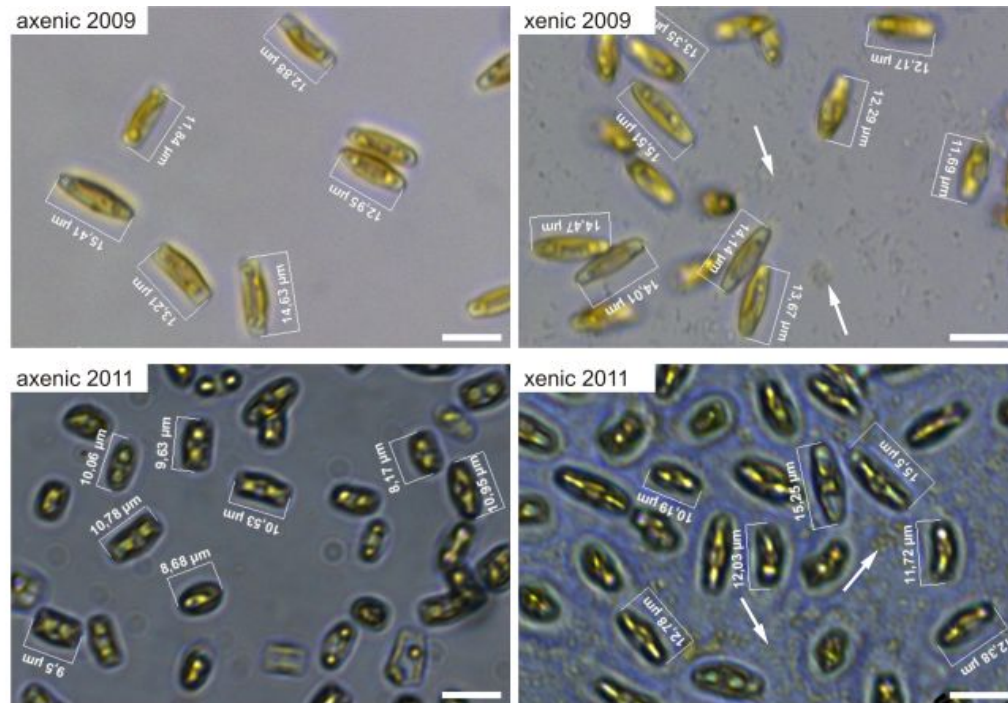


Figure S3.1: Cells of xenic and axenic *Achnanthydium minutissimum* cultures at the beginning of the experiment in April 2009 and close to the end of the experiment in August 2011. Arrows exemplarily mark bacteria cells in the xenic cultures. Scale bars represent 10 µm.

Supplementary Data Chapter 4

S4.1 Growth of *Bacteroidetes* strain 32 in diatom full medium

Bacteroidetes strain 32 was inoculated in the diatom full medium BM and in diluted LB (50% v/v). The bacterium was scrapped from an agar plate containing diluted LB, resuspended in 1 ml BM and 10 μ l were added to 5 ml of the respective medium in test glasses. Cultures were incubated at 22°C and 112 rpm. OD₆₀₀ was measured with the Camspec M107 Spectrophotometer (Camspec Ltd, Cambridge, UK). The bacterium was not able to grow in BM without *A. minutissimum* or glucose supplement (Figure S4.1).

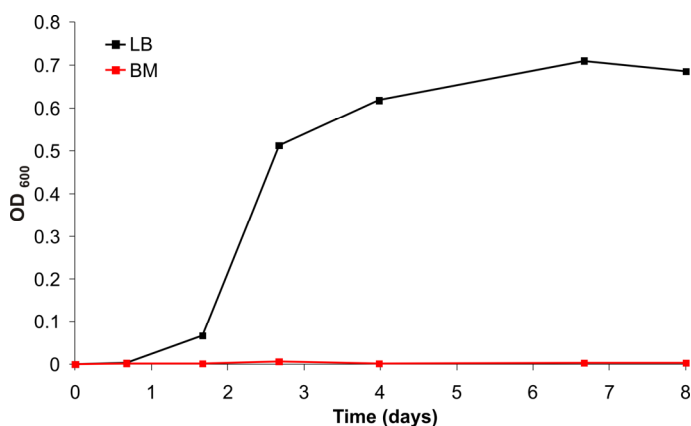


Figure S4.1: Growth of *Bacteroidetes* strain 32 in diatom full medium (BM). The bacterium was cultivated in BM (n=2) and in diluted LB (n=1).

S4.2 Co-cultivation of *Achnanthyidium minutissimum* with different bacterial isolates

Bacteria isolates B-1, B-2, B-4, B-5, B-6, B-7, B-8 and B-10 were isolated from photoautotrophic epilithic biofilms from the same sampling site as *A. minutissimum* and *Bacteroidetes* strain 32 in April 2011. The biofilm was scrapped from stone surfaces, diluted in BM and roughly vortexed for 10 min before the suspension was plated in gradual 1:10 dilutions on agar plates containing diluted LB medium. Single colonies were picked and isolated by repeated smear. The bacterial isolates were cultivated as described for strain 32. For co-cultivation, 1 ml BM was inoculated with $2.8 \cdot 10^4$ diatom cells ml^{-1} and 5 μ l of the bacterial cell suspension (OD₆₀₀ of 0.1). The co-cultures and negative controls (axenic diatom and bacteria cultivated in BM, respectively) were performed in triplicates, the positive control (co-culture of *A. minutissimum* with strain 32) in duplicates. Biofilm was stained with crystal violet and the absorption of the extracted dye was measured at 580 nm.

Supplementary Data

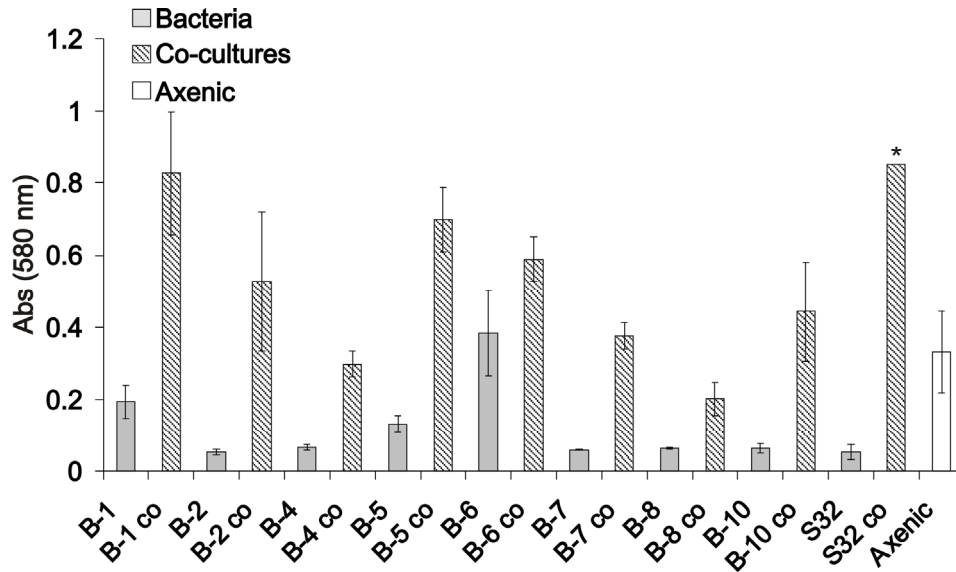


Figure S4.2: Absorption of crystal violet extracted from biofilms of co-cultures of *Achnanthydium minutissimum* with different bacterial isolates. The isolates are termed B-1 – B-10. The axenic diatom and the bacterial cultures were performed as negative controls and the co-culture of *A. minutissimum* with strain 32 as positive control. (n=3; error bars indicate standard deviation; * marks the positive control (n=2)).

Three co-cultures produce a clearly stronger biofilm than the axenic diatom culture (co-cultures with B-1, B-5 and B-6). Only the co-cultures with B-1 and B-5 achieved a biofilm quantity comparable to the co-culture with strain 32. Co-cultures with B-4, B-7 or B-8 showed comparable or even less crystal violet absorptions as the axenic diatom culture. Pure bacteria cultures in BM resulted in no or even low biofilm formation as they presumably did not grow in the used medium without the diatom or additional carbon sources. Capsule formation was only visible in the co-culture with strain 32.

Supplementary Data Chapter 5

S5.1 Localization of transposon insertion conducted by the Trenzyme company

Sequence analysis of the transposon insertion sites of *Bacteroidetes* strain 32 mutants were conducted partially by the Trenzyme Company (Trenzyme GmbH, Byk-Gulden-Strasse 2, 78465 Konstanz (info@trenzyme.com)). The following report was prepared by Armin Günther and checked by Dr. Reinhold Horlacher (Trenzyme GmbH, Konstanz, Germany):

Aim: Localization of the transposon within the genomic DNA of three strains as follows:

Sample	Barcode	Name
1	3041	Mut B16
2	3042	Mut 21.20
3	3043	Mut 31.90

Performance:

Restriction digests

Different restriction digests of the genomic DNA were set up using the restriction endonucleases BamHI, BglII, StyI, HindIII. After appropriate time points, restriction digest reactions were stopped. Self-Ligation reactions were set up. PCR reactions were set up using the oligonucleotides of table 1 and diluted ligation reactions as template. PCR reactions were again diluted and used as template for nested PCR.

Oligonucleotides

Table S5.1: Oligonucleotides for gene identification and PCR cloning.

Task	Oligonucleotide	Sequence
Nested PCR, first reaction	1475_FP1	CGATAGCTCCGCTATTG
	1475_RP2	ACACCAACGGGCTTATC
Nested PCR, second reaction	1475_FP2	GGACGGACAATTAAGCAAAG
	1475_RP1	ATACCTCACGCCAAACG
Sequencing reaction	1475_FP2	
	1475_RP1	

Supplementary Data

Table S5.2: PCR products obtained and sequenced.

Bd#	template	Enzyme	Approx. size
1	3041 – MutB16	HindIII	1350
4	3042 – Mut21.20	StyI	750bp
8	3043 – Mut 31.90	HindIII	1350bp

Table S5.3: Sequencing reactions.

PCR product	Oligonucleotide	Sequence file (*.abi)
1475_3041_PCRbd1	1475_RP1	1475_3041_PCRbd1-1475_RP1
	1475_FP2	1475_3041_PCRbd1-1475_FP2
1475_3042_PCRbd4	1475_RP1	1475_3042_PCRbd4-1475_RP1
1475_3043_PCRbd8	1475_RP1	1475_3043_PCRbd8-1475_RP1
	1475_FP2	1475_3043_PCRbd8-1475_FP2

S5.2 Nucleotide and protein sequences of the up- and downstream regions of the transposon insertion sites

Translations Aligned (5'→3'), plus strand

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FW5
|      |      |      |      |      |      |      |      |      |
| 10   | 20   | 30   | 40   | 50   | 60   | 70   | 80   | 90   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| TTGGAGAGTTTAGGCGCAAGATTCAGAGGCGTGACAAAGTTGAAACATGAGAAATTTTCAAAAACTTTAATTATTGTAACATCTCTGTAATGTAGTATTT 100
| L E S * R Q E S E A D K V E T * E I F Q K T L I I V T I C N V V I * 100
| W R V R G K N Q R L T K L K H E K F F K K L * L L * L S V M * L F 100
| G E L E A R I R G * Q S * N M R N F S K N F N Y C N Y L * C S Y L 100

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |ORF1
|AGTCCTGGTTTGCCTTTTCGTTATGCTGATAAATATACACCTTTTATGAGCAGGAACCTTGAAGTAATAAATAGATGTTTCATACATTAATGATATATA 200
| S W F A F R Y A D K Y T P F L * S R N F E V I N R C S Y I K C I Y 200
| S P G L L F V M L I N I H L F Y E A G T L K * * I D V H T L N V Y T 200
| V L V C F S L C * * I Y T F F M K Q E L * S N K * M F I H * M Y I 200

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|CAATGTTATTGTAAGTAAAGTTGTGCTATGTCATAGAAACCGATATTAAGCAAAAAAGATTTCGGACTCCGATCAGCGGTGGTACTGAATC 300
| N V I V L L T S C V L C Q * K P I L S K K D F G L R I S G W Y * I 300
| M L L Y Y * Q V V C Y V N R N R Y * A K K I S D S V S A V G T E S 300
| Q C Y C T T N K L C A M S I E T D I K Q K R F R T P Y Q R L V L N L 300

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |FW4
|TTATTTATACAAGTAACTGGCTTGCTACAAGCaAATAGAGatTTtGAGGAAcCATgacctCACaccgGAGCAGTaTaAtgtgcTcaGATtttgcGCGG 400
| L F I Q V T G L A T S K * R F * G N M T S H R S S I M C S G F C A G 400
| Y L Y K * L A W L Q A N R D F E G T * P H T G A V * C A Q D F A R 400
| I Y T S N W L G Y K Q I E I L R E H D L T P E Q Y N V L R I L R G 400

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|GCaGcATccCAATccgATCAaAgTGAgTGataTaTcCGaAacgtATGcttgaTAAaAATCCaacaCATcCaGgctGATCgAtAaAcTcTtTCTtAAaGA 500
| S I P I R S K * V I Y P K R M L D K N S N T S R L I D K L L L K D 500
| A A S Q S P D Q S E * Y I R N V C L I K I P T H P G * S I N F F L K I 500
| Q H P N P I K V S D I S E T Y A * * K F Q H I Q A D R * T S S * R 500

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |Rev3
|TcTtGTAAcCGGACGTcTGcCccagcgcgGcgggCAGtgGatGTAgTGatcAaTgaagCGgGTTGGCtttactTTTATTGATGaccccAAgTa 600
| L V T R T S C R B A V D V V I N E A G L A L L L L L D P K V 600
| L * P G R P A P A T G A Q W M * * S M K R G W L Y F Y * L T P K * 600
| S C N P D V L P Q R P A R S G C S D Q * S G V G F T F I D * P Q S R 600

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|gaGGATtgGAAAATAgccTTGAGTTCAACTTATAAATGCAACTTTTGGGTGCGGATAATAAGCAATAAAAAACATTTATTTTTCAGAGAGGAAAAGAGA 700
| E D W E N S P * V Q L I N A T F W V R I I S N K N I Y F S E R K E R 700
| R I G K I A L E F L * M Q L F G C G * * A I K T F I F Q R G K R 700
| G L G K * P L S S T Y K C N F L G A D N K Q * K H L F F R E E R E 700

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|GACAATGTCCCCCTTCTCTCACTCTGAATGGATAAAGTTTGCTATCTTTGCTTTAATTGTCCCGTCCAAAGAAAAAAGTTGCAGATGAGCAAAACATAT 800
| Q C P P F S H S E W I K F A I F A L I V R P K K K K L Q M S K H I 800
| D N V P L S L T L N G * S L L S L L * L S V Q R K K S C R * A N I * 800
| T M S P F L S L * M D K V C Y L C F N C P S K E K K V A D E Q T Y 800

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |Rev2
|AACCAGGAAACAAAGGTATGCAATTTCTATGATGTTGCAAAATACCGATGAGCAAAAAAGCAATACGGGAAGCTATCGGAGTAGATAAAGCACTGTTTAC 900
| T E E Q R Y A I S M M L Q I P M S K K A I A E A I G V D K S T V Y 900
| P R N K G M P F L * C C K Y R * A K K Q * R K L S E * I K A L F T 900
| N R G T K V C N F Y D V A N T D E Q K S N S G S Y R S R * K H C L Q 900

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|AGGGAGATAAAGCGCAATTTGCGACGCCGAAGTGGTAGCTATAGCATGGAGCTTGGCCAGCGAAAGCAGACAGCGGCAAGCAGCAAAAACATCGCAAGG 1000
| R E I K R N C D A R S G S Y S M E L A Q R K A D R R K Q Q K H R K E 1000
| G R * S A I A T P E V V A I A W S L P S E K Q T G A S S K N I A R 1000
| G D K A Q L R R P K W * L * H G A C P A K S R Q A Q A A K T S Q G 1000

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|AAGTGCCTACACCGGCAATGAGAAAAACGGATAATAAAGCTGTTGAAGAAAGGATTTCAGCCCGGAGCAGATTGTCGGCAGGAGCCGCTTGGAGGGAATTGC 1100
| V L T P A M R K R I I K L L K K G F S P E Q I V G R S R L E G I A 1100
| K C L H R Q * E N G * * S C * R K D S A R S R L S A G A A W R E L R 1100
| S A Y T G N E K T D N K A V E E R I Q P G A D C R Q E P L G G N C 1100

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|GATGGTATCTCACGAAACGATATATCGCTGGATTGGGAGGATAAGCGCGGGGTTGGCAAACTGCACAAATATCTTTCGACGCAAGGTCGACAGGTATGCC 1200
| M V S H E T I Y R W I W E D K R R G G K L H K Y L R R Q G R R Y A 1200
| W Y L T K R Y I A G F G R I S G V A N C T N I F A D K V A G M P 1200
| D G I S R N D I S L D L G G * A A G W Q T A Q I S S Q T R S Q V C Q 1200

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |BstNI
|AAACGTGGTTCTAAAAATGCAGGGCGAGGATTTATCCAGGCAGGGTGGATATTGATGAGCGTCCCGAGATAGTGGAACTGAAGGAGAGATTTGGTGATT 1300
| K R G S K N A G R G F I P G R V D I D E R P E I V E L K E R F G D L 1300
| N V V L K M Q G E D L S Q A G W I L M S V P R * W N * R R D L V I 1300
| T W F * K C R A R I Y P R Q G G Y * * A S R D S G T E G E I W * F 1300

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |BsoBI
|TAGAGATAGATACAATTATGGTAAAGAACACAAGGTGCCATTTCTACCATTAAACGACAGAGCAACAGCAGGCTCTGGATACGCAAGTTGTCGGGAAA 1400
| E I D T I I G K N H K G A I L T I N D R A T S R V W I R K L S R E K 1400
| * R * I Q L L V R T * K V F L P L T T E Q Q A G S G Y A S C R E K 1400
| R D R Y N Y W * E P Q R C H S Y H * R Q S N K Q G L D T Q V V G K 1400

|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                     |
|AGAAGCCATCCCGGTAGCTAAGATTGCAGTATGGGCACCTCGGAAAGTGA AAAA ACTTAATACACACAATTACGGCTGACAAATGGAAAGGAGTTTGCAAAG 1500
| E A I P V A K I A V W A L R K V K N L I H T I T A D N G K E F A K 1500
| K P S R * L R L Q Y M G H C G K * K T * Y T Q L R L T M E R S L Q S 1500
| R S H P G S * D C S M G T A E S E K L N T H N Y G * Q W K G S V K A 1500

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Supplementary Data

CACGAGGAAATTCGCGAAAAATGGAAATAAAATCTATTTTGGCAAACCATACCACTCATGGGAACGTGGTCCAATGAAAAACCAACGGGCTTATCA 1600
H E E I A Q K L E I K F Y F C K P Y H S W E R G A N E N T N G L I R 1600
T R K L R K N W K * N S I F A N H T T H G N V V P M K T P T G L S 1600
R G N C A K I G N K I L F L Q T I P L M G T W C Q * K H Q R A Y Q 1600

GGCAGTATATCCCAAGGGTAAGGACTTTAGTGAAGTAACCAACAACAGATTAGTGGATTGAAAAATAAACTCAATAATCGACCTCGTAAAAGACTTGG 1700
Q Y I P K G K D F S E V T N K Q I K W I E N K L N N R P R K R L G 1700
G S I S Q R V R T L V K * P T N R L S G L K I N S I I D L V K D L D 1700
A V Y P K G * G L * * S N Q Q T D * V D * K * T Q * S T S * K T W 1700

ATACCTCAGCCAAACGAAAAATTTAAACAATTTAATATCAGAATTCCTGTGCATTGGCAAGTTGAATTCAAATCACAGAGAAGTGAATAGACAGTATT 1800
Y L T P N E K F K Q I I N Q N S V A F A S * I Q I T E K * I D S I 1800
T S R Q T K N L N K L I R I L L H L Q V E F K S Q R S E * T V L 1800
I P H A K R K I * T N Y * S E F C C I C K L N S N H R E V N R Q Y Y 1800

ACCCGACAGAGCACTACGACAAGCAATGCCATCAAAAAATGACAGCAGACACAAGCATAGACTGATTGTCTTGCCTAAAAGATTGATTGGAATT 1900
T R Q S N Y D K A M P S K K M T A H T K H R L I V L P * K I D W N L 1900
P D R A T T T K Q C H Q K K * Q H T Q S I D * L S C P K R L I G I 1900
P T E Q L R Q S N A I K K N D S T H K A * T D C L A L K D * L E F 1900

TATTCAGGACTGCCTATCACTTAGATAGAAGTCCAAGTCTGCTGAAATACATAATTCAAAAATGAAATGCTTGACTGCAAAATTTAGTAACTTTGCTT 2000
F R T A Y H L D R S P R S A E I H N S K I E I A * L Q I * * L C L 2000
Y S G L P I T * I E V Q G L L K Y I I Q K L K L L D C K F S N F A * 2000
I Q D C L S L R * K S K V C * N T * F K N * N C L T A N L V T L L 2000

Tetfw
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T N * R E R R E N E S R P H N I T Y T Q A G G S C F V R Q E S G K 2100
Q I N E K E E K M K A D R I T S P I R K Q G V R A S * D R K V V N 2100
D K L T R K K R K * K P T A * H H L Y A S R G F V L R K T G K W * I 2100

TTTAAAGTTCAGTTCATCGTAGGTAGCTTAGTGGTAAAAATCCCTGCCTGCGTATAGCTGCAAAACCGTGTAGCAATCCGCTGGCGCTCCTTGCTAACAA 2200
F K V Q F I V G S L V V K I P A C V * L Q T V V S N P L R S L L T M 2200
L K F S S S * V A * W * K S L P A Y S C K P L L A I R C A P C * Q 2200
* S S V H R R * L S G K N P C L R I A A N R C * Q S A A L L A N N 2200

TGGCTCGCGGACTTACGTCGCTTGCCTGTAAGCCGCGCTTCGCGGACTGCTTACACAAGCGACTTGGCTAAATAGCTCCGCTTTTGTATCTTCGATACA 2300
A R R L T S L A V S R R F A D C L Q Q A T W L N S S A F V Y S S I Q 2300
W L G D L R R L P * A A A S R T A Y N K R L G * I A P L L Y L R Y S 2300
G S A T Y V A C R K P P L R G L L T T S D L A K * L R F C I F D T 2300

GCTTCGCTACTTCGCCAATTCGCCGAGCCATTGTTATGGCCATTTTGAATGACAATGCGAATAGATACAGACAAAATAAATGAATTTACTTAGTGATAA 2400
L R Y F A N S P S H C Y G P F L N D N A N R Y R Q T N E F T * * * 2400
F A T S P I R R A I V M G H F * M T M R I D T D K Q M N L L S D K 2400
A S L L R Q F A E P L L W A I F E * Q C E * I Q T N K * I Y L V I R 2400

Tetrev
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E R C N N W W T R W T D Y G K I I T A K R H R R F S L R K R Q R P 2500
N V A I I G G G P V G L T M A K L L Q Q N G I D V S V Y E R D N D 2500
T L Q * L V V D P L D * L W Q N Y Y S K T A * T F Q F T K E T T T 2500

CGAGAGGCAAGAATTTTGGTGAACCCCTTGACCTACACAAGGTTTCAGTTCAGGAAGCAATGAAAAAGCGGGATTGTTACAACTTATTATGACTTAG 2600
R G K N F W N P * P T Q R F R S G S N E K S G I V T N L L * L S 2600
R E A R I F G G T L D L H K G S G Q E A M K K A G L L Q T Y Y D L A 2600
E R Q E F L V E P L T Y T K V Q V R K Q * K K R D C Y K L I M T * 2600

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L T N G C K Y C * * K R Q Y F I H K K C K A R K S I * Q S * N K Q 2700
L P M G V N I A D K K G N I L S T K N V K P E N R F D N P E I N R 2700
P Y Q W V * I L L I K K A I F Y P Q K M * S P K I D L T I L K * T E 2700

BstNI
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K * L K G Y L V E * F R K R H G Y L G * K T C Y A * T W * E E V D T 2800
N D L R A I L L N S L E N D T V I W D R K L V M L E P G K K W T 2800
M T * G L S C * I V * K T T R L F G I E N L L C L N L V R R S G H 2800

CTAACTTTGAGAATAAACCGAGTGAACAGCAGATTGGTTATCTTGCCAAATGGCGGGATGCCAAGGTAAGAAAATTTGTTACCGACACGGAAGTTG 2900
N F * E * T E * N S R F G Y S C Q W R D V Q G K K I C Y R H G S * 2900
L T F E N R I P S E T A D L V I L A N G M S K V R K F V T D T E V E 2900
* L L R I N R V E Q Q I W L F L P M A G C P R * E N L L P T R K L 2900

BstNI
AAGAAACAGGTACTTTCAATATACAAGCCGATATTCATCAACAGAGATAAATGTT **CCTGG**ATTTTTTTCAGCTATGCAATGGAAAACCGGCTAATGGCATC 3000
R N R Y F Q Y T S R Y S S T R D K L S W I F S A M Q W K P A N G I 3000
E T G T F N I Q A D I H Q P E I N C P G F F Q L C N G N R L M A S 3000
K K Q V L S I Y K P I F I N Q R * T V L D F F S Y A M E T G * W H L 3000

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S P R * F I I C * P Q * * W C I A F W N K F * N T * * M E K P N A G 3100
H Q G N L L F A N P N N G A L H F G I S F K T P D E W K N Q T Q 3100
T K V I Y Y L L T P I I M V H C I L E * V L K H L M N G K T K R R 3100

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R F S K Q K * C R * F S S E R I F R L G R T L Q R I D S Y D V V I 3200
V D F Q N R N S V V D F L L K E F S D W D E R Y K E L I H T T L S F 3200
* I F K T E I V S L I F F * K N F P I G T N A T K N * F I R R C H 3200

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 V G L A T R I F P L E K P W K S K R P L P I T M I G D A A H L M P 3300
 L * D W L H G Y F L * K S L G K A S A H Y P * Q * L G M P H I * C R 3300

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 P F A G Q G V N S G L V D A L I L S D N L A D G K F N S I E E A V 3400
 L L Q G R E * I V G W W M P * Y C L I I * P M E N L I A L K R L L 3400

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 K N Y E Q Q M F M Y G K E A Q E E S T Q N E I E M F K P D F T F Q Q 3500
 K I M N S K C L C M A K K H K K N Q L K T K L K C L N P T L R F S 3500

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 L L N V * N G I K R H I T N K L A I E P V K Y F Y * T F V Q C * R 3600
 N C * M Y K M E * N G T * Q T N W Q * S R * S T S I E L L C N V E D 3600

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 I S N S I Q H L C * K S A P S P I C Q P L C G S L K K D Y T C F P K K 3700
 L V I L F N I C A K S R L H R Q F A S R Y A A A * K K T T P N Q K 3700
 * * F Y S T F V L K V G S I A N L P A V M R Q L K K R L H Q T K K 3700

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 S S * Q P P D F E L R R M K F F R D N F Q H F Q K T V * P L T W R 3800
 N H L D N H P T L N Y E G * N F S G T T S S I S K K Q F N H * L G D 3800
 I I L T T T R L * T T K D E I F Q G Q L P A F P K N S L T I D L E 3800

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 Q T N N L S I K V * T E F F R N * P D L T F P E N R L * S C F Y R 3900
 K Q T I * A L R F K P N F S E I D L T * L F L K I D F K A V F T D 3900
 T N K Q F E H * G L N R I F P K L T * P D F S * K S T L K L F L Q I 3900

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 * I R F L * Q I G N K C * V F G F K L K I K K * V F S F N V * Q G * 4000
 K S G F S N R * E I N A K Y L A L N L K S K N K C F L L M F N R A 4000
 N Q V S L T D R K * M L S I W L * T * N Q K I S V F F * C L T G L 4000

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 F D S W R W Q E T F R T Y L I D K F Q I K K S M I E N G I W I V K 4100
 D L T V G G K K L S G P T S * T S F K S K K V * * K T V Y M G L * S 4100
 I * Q L A V A R N F Q D L P H R Q Q V S N Q K K Y D R K R Y M D C K 4100

GCTTCCCGAAAATACTTTGTGTAGTTCTAATGGAAGGCAATGGAACCTCCAGAAAATTTCCAAGACTCTCAAAACATCAGGATTTGAAAATC 4200
 L P R K * L L C R F * L K D N G T S Q K I S K T L K H Q D F E N I 4200
 F L E N N F C V G S N * R T M E P P R K F P R L S N I R I L K I S 4200
 A S S K I T F V * V L I E G Q W N L P E N F Q D S Q T S G F * K Y R 4200

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 G S N A I R N I * H H F E R K F R N C K I P K I T T D N L N I S G I 4300
 E V M P * G I F D T T L K G N F G T A K F L K S Q P T T * T F R A 4300
 K * C H K E Y L T P L * K E I S E L Q N S * N H N R Q L E H F G H 4300

TCAGAAAATAATTTTCGTAATGTTCAACCAAGCTGTGCTGTTTTCAATAGCAACAACATTGTTGGCGATTTTTAATAAATGAACAGTAAGAAACCCCT 4400
 R K * F S * M F N Q S C V V F N S N N I V G D F * * M N S K K P L 4400
 S E N N F R K C S T K A V S F S I A T T L L A I F N K * T V R N P L 4400
 Q K I I F V N V Q P K L C R F Q * Q Q H C W R F L I N E Q * E T P 4400

TGCCTGCCCAATATCTAAACCGTATCCTGATTAATGCTTATGCTTATTGCATCTTTTATTAGCACTTTATCAATAGTAAGTGGTGCACCCGT 4500
 A C P N I * N R I L I T Y I C L S Y C I F Y * H F I N S K V L T R 4500
 P A P I S K R T V S * L L I F A C L I A S F I S T L S I V K C * P V 4500
 C L P Q Y L K P Y P D Y L Y L L V L L H L L L A L Y Q * * S A D P * 4500

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 K R T G N F F V I S N F L Q V N T S * V Q L I N A T F W V R I I 4600
 N E R A I S F L S L V T S Y R * I L L E F N L * M Q L F G C G * * 4600

1475_FP2
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 Q * K H L F F R E E R E T M S P F L S L * M D K V C Y L C F N C P 4700
 S N K N I Y F S E R K E R Q C P P F S H S E W I K F A I F A L I V R 4700
 A I K T F I F Q R G K R D N V P L S L T L N G * S L L S L L * L S 4700

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 P K K K L Q M S K K H I T E E Q R Y A I S M M L Q I P M S K K A I 4800
 V Q R K K S C R * A N I * P R N K G M Q F L * C C K Y R * A K K Q * 4800

Rev2
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 S G S Y R S R * K H C L Q G D K A Q L R R P K W * L * H G A C P A K 4900
 A E A I G V D K S T V Y R E I K R N C D A R S G S Y S M E L A Q R 4900
 R K L S E * I K A L S E F L T G R * S A I A T P E V V A I A W S L P S E 4900

Supplementary Data

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K A D R R K Q Q K H R K E V L T P A M R K R I I K L L K K G F S P E 5000
K Q T G A S S K N I A R K C L H R Q * E N G * * S C * R K D S A R 5000

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Q I V G R S R L E G I A M V S H E T I Y R W I W E D K R R G G K L 5100
S R L S A G A A W R E L R W Y L T K R Y I A G F G R I S G G V A N C 5100

BstNI
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A Q I S S Q T R S Q V C Q T W F * K C R A R I Y P R Q G G Y * * A S 5200
H K Y L R R Q G R R Y A K R R G S K N A G R G F I P G R V D I D E R 5200
T N I F A D K V A G M P N V V L K M Q G E D L S Q A G W I L M S V 5200

BsoBI
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P E I V E L K E R F G D L E I D T I I G K N H K G A I L T I N D R A 5300
P R * W N * R R D L V I * R * I Q L L V R T T K V P F L P L T T E 5300

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N K Q G L D T Q V V G K R S H P G S * D C S M G T A E S E K L N T 5400
T S R V W I R K L S G K E A I P V A K I A V W A L R K V K N L I H 5400
Q Q A G S G Y A S C R E K K P S R * L R L Q Y G H C G K * K T * Y T 5400

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H N Y G * Q W K G V C K A R G N C A K I G N K I L F L Q T I P L M G 5500
T I T A D N G K E F A K H E E I A Q K L E I K F Y F C K P Y H S W 5500
Q L R L T M E R S L Q S T R K L R K N W K * N S I F A N H T T H G 5500

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T W C Q * K H Q R A Y Q A V Y P K G * G L * * S N Q Q T D * V D * 5600
E R G A N E N T N G L I R Q Y I P K G K D F S E V T N K Q I K W I E 5600
N V V P M K T P T G L S G S I S Q R V R T L V K * P T N R L S G L 5600

1475_RP1
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N K L N N R P R K R L G Y L T P N E K F K Q I I N Q N S V A F A S 5700
K I N S I I D L V K D L D T S R Q T K N L N K L L I R I L L H L Q V 5700

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L N S A P E Y H Y P * R S R C N Q C T A G Q A Q K I R I E Y N * K P 5800
* I Q R L I N T H E A D A I S A L L D K L R K S E * N T I E N 5800
E F S A * I P L P M K Q M Q S V H C W T S S E N Q N R I Q L K T 5800

ORF2
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N K Q Q T K L N L N E Q Y N E I F E S S S S I P G S I F I R F I F 5900
Q I N S K P S * I * M N S T M K S L K A V A A S L A V S L F V S F S 5900
K * T A N Q V K F K * T V Q * N L * K Q * Q H P W Q Y L Y S F H F 5900

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V S A A D G X K G K A T N L K V N A S K S D L T W N A K K V T G E 6000
P C L P R M A X K E K R P I * K * T P P K A I * H G M Q K K * P V S 6000

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A H G * D C P * R R Y H Y N E W G Q I D R W K F R S R S E Q Y H L Y 6100
H T G K I A L K E G T I T M N G A K L T G G S F V A D L N S I T C 6100
T R V R L P L K K V P L Q * M G P N * Q V E V S * Q I * T V S P V 6100

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T D L T D K T I N D K L I G H L K S D D F F S V A K H P T A K F V V 6200
Q I L P T K H T M T S * L G I * N R M I S F P L R S I L R L S L W 6200

FW2; Rev5
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T K A T A K S A G Q Y D V T G D L T I K G I T K P V T F P V T V K 6300
L P R Q L R R V R D S M T * Q E T L P S K E L P N R L L F L * P * K 6300

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T T P S G A E A T G K L V V D R S K F D I K Y N S K S F F D V S T 6400
L H R Q V Q K L L V N W L L T V R N S I L N T I Q N R F L M * V L 6400

CTGGGAGCAAAATGATTGATGACTTTACTATTGATGTAAGCTTGTGCCGAAAATAGTTGTAGCCAAAGGCTGTAATCACTTCCTCATTGAGCAT 6500
G R Q N D S * * L Y Y * C K A C R K I V V A K A V I T S S F S M 6500
L G D K M I H D D F T I D V K L V A A K * L * P R L * S L P H S A C 6500
W E T K * F M M T L L L M * S L L P Q N S C S Q G C N H F L I Q H 6500

GTGTTCCGTCGGCCTAATTTGCGACGGTTTTTTTTGTGCAATCTTTTGGCGTATCGTCCCAATTTGGACTAGATCAGGTGCTCCCGATAGACGGGA 6600
C C R R A N C A T V F L C N L L R D R L Q F W T R S G A P D R A G 6600
V A V A L I V R R F F C A I F C V I S V S N F G L D Q V L P I E R E 6600
V L P S R * L C D G F F V Q S F A * I S P I L D * I R C S R * S G K 6600

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AGTCGTC AAGGTAGTGGATTGCCCCCGATCGCTGAAAGCAATAGTATTGAGACCATTGGTAAACAGCCAGATAGGTGGGTGTAAATGTA 6700
S R Q G S G F A P R S P E S N S D * D H W * Q P D R W V V M * N C N 6700
V V K V V D L P P D R L K A I V I E T I G N S Q I G G L * C K I V 6700
S S R * W I C P P I A * K Q * * L R P L V T A R * V G C N V K L * 6700

ACGGCTTATCTCGCGGAAGTGGCTTCTCAACCGCAACAAAAGCGCCTTACATTCCACTAAAAGAAACGGGACCTGTGTCGACAGAATCATGATA 6800
G L S A R R W L L Q P Q Q K A P Y I P L K E T G T L C S T E S * Y 6800
T A Y L R E G G F S N R N K R R L T F H * K K R G P C V R Q N H D I 6800
R L I C A K V A S P T A T K G A L H S T K R N G D P V F D R I M I 6800

TCGGTTCGTTGGCAAGAGAATTGTAGCGCATAACCGGTTTCAATAGCAATAGGGATCTCGGATAATTGTAGTCCGAATGAGCAAGTGGATAAAATGCT 6900
R F A W Q E N C S A Y R F Q * Q I G I S D N C S A K * A S G * N A 6900
G S L G K R I V A H T G F N S K * G S R I I V V R N E Q V D K M L 6900
S V R L A R E L * R I P V S I A N R D L G * L * C E M S K W I K C C 6900

GCCGACCCATCTCAGGGGTGAGTGAGACGAATTTTGGCGATAATATCGAAGATATATGGTTTACCGTTTACGTCCTTAACTTTGTAACCAACGCT 7000
A A P I P Q G * V R R I F C G * Y R R Y M V Y R L R P * L C N Q T W 7000
P H P F L R G E * D E F F A D N I E D I W F T V Y V L N F V T K R 7000
R T H S S G V S E T N F L R I I S K I Y G L P F T S L T L * P N V 7000

GGGAAGATTAATGATCCATAGACGAAGATACGCATCACAGCAAAACCAACCTATAATAGCGCCAAAACATGACGACCAAGAACAATTTAGAGAA 7100
E D L M I P * T K I R I T A N Q P Y N S A K N M T T K E Q I V E N 7100
G K I * * F H R R R Y A S Q Q T N P I I A P K T * R P K N K L * R T 7100
G R F N D S I D E D T H H S K P T L * * R Q K H D D Q R T N C R E 7100

CTGGCTTCCAGATATACCGGCACACCTGTGGAGGATTCGGAATATATATGCTTACCAATTTGGAAACTATGTAAGCATGTTGGCGGACAATTT 7200
W L P R Y T G T P V E E F G N Y I L L T N F G N Y V S M F A D K F 7200
G F P D I P A H L W R S S E I I Y C L P I L E T M * A C L R T N L 7200
L A S Q I Y R H T C G G V R K L Y I A Y Q F W K L C K H V C G Q I W 7200

GGGGTAGAGATCAAGGGGAGGGTAGGGCCATGCAAACGGCTACGGCTAATGATATCACCATTATCAATTTGGCATGGGTAGTCCGATGGCGGTACCG 7300
G V E I K G Q G R A M Q T A T A N D I T I I N F G M G S P M A A T V 7300
G * R S R G R V G P C K R L R L M I S P L S I L A W V V R W R L P 7300
G R D Q G A G * G H A N G Y G * * Y H H Y Q F W H G * S D G G Y R 7300

TCATGGACCTTTTGTCTGCCATAAGCCCAAGCGGTTTGTCTTGGGAAAATGCGGAGGA 7400
M D L L S A I S P K A V L F L G K C G G 7400
S W T F C L P * A P R R F C F W E N A E G 7400
H G P F V C H K P Q G G F V S G K M R R 7400

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Translations Aligned (5'→3'), minus strand

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10 20 30 40 50 60 70 80 90
TCC TCCG CATT TCC CAG AACA AAAACCG CCTTGGGG CTTATGG CAGACA AAAAGTCC ATGACGG TAGCGCC ATCGGACT ACCCATGCC AAAAATGATA 100
S A F S Q K Q N R L G A Y G R Q K V H D G S R H R T T H A K I D N 100
P P H F P R N K T A L G L M A D K R S M T V A A I G L P M P K L I 100
L R I F P E T K P P W G L W Q T K G P * R * P P S D Y P C Q N * * 100

ATG TG TGA TATC ATTAG CCGT AGCCG TTTG CATGG CCGT ACCCTG CCCCCT TGATCT CTACCC CAAATTTG TCCG CAAACATG CTTACATAG TTTTCC AAAAT 200
G D I I S R S R L H G P T L P L D L Y P K F V R K H A Y I V S K I 200
M V I S L A V A V C M A L P C P L I S T P N L S A N M L T * F P K L 200
W * Y H * P * P F A W P Y P A P * S L P Q I C P Q T C L H S F Q N 200

TG TGAAGCA ATATATA ATTTCCG AACCTCC ACAGGTG TGCCGG TATATCTGGG AAGCCAGT TCTCTACA ATTTGTTCT TTTGGT CGTCA TGTTTTTGGC 300
G K Q Y I I S E L L H R C A G I S G K P V L Y N L F F G R H V F G 300
V S N I * F P N S S T G V P V Y L G S Q F S T I C S L V V M F L A 300
W * A I Y N F R T P P Q V C R Y I W E A S S L Q F V L W S S C F W R 300

GCT AT TATAG GGTGG TTTG CFTG TATCTG CTCTA TGAATC ATTAATCT CCCACG TTTGGT TACAAG TTAAGG CCGTAA CCGSTA AAC 400
A I I G L V C D A Y L R L W N H * I F P R L V T K L R T * T V N H 400
L L * G W F A V M R I F R V Y G I I K S S H V W L Q S * G R K R * T 400
Y Y R V G L L * C V S S S M E S L N L P T F G Y K V K D V N G K P 400

AT ATATCTTCGATATATTCGCAAAAAATTCGTCTCACTCACCCCTGAGGAATGGGTGCGGCAGCATTATCCACTTGCTCATTTCGCACTACAATTAT 500
I S S I L S A K N S S H S P L R M G C G S I L S T C S F R T T I I 500
I Y L R Y Y P Q K I R L S T H P * G M G A A A F Y P L A H F A L Q L S 500
Y I F D I I R K F V S L T P E E W V R Q H F I H L L I S H Y N Y 500

CCGAGATCCCTATTGCTATTGAAACCGGTATGCGCTACAATTTCTTGGCCAAGCGAACCAGATATCATGATTCTGTGCAACACAGGGTCCCGTTTCTTT 600
R D P Y L L L K P V C A T I L L P S E P I S * F C R T Q G R P F F 600
E I P I C Y * N R Y A L Q F S C Q A N R Y H D S V E H R V P V S F 600
P R S L F A I E T G M R Y N S L A K R T D I M I L S N T G S P F L L 600

TAGTGGAAATGTAAGGCGCCTTTTGTGCGGTTGGAGAAGCCACCTTCGCGCAGATAAGCCGTTACAATTTTACATTACAACCCACCTATCTGGCTGTTAC 700
* N V N R R L L L R L E K P P S R R * A V T I L H Y N P P I W L L P 700
S G M * G A F C G W R S H L R A D K P L Q F Y I T T H L S G C Y 700
V E C K A P F V A V G E A T F A Q I S R Y N F T L Q P T Y L A V T 700

CAATGGTCTCAATCACTATTGCTTTCCAGGCGATCGGGGGCAAAATCCACTACCTTGACGACTTCCCGCTCTATCGGGAGCACCTGATCTAGTCCAAAAT 800
M V S I T I A F R R S G G K S T L T T S R S I G S T * S S P K L 800
Q W S Q S L L L S G D R G A N P L Y * R L P A L S G A P D L V Q N W 800
N G L N H Y C F Q A I G G Q I H Y L D D F P L Y R E H L I * S K I 800

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Supplementary Data

GGAGACGATCACGCAAAAGATTGCACAAAAAACCGTCGCACAATTAGCGCGACGGCAACACATGCTGAATGAGGAAGTATTACAGCCTGGCTACAAAC 900
E T I T Q K I A Q K N R R T I S A T A T H A E * G S D Y S L G Y L I 900
R R S R K R L H K K T V A Q L A R R Q H M L N E E V I T A L A Y N 900
G D D H A K D C T K K P S H N * R D G N T C * M R K * L Q P W L Q L 900

TATTTTCGGCAACAAGCTTTACATCAATAGTAAAGTCATCATGAATCAATTTTGTCTCCAGAGTACTTACATCAAAAAACGATTTTGAATTTGATTTAA 1000
Y F A A T S F T S I V K S S * I I L S P R V L T S K N D F E L Y L I 1000
I L R Q Q A L H Q * * S H H E S F C L P E Y L H Q K T I L N C I * 1000
F C G N K L Y I N S K V I M N H F V S Q S T Y I K K R F * I V F N 1000

TATCGAATTCGACCGTCAACAACCGATTACCAGTAGCTTCTGCACCTGACGGTGTAGTTTTTACGGTTACAGGAAAAGTAAACCGGTTGGTAATTC 1100
S N F E R S T T S L P V A S A P D G V V F T V T G K V T G L V I P 1100
Y R I S N G Q Q P V Y Q * L L H L T V * F L R L Q E K * P V W * F L 1100
I E F R T V N N Q F T S S F C T * R C S F Y G Y R K S N R F G N S 1100

|FW2; Rev5|
TTTGTAGGTAAGGTCCTCGTTCAGTCTCGGCACTCTTCGAGTTGCCTTGGTAACCACAACTTAGCCGTAGGATGCTTCGCAACGGAAAAAG 1200
L M V R S P V T S Y C F A L F A V A L V T T N L A V G C F A T E K 1200
* W * G L L L R H T V P H S S Q L P W * P Q T * P * D A S Q R K R 1200
F D G K V S C Y V I L S R T L R S C L G N H K L S R R M L R N G K E 1200

AAATCATCGGATTTCAAATGCCCAATCAACTTGTCAATGATGTTTGTGCGTAAGATCTGTACAGGTGATACTGTTGAGATCTGCTACGAACTTCCAC 1300
K S S D F K C P I N L S L Y L V L S V R S V Q V I L F R S A T K L P P 1300
N H P I S N A Q S T C H C M F C R * D L Y R * Y C S D L L R N F H 1300
I I R F Q M P N Q L V I V C F V G K I C T G D T V Q I C Y E T S T 1300

CTGCAATTTGGCCCAATCATTGTAATGGTACCTTCTTTAAGGGCAATCTTACCCTGTGCTCACCGGTTACTTTTTTTCATCCATGTCAGATCGCT 1400
V N L A P P F I V M V P S L R A I L P V C S P V T F F A F H V R S L 1400
L S I W P H S L * W Y L L * G Q S Y P C A H R L L F L H S M S D R F 1400
C Q F G P I H C N G T F F K G N L T R V L T G Y F F C I P C Q I A 1400

TTTGGAGCGTTTACTTTCAAATGGTTCGCTTTTCTTTGkCGCCATCCGCGGACAGACCGGAAAATGAAACGAATAAAGATACTGCCAGGGATGCTGCT 1500
L E A F T F K L V A F P L X P S A A D T E N E T N K D T A R D A A 1500
W R R L L S N W S L F L X R H P R Q T R K M K R I K I L P G M L L 1500
F G G V Y F Q I G R F S F X A I R G R H G K * N E * R Y C Q G C C Y 1500

ACTGCTTCAAAGATTTCAATGACTGTCATTAATTAATTAAGTTCGTTTATTTGGTTTCAAATGATTTCTATTCTGATTTCTGAGCTTGT 1600
T A F K D I V L F I * I * L G L L F I W F S I V F Y S D F L S L S 1600
L L S F I S L Y C S F K F N L V C L F G F Q L Y S I L I F * A C 1600
C F Q R F H C T V H L N L T W F A V Y L V F N C I L F * F S E L V 1600

CCAGCAGTGCAGTGAATGcatcTGCTTCTTCATGGGTAATGGTATTTCAGGCCTGAATTCACCTGCAAAATGCAACAGAATTCGATTAATAATTTGTTT 1700
S S A L I A S A S S W V M V F R R * I Q L A N A T E F * L I I C L 1700
P A V H * L H L L H G * W Y S G A E F N L Q M Q Q N S D * * F V * 1700
Q C T D C I C F F M G N G I Q A L N S T C K C N R I L I N N L F 1700

|1475_RP1|
AAATTTTCTGTTGGCGTGAAGTATCCCAAGTCTTTTACGAGTTCGATTATGAGTTTATTTTCAATCCACTTAATCTGTTTGTGGTTACTTCTACTAAAG 1800
N F S F G V R Y P S L L R G R L L S L F S I H L I C L L V T S L K 1800
I F R L A * G I Q V F Y E V D Y * V Y F Q S T * S V C W L L H * S 1800
K F F V W R E V S K S F T R S I I E F I F N P L N L F V G Y F T K V 1800

TCCTTACCCTTTGGGATATACTGCTGATAAGCCCGTTGGTGTTCATTTGGCACCACGTTCCCATGAGTGGTATGGTTTGCAAAAATAGAATTTTATTT 1900
S L P F G I Y C L I S P L V F S L A P R S H E W Y G L Q K * N F I S 1900
P Y P L G Y T A * * A R W C F H W H V P M S G M V C K N R I L F 1900
L T L W D I L P D K P V G V F I G T T F P * V V W F A K I E F Y F 1900

CCAATTTTGGCAAAATTCCTCGTCTTTGCAAACTCCTTTCCATTGTCAGCCGTAATTTGTGTGATTAAGTTTTCACCTTCCGCGAGTCCCATACTGC 2000
N F C A I S S C F A N S F P L S A V I V C I K F F T F R S A H T A 2000
P I F A Q F P R A L Q T P F H C Q P * L C V L S F S L S A V P I L Q 2000
Q F L R N F L V L C K L L S I V S R N C V Y * V F H F P Q C P Y C 2000

AATCTTAGTACCAGGATGGCTTCTTTCCGACAACCTGCGTATCCAGACCCCTGCTGTTGCTCTGCTTAATGGTAAGATGGCACCTTTGTGGTTC 2100
T L A T G M A S F P D N L R I Q T L L V A L S L M V R M A P L W F 2100
S * L P G W L L F P T T C V S R P C L L L C R * W * E W H L C G S 2100
N L S Y R D G F F S R Q L A Y P D P A C C S V V N G K N G T F V V L 2100

|BsoBI **|BstNI**
TTACCAATAATGATATCTATCTCTAAATCACCAAATCTCTCCTTCAGTTCACATCTCGGGACGCTCATCAATATCCACCCTGCTGGGATAAATCTCTC 2200
L P I I V S I S K S P N L S F S S T I S G R S S I S T L P G I N P R 2200
Y Q * L Y L S L N H Q I S P S V P L S R D A H Q Y P P C L G * I L 2200
T N N C I Y L * I T K S L L Q F H Y L G T L I N I H P A W D K S 2200

GCCCTGCATTTTGAACACCGTTTGGCATACTGCGACCTTGTCTGCGAAGATATTTGTGCAAGTTGCGACCCCGCGCTTATCTCCCAATCCAGCG 2300
P A F L E P R L A Y L R P C L R R Y L C S L P P R R L S S Q I Q R 2300
A L H F * N H V W H T C D L V C E D I C A V C H P A A Y P P K S S D 2300
P C I F R T T F G I P A T L S A K I F V Q F A T P P L I L P N P A 2300

ATATATCGTTTTCGTGAGATACCATCGCAATTCCTCCAGCGGCTCCTGCCGACAATCTGCTCCGGGCTGAATCCTTTCTTCAACAGCTTTATATTACCGT 2400
Y I V S * D T I A I P S K R L L P T I C S G L N P F F N S F I I R 2400
I S F R E I P S Q F P P S G S C R Q S A P G * I L S S T A L L S V 2400
I Y R F V R Y H R N S L Q A A P A D N L L R A E S F L Q Q L Y Y P F 2400

TTTCTCATTTGCCGGTGAAGCACTTCCCTGGCATGTTTTGCTGCTTCCGCTGCTGCTTTTCCGTTGGGCAAGCTCCATGCTATAGTACCCTTCCGG 2500
F L I A G V S T S L R C F C C L R L S A F R W A S S M L * L P L R A 2500
F S L P V * A L P C D V F A A C A C L L F A G Q A P C Y S Y H F G 2500
S H C R C K H F L A M F L A C P V C F S L G K L H A I A T T S G 2500

Rev2

CGTGCAGATTGCGCTTTATCTCCCTGTAACACAGTGCTTTATCTACTCCGATAGCTTCCGCTATTGCTTTTTGCTCATCGGTATTGCAACATCATAGA 2600
 S Q L R F I S L * T V L L S T P I A S A I A F L L I G I C N I E 2600
 R R N C A L S P C K Q C F Y L L R * L P L L L F C S S V F A T S * K 2600
 V A I A L Y L P V N S A F I Y S D S F R Y C F F A H R Y L Q H H R 2600

1475_Fp2

AATTGCATACCTTTGTCCTCGGTTATATGTTTGTCTACTGCAACTTTTTTTTCTTTGGACGGACAATTAAGCAAGATAGCAAACTTTATCCATTC 2700
 I A Y L C S S V I C L L I C N F F F F G R T I K A K I A N F I H S 2700
 L H T F V P R L Y V C S S A T F F S L D G Q L K Q R * Q T L S I Q 2700
 N C I P L F L G Y M F A H L Q L F F L W T D N * S K D S K L Y P F R 2700

GAGTGAGAGAAAGGGGACATGTCTCTCTTCCCTCTCTGAAAATAAATGTTTTATTGCTTATTATCCGCACCCAAAAAGTGCATTTATAAGTTGAA 2800
 E * E K G G H C L S F L S E K * M F L L L I I R T Q K V A F I S * T 2800
 S E R K Q D I V S L S L K N K C F Y C L L S A P K K L H L * V E 2800
 V R E R G T L S L F P L * K I N V F I A Y Y P H P K S C I Y K L N 2800

CTCAAGAAGTATTACCTGTAAGAAGTACTAATGACAAAAAAGAAATGCCCGTTCGTTTTACGGGTCAGCACTTTACTATTGATAAAGTGCATAATA 2900
 Q E V F T C K K L L M T K K K L P V R F T G Q H F T I D K V L I K 2900
 L K K Y S P V R S Y * * Q K R N C P F V L R V S T L L L I K C * * K 2900
 S R S I H L * E V T N D K K E I A R S F Y G S A L Y Y * * S A N K 2900

AGATGCAATAAGACAAGCAAAATATAAGTAATCAGGATACGGTTTTAGATATTGGGGCAGGCAAGGGGTTCTTACTGTTCAATTATAAAATCGCCAAC 3000
 D A I R Q A N I S N Q D T V L D I G A G K G F L T V H L L K I A N 3000
 M Q * D K Q I * V I R I R F * I L G Q A R G F L L F I Y * K S P T 3000
 R C N K T S K Y K * S G Y G F R Y W G R Q G V S Y C S F I K N R Q Q 3000

AATGTGTTGCTATTGAAAACGACACAGCTTTGGTTGAACATTACGAAAATATTTTTCTGATGCCCGAAATGTTCAAGTTGTCGGTTGCGATTTAGGA 3100
 N V V A I E N D T A L V E H L R K L F S D A R N V Q V V G C D F R N 3100
 M L L L L K T T Q L W L N I Y E N Y F L M P E M F K L S V V I L G 3100
 C C C Y * K R H S F G * T F T K I I F * C P K C S S C R L * F * E 3100

ATTTTGCAGTCCGAAATTCCTTCAAGTGGTGTCAAAATTCCTTATGGCATTACTTCCGATATTTTCAAAATCCTGATGTTGAGAGTCTTGAAA 3200
 F A V P K F P F K V V S N I P Y G I T S D I F K I L M F E S L G N 3200
 I L Q F R N F L S K W C Q I F L M A L L P I F S K S * C L R V L E I 3200
 F C S S E I S F Q S G V K Y S L W H Y F R Y F Q N P D V * E S W K 3200

HindIII

TTTTCTGGGAGTTCATGTCTTCAATTAGAACCTACACAAAAGTATTTTTCGAGGAAAGCTTACAATCCATATACCGTTTTCTATCATACTTTTTT 3300
 F L G G S I V L Q L E P T Q K L F S R K L Y N P Y T V F Y H T F F 3300
 F W E V P L S F N * N L H K S Y F R G S F T I H I P F S I I L F L 3300
 F S G R F H C P S I R T Y T K V I F E E A L Q S I Y R F L S Y F F * 3300

GATTGAACTTGTCTATGAGTAGGTCTGAAAGTTTCTGCCACCGCAACTGTCAAATCAGCCCTGTTAAACATTAAGAAAACACTTATTTTTTG 3400
 D L K L V Y E V G P E S F L P P P T V K S A L L N I K R K H L F F D 3400
 I * N L S M R * V L K V S C H R Q L S N Q P C * T L K E N T Y F L 3400
 F E T C L * G R S * K F L A T A N C N Q I S P V K H * K K T L I F * 3400

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 F K F K A K Y L A F I S Y L L E K P D L S V K T A L K S I F R K S 3500
 I L S L K P N T * H L F P I C * R N L I Y L * K Q L * S R F S G K V 3500
 F * V * S Q I L S I Y F L S V R E T * F I C K N S F K V D F Q E K 3500

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 Q V R S I S E K F G L N L N A Q I V C L S P S Q W L N C F L E M L 3600
 R S G Q F R K N S V * T L M L K L L F V C L Q V N G * T V F W K C W 3600
 S G Q V N F G K I R F K P * C S N C L F V S K S M V K L F F G N A G 3600

GAAGTGTCCCTGAAAAATTCATCCTCGTAGTCAAAAGTCCGGTGGTGTCAAGATGATTTTTTGGTGGTGTAGTCTTTTTTAAAGTCGCCGAT 3700
 E V V P E K F H P S * F K V G W L S R * F F W F G V V F F * A A A * 3700
 K L S L K N F I L R S S K S G G C Q D D F F G L V * S F F K L P H 3700
 S C P * K I S S F V V Q S R V V V K M I F L V W C S L F L S C R I 3700

AACGGTCGCAAAATGGCGATGGAGCCGACTTTAGCACAAATGTTGAATAGAATTACTAATCTCAACATGACAAAAGTCAATAGAAGTACTTCAC 3800
 R L A N I W R W S R L L A Q M L N R I T N L Q H C T K V Q * K Y F T 3800
 N G W Q I G D G A D F * H K C * I E L L I F N I A Q K F N R S T S P 3800
 T A G K L A M E P T F S T N V E * N Y * S S T L H K S S I E V L H 3800

CGGCTCATTGCCAATTTGTTGTTATGTGCGCTTTTATCCATTTTATACATTTAACAATGCTGAAACGTAAGTCGGGTTTAAACATTTCAATTTG 3900
 G S I A N L F V M C R F I P F Y T F N N C * N V K S G L N I S I S 3900
 A L C P I C L C A V P F Y S I L Y I * Q L L K R K V G F K H F N F V 3900
 R L Y C Q F V C Y V P F Y S I L Y I * Q L L K R K V G F K H F N F V 3900

TTTTGAGTTGATTCTTCTGCTTCTTGGCCATACATAAAACATTTGCTGTTCAATTTTTTAACAGCCTTCTCAATGCTATTAAATTTCCATCGGCCTA 4000
 F * V D S S C A S L P Y I N I C C S * F L T A S S M L L N F P S A R 4000
 F E L I L L V L L C H T * T F A V H N F * Q P L Q C Y * I F H R L 4000
 L S * F F L C F F A I H K H L L F I I F N S L F N A I K F S I G * 4000

GATTATCAGACAATATCAGGCATCCACCAACCCACTATTTACTCCCTGCCCTGCAAAAGGGCGCATCAAATGTCGGCATCCCCAATCATGTTATGGG 4100
 L S D N I K A S T N P L F T P C P A K G G I K C A A S P I I V M G 4100
 D Y Q T I S R H P P T H Y L L P A L Q K A A S N V R H P Q S I L W V 4100
 I I R Q Y Q G I H Q P T I Y S L P C K R R H Q M C G I P N H C Y G 4100

TAATGGCGCTTGTCTTTTCAAGGCTTTTCTAAAGGAAATATCCGTGTAGCCAATCTCAAAATGACAACGTCGTATGAATCAATCTTTGTAGCGTTG 4200
 N G R L L F Q G F S K G N I R V A N P T N D N V V * I N S L * R S 4200
 M G A C F S K A F L K E I S V * P I L Q M T T S Y E S I L C S V R 4200
 * W A L A F P R L F * R K Y P C S Q S Y K * Q R R M N Q F L V A F V 4200

Supplementary Data

TCCCAATCGGAAATCTTTCAGAGAAATCAACGACACTATTCTGTTTGGAAATCTACCTCGGTTTGGTTTTCCATTTCATCAGGTGTTTTAAAC 4300
S Q S E N S F R R K S T T L F L F * K S T C V W F F H S S G V L K L 4300
P N R K I L S E E N Q R H Y F C F E N L P A F G F S I H Q V F * N 4300
P I G K F F Q K K I N D T I S V L K I Y L R L V F P F I R C F K T 4300

TTATTCCAAATGCAATGACCATTATTGGGGTTAGCAAATAAATAACCTTGGTGAGATGCCATTAGCCGGTTTCCATTGCATAGCTGAAAAA 4400
I P K C N A P L L L G L A N N K L P W * D A I S R F P L H S * K N 4400
L F Q N A M H H Y Y W G * Q I I N Y L G E M P L A G F H C I A E K I 4400
Y S K M Q C T I I I G V S K * * I T L V R C H * P V S I A * L K K 4400

BstNI
TCCAGCAGCTTTATCTCTGGTTGATGAATATCGGCTGTATATTGAAATACCTGTTTCTCAACTCCGTCGTAACAAATTTCTTACCTGGAC 4500
P G Q F I S G * * I S A C I L K V P V S S T S V S V T N F L T L D 4500
Q D S L S L V D E Y R L V Y * K Y L F L Q L P C R * Q I F L P W T 4500
S R T V Y L W L M N I G L Y I E S T C F F N F R V G N K F S Y L G H 4500

BstNI
ATCCCGCATGGCAAGAATAACCAATCTGCTGTTTACTCGGTTTATTCTCAAAGTTAGTGCCACTTCTTCTTCCAGGTTCAAGCATAAAGTT 4600
I P P L A R I T K S A V S L G L F S K V S V H F F L P G S S I T S F 4600
S R H W Q E * P N L L F H S V Y S Q K L V S T S S Y Q V Q A * Q V 4600
P A I G K N N Q I C C F T R F I L K S * C P L L L T R F K H N K F 4600

TCTATCCCAATAACCGTGTCTGTTTCTAAACTATTCAACAAGATAGCCCTTAAGTCATTCTGTTTATTTCAGGATTGTCAAATCGATTTTCGGGCTT 4700
L S Q I T V S F S K L F N K I A L K S F L F I S G L S N R F A F F 4700
F Y P K * P C R F L N Y S T R * P L S H F C L F Q D C Q I D F R A L 4700
S I P N N R V V F * T I Q Q D S P * V I S V Y F R I V K S I F G L 4700

TACATTTTTGTGGATAAAATATGCCTTTTTATCAGCAATATTACACCCATTGGTAAGGCTAAGTCATAAAGTTGTAACAATCCCGCTTTTTTC 4800
T F F V D K I L P F L S A I F T P I G K A K S * * V C N N P A F F 4800
H F L W I K I Y Q Q Y L H P L V R L S H N K F V T I P L F S 4800
Y I F C G * N I A F F I S N I Y T H W * G * V I I S L * Q S R F F H 4800

Tetrev
ATTGCTTCTGACCTGAACTTTGTGTAGGTCAAGGGTCCACCAAAATCTTGCCTCTCGGTCGTTCTCTCGTAAACTGAAACGCTATGCCGT 4900
I A S * P E P L C R S R V P P K I L A S R R S L S L S * T E T S M P F 4900
L L P D L N L C V G Q G F H Q K F L P L G R C L F R K L K R L C R 4900
C F L T * T F V * V K G S T K N S C L S V V V S F V N * N V Y A V 4900

TTTGTGTAATAATTTGCCATAGTCAGTCCACCGGGTCCACCACCAATATTGCAACGTTCTTATCACTAAGTAAATTCATTGTTTGTCTGTATCTAT 5000
C C N N F A I V S P T G P P P I A T F L S L S K F I C L S V S I 5000
F A V I I L P * S V Q R V H H Q L L Q R S Y H * V N S F V C L Y L F 5000
L L * * F C H S Q S N G S T T N Y C N V L I T K * I H L F V C I Y 5000

TGCGATTGTCATTCAAATGCGCCATAAATGGCTCGGCGAATGGCGAAGTAGCGAAGCTGTATCGAAGATACAAAAGCGGAGCTATTTAGCCAAGT 5100
R I V I Q K W P I T M A R R I G E V A K L Y R R Y K S G A I * P S 5100
A L S F K N G P * Q W L G E L A K * R S C I E D T K A E L F S Q V 5100
S H C H S K M A H N N G S A N W R S S E A V S K I Q K R S Y L A K S 5100

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R L L * A V R E A A A Y G K R R K S P S H C * Q G A Q R I A N N G L 5200
A C C K Q S A K R R L T A S D V S R R A I V S K E R S G L L T T V 5200
L V V S S P R S G G L R Q A T * V A E P L L A R S A A D C * Q R F 5200

Tetfw
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Q L Y A G R D F Y H * A T Y D E L N F K F T T F L S Y E A R T P C 5300
C S Y T Q A G I F T T K L P T M N * T L N L P L S C L T K H E P P A 5300
A A I R R Q G F L P L S Y L R * T E L * I Y H F P V L R S T N P L 5300

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C V * V M L C G R L S F S L L S R * F V K Q S Y * I C S Q A I S I 5400
L A Y R * C Y A V G F H F L F F L V N L S S K V T K F A V K Q F Q F 5400

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F * I M Y F S R P W T S I * V I G S P E * I P I N L L G Q D N Q S M 5500
F E L C I S A D L G L L S K * * A V L N K F Q S I F * G K T I S L 5500
L N Y V F Q Q T L D F Y L S D R Q S * I N S N Q S F R A R Q S V Y 5500

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L C V C C H F F * W H C F V V V A L L S G N T V Y S L L C D L N S T 5600
C F V C A V I F F D G I A L S * L L C R V I L S I H F S V I * I Q L 5600
A L C V L S F F L M A L L C R S C S V G * Y C L F T S L * F E F N 5600

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A N A T E F * L I I C L N F S F G V R Y P S L L R G R L L S L F S 5700
L Q M Q Q N S D * * F V * I F R L A * G I Q V F Y E V D Y * V Y F Q 5700

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I H L I C L L V T S L K S L P F G I Y C L I S P L V F S L A P R S 5800
S T * S V C W L L H * S P Y P L G Y T A * * A R W C F H W H H V P 5800

CATGAGTGGTATGGTTTGCAAAAATAGAATTTTATTTCCAATTTTTCGCAATTTCTCGTGTCTTGGCAACTCTTTCCATTGTCAGCCGTAATTTGTGT 5900
* V V W F A K I E F Y F Q F L R N F L V L C K L L S I V S R N C V 5900
H E W Y G L Q K * N F I S N F C A I S S C F A N S F P L S A V I V C 5900
M S G M V C K N R I L F I F A Q Q P R A L Q T P F H C Q P * L C 5900

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 I K F F T F R S A H T A I L A T G M A S F P D N L R I Q T L L V A 6000
 V L S F S L S A V P I L Q S * L P G W L L F P T T C V S R P C L L L 6000

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 S V V N G K N G T F V V L T N N C I Y L * I T K S L L Q F H Y L G T 6100
 L S L M V R M A P L W F L P I I V S I S K S P N L S F S S T I S G 6100
 C R * W * E W H L C G S Y Q * L Y L S L N H Q I S P S V P L S R D 6100

CGCTCATCAATATCCACCCTGCTGGGATAAATCCTCGCCCTGCATTTTTAGAACCCAGTTTGGCATACTGCGACCTTGTCTCGAAGATATTTGTGCA 6200
 L I N I H P A W D K S S P C I F R T T F G I P A T L S A K I F V Q 6200
 R S S I S T L P G I N P R P A F L E P R L A Y L R P C L R R Y L C S 6200
 A H Q Y P P C L G * I L A L H F * N H V W H T C D L V C E D I C A 6200

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 F A T P P L I L P N P A I Y R F V R Y H R N S L Q A A P A D N L L 6300
 L P P R R L S S Q I Q R Y I V S * D T I A I P S K R L L P T I C S 6300
 V C H P A A Y P P K S S D I S F R E I P S Q F P P S G S C R Q S A P 6300

CGGGCTGAATCCTTCTCAACAGCTTTATTATCCGTTTCTCATGCGGGTGAAGCACTTCCCTGCGATGTTTTGCTGCTGCGCCTGCTGCTTTT 6400
 R A E S F L Q Q L Y Y P F S H C R C K H F L A M F L L A P V C F S 6400
 G L N P F F N S F I I R F L I A G V S T S L R C F C C L R L S A F 6400
 G * I L S S T A L L S V F S L P V * A L P C D V F A A C A C L L F 6400

CGCTGGGCAAGCTCCATGCTATAGCTACCACTTCGGGCGTCGCAATTCGGCTTTATCTCCCTGTAACAGTGTCTTTATCTACTCGATAGCTCCCGTA 6500
 L G K L H A I A T T S G V A I A L Y L P V N S A F I Y S D S F R Y 6500
 R W A S S M L * L P L R A S Q L R F I S L * T V L L S T P I A S A I 6500
 A G Q A P C Y S Y H F G R R N C A L S P C K Q C F Y L L R * L P L 6500

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 C F F A H R Y L Q H H R N C I P L F L G Y M F A H L Q L F F L W T 6600
 A F L L I G I C N I I E I A Y L C S S V I C L L I C N F F F F G R 6600
 L L F C S S V F A T S * K L H T F V P R L Y V C S S A T F F S L D G 6600

GACAATTAAGCAAAGATAGCAAACCTTTATCCATTCAGAGTGAAGAAAGGGGACATTTGCTCTCTTTCTCTCTGAAAAATAAATGTTTTATGCTT 6700
 D N * S K D S K L Y P F R V R E R G T L S L F P L * K I N V F I A Y 6700
 T I K A K I A N F I H S E * E K G G H C L S F L S E K * I V L L A 6700
 Q L K Q R * Q T L S I Q S E R K G D I V S L S S L K N K C F Y C L 6700

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 Y P H P K S C I Y K L N S R A I F P I L Y F G V N Q * K * S Q P R 6800
 I I R T Q K V A F I S * T Q G L F S S T L G S I N K S K A N P A 6800
 L S A P K K L H L * V E L K G Y F P N P L L W G Q S I K V K P T P 6800

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 F I D H Y I H C A P V A G A G R P G Y K I F K K K F I D Q P G C V 6900
 S L I T T S T A R R S L G Q D V R V T R S L R R S L S I S L D V L 6900
 L H * S L H P L R A G R W G R T S G L Q D L * E E V Y R S A W M C W 6900

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 G I F I K H T F R I Y H S L * S D W D A A R A K S * A H Y T A P V * 7000
 E F L S S I R F G Y I T H F D R I G M L P A Q N P E H I I L R C 7000
 N F Y Q A Y V S D I S L T L I G L G C C P R K I L S T L Y C S G V 7000

GaggtcATGtTCCCTCaaatCTCTaTtTgCtTGTAGCCAAGCCAGTACTGTATATAAAGATTCAATCAACCGCTGATACGGAGTCCGAAATCTT 7100
 G H V P S K S L F A C S Q A S Y L Y K * D S V P T A D T E S E I F 7100
 E V M F P Q N L Y L L V A K P V T C I N K I Q Y Q P L I R S P K S F 7100
 R S C S L K I S I C L * P S Q L L V * I R F S T N R * Y G V R N L 7100

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 F A * Y R F L L T * H T T C * * Y N N I V Y T F N V * T S I Y Y F 7200
 L L N I G F Y * H S T Q L V S S T I T L Y I H L M Y E H L F I T S 7200
 F C L I S V S I D I A H N L L V V Q * H C I Y I * C M N I Y L L L Q 7200

AAAGTTCCTGCTCATAAAAAAGGTGTATATTTATCAGCATAACGAAAAGCAAACCGAGTAAATAACTACATTACAGATAGTTACAATAATTAAGTT 7300
 K V P A S * K R C I F I S I T K S K P G L N N Y I T D S V T I N * S F 7300
 K F L L H K K G V Y L S A * R K A N Q D * I T T L Q I V T I I K V 7300
 S S C F I K K V Y I Y Q H N E K Q T R T K * L H Y R * L Q * L K F 7300

TTTTGAAAAATTTCTCATGTTTCAACTTTGTCAGCCTCTGATTCTGCTCTAACTCTCCAA 7400
 L K N F S C F N F V S L * F L P L T L Q 7400
 F * K I S H V S T L S A S D S C L * L S A 7400
 F E K F L M F Q L C Q P L I L A S N S P 7400

Figure S5.1: Nucleotide and amino acid sequences of the Tn4351-transposon insertion site of mutant B-16. Nucleotide sequence was revised manually via comparison with the electropherogram. Manually changed nucleotides are written in small letters. For nucleotides without clear peaks in the chromatogram, letters according to the IUPAC Ambiguity Code were used. The nucleotide sequence was simple sequenced from nucleotides 0-68 bp and 6449-7362 bp (plus strand). All other sequences were repeatedly sequenced. Sequence of the Tn4351-transposon was received from Prof. M. J. McBride (University of Wisconsin – Milwaukee, USA) and the transposon border is flanked by direct repeats marked with underlined letters (XXX). First and last

Supplementary Data

nucleotides of the transposon are highlighted in red (X). Colour code: (xxx) sequence obtained from the Trenzyme report; (xxx) primers: B16-FosmidprimerFW5 (FW5), B16-FosmidprimerFW4 (FW4), B16-FosmidprimerRev3 (Rev3), 1475_FP2, B16-FosmidprimerRev2 (Rev2), Tn4351_Tet_fw (Tetfw), Tn4351_Tet_rev (Tetrev), 1475_RP1, B16-FosmidprimerFW2 (FW2), B16-FosmidprimerRev5 (Rev5), B16-FosmidprimerFW3 (FW3); (xxx) restriction sites located in the transposon for BsoBI, BstNI and HindIII. Nucleotide sequence was transcribed in amino acid sequence using the DNA to protein translation tool of insilico.ehu.es (Bikandi et al. 2004). Open reading frames (ORF1-4) in the amino acid sequences which led to hits when blasted against the NCBI data base are highlighted in blue (xxx).

Translations Aligned (5'→3'), plus strand

```

ORF1
10      20      30      40      50      60      70      80      90
ACAAAACCAGGAGAAGATTTACGCATGACGAAAACCTGACCTATGCCGATTGATCGGATTTGGGATCCGCCACGTAAGGAAGTAAACCAAGTCATTGA 100
T K P G E D F T H D E N L T Y A G L I G F W D P P R K E V T P K S L N 100
Q N Q E K I S R M T K T * P M P D * S D F G I R H V R K * P S H * 100
K T R R R F H A * R K P D L C R I D R I L G S A T * G S N Q V I E 100

ACCCCTGTCATAAGGCTGGTATAAAAAGTCATTATGGTTACCCGGTGATCACCCTGCTACTTCGCTGAAAATTGCAAAGCAGTCCGCCCTGGTGAAGAAGA 200
A C H K A G I K V I M V T G D H P A T S L K I A K Q V G L V E E D 200
T P V I R L V * K S L W L P V I T R L L R * K L Q S R S A W L K K T 200
R L S * G W Y K S H Y G Y R * S P G Y F A E N C K A G R P G * R R 200

CGAGTACTGGTACTACCGGAAAAGATCTTGAATCGTTTATTTTCATCGCGGAATGACCAGAAAAAATGATGGAATGCCATGTATTGCCCGGGTA 300
E L L V L T G K D L E S F D F S S A N D Q K K M M E C H V F A R V 300
S Y W Y S P G K I L N R L I F H R R M T R K K * W N A M Y L P G * 300
R A T G T H R E R S * I V * F F I G E * P E K N D G M P C I C P G K 300

AATCCTGCTCAGAACTGGACATGATTATTTTATCAAAAACAGGGAGATATCGTGGGCATGACCCGGCAGCGGGTAAACGACGCTCCCGCCTGAAGA 400
N P A Q K L D M I D F Y Q K Q G D I V G M T G D G V N D A P A L K K 400
I L L R N W T * L I F I K N R * E I S W A * P A T G * T T L P P * R 400
S C S E T G H D * F L S K T G R Y R G H D R R R G K R R S R P E E 400

AATCCGATATCGGTATTGCCATGGGACTCCGGGAACACCCGTCCCGCGAAGCTGCTGATATGGTCTCAAAGATGACTCGTTTGTCCATCGTGCA 500
S D I G I A M G L R G T A V A A E A A D M V L K D D S F A S I V H 500
N P I S V L P W D S G E Q P S P P K L L I W C S K M T R L L P S C M 500
I R Y R Y C H G T P G N S R R R R S C * Y G A Q R * L V C F H R A 500

TGCCATTGAACAGGGAAGGGTCATTTTTGAAAATATCAGGAAGTTCATCGTGTTCCTGTTGCTGCAACATGAGTGAATTTTCGTGGTCACTTTTGGC 600
A I E Q G R V I F E N I R K P I V F L L S C N M S E I F V V T F A 600
P L N R E G S F L K I S G S S C F L C C P A T * V K F S W S L L R 600
C H * T G K G H F * K Y Q E V H R V S V V L Q H E * N F R G H F C G 600

GGTTTTCTCAATGTTGGCAGCCCGCTTCTGCCCTTCAAATCTCTTCAATCAACATCrTkACCGATGTGTTCCCGGCGCTTGCOCCTGGGTAGGTAAG 700
G F L N V G S P L L P L Q I L F I N I X T D V F P A L A L G V G K D 700
V F S M W A A R F C P F K F S S S T S X P M C S R R L P W V * V K 700
F S Q C G Q P A S A P S N S L H Q H X X R C V P G A C P G C R * R 700

ACAACAAGAACTGATGAAATyykwaAsrrGGGATCCCAAGCAGCCCATATtACAGGCAGCGGATtGGAGGAGAATTGTGTTTTATGCATTGGTCATGAC 800
N K N L M K X X X D P K Q P I L Q A A D W R R I V F Y A L V M T 800
T T R I * * N X X X G I P S S P Y Y R Q R I G G E L C F M H W S * P 800
Q Q E S D E X X X X G S Q A A H I T G S G L E E N C V L C I G H D 800

|Rev2
CGCATCCGTTTTGGTGTACTGGTATGCAATGGATCAACTGGGTTTTAGTCCCGAAGAAAGAAACACCGTTACCTTTTTATGCACCTTCACTGGCACAG 900
A S V L G V Y A I D Q L G F S P E E G N T V T F Y A L S L A Q 900
H P F W V C T G M Q L I N W V L V P K K E T P L P F M H F H W H S 900
R I R F G C V L V C N * S T G F * S R R R K H R Y L L C T F T G T A 900

CTGCTTCATGTTTTCAACATGATTTCAGGCAAGCTGCATTTTTCAATAATGAAATCACCCGGAACAAATTTATCTGGATGGCTTGGACACTTCGCATTG 1000
L L H V F N M Y S G K L H F F N N E I T R N K F I W M A L T L C I A 1000
C F M F S T C I Q A S C I F S I M K S P G T N L S G W L * H S A L 1000
A S C F Q H V F R Q A A F F Q * * N H P E Q I Y L D G F D T L H C 1000

CCATTATGCTGTGCACCTATTACACACCCCTTCTCCGACAATACTCTCACTTCAGTACTTGATGTACAGCTCTGGAATTAATAETGAGTCAACTT 1100
I M L V T Y Y T P F F R Q I L S L Q L L D V Q A L E L I L E F S N L 1100
P L C L S P I T H P S S D K Y S H F S Y L M Y K L W N * Y L S S T Y 1100
H Y A C H L L H T L L P T N T L T S V T * C T S S G I N T * V Q L 1100

ATAAATGCAACTTTTTGGGTGCGGATAATAAGCRATAAAAACATTTATTTTTTCAGAGAGGAAAGAGACAAATGTCGCCCTTCTCTCACTCTGAATGGA 1200
* M Q L F G C G * * A I K T F I F Q R G K R D N V P L S L T L N G 1200
K C N F L G A D N K Q * K H L F F S E R E R E T M S P F L S L * M D 1200
I N A T F L V R I I S N K N I Y F S E R K E R Q C P P F S H S E W I 1200

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* S L L S L L * L S V Q R K K S C R * A N I * P R N K G M Q F L * C 1300
K V C Y L C F N C P S K E K K V A D E Q T Y N R G T K V C N F Y D 1300
K F A I F A L I V R P K K K K L Q M S K H I T E E Q R Y A I S M M 1300

```

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 V A N T D E Q K S N S G S Y R S R * K H C L Q G D K A Q L R R P K W 1400
 L Q I P M S K K A I A E A I G V D K S T V Y R E I K R N C D A R S 1400

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 V A I A W S L P S E K Q T G A S S K N I A R K C L H R Q * E N G * 1500
 * L * H G A C P A K S R Q A Q A A K T S Q G S A Y T G N E K T D N 1500
 G S Y S M E L A Q R K A D R R K Q Q K H R K E V L T P A M R K R I I 1500

TAAAGCTGTTGAAGAAAGATTTCAGCCCGGAGCAGATTTCGGCAGGAGCCGCTTGGAGGAATTGCGATGGTATCTCACGAAAACGATATATCGTGGAT 1600
 * S C * R K D S A R S R L S A G A A W R E L R W Y L T K R Y I A G F 1600
 K A V E E R I Q P G A D C R Q E P L G G N C D G I S R N D I S L D 1600
 K L L K K G F S P E Q I V G R S R L E G I A M V S H E T I Y R W I 1600

TTGGGAGGATAAGCGCGGGTGGCAAACTGCACAAATCTCTCGCAGACAAGGTCGAGGTATGCCAAACGGTGTCTAAAAATGCAGGCGGAGGATTT 1700
 G R I S G G V A N C T N I F A D K V A G M P N V V L K M Q G E D L 1700
 L G G * A A G W Q T A Q I S S Q T R S Q V C Q T W F * K C R A R I Y 1700
 W E D K R R G G K L H K Y L R R Q G R R Y A K R G S K N A G R G F 1700

BstNI | | | | **BsoBI** | | | |
 ATCCAGGCAGGGTGGATATTGATGAGCGTCCCGAGTAGTGAAGTGAAGGAGAGATTTGGTGATTTAGAGATAGATACAAATTATTGGTAAAGACCACA 1800
 S Q A G W I L M S V P R * W N * R R D L V I * R * I Q L L V R T P 1800
 P R Q G G Y * * A S R D S G T E G E I W * F R D R Y N Y W * E P Q 1800
 I P G R V D I D E R P E I V E L K E R F G D L E I D T I I G K N H K 1800

AAGGTGCCATCTTACCATTAAACGACAGCAACAAGCAGGGTCTGGATACGCAAGTGTTCGGGAAAAGAACCCATCCCGGTAGCTAAGATTGCGATG 1900
 K V P F L P L T T E Q Q A G S G Y A S C R E K K F S R * L R L Q Y G 1900
 R C H S Y H * R Q S N K Q G L D T Q V V G K R S H P G S * D C S M 1900
 G A I L T I N D R A T S R V W I R K L S G K E A I P V A K I A W 1900

GGCAGTCCGGAAGTGAAGAACTTAATACACACAATTACGGCTGACAATGGAAGGAGTTGCAAAAGCAGGAAATTCGCCAAAATTCGAAATAAAA 2000
 H C G K * K T * Y T Q L R L T M E R S L Q S T R K L R K N W K * N 2000
 G T A E S E K L N T H N Y G * Q W K G V C K A R G N C A K I G N K I 2000
 A L R K V K N L I H T I T A D N G K E F A K H E E I A Q K L E I K 2000

TTCTATTTTTCGAAACCATACCCTCATGGGAACGTGGTCCCAATGAAAACACCAACGGGCTTATCAGGCAGTATATCCCAAAGGGTAAGACTTTAGTG 2100
 S I F A N H T T H G N V V P M K T P T G L S G S I S Q R V R T L V 2100
 L F L Q * I P L M G T W C Q * K H Q R A Y Q A V Y P K G * G L * * 2100
 F Y F C K P Y H S W E R G A N E N T N G L I R Q Y I P K G K D F S E 2100

AAGTAACCAACAACAGATTAAAGTGGATTGAAAATAAACTCAATAATCGACCTCGTAAAAGACTTGGATACCTCAGCCAAAGCAGAAAATTTAAACAAAT 2200
 K * P T N R L S G L K I N S I I D L V K D L D T S R Q T K N L N K L 2200
 S N Q Q T D * V D * K * T Q * S T S * K T W I P H A K R K I * T N 2200
 V T N K Q I K W I E N K L N N R P R K R L G Y L T P N E K F K Q I 2200

TATTAATCAGAATCTGTTGCATTTCGCAAGTGAATTCAAATCACAGAGAAGTGAATAGACAGTATTACCCGACAGCAACTACGCAAAAGCAATGCCA 2300
 L I R I L L H L Q V E F K S Q R S E * T V L P D R A T T T K Q C H 2300
 Y * S E F C C I C K L N S N H R E V N R Q Y Y P T E Q L R Q S N A I 2300
 I N Q N S V A F A S * I Q I T E K * I D S I T R Q S N Y D K A M P 2300

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 Q K K * Q H T Q S I D * L S C P K R L I G I Y S G L P I T * I E V 2400
 K K N D S T H K A * T D C L A L K D * L E F I Q D C L S L R * K S 2400
 S K K M T A H T K H R L I V L P * K I D W N L F R T A Y H L D R S P 2400

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 Q G L L K I I I Q K L L D C K F S N F A * Q I N E K E K E M K A 2500
 K V C * N T * F K N * N C L T A N L V T L L D K L T R K K R K * K 2500
 R S A E I H N S K I E I A * L Q I * * L C L T N * R E R R E N E S 2500

CCGACCCATAACATCACCTATA **CGCAAGCAGGGGGTCTGTC** TCCGTAAGACAGGAAGTGGTAAATTTAAAGTTCAGTTCATCGTAGCTAGCTAGTG 2600
 D R I T S P I R K Q G V R A S * D R K V V N L K F S S S * V A * L W 2600
 P T A * H H L Y A S R G F V L R K T G K W * I * S S V H R R * L S G 2600
 R P H N I T Y T Q A G G S C F V R Q E S G K F K V Q F I V G S L V 2600

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 * K S L P A Y S C K P L L A I R C A P C * Q W L G D L R R L P * A 2700
 K N P C L R I A A N R C * Q S A A L S L A N N G S A T Y V A C R K P 2700
 V K I P A C V * L Q T V V S N P L R S L L T M A R R L T S L A V S R 2700

GCCGCTTCGGGACTGCTTACAACAAGCGACTTGGCTAAATAGCTCCGCTTTTGTATCTTCGATACAGCTTCGCTACTTCGCCAATTCGCCGAGCCATTG 2800
 A A S R T A Y N K R L G * I A P L L Y L R Y S F A T S P I R A I V 2800
 P L R G L L T T S D L A K * L R F C I F D T A S L L R Q F A E P L 2800
 R F A D C L Q Q A T W L N S S A F V S S I Q L R Y F A N S P S H C 2800

TTATGGCCATTTTGAATGACAATGCGAATAGATACAGACAAAATAAATGAAATTTACTTACTGATAAGAAGTTCGAATAATGGTGGTGGACCCGCTGG 2900
 M G H F * M T M R I D T D K Q M N L L S D K N V A I I G G G P V G 2900
 L W A I F E * Q C E * I Q T N K * I Y L V I R T L Q * L V V D P L D 2900
 Y G A P F L N D N A N R Y R Q T N E F T * * * E R C N N W W W T R W 2900

ACTGACTATGGCAAAATTTATACAGCAAAACGGCATAGACGTTTCA **GTTTACGAAAGACACACCGCAGAG** CCAAGAATTTTGGTGAACCCCTTGAC 3000
 L T M A K L L Q Q N G I D V S V Y E R D N D R E A R I F G G T L D 3000
 * L W Q N Y Y S K T A * T F Q F T K E T T T E R Q E F L V E P L T 3000
 T D Y G K I I T A K R H R R F S L R K R Q R P R G K N F W W N P * P 3000

Supplementary Data

CTACACAAGGTTTCAGTCCAGGAAGCAATGAAAAAGCGGGATTGTTACAACTTATTACTAGCCTTACCAATGGGTGTAATAATTGCTGATAAAA 3100
L H K G S G Q E A M K K A G L L Q T Y Y D L A L P M G V N I A D K K 3100
Y T K V Q V R K Q * K K R D C Y K L I M T * P Y Q W V * I L L I K 3100
T Q R F R S G S N E K S G I V T N L L * L S L T N G C K Y C * * K 3100

AAGGCAATATTTTCCACAAAAATGTAAGCCCGAAAATCGATTGCAATCCTGAAATAACAGAAATGACTTAAGGGCTATCTGTGTAATGTTT 3200
G N I L S T K N V K P E N R F D N P E I N R N D L R A I L L N S L 3200
K A I F Y P Q K M * S P K I D L T I L K * T E M T * G L S C * I V * 3200
R Q Y F I H K K C K A R K S I * Q S * N K Q K * L K G Y L V E * F 3200

AGAAAACGACCGTTATTGGGATAGAAAACCTGTTATGCTTGAACCTGGTAAGAAGAAGTGGACACTAATTTTGAGAATAAACCGAGTGAAACAGCA 3300
E N D T V I W D R K L V M L E P G K K K W T L T F E N K P S E T A 3300
K T T R L F G I E N L L C L N L V R R S G H * L L R I N R V K Q Q 3300
R K R H G Y L G * K T C Y A * T W * E E V D T N F * E * T E * N S R 3300

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D L V I L A N G G M S K V R K F V T D T E V E E T G T F N I Q A D I 3400
I W L F L P M A G C P R * E N L L P T R K L K K Q V L S I Y K P I 3400
F G Y S C Q W R D V Q G K K I C Y R H G S * R N R Y F Q Y T S R Y 3400

TTCATCAACCAGAGATAAATCTGCTGGATTTTTCAGCTATGCAATGGAACCCGGCTAATGGCATCTCACCAAGGTAATTTATTATTGCTAACCCCAA 3500
H Q P E I N C P G F F Q L C N G N R L M A S H Q G N L L F A N P N 3500
F I N Q R * T V L D F F S Y A M E T G * W H L T K V I Y Y L L T P I 3500
S S T R D K L S W I F S A M Q W K P A N G I S P R * F I I C * P Q 3500

TAATAATGGTGCATTGTCATTTTGAATAAGTTTAAACACCTGATGAATGGAACCAACCGCAGGTAGATTTTCAAAACAGAAATAGTGTCTGTTGAT 3600
N N G A L H F G I S F K T P D E W K N Q T Q V D F Q N R N S V V D 3600
I M V H C I L E * V L K H L M N G K T K R R * I F K T E I V S L I 3600
* * W C I A F W N K F * N T * * M E K P N A G R F S K Q K * C R * F 3600

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F L L K E F S D W D E R Y K E L I H T T L S F V G L A T R I F P L E 3700
F F * K N F P I G T N A T K N * F I R R C H L * D W L H G Y F L * 3700
S S E R I F R L G R T L Q R I D S Y D V V I C R I G Y T D I S F R 3700

AAAAGCCTTGGAAAAGCAAGCCCATACCCATAACAATGATTGGGGATGCCGCACATTGATGCCGCTTTTGACGGCAGGGAGTAAATAGTGGGTT 3800
K P W K S K R P L P I T M I G D A A H L M P P F A G Q G V N S G L 3800
K S L G K A S A H Y P * Q * L G M P H I * C R L L Q G R E * I V G W 3800
K A L E K Q A P I T H N N D W G C R T F D A A F C R A G S K * W V 3800

GGTGGATGCCTTGATATGTCTGATAATCTAGCCGATGGAATAATTAATAGCATTGAAGAGGCTGTAAAAATTAAGACAGCAAAATGTTATGTATGGC 3900
V D A L I L S D N L A D G K F N S I E E A V K N Y E Q Q M F M Y G 3900
W M P * Y C L I I * P M E N L I A L K R L L K I M N S K C L C M A 3900
G G C L D I V * * S S R W K I * * H * R G C * K L * T A N V Y V W Q 3900

AAAGAGCACAAGAAGAACTCAAAACGAAATGAAATGTTAAACCCGACTTACGTTTCAGCAATTGTTAAATGTATAAAATGGAATAAAACCGC 4000
K E A Q E E S T Q N E I E M F K P D F T F Q Q L L N V * N G I K R H 4000
K K H K K N Q L K T K L K C L N P T L R F S N C * M Y K M E * N G 4000
R S T R R I N S K R N * N V * T R L Y V S A I V K C I K W N K T A 4000

ACATAACAACAAATTTGGCAATAGAGCCGGTGAAGTACTTCTATTGAACCTTTTGTGCAATGTTGAAGATTAGTAATCTATTCAACATTTGTGCTAAAAG 4100
I T N K L A I E P V K Y F Y * T F V Q C * R L V I L F N I C A K S 4100
T * Q T N W Q * S R * S T S I E L C N V E D * * F Y S T F V L K V 4100
H N K Q I G N R A G E V L L N F C A M L K I S N S I Q H L C * K 4100

TCGGCTCCATCGCAATTTGCCAGCCGTTATCGCGCAGCTTAAAAAAGACTACCAACCAAAAAATCATCTTGACAAACCCCGACTTTGAACTAC 4200
R L H R Q F A S R Y A A A * K K T T P N Q K N H L D N H P T L N Y 4200
G S I A N L P A V M R Q L K K R L H Q T K K I I L T T T R L * T T 4200
S A P S P I C Q P L C G S L K K D Y T K P K K S S * Q P P D F E L R 4200

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E G * N F S G T T S S I S K K Q F N H * L G D K Q T I * A L R F K P 4300
K D E I F Q G Q L P A F P K N S L T I D L E T N K Q F E H * G L N 4300
R M K F F R D N F Q H F Q K T V * P L T W R Q T N N L S I K V * T 4300

CGAATTTTCCGAAATGACCTGACCTGCTTTTCTGAAATCGACTTTAAAGCTGTTTTTACAGATAAATCAGGTTTCTCTAACAGATAGGAAATAAA 4400
N F S E I D L T * L F L K I D F K A V F T D K S G F S N R * E I N 4400
R I F P K L T * P D F S * K S T L K C L F L Q I N Q V S L T D R K * M 4400
E F F R N * P D L T F P E N R L * S C F Y R * I R F L * Q I G N K 4400

TGCTAAGTATTTGGCTTAAACTTAAATCAAAAAATAAGTGTTTTCTTTAATGTTTAAACAGGCTGATTGACAGTTGGCGGTGGCAAGAACTTTCA 4500
A K Y L A L K S K N K F L L M F N R A D L T V G G G K K L S 4500
L S I W L * T * N Q K I S V F F * C L T G L I * Q L A V A R N F Q 4500
C * V F G F K L K I K K * V F S F N V * Q G * F D S W R W Q E T F R 4500

GGACCTACCTCATAGACAAGTTTCAAATCAAAAAAGTATGATAGAAAACGGTATATGGATTGTAAGCTTCTCGAAAATAACTTTGTGTAGTTCTA 4600
G P T S * T S F K S K K V * * K T V Y G L * S E F L E N N F C V G S N 4600
D L P H R Q V S N Q K K Y D R K R Y M D C K A S S K I T F V * V L 4600
T Y L I D K F Q I K K S M I E N G I W I V K L P R K * L L C R F * 4600

ATTGAAGGCAATGGAACCTCCAGAAAATTTCCAAGACTCTCAACACTCAGGATTTTGAATAATCGGAAGTAATGCCATAAGGAATATTTGACACCAC 4700
* R T M E P P R K F P R L S N I R I L K I S E V M P * G I F D T 4700
I E G Q W N L P E N F Q D S Q T S G F * K Y R K * C H K E Y L T P L 4700
L K D N G T S Q K I S K T L K H Q D F E N I G S N A I R N I * H H 4700

Supplementary Data

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ORF2
ACACATTATGGACGAAAAGAAAATGCCACCGAAATGGAGAAGTCAAAGCCGATATCATCGTTGAAATCATAGAATATGTTCCAAAACCGCGTGTGAGC 6500
T L W T E K K M P P K W R S Q K R I S S L K S * N M F Q T P C * A 6500
T H Y G P K R K C H R N G E V K S A Y H R * N H R I C S K R A E Q 6500
H I M D R K E N A T E M E K S K A H I I V E I I E Y V P N A V L S 6500

AGAACCATCATTCGCAAGACCACCGGTAACATTACCGTCTCTTCATTGATACAGGGGAGGAGCTTCCAGAGAAGTCATTCGCCTTTGATACCTACATCC 6600
E P S F A R P P V T L P S L H L I Q A R S F Q R S H L P L I P T S 6600
N H H S Q D H R * H Y R L F I * Y R R G A S R E V I C L * Y L H P 6600
R T I I R K T T G N I T V S S F D T G E E L P E K S S A F D T Y I Q 6600

AGATCATCGACGGGTCTGCTGAAATTTGATAGACGGGACCAAGCACAACACTGAAATAGGGGAGGAATAATCATTCCGGCTCACGCCAGAAATTCCTT 6700
R S S T G L L K L L * T G P S T N * N * G R E * S F R L T P E I P F 6700
D H R R V C * N C Y R R D Q A Q T E I R G G N N H S G S R Q K F L 6700
I I D G S A E I V I D G T K H K L K L G E G I I I P A H A R N S F 6700

TTGGCCAAAGAGCAGTTCAAATGATCTCGACGGTAATCAAGAGCGGGTATGAATGATTGAAATCATGCTCAACAACAACCAATCTCAAGGATATGAT 6800
A P K S S S K * S R R R * S R A G M N D * N H A Q Q Q T N L K D M I 6800
L R Q R A V Q N D L D G N Q E R V * M I E I M L N N K P I S R I * Y 6800
C A K E Q F K M I S T V I K S G Y E * L K S C S T T N Q S Q G Y D 6800

ORF3

ACCAAAAAAATGAAGGCTGCTGtGGTTCGTACGTTGGACAACCCTTCAGATCGAAGAAATGCCGTGAAAgAACACGGTAAAAATCAAATCTCGGTT 6900
P K K M K A A V V R T F G Q P L Q I E E M F V K E P G K N Q I L V 6900
Q K K * R L L W F V R L D N R F R S K K C L * K N Q V K I K F W L 6900
T K K N E G C C G S Y V W T T A S D R R N A C E R T R * K S N S G * 6900

AAGTTCATGGCAAGTGGCTTTGTCATACCGATcTccaCGCCACCGATGGGACTGGCCTGCAAAACCCAAAATGCCTCTGATCCCTGGCCATGAAGGTA 7000
K V M A S G V C H T D L H A T D D G D W P A K P K M P L I P G H E G I 7000
R S W Q V A F V I P I S T P P M A T G L Q N P K C L * S L A M K V 7000
G H G K W R L S Y R S P R H R W R L A C K T Q N A S D P W P * R Y 7000

Rev1
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G Y V V A L G P E V T G V K E G D I V G V P W L Y S A C G C C E F 7100
L A M W L H W D R R * L V * K K G I L W V Y P G C I V L V D A A S S 7100
W L C G C T G T G G D W C K R R G Y C G C T L V V * C L W M L R V 7100

CTGCATCACCG 7200
C I T 7200
A S P 7200
L H H C 7200

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Figure S5.2: Nucleotide and amino acid sequences of the Tn4351-transposon insertion site of mutant 21.20. Nucleotide sequence was revised manually via comparison with the electropherogram. Manually changed nucleotides are written in small letters. For nucleotides without clear peaks in the chromatogram, letters according to the IUPAC Ambiguity Code were used. The nucleotide sequence was simple sequenced from nucleotides 0-1087 bp, 5766-5798 bp and 6895-7111 bp (plus strand). All other sequences were repeatedly sequenced. Sequence of the Tn4351-transposon was received from Prof. M. J. McBride (University of Wisconsin – Milwaukee, USA) and the transposon border is flanked by direct repeats marked with underlined letters (XXX). First and last nucleotides of the transposon are highlighted in red (X). Colour code: (XXX) sequence obtained from the Trenzyme report; (XXX) primers: 21.20-FosmidprimerRev2 (Rev2), 1475_RP1, 21.20-FosmidprimerFW1 (FW1), Tn4351_Tet_fw (Tetfw), Tn4351_Tet_rev (Tetrev), 21.20-FosmidprimerFW2 (FW2), 21.20-FosmidprimerRev1 (Rev1); (XXX) restriction sites located in the transposon for BsoBI, BstNI, HindIII and StyI. Nucleotide sequence was transcribed in amino acid sequence using the DNA to protein translation tool of insilico.ehu.es (Bikandi et al. 2004). Open reading frames (ORF1-3) in the amino acid sequences which led to hits when blasted against the NCBI data base are highlighted in blue (XXX). No ORFs were found in the minus strand.

Translations Aligned (5'→3'), plus stand

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10      20      30      40      50      60      70      80      90
|      |      |      |      |      |      |      |      |
CCCGTGATTTACGACTTACAGTTATTACAAAAAACGATGATACACACTGATTCCCAACGGATACCGGATTTGTCCAGAAATAATCCTGTACGTCGAACCA 100
P V I Y D L Q L L Q K T M I H T D S Q R I P D F V T N N P V R R T N 100
P * F T T Y S Y Y K K R * Y T L I P N A Y R I L S R I I L Y V E P 100
R D L R L T V I T K N D D T H * F P T H T G F C H E * S C T S N Q 100

ACGGTTGCCGGAGACGCTGTTCCCGGCATGAACCTTTAAACTTTATTTATTCAAAGAACCACCAACTCACTGGCATAAATCCGGACCAGCAGCAGAAAATA 200
G C R R R C S R H E P F K L Y L F K E P N S L A * I R T S S R K * 200
T V A G D A V P G M N P L N F I Y S K N Q T H W H K S G P A A E N S 200
R L P E T L F P A * T L * T L F I Q R T K L T G I N P D Q Q Q K I 200

GCTGATCATCAGCGGCCAAAGaCAAAACCCGCAAAACCAACAGCTTGAGCCCAACAATTAATCCAAGTACTGCCATAACCGGATGAACATTGCCGATT 300
L I I S G P K T K P Q K P N S L S P T I N P S T A I T G * T L P I 300
* S S A G Q R Q N P R N Q T A * A Q Q L I Q V L R * R D E H C R F 300
A D H Q R A K D K T P E T K Q L E P N N * S K Y C D N G M N I A D F 300

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TTCTGAAGTAAAGTATCGCGCCAGATAATCAACGTTGCTCGTGACAACGATGCTGTAATGGCCAGTCCGATGGCTTGTCCACTTTCACGGGATGCAT 400
 F * S K V I R A R * S T L L V T T M L * M A S P M A C P L S P D A Y 400
 S E V K * S A P D N Q * R C S * Q R C C K W P V R W L V H F R M H 400
 L K * S D P R Q I I N V A R D N D A V N G Q S D G L S T F T G C I 400

ACAGATAGATGATCAGCGGAATCCAGATAAAGGAGGTGCCACGAGGAAAAATGCAAAAATGGCAGTAATAAAACCCCATACAGATAATCCTTTAC 500
 R * M I S G I Q I K E V P T T G K N A K M A V I K P H N R * S F T 500
 T D R * S A E S R * R R C P R Q G K M Q K W Q * * N P I T D N P L R 500
 Q I D D Q R N P D K G G A H D R E K C K N G S N K T P * Q I I L Y 500

GCCAAAAATCCAGTATCCAAGTACGGCAATTACCCCTGAACAATAGAGATCAGTGAATACCCAGGCGTTGGCAGGACCATGTTTTAGTTTCGATG 600
 P K I Q Y P S T A I T P * T I E I S G I P K A L A R T M F L V S M 600
 Q K S S I Q V R Q L P L E Q * R S V E Y P R R W H G P C F * F R W 600
 A K N P V S K Y G N Y P L N N R D Q W N T Q G V G T D H V F S F D G 600

GCCAGCACATCTACATTTTCCCTCTTTAATGGGATCATATGCGAAATTTCCCTTTCCATTTCCGGTTCGGTGGTAAAGCATAAAAAGAAAAGAA 700
 A S T S T F S S F N G I I C E I F L S I S V P L V S I K * K R K R I 700
 P A H L H F P L L M G S Y A K F S F P F R F R W * A * N R K E K G 700
 Q H I Y I F L F * W D H M R N F P F H F G S V G K H K I E K K D 700

TCATGAGCAGATTGCCCGCCAGCAAGCAGAATATTAGAAAGGAAGAAATGAAATTTGTGATCTGCTGTATGCGCTCAGGCTTTGGTCCCGTAG 800
 M S R L P A T E A E L F R K E G M K F V I C L C M S L R L W S V S 800
 S * A D C P P P K Q N Y S E R K E * N L * S V S V C R S G F G P * A 800
 H E Q I A R H R S R T I Q K G R N E I C D L S L Y V A Q A L V R E 800

CATGTCAAACCCCTCCACTGTTCTATTGAGCCGATCGGATTTAAAAACCAATGATCTGATCAGAATATGACAGAAGTACATGATCTTAGACGAA 900
 M S K P V H C S I * A D A D L K T T M I * S E L C R S T L I L D E 900
 C Q N P S T V L F E P M R I * K P Q * S D Q N Y A E V H * S * T K 900
 H V K T R P L F Y L S R C G F K N H N D L I R I M Q K Y I D L R R N 900

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 I K * M A K D T G R P I P I R N A S K N N S A V P I L F Q C L R M V 1000
 S N E W Q R I P A D Q Y Q S E T Q V K I T V R F P F Y S N A C G W 1000
 Q M N G K G Y R Q T N T N Q K R K * K * Q C G S H F I P M L A D G 1000

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 K V K Y N S L L S X Y K A R A P K K P G R N P F S S N R S N S A R 1100
 L R * S T T P X S V X T K Q G H L K S R E E I H L A Q T G A T A P G 1100
 * G E V Q L P X Q X I Q S K G T * K A G K K S I * L K Q E Q Q R Q 1100

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 R R A * S V I R F V X S Y C * R L A W C W X * X X V C N X S * V Q 1200
 G D E H D Q * S D L S X P I V E G W H G C V G X E X P X V I X L E F N 1200

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 L I N A T F W V R I I S N K N I Y F S E R K E R Q C P P F S H S E 1300
 L * M Q L F G C G * * A I K T F I F Q R G K R D N V P L S L T L N 1300

1475_FP2
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 W I K F A I F A L I V R P K K K L Q M S K H I T E E Q R Y A I S M 1400
 G * S L L S L L * L S V Q R K K S C R * A N I * P R N K G M Q F L 1400

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 M L Q I P M S K K A I A E A I G V D K S T V Y R E I K R N C D A R 1500
 * C C K Y R * A K K Q * R K L S E * I K A L F T G R * S A I A T P E 1500

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 S G S Y S M E L A Q R K A D R R K Q Q K H R K E V L T P A M R K R 1600
 V V A I A W S L P S E K Q T G A S S K N I A R K C L H R Q * E N G 1600

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 I I K L L K K G F S P E Q I V G R S R L E G I A M V S H E T I Y R W 1700
 * * S C * R K D S A R S R L S A G A A W R E L R W Y L T K R Y I A 1700

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 I W E D K R R G G K L H K Y L R R Q G R R Y A K R G S K N A G R G 1800
 G F G R I S G G V A N C T N I F A D K V A G M P N V V L K M Q G E D 1800

BstNI | **BsoBI**
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 I Y P R Q G G Y * * A S R D S G T E G E I W * F R D R Y N Y W * E P 1900
 F I P G R V D I D E R P E I V E L K E R F G D L E I D T I I G K N 1900
 L S Q A G W I L M S V P R * W N * R R D L V I * R * I Q L L V R T 1900

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 Q R C H S Y H * R Q S N K Q G L D T Q V V G K R S H P G S * D C S 2000
 H K G A I L T I N D R A T S R V W I R K L S G K E A I P V A K I A V 2000
 T K V P F L P L T T E Q Q A G S G Y A S C R E K K P S R * L R L Q 2000

Supplementary Data

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W A L R K V I K W I E N K L N N R P R K R L G Y L T P N E K F K Q
Y G H C G K * K T * Y T Q L R L T M E R S L Q S T R K L R K N W K *
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K F Y F C K P Y H S W E R G A N E N T N G L I R Q Y I P K G K D F
N S I F A N H T T H G N V V P M K T P T G L S G S I S Q R V R T L
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S E V T N K Q I K W I E N K L N N R P R K R L G Y L T P N E K F K Q
V K * P T N R L S G L K I N S I I D L V K D L D T S R Q T K N L N
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I I N Q N S V A F A S * I Q I T E K * I D S I T R Q S N Y D K A M
K L L I R I L L H L Q V E F K S Q R S E * T V L P D R A T T T K Q C
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K A D R I T S P I R K Q G V R A S * D R K V V N L K F S S S * V A *
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S R R F A D C L Q Q A T W L N S S A F V S S I Q L R Y F A N S P S H
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C Y G P F L N D N A N R Y R Q T N E F T * * * E R C N N W W W T R
I V M G H F * M T M R I D T D K Q M N L L S D K N V A I I G G G P V
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G L T M A K L L Q Q N G I D V S V Y E R D N D R E A R I F G G T L
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* P T Q R F R S G S N E K S G I V T N L L * L S L T N G C K Y C * *
D L H K G S G Q E A M K K A G L L Q T Y Y D L A L P M G V N I A D
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K R Q Y F I H K K C K A R K S I * Q S * N K Q K * L K G Y L V E *
K K G N I L S T K N V K P E N R F D N P E I N R N D L R A I L L N S
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F R K R H G Y L G * K T C Y A * T P W * E E V D T N F * E * T E * N
L E N D T V I W D R K L V M L E T P G K K K W T L T F E N K P S E T
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S R F G Y S C Q W R D V Q G K K I C Y R H G S * R N R Y F Q Y T S R
A D L V I L A N G G M S K V R K F V T D T E V E E T G T F N I Q A
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Y S S T R D K L S W I F S A M Q W K P A N G I S P R * F I I C * P
D I H Q P E I N C P G F F Q L C N G N R L M A S H Q G N L L F A N P
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Q * * W C I A F W N K F * N T * * M E K P N A G R F S K Q K * C R
N N N G A L H F G I S F K T P D E W K N Q T Q V D F Q N R N S V V

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 * F S S E R I F R L G R T L Q R I D S Y D V V I C R I G Y T D I S F 3800
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 R K A L E K Q A P I T H N N D W G C R T F D A A F C R A G S K * W 3900
 L E K P W K S K R P L P I T M I G D A A H L M P P F A G Q G V N S G 3900

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 V G G C L D I V * * S S R W K I * * H * R G C * K L * T A N V Y V 4000
 L V D A L I L S D N L A D G K F N S I E E A V K N Y E Q Q M F M Y 4000

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 W Q R S T R R I N S K R N * N V * T R L Y V S A I V K C I K W N K T 4100
 G K E A Q E E S T Q N E I E M F K P D F T F Q Q L L N V * N G I K 4100

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 A H N K Q I G N R A G E V L L L N F C A M L K I S N S I Q H L C * 4200
 R H I T N K L A I E P V K Y F Y * T F V Q C * R L V I L F N I C A K 4200

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 K S A P S P I C Q P L C G S L K K D Y T K P K K S S * Q P P D F E 4300
 S R L H R Q F A S R Y A A A * K K T T P N Q K N H L D N H P T L N 4300

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 L R R M K F F R D N F Q H F Q K T V * P L T W R Q T N N L S I K V * 4400
 Y E G * N F S G T T S S I S K K Q F N H * L G D K Q T I * A L R F 4400

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 T E F F R N * P D L T F P E N R L * S C F Y R * I R F L * Q I G N 4500
 K P N F S E I D L T * L F L K I D F K A V F T D K S G F S N R * E I 4500

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 K C * V F G A L L K I K K * V F S F N V * Q G * F D S W R W Q E T 4600
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 F R T Y L I D K F Q I K K S M I E N G I W I V K L P R K * L L C R F 4700
 S G P T S * T S F K S K K V * * K T V Y G L * S F L E N N F C V G 4700

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 * L K D N G T S Q K I S K T L K H Q D F E N I G S N A I R N I * H 4800
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 H F E R K F R N C K I P K I T T D N L N I S G I R K * F S * M F N 4900
 T L K G N F G T A K F L K S Q P T T * T F R A S E N N F R K C S T 4900

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 E R E T M S P F L S L * M D K V C Y L C F N C P S K E K K V A D E Q 5300
 K E R Q C P P F S H S E W I K F A I F A L I V R P K K K L Q M S 5300

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 T Y N R G T K V C N F Y D V A N T D E Q K S N S G S Y R S R * K H 5400
 K H I T E E Q R Y A I S M M L Q I P M S K K A I A E A I G V D K S T 5400

Supplementary Data

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C L Q G D K R R P K W * L * H G A C P A K S R Q A Q A A K N T 5500
V Y R E I K R N C D A R S G S Y S M E L A Q R K A D R R K Q Q K H 5500

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S Q G S A Y T G N E K T D N K A V E E R I Q P G A D C R Q E P L G G 5600
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N C D G I S R N D I S L D L G G * A A G W Q T A Q I S S Q T R S Q 5700
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W * F R D R Y N Y W * E P Q R C H S Y H * R Q S N K Q G L D T Q V V 5900
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L Q S T R K L R K N W K * N S I F A N H T T H G N V V P M K T P T G 6100
C K A R G N C A K I G N K I L F L Q T I P L M G T W C Q * K H Q R 6100
A K H E E I A Q K L E I K F Y F C K P Y H S W E R G A N E N T N G 6100

GCTTATCAGCAGTATATCCAAAGGGTAAGGACTTTAGTGAAGTAACCAACAACAGATTAAAGTGGATTGAAAATAAACTCAATAATCGACCTCGTAA 6200
L S G S I S Q R V R T L V K * P T N R L S G L K I N S I I D L V K 6200
A Y Q A V I Y P K * G L * * S N Q Q T D * V D * K * T Q * S T S * K 6200
L I R Q Y I P K G K D F S E V T N K Q I K W I E N K L N N R P R K 6200

AGACTGGATACCTCAGCCCAACGAAAAATTAACAATTAATTAATCAGAATCTGTGCAATTGCAAGTTGAATTGACTGCTTTTGATCAAA 6300
D L D T S R Q T K N L N K L I R I L L H L Q V E F S T A F * S K 6300
T W I P H A K R K I * T N Y * S E F C C I C K L N S A L P F D Q R 6300
R L G Y L T P N E K I F K Q I I N Q N S V A F A S * I Q H C L D I K E 6300

AACGTAACCTTAAAGCGCACAAAATAGTTGAAAGTAAGATTATCCTAATAAATAGAGACTAAATATCATTCTCGGTCTTAATCATTACTTTCAGAACAAA 6400
N V T L K R T N K L K V R L S * * I E T K Y H S R S * S L S E Q N 6400
T * L * S A Q I S * K * D Y P N K * R L N I I L G L N H Y F Q N K 6400
R N F K A H K * V E S K I I L I N R D * I S F S V L I I T F R T K 6400

ACTATCGAAATTTACAAACATATCATGTGCAAAAACCCCGCCCGTTACCTCTGTGAAATCTGACAGCCTTCCCTTCAACAGATTTCAAACCGGATTA 6500
Y R N F T T I S C Q K N P A P L P L L N L T A F L Q Q I S K P D Y 6500
T I E I S Q Q Y H V R K T P P R Y L C * I * Q P S F N R R F Q N R I I 6500
L S K F H N N I M S E K P R P V T S A E S D S L P S T D F K T G L 6500

TCGAGTCAGGAAGCTGCTGCGCATCGCGCAGTTGGTCTGAAAGAGCTGGAGCAAGTCCGGCCCGAGAGCATCTGGTCCATCTCTGAGACAGGTA 6600
R V R K L L C A S R S L V * T S W S K S G P R A S G P S F * D R * 6600
E S G S C S A H R A V W S E R A G A S P A R E H L V H P S E T G E 6600
S S Q E A A L R I A Q F G L N E L E Q V R P E S I W S I L L R Q V N 6600

ACAGTATCATGCTGGATTCTGCGGTGGCGCCATCGTATCCTTTTCTGGGTGATAACCTGGAAGGCTTCGCGGTGATCGCGCTTATCTGATCAA 6700
T V S S S G F W R W R P S Y P F F W V I T W K A S P * S P L F * S M 6700
Q Y H R L D S G G G H R I L F S G * * P G R L R R D R R Y S D Q 6700
S I I V W I L A V A A I V S F F L G D N L E G F A V I A V I L I N 6700

TGCACTCACTGGTTTTATTCTCGAATAACAACCGCCGCAATCGATGGAGCGTTGCGAAAACCTGGACACGACACCAGCCAGGTTATCGGGATGAAAA 6800
H S L V L F S N T T P G N R W R R C E N W T R H Q P G Y C G M E K 6800
C T H W F I Y S R I Q R P A I D G V A K T G H D T S Q G I A G W K N 6800
A L T G F I L E Y N A R Q S M E A L R K L D T T P A R V L R D G K 6800

ATAAAAAGAGTTCCTTCCGATCAGCTGACCCCTGGCGCAGTCTGATCCTGGAAGCCGGGATCTGATCCCGGCTGACGCACTGATCCTGGAAGAGAAC 6900
* K K F L P I S * P W A T C * S W K P G I * S R L T H * S W K R T 6900
K R S S F R S A D P G R R A D P G S R G S D P G * R T D P G R E P 6900
I K E V P S D Q L T L G D V L I L E A G D L I P A D A L I L E E N Q 6900

AGTTAACCATTTGATGAATCCGCTCTCACGGGCAATCTGTCTTCCCAAAAAGTACGGATGCGTCTCCCGAGGATGCGCCCTGGCGCAGCCACAAA 7000
S * P L M N P L S R A N L F L P K K V R M R L P R M R P W A T T 7000
V N H * * I R S H G R I C S F P K K Y G C V S R G C A P G R P P Q 7000
L T I D E S A L T G E S V P S Q K S T D A S P E D A P L G D R H N 7000

CAGGCTGTTTAAAGGCACGGCCATCACCACGAAACGGCAAGGCCATCGTAACCCAAATAGGACAAGCAACCGAGCTGGGCAAAAATGCTCCATGGTC 7100
G C L R A R P S P T E T A R P S * P K * D K Q P S W A K L P P W S 7100
Q A V * G H G H H Q R K R Q G H R N P N R T S N R A G Q N C L H G Q 7100
R L F K G T A I N G N G K A I V T Q I G Q A T E L G K I A S M V 7100

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AGCGAAGCCAAACGCTCGGCTACGCCATTAGAAGCTAACTGGACGCCCTCGGCAAAGTACTGATCTGGGTTACGCTGGGT 7200
A K P N A R L G R H * K L N W T P S A K Y * S G L R W G 7200
R S Q T L G Y A I R S * T G R P R Q S T D L G Y A G 7200
S E A K R S A T P L E A K L D A L G K V L I W V T L G 7200

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Translations Aligned (5'→3'), minus strand

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      10      20      30      40      50      60      70      80      90
ACCCAGCGTAACCCAGATCAGTACTTTGCCGAGGGCGTCCAGTTTAGCTTCTAATGGCGTAGCCGAGCGTGTGGCTTCGCTGACCATGGAGGCAATTTTG 100
T Q R N P D Q Y F A E G V Q F S F * W R S R A F G F A D H G G N F A 100
P S V T Q I S T L P R A S S L A S N G V A E R L A S L T M E A I L 100
P A * P R S V L C R G R P V * L L M A * P S V W L R * P W R Q F C 100

      |      |      |      |      |      |      |      |      |
CCCAGCTCGGTTGCTTGTCTATTGGGTTACGATGGCCTTCCGCTTTCGCTGGTATGGCCGTCGCCCTTAACAGCGCTGTTGGCGGTCGCCAGGG 200
Q L G C L S Y L G Y D G L A V S V G D G R A L K Q P V V A V A Q G 200
P S S V A C P I P G F Q D Q H V A Q G Q L I G R N F F Y F S I P Q Y P 200
P A R L L V L F G L R W P C R F R W * W P C P * T A C C G G R P G 200

      |      |      |      |      |      |      |      |      |
GCGCATCTCGGAGACGATCCGTACTTTTTGGGAAGAACAGATTCCGCCGTGAGAGCGGATTCAATGGTTAACTGGTTCTCTCCAGGATCAG 300
R I L G R D I R T F L G R N R F A R E S G F I N G * L V L F Q D Q 300
A S S G D A S T D S P V R A D S S M V N W F S S R I S 300
A H P R E T H P Y F F W G K E Q I R P * E R I H Q W L T G S L P G S V 300

      |      |      |      |      |      |      |      |      |
TGCCTCAGCCGGGATCAGATCCCGGCTTCCAGGATCAGCAGCTCGCCAGGGTCAGCTGATCGGAAGGAACCTCTTTTATTTTCCATCCCGCAATAC 400
C V S R D Q I P G F Q D Q H V A Q G Q L I G R N F F Y F S I P Q Y P 400
A S A G I R S P A S R I S T S P R V S * S E G T S F I F P S R N T 400
R Q P G S D P R L P G S A R R P G S A D R K E L L L F F H P A I P 400

      |      |      |      |      |      |      |      |      |
CTGGCTGTGCTGTCAGTTTTCGCAACGCCCTCCATCGATTGCCGGCGTGTATTTCGAGAATAAAACAGTGGTGCATTGATCAGAATAACGGCGA 500
G W C R V Q F S Q R L H R L P G V V F E N K T S E C I D Q N N G D 500
L A G V V S S F R N A S I D C R A L Y S R I K P V S A L I R I T A I 500
W L V S C P V F A T P P S I A G R C I R E * N Q * V H * S E * R R 500

      |      |      |      |      |      |      |      |      |
TCACGGCGAAGCTTCCAGTTATCACCAGAAAAAGGATACGATGGCCGCCACCGCCAGAATCCAGACGATGATACTGTTCCACCTGTCTCAGAAGGAT 600
H G E A F Q V I T Q K K G Y D G R H R Q N P D D D T V H L S Q K D 600
T A K P S R L S P R K K D T M A A T A R I Q T M I L F T C L R E M 600
S R R S L P G Y H P E K R I R W P P P P E S R R * Y C S P V S E G W 600

      |      |      |      |      |      |      |      |      |
GGACAGATGCTCTCGGCGCGGACTTGTCCAGCTCGTTCCAGACCAACTGCGCGATGCGCAGAGCAGCTTCTGACTCGATAATCCGGTTTTGAAATCT 700
G P D A L G P D L L Q L V Q T K L R D A Q S S F L T R * S G F E I C 700
D Q M L S G R T C S S S F R P N C A M R R A A S * L D N P V L K S 700
T R C S R A G L A P A R S D Q T A R C A E Q L P D S I I R F * N L 700

      |      |      |      |      |      |      |      |      |
GTTGAAGGAAGGCTGTGATTCAGCAGAGGTAACGGGGCGGGTTTTTCTGACATGATATTGTTGTGAAATTCGATAGTTTTGTTCTGAAAGTAATGA 800
* R K A V R F S R G N G A G F F * H D I V V K F R * F C S E S N D 800
V E G R L S D S A E V T G R G F S D M I L L * N F D S F V L K V M I 800
L K E G C Q I Q Q R * R G G V F L T * Y C C E I S I V L F * K * * 800

      |      |      |      |      |      |      |      |      |
TTAAGCCGAGAATGATATTTAGTCTCTATTTATTAGGATAATCTTACTTTCAACTTATTGTCGCGCTTTAAAGTTACGTTCTTTGATCAAAAAGCAGT 900
* D R E * Y L V S I Y * D N L T F N L F V R F K V T F F D Q K A V 900
K T E N D I * S L F I R I I L L S T Y L C A L K L R S L I K R Q C 900
L R P R M I F S L Y L L G * S Y F Q L I C A L * S Y V L * S K G S A 900

      |      |      |      |      |      |      |      |      |
CTGAATTCAACTTGCAATGCAACAGAATTCGATTAATAATTTGTTAAATTTTTCGTTTGGCGTGAGGTATCCAAGTCTTTTACGAGGTCGATTATTG 1000
L N S T C K C K N R I L I N N L F K F F V W R E V S K S F T R S I I E 1000
* I Q L A N A T E F * L I I C L N F S F G V R Y P S L L R G R L L 1000
E F N L Q M Q Q N S D * * F V * I F R L A * G I Q V F Y E V D Y * 1000

      |      |      |      |      |      |      |      |      |
AGTTATTTTCAATCCACTTAATCTGTTTGGTTACTTCACTAAAGTCTTACCTTTGGGATACTGCCTGTGATAAGCCCGTGGTGTGTTTCATTGG 1100
F I F N P L N L F V G Y F T K V L T L W D I L P D K P V G V F I G 1100
S L F S I H L I C L L V T S L K S L P F G I Y C L I S P L V F S L A 1100
V Y F Q S T * S V C W L L H * S P Y P L G Y T A * * A R W C F H W 1100

      |      |      |      |      |      |      |      |      |
CACCAGTTCCCATGAGTGGTATGGTTGCAAAAATAGAATTTTATTTCCAATTTTTGCGCAATTTCTCGTCTTTCGCAAACTCCTTCCATTGTCAGC 1200
T T F P * V V W F A K I E F Y F Q F L R N F L V L C K L L S I V S 1200
P R S H E W Y G L Q K * N F I S N F C A I S S C F A N S F P L S A 1200
H H V P M S G M V C K N R I L F P I F A Q F P R A L Q T P F H C Q P 1200

      |      |      |      |      |      |      |      |      |
CGTAATGTGTGATTAAGTTTTTCACTTTCCGCAAGTCCATACTGCAATCTTAGCTACCGGGATGGCTTCTTTCCCGACAACCTGCGTATCCAGACC 1300
R N C V Y * V F H F P Q C P Y C N L S Y R D G F F S R Q L A Y P D P 1300
V I V C I K F F T F R S A H T A I L A T G M A S F P D N L R I Q T 1300
* L C V L S F S L S A V P I L Q S * L P G W L L F P T T C V S R P 1300

      |      |      |      |      |      |      |      |      |
CTGCTTGTGCTGCTGCTTAATGGTAAGAATGGCACCTTTGTGGTTCTTACCAATAATGTATCTATCTCTAAATCACCAAATCTCTCCTCAGTTCCA 1400
A C C S V N G K N G T F V V L T N N C I Y L * I T K S L L Q F H 1400
L L V A L S L M V R M A P L W F L P I I V S I S K S P N L S F S S T 1400
C L L L C R * W * E W H L C G S Y Q * L Y L S L N H Q I S P S V P 1400

BsoBI | BstNI
CTATCTCGGGACGCTCATCAATATCCACCCTCCTCGGATAAATCCTCGCCCTGCATTTTTAGAACACGTTTGGCATACTGCGACCTTGTCTGCGAAG 1500
Y L G T L I N I H P A W D K S S P C I F R T T F G I P A T L S A K 1500
I S G R S I S T L P G I N P R P A F L E P R L A Y L R P C L R R 1500
L S R D A H Q Y P P C L G * I L A L H F * N H V W H T C D L V C E D 1500

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Supplementary Data

ATATTTGTGCAGTTTGGCCACCCCGCGTTATCCCTCCCAATCCAGCGATATATCGTTTCGTGAGATACCATCGCAATCCCTCCAAGCGGCTCTCGCCG 1600
 I F V Q F A T P P L I L P N P A I Y R F V R Y H R N S L Q R A L P A D 1600
 Y L C S L P P R R L S S Q I Q R Y I V S * D T I A I P S K R L L P 1600
 I C A V C H P A A Y P P K S S D I S F R E I P S Q F P P S G S C R 1600

ACAATCTGCTCCGGGCTGAATCCTTTCTCAACAGCTTTATTATCCGTTTCTCATTGCCGGTGAAGCACTTCTTCCGATGTTTTGTTGCTTGGCGCC 1700
 N L L R A E S F L Q Q L Y Y P F S H C R C K H F L A M F L L L A P 1700
 T I C S G L N P F F N S F I I R F L I A G V S T S L R C F C C L R L 1700
 Q S A P G * I L S S T A L L S V F S L P V * A L P C D V F A A C A 1700

TGCTGCTTTTCGCTGGGCAAGCTCCATGCTATAGCTACCACCTCGGGCTCGCAATTCGCGTTTATCTCCCTGTAACAGTGTCTTTTACTACTCCGAT 1800
 V C F S L G K L H A I A T T S G V A I A L Y L P V N S A F I Y S D 1800
 S A F R W A S S M L * L P L R A S Q L R F I S L * T V L L S T P I 1800
 C L L F A G Q A P C Y S Y H F G R R N C A L S P C K Q C F Y L L R * 1800

AGCTCCGCTATTGCTTTTTTGTCTCATCGGTATTGCAACATCATAGAAATGCATACCTTTGTTCCCTCGGTTATATGTTTGTCTCATCTGCAACTTTTT 1900
 S F R Y C F F A H R Y L Q H H R N C I P L F L G Y M F A H L Q L F F 1900
 A S A I A F L L I G I C N I I E I A Y L C S S V I C L L I C N F F 1900
 L P L L L F L C S S V F A T S * K L H T F V P R L Y V C S S A T F F 1900

TCTTTGGAGCGACAATTAAGCAAAGATAGCAAACCTTATCCATTGAGAGTGAAGAAAGGGGACATTGCTCTCTTCTCCTCTGAAAAATAAATGT 2000
 L W T D N * S K D S K L Y P F R V R E R G T L S L F P L * K I N V 2000
 F F G R T I K A K I A N F I H S E * E K G G H C L S F L S E K * M F 2000
 S L D G Q L K Q R * Q T L S I Q S E R K G D I V S L S S L K N K C 2000

TTTTATGCTTATTATCCGACCCCAAAAGTTGCATTTAAGTTGAACTCGAAGTATTACACCTGTAAGAAGTACTAATGACAAAAAAGAAATGCC 2100
 F I A Y Y P H P K S C I Y K L N S R S I H L * E V T N D K K E I A 2100
 L L L I R T Q K V A F I S * T Q E V F T C K K L L M T K K L P 2100
 F Y C L L S A P K K L H L * V E L K K Y S P V R S Y * * Q K R N C P 2100

CGTTCGTTTTACGGGTCAGCACTTTACTATTGATAAAGTGCTAATAAAGATGCAATAAGACAAGCAAATATAAGTAATCAGGATACGGTTTTAGATATT 2200
 R S F Y G S A L Y Y * * S A N K R C N K T S K Y K * S G Y G F R Y W 2200
 V R F T G Q H F T I D K V L I K D A I R Q A N I S N Q D T V L D I 2200
 F V L R V S T L L L I K C * * K M Q * D K Q I * V I R I R F * I L 2200

GGGCGAGCAAGGGTTTCTTACTGTTCAATTTATAAAAATCGCAACAATGTTGTTGCTATTGAAAACGACACAGCTTTGGTTGAACATTTACGAAAAT 2300
 G R Q G V S Y C S F I K N R Q Q C C Y * K R H S F G * T F T K I 2300
 G A G K G F L T V H L L K I A N N V V A I E N D T A L V E H L R K L 2300
 G Q A R G F L L F I Y * K S P T M L L L L K T T Q L W L N I Y E N 2300

TATTTTCGTAGCCCGAAATGTTCAAGTTGTCGGTTGTGATTTAGGAATTTGCAAGTCCGAAATTCCTTTCAAAGTGGTCAATATTCCTTATGG 2400
 I F * C P K C S S C R L * F * E F C S S E I S F Q S G V K Y S L W 2400
 F S D A R N V Q V V G C D F R N F A V P K F P F K V V S N I P Y G 2400
 Y F L M P E M F K L S V V I L G I L Q F R N F L S K W C Q I F L M A 2400

CATTACTCCGATATTTCAAATCCTGATGTTGAGAGTCTTGGAAATTTTGGGAGGTTCCATTGTCCTTCAATAGAACCTACACAAAAGTTATTT 2500
 H Y F R Y F Q N P D V * E S W K F S G R F H C P S I R T Y T K V I F 2500
 I T S D I F K I L M F E S L G N F L G G S I V L Q L E P T Q K L F 2500
 L L P I F S K S * C L R V L E I F W E V P L S F N * N L H K S Y F 2500

HindIII
 TCGAGCAAGCTTTACAATCCATATACCGTTTTTCTATCATACTTTTTTTGATTTGAAACTTGTCTATGAGTAGGTCCTGAAAGTTTCTGCCACCGCCAA 2600
 E E A L Q S I Y R F L S Y F F * F E T C L * G R S * K F L A T A N 2600
 S R K L Y N P Y T V F Y H T F F D L K L V Y E V G P E S F L P P P T 2600
 R G S F T I H I P F S I I L F L I * N L S M R * V L K V S C H R Q 2600

CTGCAAAATCAGCCCTGTTAAACATTAAGAAAGCAACTTATTTTTGATTTAAGTTTAAAGCCAAATCTTAGCATTATTTCTCTATCTGTAGAGAA 2700
 C Q I S P V K H * K K T L I F * F * V * S Q I L S I Y F L S V R E 2700
 V K S A L L N I K R K H L F F D F K F K A K Y L A F I S Y L L E K 2700
 L S N Q P C * T L K E N T Y F L I L S L K P N T * H L F P I C * R N 2700

ACCTGATTTATCTGTAACACAGCTTTAAAGTCGATTTTCAGGAAAGTCAGGTCAGGTCATTTCCGAAAAATTCGGTTTAAACCTTAATGCTCAAATT 2800
 T * F I C K N S F K V D F Q E K S G Q V N F G K I R F K P * C S N C 2800
 P D L S V K T A L K S I F R K S Q V R N I S E K I F G L N L N A Q I 2800
 L I Y L * K Q L * S R F S G K V R S G Q F R K N S V * T L M L K L 2800

GTTGTTTGTCTCCAAGTCAATGGTTAACTGTTTTTTGAAATGCTGGAAGTTGTCCTGAAAAATTCATCCTTCGTAGTTCAAAGTCCGGTGGTTGT 2900
 L F V S K S M V K L F F G N A G S C P * K I S S F V V Q S R V V L 2900
 V C L S P L S Q W L N C F L E V V P E K F H P S * F K V G W L S 2900
 F V C L Q V N G * T V F W K C W K L S L K N F I L R S S K S G G C 2900

CAAGATGATTTTTTGGTTGAGTCTTTTTTAAAGTCCGCATACCGGCTGGCAAATTCGCGATGGAGCCGACTTTTAGCACAAATGTTGAATAG 3000
 K M I F L V W C S R I T A G K L A M E P T F S T N V E * 3000
 R * F F W F G V V F F * A A A * R L A N W R W S R L L A Q M L N R 3000
 Q D D F F G L V * S F F K L P H N G W Q I G D G A D F * H K C * I E 3000

AATTACTAATCTTCAACATTCGACAAAAGTTCAATAGAAGTACTTACCAGGCTCTATTGCCAATTTGTTGTTATGTCGGTTTTTATCCATTTTATACA 3100
 N Y * S S T L H K S S I E V L H R L Y C Q F V C Y V P F Y S I L Y I 3100
 I T N L Q H C T K V Q * K Y F T G S I A N L F V M C R F I P F Y T 3100
 L L I F N I A Q K F N R S T S P A L L P I C L L C A V L F H F I H 3100

TTTAAACAAATGCTGAAACGTAAGTCGGGTTTAAACATTTCAATTCGTTTTGAGTGATTCTTCTGTGCTTCTTGGCATAACAACATTTGCTGTT 3200
 * Q L L K R K V G F K H F N F V L S * F F L C F F A I H K H L L F 3200
 F N N C * N V K S G L N I S I S F * V D S S C A S L P Y I N I C C S 3200
 L T I A E T * S R V * T F Q F R F E L I L L V L L C H T * T F A V 3200

CATAATTTTAAACAGCCTCTCAATGCTATTAATTTTCCATCGGCTAGATTATCAGACAATATCAAGGCATCCACCAACCCACTATTTACTCCCTGCC 3300
I I F N S L F N A I K F S I G * I I R Q Y Q G I H Q P T I Y S L P 3300
* F L T A S S M L L N F P S A R L S D N I K A S T N P L F T P C P 3300
H N F * Q P L Q C Y * I F H R L D Y Q T I S R H P P T H Y L L P A L 3300

TGCAAAAGCGGCATCAAAATGTGGCGCATCCCCAATCATTTGTTATGGTAATGGCGCTTGCTTTTCAAGGCTTTTCTAAAGGAAATATCCGTGTAGCC 3400
C K R R H Q M C G I P N H C Y G * W A L A F P R L F * R K Y P C S Q 3400
A K G G I K C A A S P I I V M G N G R L L F Q G F S K G N I R V A 3400
Q K A A S N V R H P Q S L L W V M G A C F S K A F L K E I S V * P 3400

AATCCTACAATGACAACGTCGTATGAATCAATCTTTGTAGCGTTCGTCCTCAATCGGAAATCTTTCCAGAAGAAATCAACGCACTATTTCTGTTTT 3500
S Y K * Q R R M N Q F F V A F V P I G K F F Q K K I N D T I S V L 3500
N P T N D N V V * I N S L * R S S Q S E N S F R R K S T T L F L F * 3500
I L Q M T T S Y E S I L C S V R P N R K I L S E E N Q R H Y F C F 3500

GAAATCTACCTGCGTTGGTTTTTCCATTTCATCAGGTGTTTTAAACTTATCCAAAATGCAATGCACCATTTATTTGGGGTTAGCAATAATAAAT 3600
K I Y L R L V F P F I R C F K T Y S K M Q C T I I I G V S K * * I 3600
K S T C V W F F H S S G V L K L I P K C N A P L L L G L A N N K L 3600
E N L P A F G F S I H Q V F * N L F Q N A M H H Y Y W G * Q I I N Y 3600

BstNI

ACCTTGGTGAATGCCATTAGCCGGTTTCCATTGCATAGCTGAAAAATCCAGGACAGTTTATCTCTGTTGATGAATATCGGCTTGTATTTGAAAGTA 3700
T L V R C H * P V S I A * L K K S R T V Y L W L M N I G L Y I E S T 3700
P W * D A I S R F P L H S * K N P G Q F I S G * * I S A C I L K V 3700
L G E M P L A G F H C I A E K I Q D S L S L V D E Y R L V Y * K Y 3700

CCTGTTTCTTCAACTCCGTGTCGGTAACAAATTTTCTACCTGGACATCCCGCCATTGGCAAGAATAACCAAAATCTGCTGTTTCACTCGGTTTATCT 3800
C F F N F R V G N K F S Y L G H P A I G K N N Q I C C F T R F I L 3800
P V S S T S V S V T N F L T L D I P P L A R I T K S A V S L G L F S 3800
L F L Q L P C R * Q I F L P W T S R H W Q E * P N L L F H S V Y S 3800

BstNI

CAAAAGTTAGTCCACTTCTTCTTCCAGGTTCAAGCATAACAAGTTTTCTATCCAAATAACCGTTCGTTTTCTAAACTATTCAACAAGATAGCCCT 3900
K S * C P L L L T R F K H N K F S I P N N R V V F * T I Q Q D S P 3900
K V S V H F F L P G S S I T S F L S Q I T V S F S K L F N K I A L 3900
Q K L V S T S S Y Q V Q A * Q V F Y P K * P C R F L N Y S T R * P L 3900

TAAGTCATTTCTGTTTATTTCCAGGATTGCAAAATCGATTTTCGGGCTTACATTTTTTGTGGATAAAATATTGCCTTTTTTATCAGCAATATTTACACC 4000
* V I S V Y F R I V K S I F G L Y I F C G * N I A F F I S N I Y T H 4000
K S F L F I S R G L S N R F S G F T Y F V D K I L P F L S A I F T P 4000
S H F C L F Q D C Q I D F R A L H F L W I K Y C L F Y Q Q Y L H P 4000

ATTGGTAAGGCTAAGTCATAAAGTTTGTAAACATCCCGCTTTTTTCAATGCTTCCTGACCTGAACTTTGTGTAGTCAAGGGTTCCACCAAAAATTC 4100
W * G * V I I S L * Q S R F F H C F L T * T F V * V K G S T K N S 4100
I G K A K S * * V C N N P A F F I A S * P E P L C R S R V P P K I L 4100
L V R L S H N K F V T I P L F S L L P D L N L C V G Q G F H Q K F 4100

Tetrev

TGCTCCTCGGTCGTCTCTCTTTCGTAACGAAACGCTATGCGGTTTGTCTGTAATAATTTGCCATAGTCAGTCCAAACGGGTCCACCACCAATAT 4200
C L S V V V S F V N * N V Y A V L L * * F C H S Q S N G S T N Y 4200
A S R S L S L S * T E T S M P F C C N N F A I V S P T G P P P I I 4200
L P L G R C L F R K L K R L C R F A V I I L P * S V Q R V H H Q L L 4200

TGCAACGTTCTTATCACTAAGTAAATTCATTTGTTGTCTGTATCTATTCGATTCATTCAAAAATGGCCATAACAATGGCTCGGCCAATGGCGAA 4300
C N V L I T K * I H L F V C I Y S H C H S K M A H N N G S A N W R S 4300
A T F L S L S K F I C L S V S I R I V I Q K W P I T M A R R I G E 4300
Q R S Y H * V N S F V C L Y L F A L S F K N G P * Q W L G E L A K 4300

GTAGCGAAGCTGTATCGAAGATACAAAAGCGGAGCTATTTAGCCAAGTCCGCTTGTGTGAAGCAGTCCGGAAGCGCGGCTTACGGCAAGCGAGTAA 4400
S E A V S K I Q K R S Y L A K S L V V S S P R S G G L R Q A T * V 4400
V A K L Y R R Y K S G A I * P S R L L * A V R E A A A Y G K R R K S 4400
* R S C I E D T K A E L F S Q V A C C K Q S A K R R L T A S D V S 4400

CGCCAGCCATTGTAGCAAGGAGCGCAGCGGATTGCTAACACGGTTTCAGCTATACGACGCGAGGATTTTTACCACCTAAGCTACCTACGATGA 4500
A E P L L A R S A A D C * Q R F A A I R R Q G F L P L S Y L R * T 4500
P S H C * Q G A Q R I A N N G L Q L Y A G R D F Y H * A T Y D E L 4500
R R A I V S K E R S G L L T T V C S Y T Q A G I F T T K L P T M N * 4500

Tetfw

GAACTTAAATTTACCACTTCTCTGCTTACGAAACGCAACCCCTGCTGCGTATAGTGATGTTATGCGGTCGGCTTTCAATTTCTCTTCTCTCG 4600
E L * I Y H F P V L R S T N P L L A Y R * C Y A V G F H F L F F L V 4600
N F T T F L S Y E A R T P C L R I G D V M R S A F I F S S A 4600
T L N L P L S C L T K H E P P A C V * V M L C G R L S F S L S R 4600

TAAATTTGCAAGCAAGTTACTAAATTTGCAGTCAAGCAATTTCAATTTTGAATTATGATTTTCAGCAGACCTTGACTTCTATCTAAGTATAGGCA 4700
N L S S K V T K F A V K Q F Q F L N Y V F Q Q T L D F Y L S D R Q 4700
L I C Q A K L L N L Q S S N F N F * I M Y F S R P W T S I * V I G S 4700
* F V K Q S Y * I C S Q A I S I F E L C I S A D L G L L S K * * A 4700

GTCCTGAAATAATTCAAATCAATCTTTAGGGCAAGACAAATCAGTCTATGCTTTGTGTGTGCTGTCATTTTTTTTATGGCATTGCTTTGTCGTAGTTGC 4800
S * I N S N Q S F R A R Q S V Y A L C V L S F F L M A L L C R S C 4800
P E * I P I N L L G Q D N Q S M L C V C C H F F * W H C F V V A 4800
V L N K F Q S I F * G K T I S L C F V C A V I F F D G I A L S * L L 4800

1475 RP1

TCTGTCGGGTAATACTGCTATTCCTCTCTGATTTGAATTCACCTGCAATGCAACAGAATCTGATTAATAATTTGTTAAATTTTCTGTTGG 4900
S V G * Y C L F T S L * F E F N L Q M Q Q N S D * * F V * I F R L A 4900
L S G N T V Y S L L C D L N S T C K C N R I L I N N L F K F F V W 4900
C R V I L S I H F S V I * I Q L A N A T E F * L I I C L N F S F G 4900

Supplementary Data

CGTGAGGTATCCAAGTCTTTACGAGGTCGATTATTGAGTTTATTTTCAATCCACTTAATCTGTTTGGTTACTTCACTAAAGTCTTACCCCTTGGG 5000
* G I Q V F Y E V D Y * V Y F Q S T * S V C W L L H * S P Y P L G 5000
R E V S K S F T R S I I E F I F N P L N L F V G Y F T K V L T W D 5000
V R Y P S L L R G R L L S L F S I H L I C L L V T S L K S L P F G 5000

ATATACTGCTGATAAGCCCGTTGGTGTTTTCATTGGCACCACGTTCCCATGAGTGGTATGGTTTGCAAAAAATAGAATTTTATTCCAATTTTGGCGAA 5100
Y T A * * A R W C F H W H H V P M S G M V C K N R I L F P I F A Q 5100
I L P D K P V G V F I G T T F P * V V W F A K I E F Y F Q F L R N 5100
I Y C L I S P L V F S L A P R S H E W Y G L Q K * N F I S N F C A I 5100

TTTCTCGTGCTTTGCAAACTCCTTTCCATTGTCAGCCGTAATTGTGTGATTAAGTTTTTCACTTTCCGAGTGGCCATACGCAATCTTAGCTACCGG 5200
F P R A L Q T P F H C Q P * L C V L S F S L S A V P I L Q S * L P G 5200
F L V L N H Q I S P S V P L S R D A H Q Y P P C L G * I L A L H F * 5200
S S C F A N S F P L S A V I V C I K F F T F R S A H T A I L A T G 5200

GATGGCTTTTCCGACAACCTTGCAGACCTGCTTGTGCTCTGCTGTTAATGGTAAGAATGGCACCTTTGGTGTCTTACCAATAATGTA 5300
W L L F P T T C V S R P C L L L C R * W * E W H L C G S Y Q * L Y 5300
D G F F S R Q L A Y P D P A C C S V V N G K N G T F V V L T N N C I 5300
M A S F P D N L R I Q T L L V A L S L M V R M A P L W F L P I I V 5300

TCTATCTAAATCACCAAATCTCCTCAGTTCACACTCTCGGGACGCTCATCAATATCCACCCTGCTGGGATAAATCCTCGCCCTGCATTTTAG 5400
L S L N H Q I S P S V P L S R D A H Q Y P P C L G * I L A L H F * 5400
Y L * I T K S L L Q F H Y L G T L I N I H P A W D K S S P C I F R 5400
S I S K S P N L S F S S T I S G R S S I S T L P G I N P R P A F L E 5400

AACCAGTTTGGCATACTGCGACCTTGTCTGCGAAGATATTTGTCAGTTTGGCCACCCCGCGCTTATCCTCCAATCCAGCGATATATCGTTTCGTG 5500
N H V W H T C D L V C E D I C A V C H P A A Y P P K S S D I S F R E 5500
T T F G I P A T L S A K I F V Q F A T P P L I L P N P A I Y R F V 5500
P R L A Y L R P C L R R Y L C S L P P R R L S S Q I Q R Y I V S * 5500

AGATACCATCGCAATTCCTCCAAGCGGCTCCTGCCGACAATCTGCTCCGGGCTGAATCCTTCTCAACAGCTTTATTATCCGTTTCTCATTGCCGGT 5600
I P S Q F P P S G S C R Q S A P G * I L S S T A L L S V F S L P V 5600
R Y H R N S L Q A A P A D N L L R A E S F L Q Q L Y Y P F S H C R C 5600
D T I A I P S K R L L P T I C S G L N P F F N S F I I R F L I A G 5600

GTAAGCACTTCTTGGCAGTGTTTTGTGCTTGGCCCTGCTGCTTTTCGCTGGGCAAGCTCCATGCTATAGTACCCTTCCGGGCTGCAATTCGCGT 5700
* A L P C D V F A A C A C L L F A G Q A P C Y S Y H F G R R N C A 5700
K H F L A M F L L A P V C F S L G K L H A I A T T S G V A I A L 5700
V S T S L R C F C C L R L S A F R W A S S M L * L P L R A S Q L R F 5700

TTATCTCCCTGTAACAGTGTCTTTTATCTACTCCGATAGCTTCCGCTATTGCTTTTTTGTGTCATCGGTATTGCAACATCATAGAAATGCATACCTTTG 5800
L S P C K Q C F Y L L R * L P R L L F C S S V F A T S * K L H T F V 5800
Y L P V N S A F I Y S D S F R Y C F F A H R Y L Q H H R N C I P L 5800
I S L * T V L L S T P I A S A I A F L L I G I C N I I E I A Y L C 5800

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P R L Y V C S S A T F F S L D G Q L K Q R * Q T L S I Q S E R K G 5900
F L G Y M F A H L Q L F F L W T D N * S K D S K L Y P F R V R E R G 5900
S S V I C L L I C N F F F F G R T I K A K I A N F I H S E * E K G 5900

GGACATGTCTCTTCTCTGAAAAATAAATGTTTTTATTGCTTATTATCCGACCCAAAAAGTTGCATTTATAAGTTGAAGTCAAGAGkTATTAC 6000
D I V S L S S L K N K C F Y C L L S A P K K L H L * V E L K X Y Y 6000
T L S L F P L * K I N V F I A Y Y P H P K S C I Y K L N S R X I T 6000
G H C L S F L S E K * M F L L L I I R T Q K V A F I S * T Q E X L X 6000

AKACGGkmTTTCyAkCACAACCCATCCCAACCTTCAACAATAGGATsAGACAATCGGATTACTGATCATGCTCGCCCTGGCGCTGTTGCTCCTGT 6100
X R X F X X N T M P T F N N R X R Q I G L L I M L V A L A L L L L F 6100
X G X S X P T P C Q P S T I G X D K S D Y * S C S S P W R C C S C 6100
T X F X X Q H H A N L Q Q * D X T N R I T D H A R R P G A V A P V 6100

TTGAGCTAAATGGATTTCTCCCGGCTTTTAGGTGCCCTGCTTGTATDACTAGmAGGGAGTGTACTTCCACCTTAACCATCCGCAAGCATTGGAA 6200
E L N G F L P G F L G A L A L Y X L X R E L Y F F L T I R K H W N 6200
L S * M D F L P A F * V P L L C X Y * X G S C T S P * P S A S I G I 6200
* A K W I S S R L F R C P C F V X T E X G V V L H L N H P Q A L E 6200

TAAAATGGGAACCCAGCTGTTATTTTACTTGCCTTTCTGATTGGTATGGTCTGCGGATCCTTTGCCATTTCATTGATTTCGCTCAAGATCAATGTA 6300
K M G T A L L F L L A F L I G I G L P V S F A I H L I S S K I N V 6300
K W E P H C Y L R F * L V L V C R Y P L P F I * F R L R S M Y 6300
* N G N R T V I F T C V S D W Y W S A G I L C H S F D F V * D Q C T 6300

CTTCTGCATAAATCTGATCAGATCATTGGTTTTTAAATCCGCATCGCTCAAATAGAACAGTGGACGGGTTTGACATGCTCACGGACCAAAGCCTGA 6400
L L H N S D Q I I V F K S A S A Q I E Q W T G F D M L T D Q S L S 6400
F C I I L I R S L W F L N P H R L K * N S G R V L T C S R T K A * 6400
S A * F * S D H C G F * I R I G S N R T V D G F * H A H G P K P E 6400

GGCAGATCAGAGACAGATCACAAATTTTCTCCTTCTGAAATGTTCTGCTTCCGTTGGCGGGCAATCTGCTCATGATCCTTTTCTTTCTATT 6500
D I Q R Q I T N F I P S F L N S S A S V A G N L L M I L F L F Y F 6500
A T Y R D R S Q I S F L P P * I V L L R W R A I C S * S F F F S I L 6500
R H T E T D H K F H S F L S E * F C F G G G Q S A H D P F S F L F 6500

TATGCTTACCAACGGAAACCGAAATGGAAAGGAAATTCGCATATGATCCCATTAAGAGGAAATGTAGATGTCTGCGCCATCGAACAATAAAACATG 6600
M L T N G T E M E R K I S H M I P L K E E N V D V L A I E T K N M 6600
C L P T E P K W K G K F R I * S H * K R K M * M C W P S K L K T W 6600
Y A Y Q R N R N G K E N F A Y D P I K R G K C R C A G H R N * K H G 6600

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GTCGGTGCCAAACGCTTGGGATTCACCTGATCTCTATTGTTCAAGGGTAATGCGGTACTGGATACTGGATTTTGGCGTAAAGGATTATCTGTTAT 6700
V R A N A L G I P L I S I V Q G V I A V L G Y W I F G V K D Y L L W 6700
S V P T P W V F H * S L L F G * L P Y L D T G F L A * R I I C Y 6700
P C Q R L G Y S T D L Y C S R G N C R T W I L D F W R K G L S V M 6700

GGGTTTTTACTGATCCATTTTTCGATTTTCCTGTCGTGGGCACCTCCTTTATCTGGATCCGCTGATCATCTATCTGTATGCATCCGGTGAAGTGG 6800
G F I T A I F A F F P V V G T S F I W I P L I I Y L Y A S G E S G 6800
G V L L L P F L H F S L S W A P P L S G F R * S S I C M H P V K V D 6800
G F Y Y C H F C I F P C R G H L L Y L D S A D H L S V C I R * K W 6800

ACAAGCCATCGGACTGGCCATTTACAGCATCGTTGTACAGGACACCGTTGATTATCTGGCGGGATCACTTTACTTCAGAAAATCGGCAATGTTTCATCCC 6900
Q A I G L A I F Y S I V V T S N V D Y L A R I T L L Q K I G N V H P 6900
K P S D W L P F T A S L S R A T L I I W R G S L Y F R K S A M F I P 6900
T S H R T G H L Q H R C H E Q R * L S G A D H F T S E N R Q C S S R 6900

GTTATCGCAGTACTGGATTAATGTTGGGCTCAAGCTGTTGGTTTCTGGGGTTTGTCTTTGGCCCGCTGATGATCAGCTATTTCCTGCTGCTGGTCC 7000
V I A V L G L I V G L K L F G F W G F V F G P L M I S Y F L L L V R 7000
L S Q Y L D * L L G S S C L V S G V L S L A R * * S A I F C A C W S 7000
Y R S T W I N C W A Q A V W F L G F C L W P A D D Q L F S A A G P 7000

GGTTTATGCCAGTGAAGTTGGTCTTGAATAAATAAAGTTTAAAGGGTTCATGCCGGGAACAGCGTCTCCGGCAACCGTTGGTTCGACGTACAGGATT 7100
I Y A S E F G S L N K * S L K G S C R E Q R L R Q P L V R R T G L 7100
G F M P V S L V L * I N K V * R V H A G N S V S G N R W F D V Q D Y 7100
D L C Q * V W F F E * I K F K G F M P G T A S P A T V G S T Y R I 7100

ATTTCGTGACAAAATCCGGATTCGCTTGGGAATCAGTGTGATCATCGTTTTTTGTAATAACTGTAAGTCGTAATACACGGG 7200
F V T K S G M R W E S V C I I V F C N N C K S * I T G 7200
S * Q N P V C V G N Q C V S S F F V I T V S R K S R 7200
I R D K I R Y A L G I S V Y H R F L * * L * V V N H G 7200

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Figure S5.3: Nucleotide and amino acid sequences of the Tn4351-transposon insertion site of mutant 31.90. Nucleotide sequence was revised manually via comparison with the electropherogram. Manually changed nucleotides are written in small letters. For nucleotides without clear peaks in the chromatogram, letters according to the IUPAC Ambiguity Code were used. The nucleotide sequence was simple sequenced from nucleotides 0-262bp, 1164-1268 and 6360-7258 (plus strand). All other sequences were multiple sequenced. Sequence of the Tn4351-transposon was received from Prof. M. J. McBride (University of Wisconsin – Milwaukee, USA) and the transposon border is flanked by direct repeats marked with underlined letters (XXX). First and last nucleotides of the transposon are highlighted in red (X). Colour code: (XXX) sequence obtained from the Trenzyme report; (XXX) primers: 1475_FP2, 1475_RP1, Tn4351_Tet_fw (Tetfw), Tn4351_Tet_rev (Tetrev), 31.90-FosmidprimerFW1 (FW1); (XXX) restriction sites located in the transposon for BsoBI, BstNI and HindIII. Nucleotide sequence was transcribed in amino acid sequence using the DNA to protein translation tool of insilico.ehu.es (Bikandi et al. 2004). Open reading frames (ORF1 and 2) in the amino acid sequences which led to hits when blasted against the NCBI data base are highlighted in blue (XXX).

S5.3 Alignments of the open reading frames with similar protein sequences from other bacteria

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gi|517451378|ref|WP_018622181.  -----MSIETDIKQNPFRTPYQRVVNMVLYTSNWIATNQT 36
gi|522091384|ref|WP_020602593.  -----MSIETDIKQNTFFRTPYQRVVNMVLYTSNWIATNQT 36
gi|522089383|ref|WP_020600592.  -----MSIETDIKQTTFFKPNYHRVNMVLYTSNWIATNQT 36
gi|284035298|ref|YP_003385228.  -----MSIETDIKQSTPFKSPYQRVLVNLMVLYTSNWIATNQT 36
gi|496582628|ref|WP_009282467.  -----MSIETDIKQPT-FRNPYHRLIVNLMFTSNWVANAQM 35
gi|518830386|ref|WP_019986335.  -----MSIETDIRQSA-FSSPYHRVNVVLYSGNWNHESQM 35
gi|436837460|ref|YP_007322676.  -----MRIEDEIRQAT-FRSPYHRVNVNLMFTSNWVANAQM 35
gi|338214546|ref|YP_004658609.  -----MSIETDIKQSK-FRNAYHKMALNLIYTSNWIATNQT 36
gi|408675081|ref|YP_006874829.  -----MSIEKDIKQKPKFSSYQKLVNLIYTSNWIATNQT 36
gi|518783561|ref|WP_019940850.  -----MSIETDIKQKK-FRSPYQKLVNLIYTSNWIATNQT 35
gi|255036185|ref|YP_003086806.  -----MSIETDIKQKK-FRSPYQKLVNLIYTSNWIATNQT 35
mutant  MYIQCYCTTNKLCMSIETDIKQKR-FRTPYQRLVNLNLIYTSNWIATNQT 49
      * * * : * * * * * : : : * : * : * : * : *
gi|517451378|ref|WP_018622181.  QLLKPSGLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 85
gi|522091384|ref|WP_020602593.  QLLKPSGLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 85
gi|522089383|ref|WP_020600592.  KLLKPSGLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 85
gi|284035298|ref|YP_003385228.  QLLKPFKLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 85
gi|496582628|ref|WP_009282467.  HLLKPYDLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 84
gi|518830386|ref|WP_019986335.  RILKPFGLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 84
gi|436837460|ref|YP_007322676.  RLLKPFGLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 84
gi|338214546|ref|YP_004658609.  CLLKPYDLTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 84
gi|408675081|ref|YP_006874829.  GLLKPFDLSSQYVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 85
gi|518783561|ref|WP_019940850.  ESFKEHDTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 84
gi|255036185|ref|YP_003086806.  ESFKEHDTPOQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 84
mutant  EILREHDLTPEQYNVLRILRGQYVNPVKVSDITE-RMLDKMSNASRLVDK 99
      : : * * * * * : * : : * : * * * * * * *

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gi|255036182|ref|YP_003086803.1|mutant|YWTGTVYTTNRRRIWEHDDSFKEYLRAIRAMAIDMETATIFIVGFANSIPH 199
gi|518783564|ref|WP_019940853.1|mutant|YWTGTVYTTNRRRIWEHDASFKEYLQAVRAMAIDMETATIFIVGFANSIPH 199
-----
gi|522089380|ref|WP_020600589.1|mutant|YWTGTVYTTNRRRIWEHDEQFKDYLRREIRTAIDMETATIFIVGFVNSIPH 199
gi|284035301|ref|YP_003385231.1|mutant|YWTGTVYTTNRRRIWEHDETFKDYLRRELRAMAIDMETATVFTVGFVNSIPH 199
gi|517451375|ref|WP_018622178.1|mutant|YWTGTVYTTNRRRIWEHDEAFKEYLVELRMAIDMETATIFIVGFVNSIPH 199
gi|522091380|ref|WP_020602589.1|mutant|YWTGTVYTTNRRRIWEHDEAFKEYLVELRMAIDMETATIFIVGFVNSIPH 199
gi|496583181|ref|WP_009283020.1|mutant|YWTGTVYTTNRRRIWEHDETFKEYLREIRAMAIDMETATVFTVGFVNSIPH 199
gi|338210530|ref|YP_004654579.1|mutant|YWTGTVYTTNRRRIWEHDEVFKEYLREIRTAIDMETATIFIVGFVNSIPH 199
gi|518831172|ref|WP_019987112.1|mutant|YWTGTVYTTNRRRIWEHDEQFKEYLRRETRAMAIDMETATIFIVGFANSIPH 200
gi|312130416|ref|YP_003997756.1|mutant|YWTGTVYTTNRRVWEHDEEFKAYLREIRALGIDMETATIFIVGFMSIPH 199

gi|255036182|ref|YP_003086803.1|mutant|GALLLVSDNPLVPEGVKTEESDKKVTSSVVMQHLEIGIDALTELARSGES 249
gi|518783564|ref|WP_019940853.1|mutant|GALLLVSDNPLVPEGVKTEESDKKVTSSVVKQHLDIGIEALTELARSGES 249
-----
gi|522089380|ref|WP_020600589.1|mutant|GALLLVSDNPLVPEGVKTEESDKRVTSQFVNVHLEIGIDALVELESSGDS 249
gi|284035301|ref|YP_003385231.1|mutant|GALLLVSDNPLVPEGVKTEESDKRVTQGFVNVHLEIGIDALLELESSGDS 249
gi|517451375|ref|WP_018622178.1|mutant|GALLLVSDNPLVPEGVKTEESDKIVTQFVNIHLEIGIDALLELESSGDT 249
gi|522091380|ref|WP_020602589.1|mutant|GALLLVSDNPLVPEGVKTEESDKIVTAQFVNIHLEIGIDALVELESSGDT 249
gi|496583181|ref|WP_009283020.1|mutant|GALLLVSDNPLVPEGVKTEESDKVVTQFVNVKHLLEIGIDALQELASSGES 249
gi|338210530|ref|YP_004654579.1|mutant|GALLLVSDNPLVPEGVKTEESDKKVTSTFVERHLQIGIDSLIELANSGES 249
gi|518831172|ref|WP_019987112.1|mutant|GALLLVSDNPMIPEGVKTEESDKKVTTFVERHLQIGIDSLHELASSGES 250
gi|312130416|ref|YP_003997756.1|mutant|GALLLVSDNPMTPPEGVKTEASDKKVTASFVEKHLQIGIDALDELANS GAS 249

gi|255036182|ref|YP_003086803.1|mutant|VKHLRFES 257
gi|518783564|ref|WP_019940853.1|mutant|VKHLRFEN 257
-----
gi|522089380|ref|WP_020600589.1|mutant|VKHLRFE- 256
gi|284035301|ref|YP_003385231.1|mutant|VKHLRFE- 256
gi|517451375|ref|WP_018622178.1|mutant|VKHLRFE- 256
gi|522091380|ref|WP_020602589.1|mutant|VKHLRFE- 256
gi|496583181|ref|WP_009283020.1|mutant|VKHLRFE- 256
gi|338210530|ref|YP_004654579.1|mutant|VKHLRFE- 256
gi|518831172|ref|WP_019987112.1|mutant|VKHLRFE- 257
gi|312130416|ref|YP_003997756.1|mutant|VKHLRFEN 257

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Figure S5.7: Alignment B-16-ORF4. Alignment of ORF4 of mutant B-16 with protein sequences of the NCBI database: AMP nucleosidase of *Dyadobacter fermentans* (Sequence ID: ref|YP_003086803.1; max. ident. 93%), AMP nucleosidase of *Dyadobacter beijingsensis* (Sequence ID: ref|WP_019940853.1; max. ident. 92%), AMP nucleosidase of *Runella slithyiformis* (Sequence ID: ref|YP_004654579.1; max. ident. 86%), AMP nucleosidase of *Rudanella lutea* (Sequence ID: ref|WP_019987112.1; max. ident. 84%), AMP nucleosidase of *Spirosoma luteum* (Sequence ID: ref|WP_018622178.1; max. ident. 82%), AMP nucleosidase of *Spirosoma panaciterrae* (Sequence ID: ref|WP_020600589.1; max. ident. 82%), AMP nucleosidase of *Spirosoma linguale* (Sequence ID: ref|YP_003385231.1; max. ident. 82%), AMP nucleosidase of *Leadbetterella byssophila* (Sequence ID: ref|YP_003997756.1; max. ident. 81%), AMP nucleosidase of *Fibrisoma limi* (Sequence ID: ref|WP_009283020.1; max. ident. 82%), AMP nucleosidase of *Spirosoma spitsbergense* (Sequence ID: ref|WP_020602589.1; max. ident. 81%). Other BLAST hits were below 81% of maximum identity.

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gi|495552716|ref|WP_008277295.1|mutant|-----MSQVAPKVDLEEASS 15
gi|172038168|ref|YP_001804669.1|mutant|-----MSQVAPKVDLEEASS 15
gi|494423700|ref|WP_007221740.1|mutant|-----MVLEP-----IAPWT 10
gi|523471444|gb|EPR43241.1|mutant|-----MIETP-----WS 7
gi|451946897|ref|YP_007467492.1|mutant|-----MGKIHVPTSTENVTIIGIPDSPA 24
gi|496585888|ref|WP_009285602.1|mutant|-----MTATATNNLSVPELPHT 17
gi|522086765|ref|WP_020597974.1|mutant|-----MPKLTILHNP HQ 12
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gi|319954445|ref|YP_004165712.1|mutant|-----MTTFKDTLDIIENTYS 16
gi|494047972|ref|WP_006990088.1|mutant|-----MKNGSKRIKKNASL 14
gi|516341118|ref|WP_017731151.1|mutant|MVGEIKQRRRIIPTTLKSNISWFRNPLRHMASEKTTSGRKLPLVENAYR 50
gi|497264445|ref|WP_009578662.1|mutant|-----MAAQLYHA 8

gi|495552716|ref|WP_008277295.1|mutant|LQTNVDLQRLSVDIKQGLDSSTVKKRRKRYGENRLQEFQRRSAWQILIAQ 65
gi|172038168|ref|YP_001804669.1|mutant|LQTDVVLQRLSVDLQGLDPSTVKKRREYKGNRLEEFQRRSAWQILIAQ 65
gi|494423700|ref|WP_007221740.1|mutant|LPWQDVIQDLHVSFNRGLDTVEVKKRYKFGSNRLEEFQRRSAWQILIAQ 60
gi|523471444|gb|EPR43241.1|mutant|HPIDEVLSAFEVDRDGLKRPQVSARKKRYGRNRLREIQKKQAWRILLDQ 57
gi|451946897|ref|YP_007467492.1|mutant|DSLEDSVKKLQVEPSQGLSTTEASARLQTFGHNMLREVKSKSLTIFINQ 74
gi|496585888|ref|WP_009285602.1|mutant|LPAEQLVKSLAVDAKSGLTGDEANRRLEQYGNALQEAQRESAWIAVRQ 67
gi|522086765|ref|WP_020597974.1|mutant|LDFKQLFDNLATDPRKGLTADAEARSLEEFQGNALQETKRESALTI LLRQ 62
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gi|319954445|ref|YP_004165712.1|mutant|KKVDVIVNLLDVLKTLGQERSINDRIAKYGLMSYREQQKSKSILLILFEQ 66
gi|494047972|ref|WP_006990088.1|mutant|KSIEEVLKELSDADTEKGLSDKEVEKPKKYGNPILLEEQSKSIVRILFSQ 64
gi|516341118|ref|WP_017731151.1|mutant|RSAAEVAQAVEAHPKQLSGSEVEERIKKYGRNELEKQERSWQILVAQ 100
gi|497264445|ref|WP_009578662.1|mutant|KEVEELLTSFNTDLNKLGSNAEARKRLVQYGNPILIEKKQKSGRILLDQ 58

gi|495552716|ref|WP_008277295.1|mutant|FKSPIIALLAVAVALSFAFQEWIEGIAIIIAILLNNAIIGFVTTETKAVRSM 115
gi|172038168|ref|YP_001804669.1|mutant|FKSPIIALLAVAVALSFAFQEWIEGIAIIIAILLNNTVIGFVTTETKAVRSM 115
gi|494423700|ref|WP_007221740.1|mutant|LKSLLIILLGVTTIVSFAFGEWVEGMAIGVVIVINTAIGFTELKAVRSM 110
gi|523471444|gb|EPR43241.1|mutant|FRNLIVVLLGAAAALSYFFGDWLEGSAILAVIFINAAIGFATLKA VRSM 107
gi|451946897|ref|YP_007467492.1|mutant|FRNLIVVFLIAASLLSFSFGYVEGLAIGVIVINAAIIGFVTELKARS I 124
gi|496585888|ref|WP_009285602.1|mutant|FNSVIVWILAAAAIISPLLGETVEGWAIIVAVILINAITGFVLEFNASQSM 117
gi|522086765|ref|WP_020597974.1|mutant|FTGVIVYILVAAAGISFFLGDVTEGVALLVLLINAVTGFGLWNAQSM 112
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gi|319954445|ref|YP_004165712.1|mutant|FKSPIIILLVVAAGSFFFDWLEGFSAIIVAVITVVLGPIMLQARNSM 116
gi|494047972|ref|WP_006990088.1|mutant|INNPIYLLTAAAVLAFSFDNIAEGIAIVVLLINTIIGFWMYNAQKSM 114
gi|516341118|ref|WP_017731151.1|mutant|ITNPVVYLLTAAATMAFVFGDIEGIAITVLLVNTIIGFWMYNAQKSM 150
gi|497264445|ref|WP_009578662.1|mutant|FTSPIVWLLIAGTLAFLGIEPEGIAIIVVINTPIFGFMWQALRSM 108

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gi|494423700|ref|WP_007221740. |EALRKLGSVTKVIRNSQLREIPADELVPGDIVVVEGGDVVPADLRLLLEA 160
gi|523471444|gb|EPR43241.1| |EALRRMGSTKTRVRRDRVMAVPADTLVPGDIVVIEGGDVITADLRLLIAA 157
gi|451946897|ref|YP_007467492. |EALRKLGSVSSRVRDRGRISEILANDLVPGDIIILEGGDVISADLRLLIA 174
gi|496585888|ref|WP_009285602. |DALRKMDTNPARVWRDRGVHEIISSEDDVTLGDILMVEAGDLVAADASLLEA 167
gi|522086765|ref|WP_020597974. |DALRKL DSTPARVLRDRGHVREIASEDDVTIVGDILVVEAGDLVAADANLFQV 162
mutant
gi|319954445|ref|YP_004165712. |KALKKMDVSVSKVWRDNLKEIQAEIRVPGDVLVLEAGDIVMADARLLIEV 166
gi|494047972|ref|WP_006990088. |KSLKKLDKIQARVIRNGKQRI DA EKLVPGDILIVESGDLPADARILEA 164
gi|516341118|ref|WP_017731151. |EALKAMDKIQARVLRDRGKQIDAEIIVPGDILLVEAGDLVAADARLLIEV 200
gi|497264445|ref|WP_009578662. |LELKKMGRATKVFREGTRKELDSVQLVPGDIIYFEGGDLITADARLLIVQ 158

gi|495552716|ref|WP_008277295. |SKLQADESALTGESVFPVSKTIEPLEKLEIADRNNMVFKGTAITRSGSEG 215
gi|172038168|ref|YP_001804669. |SKLQADESALTGESVFPVSKTIEPLEKLEIADRNNMVFKGTAITRSGSEG 215
gi|494423700|ref|WP_007221740. |SKLQADESALTGESVFPVSKGVEHLEEDTSLAERTNMLFKGTSVTRGSAKG 210
gi|523471444|gb|EPR43241.1| |SKLQADESALTGESLPEVKSVAIPLVKASLAERTNMIYKGTAITRSGAGEG 204
gi|451946897|ref|YP_007467492. |SRLQSDSVLTGESLPEVKGSEVLEGGTSLADRDNMLFKGTALTTRGSGEA 227
gi|496585888|ref|WP_009285602. |NQLQVDESALTGESLPEVKATAFTADAPLGDQHNRLFKGTAVTGGNGRA 217
gi|522086765|ref|WP_020597974. |NQLQVNESALTGESLFPVKNKNTQIPADDAPLGDQHNRLFKGTAATDGNGRA 212
mutant
gi|319954445|ref|YP_004165712. |NQFEIDESALTGESLPEVKKIEVINKNEVPLADKINILFKGTSVVRGNARA 216
gi|494047972|ref|WP_006990088. |TELQIDESPLTGESVPTIKSPEVLKKEKSVADRTNIYKGTAVVSGGTSKA 214
gi|516341118|ref|WP_017731151. |SELGVDESPLTGESLFPVTKSPDVEEKEKGVDRRLNLFKGTAVVSGTGKA 250
gi|497264445|ref|WP_009578662. |HNLAIKEAALTGESAQVSKHTEKLPEDTVLADRTNSVFKGTLVARGNGKA 208

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gi|172038168|ref|YP_001804669. |VVVSTGMDTELGHISSLTAQAEIEEVTPLEKRLDSLGRKLIWITLLIAVVI 265
gi|494423700|ref|WP_007221740. |VVVATGMKTELGKISSLVESAKEEITPLEKRLNKLGNKLIWVTLAVTAVI 260
gi|523471444|gb|EPR43241.1| |VVVATGMETELGRISALVAEAEDEITPLEKRLDQLGHKLIWVTLAVTAVI 257
gi|451946897|ref|YP_007467492. |VVIATGMETELGKISSLVAGTKEDATPLEKRLNQLGNLIWVTLIAVVFV 274
gi|496585888|ref|WP_009285602. |VITGIGQOQTELGKIATMVQEARTATPLEAKLDSLKLVWVTLVGLAGLF 267
gi|522086765|ref|WP_020597974. |IVTIGIQOQTELGKIATMVQEAARTATPLEAKLDSLKLVWVTLVGLAVLF 262
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gi|516341118|ref|WP_017731151. |VVVGTGMDTELGNISAMVGEQDEDEITPLNRKLNKLNKLIWVTLGLAVAF 300
gi|497264445|ref|WP_009578662. |IVVATGNDTELGRISEITQEAEKHATPLDKRLSKLSKLIWVTLAMTFLI 258

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gi|494423700|ref|WP_007221740. |ATAGITGEDIILMKIISIALAVATIPEGLPIVATLALARGMWRMAKRNA 310
gi|523471444|gb|EPR43241.1| |GAAGLVGRGKDLITLTAIALAVASIEPEGLPIVATLALARGMWRMAKRNA 307
gi|451946897|ref|YP_007467492. |VVTGLMAGQELYLMIETGIALAVASIEPEGLPIVATLALARGMWRMAKRNA 324
gi|496585888|ref|WP_009285602. |LIVGLIRGEVRLQIETSIALAAIAAIPEGMSVVVATIALAYGMLRLAEKKV 317
gi|522086765|ref|WP_020597974. |LIVGLIRGEVRLRLVETALALAAIAAIPEGMSVVVATIALAYGMLRLANKKV 312
mutant
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gi|516341118|ref|WP_017731151. |FIFGWIAGKELYLLIQTSAIWTIAAIPEGLPIVASIALARGMWRMAKRNA 350
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gi|172038168|ref|YP_001804669. |IINRLSAVETLGATSIICTDKTGTLTENRMTAREVRLNSDSIEITGEEQ- 364
gi|494423700|ref|WP_007221740. |LINRLSSVETLGATSIICTDKTGTLTENKMTVTRIILDSGEIKISGEGLN 360
gi|523471444|gb|EPR43241.1| |LINRLSSVETLGATTVIFTDKGTGTLENRMTVSRVLDVAGEIRTDKAEKG- 356
gi|451946897|ref|YP_007467492. |LIKELSAVETLGATSIICTDKTGTLTENRLTAVKFAIATGSDIHIINSPE 374
gi|496585888|ref|WP_009285602. |IVKRLSAVETLGGTNVIFTDKGTGTLENRMTVSRVLDVAGEIRTDKAEKG- 367
gi|522086765|ref|WP_020597974. |IVKRLSAVETLGGTNVIFTDKGTGTLENRMTVSRVLDVAGEIRTDKAEKG- 362
mutant
gi|319954445|ref|YP_004165712. |LIKRLASVETLGGVNIIFTDKGTGTLENKIDVKNLMPFDEDI IAKKGDD 366
gi|494047972|ref|WP_006990088. |IVKLEAVETLGETTIIIFTDKGTGTLENKIDVKNLMPFDEDI IAKKGDD 363
gi|516341118|ref|WP_017731151. |VVKKLAAVETLGETTVIFTDKGTGTLENRMTVSRVLDVAGEIRTDKAEKG- 399
gi|497264445|ref|WP_009578662. |IVKTLKAVQTLGETNVILTDKGTGTLENRMTVSRVLDVAGEIRTDKAEKG- 358

gi|495552716|ref|WP_008277295. |TRGQFRRDDQITID-PLDNSILRNLNVCVLCNNATLAV----- 401
gi|172038168|ref|YP_001804669. |TQGFRRNDQITID-PLDHPILRNLNVCVLCNNATLAA----- 401
gi|494423700|ref|WP_007221740. |TEGEFRKKGPELD-PLREKTLQELLHAGVLCNNAASVEP----- 397
gi|523471444|gb|EPR43241.1| |----YLLDDRQVH-PEDFSTLQCLLRVAVLCNNAAYEE----- 389
gi|451946897|ref|YP_007467492. |KEKTFVGDAGEISLPEPNRALQHVLEVGVLNNAEITL----- 412
gi|496585888|ref|WP_009285602. |QTLNVTNGDQIIII--ESDVYQQLVLAGVLCNNAADYNP----- 402
gi|522086765|ref|WP_020597974. |KNLQVIAGNSEIT--DSVDFQKLQVQVLCNNAEYDD----- 397
mutant
gi|319954445|ref|YP_004165712. |SSIVINK-----NKGAYEKLILISILCNNAASEQ----- 395
gi|494047972|ref|WP_006990088. |AKPN-LQEEHEPK--KNENFLQIFKISVLANDASLEGEENLIDKEEKRD 409
gi|516341118|ref|WP_017731151. |SQGVGLKGEPE--AKENLRHILKISVLANDARLLD----- 434
gi|497264445|ref|WP_009578662. |IEAEREN-----MESDIEILLIVGLTCLNDSTYHP----- 387

gi|495552716|ref|WP_008277295. |-----
gi|172038168|ref|YP_001804669. |-----
gi|494423700|ref|WP_007221740. |-----
gi|523471444|gb|EPR43241.1| |-----
gi|451946897|ref|YP_007467492. |-----
gi|496585888|ref|WP_009285602. |-----
gi|522086765|ref|WP_020597974. |-----
mutant
gi|319954445|ref|YP_004165712. |-----
gi|494047972|ref|WP_006990088. |VSGEKEVEHTNDESGKNKVDVNLNVAQDKVEIEAKKENAEKDKQDVLDD 459
gi|516341118|ref|WP_017731151. |-----DNSGAN----- 440
gi|497264445|ref|WP_009578662. |-----

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gi|451946897|ref|YP_007467492.-----SPE 415
gi|496585888|ref|WP_009285602.-----H 403
gi|522086765|ref|WP_020597974.-----D 398
mutant
gi|319954445|ref|YP_004165712.-----K 396
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gi|516341118|ref|WP_017731151.-----EDGQ 444
gi|497264445|ref|WP_009578662.-----

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gi|451946897|ref|YP_007467492.-----GVIKVSVDGPLEVALLAIAVKAGMTRDELAVQFPEDKETSFDSDIKMMAT 465
gi|496585888|ref|WP_009285602.-----DTDTKVEVDGPEVALLKFAVYAGQDPDI IREQYPREAEKPFSSDTRMMGT 453
gi|522086765|ref|WP_020597974.-----DGNFREVGDVVDVSLKFAVAVGLNFDAINLEYARRAEKAFSSDTRMMGT 448
mutant
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gi|494047972|ref|WP_006990088.-----GKSGKGEGLDPIEIALLNFAKKFDQDLRKNRKLRLEHDFPDSMIMGA 559
gi|516341118|ref|WP_017731151.-----DQSGKGEGLDPIEIALHFSKKNSEAYQYRQLERRLHDFPDSMIMGT 494
gi|497264445|ref|WP_009578662.-----SETESTTGDPIEIALLRSAEILGAPDDLKKYPRIAEVPFPAEIKMMGT 437

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gi|523471444|gb|EPR43241.1|-----FHEDENGVLVAVKGAPEVSLSSSTRILTEAGESELSSEARDVWTAHTERL 489
gi|451946897|ref|YP_007467492.-----YHRVDEGYRIVAVKGAPEVSI EACDTLTFGDGGTALTAEDRKQWSQLNEEM 515
gi|496585888|ref|WP_009285602.-----LHRTNDRYVAVKGAPEVIMD-----RCPDMEALQKQKQRMSEQM 494
gi|522086765|ref|WP_020597974.-----LHEHEDRYIVAVKGAPEVIMN-----RSVSLDEKARKAQYELAEKM 489
mutant
gi|319954445|ref|YP_004165712.-----LHKNNAGNFVAAKGAVEKLI DTCFSYAGDRV I PLTEAAKKDFLNRAETI 496
gi|494047972|ref|WP_006990088.-----VYKENEGFYVAGKGAHAAILERSSKILVDNELKELTDEKDYWHKKNEL 609
gi|516341118|ref|WP_017731151.-----AYEEERGGYIAGKGAADALLSRCTHILVKGEVRELTEKDKAEWLRKNDMS 544
gi|497264445|ref|WP_009578662.-----ANKANGAYLISVKGATEAVLQKCTKMLD-EDGNVPLGDHDEWHNAVNE 486

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gi|494423700|ref|WP_007221740.-----ARDGLRILVAEKTVTA-----SNSNPYQDLTL LGVGLLDPREAVKKA 545
gi|523471444|gb|EPR43241.1|-----ARLGLRILVAFAQKRESS-----VEAPPYRDLTL IGLAGLLDSPRADIRDA 534
gi|451946897|ref|YP_007467492.-----AAKGLRVLALAEKQSN-----LEEEPYAKLQLVGLVGLMDPPREDVKEA 560
gi|496585888|ref|WP_009285602.-----AADGLRILAFAYREIDE-----KPGDDFA-DSNLTYLGLIGFLDPPREVTPTA 541
gi|522086765|ref|WP_020597974.-----AADGLRILAFAYREIDKTRTPGDDFA-DRDLTYLGLIGFLDPPREVTPTS 538
mutant
gi|319954445|ref|YP_004165712.-----EAQGSKVLAFAPKAAAN--IPTNFL--SGLTLVGLIGFLDPPREVEVA 542
gi|494047972|ref|WP_006990088.-----SEEGRLVAFASYKNIDTLP EEEKEENDFIRGLNFVGLIGFLDPPREKVSNA 659
gi|516341118|ref|WP_017731151.-----ANDGLRVLGFAYQQPDELPSGAAADDFLQNLTFVGLIGFLDPPREKVASA 594
gi|497264445|ref|WP_009578662.-----AGKGLRVLGFAYKTKGS---EPDHKNFIQDLTF IGLGGFDPAKRDVKEA 533
* * * * *

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gi|494423700|ref|WP_007221740.-----LALCRDAGIKVVMVTDGQPV TARNIALAVGLIHG---ETEVIHGKDLKK 592
gi|523471444|gb|EPR43241.1|-----LNVVTCKAGLRVVMVTDGQV ILTARNIADAVGLTHDK---NAEAVEGKDLKT 581
gi|451946897|ref|YP_007467492.-----LAQCREAGIRVIMATGQVATQA IGRVAVGLVAED---NAQVIGHGLDLGR 607
gi|496585888|ref|WP_009285602.-----LESCEAGIKVIMVTDGHPATALT IARQVHLEPG---EDIALTGRDLKP 588
gi|522086765|ref|WP_020597974.-----LEACRRAGIKVIMVTDGHPATALT IASKVKLLEPG---EEIVLTKGDKLP 585
mutant
gi|319954445|ref|YP_004165712.-----LNACHKAGIKVIMVTDGHPATSLK IAKQVGLVEED---ELLVLTGKDLSE 79
gi|494047972|ref|WP_006990088.-----LQSCRNAGIKVIMVTDGHPATALN IAEKIKLSEK---DNVINGKELD- 587
gi|516341118|ref|WP_017731151.-----INICKDAGIKVIMVTDGHPGTAKNVGKEVGI DYLEEKTNVLSGKDLQA 709
gi|497264445|ref|WP_009578662.-----IETCHRAGIKVVMVTDGHPGTAVN IGRQVQLVTAENESEHTVLHGKNIQA 644
IQSCKEAGIKVVMVTDGHPETAGS IAEVGLVKDGR--EATKIHGRLDGLK 581
* * * * *

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gi|172038168|ref|YP_001804669.-----PDDIPSDQQEALRQVPIFARV SFEQKLNLI TLHQQAGAVVGMTGDGVNDA 646
gi|494423700|ref|WP_007221740.-----PEELSEEDLQRI LRVSVEFARVDPKQKLDLTAIYQKNQAVVAMTGDGVNDA 642
gi|523471444|gb|EPR43241.1|-----PETLSRADRRRLVRIPIFARV SFRQKLDLISLYQAEQEVVAMTGDGVNDA 631
gi|451946897|ref|YP_007467492.-----LVLDLSEAEKDRIASQ LFAVRSFQKLDL IAFHQERHAI VAMTGDGVNDA 657
gi|496585888|ref|WP_009285602.-----LDQLTDADRERLMACRVFARV SPTQKLDMDL YQQQDGI VGMTGDGVNDA 638
gi|522086765|ref|WP_020597974.-----IDQLRVDEKQVNLNCRV FAVRSFPAQKLDMDI ELYHQEGNI VGMTGDGVNDA 635
mutant
gi|319954445|ref|YP_004165712.-----FDFSSANDQKMMMECHV FAVRNPAQKLDMDI DFYQKQDGI VGMTGDGVNDA 129
gi|494047972|ref|WP_006990088.--------SKLSDKKLFAATIFARV TPKQKLAMVSFYQKHSI VAMTGDGVNDA 633
gi|516341118|ref|WP_017731151.-------ELDKEDDSKIINTKIFSRV DPSQKLI I KYFQRNGE I VGMTGDGVNDA 757
gi|497264445|ref|WP_009578662.-------ELDKNDNEKLVSTV FSVRDPGQKLSL I KHVQQAQGE I VGMTGDGVNDA 692
VGSKVITIQKHILDANI FARINASQKLDLVLKYQNNY I VGMTGDGVNDA 631
* * * * *

gi|495552716|ref|WP_008277295.-----PALKKADIGIAMGQRGTQVAKEAADM I LQDDAFST I VAAVEQGRAIFNNI 696
gi|172038168|ref|YP_001804669.-----PALKKADIGIAMGQRGTQVAKEAADM I LQDDAFST I VAAVEQGRAIFNNI 696
gi|494423700|ref|WP_007221740.-----PALKKADIGIAMGQCGTQVAREAA DMILKDDNFST I VAABEQGRVIFNNI 692
gi|523471444|gb|EPR43241.1|-----PALKKADIGIAMGLRGTQVAREAA DMVLQDDAFSS I VAABEQGRVIFNNI 681
gi|451946897|ref|YP_007467492.-----PALKKADIGIAMGLRGTQVAREAS DMVLKDDSFAS I VHAVKQGRVIFNNI 707
gi|496585888|ref|WP_009285602.-----PALKKSDIGIAMGQRGTQVAEETAAM VLVKDDSFST I VAVTAQGRVIFNNI 688
gi|522086765|ref|WP_020597974.-----PALKKSDIGIAMGLRGTQVAEETA D I VLVKDDSFST I VAAIAQGRVIFNNI 685
mutant
gi|319954445|ref|YP_004165712.-----PALKKSDIGIAMGLRGTQVAEETA D I VLVKDDSFST I VAVTAQGRVIFNNI 179
gi|494047972|ref|WP_006990088.-----PALKKRANVGIAMGKRGTQVAQEV S DMVLKDDSFST I VVAIEQGRVIFNNI 807
gi|516341118|ref|WP_017731151.-----PALKKRANVGIAMGKNGTQVAQEV S DMVLKDDAFS I VVAIEQGRVIFNNI 742
gi|497264445|ref|WP_009578662.-----PALKKADIGIAMGKRGTQVAEETA D I VLVKDDSFST I VVAIEQGRVIFNNI 681
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Supplementary Data

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gi|495552716|ref|WP_008277295.1|ca|Ca2+-transporting ATPase of Fibrisoma limi (Sequence ID: ref|WP_009285602.1; max. ident. 72%),
gi|172038168|ref|YP_001804669.1|ca|hypothetical protein of Spirosoma panaciterrae (Sequence ID: ref|WP_020597974.1; max. ident. 65%),
gi|494423700|ref|WP_007221740.1|ca|p-type (transporting) ATPase, had superfamily of Cellulophaga algicola (Sequence ID: ref|YP_004165712.1; max.
gi|523471444|gb|EPR43241.1|ca|ATPase of planctomycete KSU-1 (Sequence ID: ref|WP_007221740.1; max. ident. 50%),
gi|451946897|ref|YP_007467492.1|ca|p-type (transporting) ATPase, HAD superfamily of Gillisia limnaea (Sequence ID: ref|WP_006990088.1; max. ident.
gi|496585888|ref|WP_009285602.1|ca|50%), hypothetical protein of Nafulsella turpanensis (Sequence ID: ref|WP_017731151.1; max. ident. 51%),
gi|522086765|ref|WP_020597974.1|ca|translocating p-type ATPase of Desulfocapsa sulfexigens (Sequence ID: ref|YP_007467492.1; max. ident. 52%),
mutant
gi|319954445|ref|YP_004165712.1|ca|cation-transporting ATPase of Fulvivirga imtechensis (Sequence ID: ref|WP_009578662.1; max. ident. 50%);
gi|494047972|ref|WP_006990088.1|ca|ATPase of Cyanothece sp. CCY0110 (Sequence ID: ref|WP_008277295.1; max. ident. 50%),
gi|516341118|ref|WP_017731151.1|ca|cation-transporting ATPase of Cyanothece sp. ATCC 51142 (Sequence ID: ref|YP_001804669.1; max. ident. 50%) and
gi|497264445|ref|WP_009578662.1|ca|p-type (transporting) ATPase, HAD superfamily of Desulfococcus multivorans (Sequence ID: gb|EPR43241.1; max.
ident. 50%). Other BLAST hits were below 50% of maximum identity.

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Figure S5.8: Alignment 21.20-ORF1. Alignment of ORF1 of mutant 21.20 with protein sequences of the NCBI database: Ca²⁺-transporting ATPase of *Fibrisoma limi* (Sequence ID: ref|WP_009285602.1; max. ident. 72%), hypothetical protein of *Spirosoma panaciterrae* (Sequence ID: ref|WP_020597974.1; max. ident. 65%), p-type (transporting) ATPase, had superfamily of *Cellulophaga algicola* (Sequence ID: ref|YP_004165712.1; max. ident. 53%), ATPase of planctomycete KSU-1 (Sequence ID: ref|WP_007221740.1; max. ident. 50%), p-type (transporting) ATPase, HAD superfamily of *Gillisia limnaea* (Sequence ID: ref|WP_006990088.1; max. ident. 50%), hypothetical protein of *Nafulsella turpanensis* (Sequence ID: ref|WP_017731151.1; max. ident. 51%), translocating p-type ATPase of *Desulfocapsa sulfexigens* (Sequence ID: ref|YP_007467492.1; max. ident. 52%), cation-transporting ATPase of *Fulvivirga imtechensis* (Sequence ID: ref|WP_009578662.1; max. ident. 50%); ATPase of *Cyanothece* sp. CCY0110 (Sequence ID: ref|WP_008277295.1; max. ident. 50%), cation-transporting ATPase of *Cyanothece* sp. ATCC 51142 (Sequence ID: ref|YP_001804669.1; max. ident. 50%) and p-type (transporting) ATPase, HAD superfamily of *Desulfococcus multivorans* (Sequence ID: gb|EPR43241.1; max. ident. 50%). Other BLAST hits were below 50% of maximum identity.

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mutant
gi|495517626|ref|WP_008242271.1|MDR---KENATEME----KSKAHIIVEIEYVFNVAVLSRTIIRKKTGNIT 43
gi|327405058|ref|YP_004345896.1|MQD---NNFKAELE----KSKAHIIVEIEYVFNVAVLSRTIIRKASGNVT 43
gi|387790678|ref|YP_006255743.1|MKS---TTDGKELE----KAKSHIIVEIEYVFNVAVLSKTIILKKTGNVT 43
gi|489071017|ref|WP_002980986.1|-----MDRGEVE----KSKALIIVEIEYLSNAVVSRTIIRKKTGNIS 39
gi|495788621|ref|WP_008513200.1|MFKP-IAMNHRLE----KSKVVIITSDVYIPNSVVKIILEKLTGNIS 45
gi|495788621|ref|WP_008513200.1|MEK--NASDNTELE----TGKAHIIVEIEYVFNVAVVIKTIIRKKTGNIS 44
gi|495788621|ref|WP_008513200.1|MEN--NKPDNTELE----PGKAHIIVDIIEYVFNVAVVIKTIIRKKTGNIS 44
gi|510819842|ref|WP_016193354.1|MDQKYQAEPPDSIE----KAKPHMIVEIEYVFNVAVMKNIIRKKTGNIS 46
gi|489531458|ref|WP_003436190.1|-----MEKSEVE----ESEIFIIVEIEYVFNVAVVIKTIIRKKTGNVT 39
gi|327405221|ref|YP_004346059.1|MQTQNIISDEEVINSGLPKSQPHMVVEIEYVFNVAVLSRTIIRKKTGNIS 50
:: : : :*:*.::: : : : *::

mutant
gi|495517626|ref|WP_008242271.1|VSSFDTEGEELEEKSSAFDPTIYIIGDGAELVIDGTGKHLKLGEGIIIPAH 93
gi|327405058|ref|YP_004345896.1|VSSFDAGEELEEKSSPFDFYIIGDGAELIIDDSPFTLKLGEGLIIPAH 93
gi|387790678|ref|YP_006255743.1|VLSFDTEGEMLEKISPFDFYIIEGNAEVIDGKLNLLTTGQSIIPAH 89
gi|489071017|ref|WP_002980986.1|VLSFDKKEGLSEKISPFDFYIIEGKAEIIDDGASVFMEEGECIIPAH 95
gi|495788621|ref|WP_008513200.1|IMSFDSGEGLEKTSPFDFYIIEGNAEVIDHVSNHLKAGMGIIPAH 94
gi|495788621|ref|WP_008513200.1|IMSFDSGEGLSVKTSPFDFYIIEGNAEVIDHVSNHLKAGMGIIPAH 94
gi|510819842|ref|WP_016193354.1|IMSFDSGEGLEKTSVDFYIIEGNAEVIDRVSNQLKSGGQVIVIPAH 96
gi|489531458|ref|WP_003436190.1|AVSVDTGEDITERISPFDFYIIEGSAEIIDHVSQTLAKGQSIIPAH 89
gi|327405221|ref|YP_004346059.1|ATSFDEGEKLCETTESDPTIYIIGDKATVITINHDRELSLGEGLIVIPAA 100
*.* * : : * **:*.* : * : * :***

mutant
gi|495517626|ref|WP_008242271.1|ARNSFCAKEQFKMISTVIKSGYE--- 116
gi|327405058|ref|YP_004345896.1|ALHSFNASQQFKMISTVIKSGYEDQL 119
gi|387790678|ref|YP_006255743.1|TRHKFNANEQFKMISTVIKSGYENEN 119
gi|489071017|ref|WP_002980986.1|SPNSIKANERFKMISTVIKSGYE--- 112
gi|495788621|ref|WP_008513200.1|RSHSIKGNRFKMIIVTIKSGYE--- 118
gi|495788621|ref|WP_008513200.1|LPNYIKPNGRFKMIQTVIKSGYE--- 117
gi|495788621|ref|WP_008513200.1|LPNYIKPNGRFKMIQTVIKSGYE--- 117
gi|510819842|ref|WP_016193354.1|LSSFIRPNGRFKMIQTVIKSGYE--- 119
gi|489531458|ref|WP_003436190.1|SMNTIKANERFKMISTVIKSGYE--- 112
gi|327405221|ref|YP_004346059.1|TLHCFKAEEKFKMITVIASVHQV-- 124
: . :**** * * * :

Figure S5.9: Alignment 21.20-ORF2. Alignment of ORF2 of mutant 21.20 with protein sequences of the NCBI database: Cupin 2 barrel domain-containing protein of Fluviicola taffensis (Sequence ID: ref|YP_004345896.1; max. ident. 71%), cupin of Pedobacter sp. (Sequence ID: ref|WP_008242271.1; max. ident. 74%), cupin domain-containing protein of Solitalea canadensis (Sequence ID: ref|YP_006255743.1; max. ident. 65%), cupin of Mucilaginibacter paludis DSM 18603 (Sequence ID: ref|WP_008513200.1; max. ident. 65%), cupin of Mucilaginibacter paludis (Sequence ID: ref|WP_008504582.1; max. ident. 58%), hypothetical protein of Arcticibacter svalbardensis (Sequence ID: ref|WP_016193354.1; max. ident. 55%), cupin domain-containing protein of Psychroflexus gondwanensis (Sequence ID: ref|WP_003436190.1; max. ident. 62%), hypothetical protein Fluta_3248 (cupin) of Fluviicola taffensis (Sequence ID: ref|YP_004346059.1; max. ident. 50%) and cupin of Chryseobacterium gleum (Sequence ID: ref|WP_002980986.1; max. ident. 52%). Other BLAST hits were below 50% of maximum identity.

gi|489067515|ref|WP_002977507.1|MIPKTMKAAVVQGYGQPLKIEEVPVREPGRYEVLVKVMACGVCHTDLHAV 50
gi|495117461|ref|WP_007842278.1|MIPKTMKAAVVQGYGQPLKIEEVPVREPGRYEVLVKVMACGVCHTDLHAV 50
gi|496380606|ref|WP_009089596.1|MIPKTMKAAVVQGYGQPLKIEEVPVREPGRYEVLVKVMACGVCHTDLHAV 50
gi|489101178|ref|WP_003011041.1|MIPKTMKAAVARGYGQPLSIEEVPVKQPGRNQILVKVACGVCHTDLHAI 50
gi|489090486|ref|WP_003000382.1|MIPKTMKAAVARGYGQPLSIEEVPVKQPGRNQILVKVACGVCHTDLHAI 50
gi|494231392|ref|WP_007139094.1|MLPKTMKAAVIREFGSLKIEEVEVKRPGRNEILVKVIAAGVCHTDLHAT 50
gi|495788562|ref|WP_008513141.1|MIPKTMKAAVIRGFGQPLHIEEMPVKEPGENQILVKVIAAGVCHTDLHAC 50
gi|495785498|ref|WP_008510077.1|MIPKTMKAAVIRGFGQPLHIEEMPVKEPGENQILVKVIAAGVCHTDLHAC 50
mutant
gi|431798554|ref|YP_007225458.1|MIPKTMKAAVIRGFGQPLKIEEIPVKAPNENQILVQVMASGVCHTDLHAA 50
*:*.****** :.* * :*: * : * . : :*:*.*****

gi|489067515|ref|WP_002977507.1|DGDWPAKPKMPLIPGHEGVGIVVACGPEAF-VKEGDVAVGVPWLYSACGCC 99
gi|495117461|ref|WP_007842278.1|DGDWPAKPKMPLIPGHEGVGIVVACGPEAH-VKEGDVAVGVPWLYSACGCC 99
gi|496380606|ref|WP_009089596.1|DGDWPAKPKMPLIPGHEGVGIVVACGPEAD-VKEGDVAVGVPWLYSACGCC 99
gi|489101178|ref|WP_003011041.1|EGDWPVKS KMPLIPGHEGVGYVVATGPDVQNVKEGDAGVVPWLYSACGCC 100
gi|489090486|ref|WP_003000382.1|EGDWPVKS KMPLIPGHEGVGYVVATGPDVQNVKEGDAGVVPWLYSACGCC 100
gi|494231392|ref|WP_007139094.1|EGDWPVQPKMPLIPGHEGVGYVVAVGPDVKNVKEGDAGVVPWLYSACGCC 100
gi|495788562|ref|WP_008513141.1|QGDWPAKPKMPLIPGHEALGYVVALGRGVKHIKEGDIVGVPWLYSACGNC 100
gi|495785498|ref|WP_008510077.1|QGDWPAKPKIPLIPGHEAVGYVVALGAGVQKIEGDIVGVPWLYSACGHC 100
mutant
gi|431798554|ref|YP_007225458.1|DGDWPAKPKMPLIPGHEGIGYVVALGPEVTGVKEGDIVGVPWLYSACGCC 100
DGDWPKRPLPLIPGHEGIGYVVAAGSVNKNTKEGDIVGVPWLYSACGHC 100
:***. :. :***** : * * * * . **** ***** *

gi|489067515|ref|WP_002977507.1|DYCITGWETLCEAQKNGGYSVDGGFAEYVIADSRVYVGLKSDVNFLEIAP 149
gi|495117461|ref|WP_007842278.1|DYCITGWETLCEAQKNGGYSVDGGFAEYVIADSRVYVGLKSDVNFLEIAP 149
gi|496380606|ref|WP_009089596.1|DYCITGWETLCEAQKNGGYSVDGGFAEYVIADSRVYVGLKSDVNFLEIAP 149
gi|489101178|ref|WP_003011041.1|DFCITGWETLCEAQKNGGYSVDGGFAEYVIADSRVYVGLKSDVNFLEIAP 150
gi|489090486|ref|WP_003000382.1|DFCITGWETLCEAQKNGGYSVDGGFAEYVIADSRVYVGLKSDVNFLEIAP 150
gi|494231392|ref|WP_007139094.1|DQCITGWETLCEAQKNGGYSVDGSGFAEYVIADSRVYVGLLPSNVFEMEMAP 150
gi|495788562|ref|WP_008513141.1|DYCYTGWETLCEAQKNGGYSVDGSGFAEYVIVADAGYVAHFPPNINFTEMAP 150
gi|495785498|ref|WP_008510077.1|DYCYTGWETLCEAQKNGGYSVDGSGFAEYVIVADAGYVAHFPPNINFTEMAP 150
mutant
gi|431798554|ref|YP_007225458.1|EFCIT----- 105
EHCITGWETLCEAQKNGGYSVDGSGFAEYVIVADAGYVAHFPPNINFTEMAP 150
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Supplementary Data

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gi|489067515|ref|WP_002977507.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGMHVAID 199
gi|495117461|ref|WP_007842278.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGMHVAID 199
gi|496380606|ref|WP_009089596.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGMHVAID 199
gi|489101178|ref|WP_003011041.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGMHVAID 200
gi|489090486|ref|WP_003000382.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGMHVAID 200
gi|494231392|ref|WP_007139094.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGMHVAID 200
gi|495788562|ref|WP_008513141.1|IICAGVTVYKGLKQTDAKSGEWVAISGIGGLGHVAVQYAKAMGFHVAID 200
gi|495785498|ref|WP_008510077.1|IICAGVTVYKGLKQTDAKSGEWVAISGIGGLGHVAVQYAKAMGFHVAID 200
mutant
gi|431798554|ref|YP_007225458.1|ILCAGVTVYKGLKETETKPGEWVAISGIGGLGHVAVQYAKAMGLHVLAVD 200

gi|489067515|ref|WP_002977507.1|VADDKLDLAKKLGADLVVNAKNTDPGEYLHKEVGGMHGALITAVSPIAFK 249
gi|495117461|ref|WP_007842278.1|VADDKLELAKKLGADLVVNAKNTDPGEYLHKEAGGMHGALITAVSPVAFK 249
gi|496380606|ref|WP_009089596.1|VADDKLELAKKLGADLVVNAKNTDPGEYLHKEVGGMHGALITAVSPIAFK 249
gi|489101178|ref|WP_003011041.1|VADDKLDLAKKLGADITVNAKNTDPGQYIQKEIGGVHGALITAVSPIAFK 250
gi|489090486|ref|WP_003000382.1|VADDKLDLAKKLGADITVNAKNTDPGQYIQKEIGGVHGALITAVSPIAFK 250
gi|494231392|ref|WP_007139094.1|IADDKLELAKKLGADLVVNAKHNQPGFELKKEVGGMHGALITAVSPIAFK 250
gi|495788562|ref|WP_008513141.1|IADDKLALAKSLGADLVVNAKEQDPGTFLLKKEVGMHGVLVTPSPVAFK 250
gi|495785498|ref|WP_008510077.1|IADDKLALAKNLGADLVVNAKEYDPGKFLKKEVGMHGVLVTPSPVAFK 250
mutant
gi|431798554|ref|YP_007225458.1|VSDDKLNLAKEGADRVVNGKNPDEVMMARKETGGVHGVLVTPSPVAFK 250

gi|489067515|ref|WP_002977507.1|QGIDVLRKRGTIALNGLPPGSFELPIFETVLKRIIVRGSIVGTRKDLQEA 299
gi|495117461|ref|WP_007842278.1|QGIDVLRKRGTIALNGLPPGSFELPIFETVLKRIIVRGSIVGTRKDLQEA 299
gi|496380606|ref|WP_009089596.1|QGIDVLRKRGTIALNGLPPGSFELPIFETVLKRIIVRGSIVGTRKDLQEA 299
gi|489101178|ref|WP_003011041.1|QGIDVLRKRGTIALNGLPPGSFELPIFETVLKRIIVRGSIVGTRKDLQEA 300
gi|489090486|ref|WP_003000382.1|QGIDVLRKRGTIALNGLPPGSFELPIFETVLKRIIVRGSIVGTRKDLQEA 300
gi|494231392|ref|WP_007139094.1|QGLALRRKGVLSLNLPGSFDLSIFDVTINGTTIRGSIVGTRKDLQEA 300
gi|495788562|ref|WP_008513141.1|QGLSALRRKGVLSLNLPGSFDLSIFDVTINGTTIRGSIVGTRKDLQEA 300
gi|495785498|ref|WP_008510077.1|QGLSALRRKGVLSLNLPGSFDLSIFDVTINGTTIRGSIVGTRKDMEEA 300
mutant
gi|431798554|ref|YP_007225458.1|QALDILLRRKGTLMNGLPPGSFDLPFETVLNRYIVRGSIVGTRKDLQEA 300

gi|489067515|ref|WP_002977507.1|LDFANEGLVKATVTPAKLEDINDVFDKMKKQIDGRIVLDIAGSN- 344
gi|495117461|ref|WP_007842278.1|LDFANEGLVKATVTPAKLEDINDVFNKMKKQIDGRIVLDIAGSN- 344
gi|496380606|ref|WP_009089596.1|LDFANEGLVKATVTSAKLEDINDVFDKMKKQIDGRIVLDIAGSNQ 345
gi|489101178|ref|WP_003011041.1|LDFANEGLVKATVTPAKLEDINSVFDQMKAGKIDGRIVLDIAG--- 343
gi|489090486|ref|WP_003000382.1|LDFANEGLVKATVTPAKLEDINSVFDQMKAGKIDGRIVLDIAG--- 343
gi|494231392|ref|WP_007139094.1|IQFAVEGKVKATITSAKLEDVNEVFDKMKKQIDGRIVLDIAG--- 344
gi|495788562|ref|WP_008513141.1|ISFALEGKVKATVHAAKLEDINTVFDKMKKGDISGRIVLEIAKPS- 345
gi|495785498|ref|WP_008510077.1|ISFALEGKVKATVHAAKLEDINTVFDKMKKGDISGRIVLEIAKPS- 345
mutant
gi|431798554|ref|YP_007225458.1|IDFAMEGKVTITVKSAPLEDINLIIDQMKKQIDGRIVLDIAGH--- 343

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Figure S5.10: Alignment 21.20-ORF3. Alignment of ORF3 of mutant 21.20 with protein sequences of the NCBI database: alcohol dehydrogenase (GroES-like protein) of *Sphingobacterium spiritivorum* ATCC 33300 (Sequence ID: ref|WP_003011041.1; max. ident. 80%), Alcohol dehydrogenase (GroES-like protein) of *Sphingobacterium spiritivorum* ATCC 33300 (Sequence ID: ref|WP_003000382.1; max ident. 80%), alcohol dehydrogenase of *Mucilagibacter paludis* (Sequence ID: ref|WP_008513141.1; max. ident. 80%), Zn-dependent alcohol dehydrogenase of *Echinicola vietnamensis* (Sequence ID: ref|YP_007225458.1; max. ident. 78%), alcohol dehydrogenase *M. paludis* (Sequence ID: ref|WP_008510077.1; max. ident. 78%), alcohol dehydrogenase of *Chryseobacterium gleum* (Sequence ID: ref|WP_002977507.1; max. ident. 79%), alcohol dehydrogenase of *Flavobacterium frigoris* (Sequence ID: ref|WP_007139094.1; max. ident. 76%), alcohol dehydrogenase of *Chryseobacterium* sp. (Sequence ID: ref|WP_007842278.1; max. ident. 79%) and alcohol dehydrogenase of *Elizabethkingia anopheles* (Sequence ID: ref|WP_009089596.1; max ident. 76%). Other BLAST hits were below 76% of maximum identity.

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gi|518785905|ref|WP_019943194.1|--MVSDFNRRIRQVFLFLIVIVLALIVFRQLYIFPPGFLGALTYILCR 48
gi|255035092|ref|YP_003085713.1|--MAPEFNRRIRQVSVFLIVLALAIIVFKQLYIFPPGFLGALTYILCR 48
mutant
gi|517459406|ref|WP_018630163.1|---MPDFNRRRQIGLLIMLVALALLLLELNGFLPGFLGALALYXLXRE 47
gi|517456390|ref|WP_018627163.1|---MPDFNVRIRKQMLTLILLGLIFLAGKELVFPFLGALGAVTYLIISR 47
-----
gi|495863164|ref|WP_008587743.1|MNVKKIDNLRQLLILLIGLIGLIFFFNLRYFTPGILGAITLYILFRK 50
gi|312131790|ref|YP_003999130.1|MASPQISNRIINQIVLIIAIVFSCLLIFHYLNYYLPGFLAATLYIVYRE 50
gi|489091523|ref|WP_003001417.1|MKYKQIHNNISINQIMLIIIIILICLIFTNLSYLLPGFLGAVTYLIILFRN 50

gi|518785905|ref|WP_019943194.1|FYFRLTEQRKWNKSLTALIFMVLFMACIVAPVYFAAQMIQKIEGIMQNP 98
gi|255035092|ref|YP_003085713.1|YFHLTEKRKWNKSLTALIFMVLFMACIVAPVYVLAQMVFKVTKGIMDNP 98
mutant
gi|517459406|ref|WP_018630163.1|LYFTLTIRKHNKMGCTALLFLAFLIGLIPVSAIHLISSKINVLHNS 97
gi|517456390|ref|WP_018627163.1|LYFTMVFPQKWSKAWVAIGYIFFYLIIFLIPFTVAVTLISPKINKALQNP 97
-----
gi|495863164|ref|WP_008587743.1|TYFRLTEERKWRRLASIFLMLTLIIVALPLWLIIEVMIPQISGLLANK 100
gi|312131790|ref|YP_003999130.1|WYFRLTEQRKWNKSLTALLIFPFSIVFVLPVLPGLDYMIPRLSVFLDNT 100
gi|489091523|ref|WP_003001417.1|AYFKMTEQRKWNKSLVSLMLVLLTVVVFVFTWGIINITYIPEMNTLIGDK 100
: . : . * : . :

gi|518785905|ref|WP_019943194.1|EQVNKAVDAISDQLREWTGQD--LLTKEATADIRKKAASFIPIGLNSSAT 146
gi|255035092|ref|YP_003085713.1|EQVNQATAAISDQLREWTGQD--LLTKEATADMGKKAAGFIPAILNSSAS 146
mutant
gi|517459406|ref|WP_018630163.1|DQIIIVVFKSASAQIEQWTGFD--MLTDQSLSDIQRQITNFIPIPSFLNSSAS 145
gi|517456390|ref|WP_018627163.1|EAIIEETGKHVITTIQNKILGR--LLSENALSDMVKRATAAIKPLLNSTIN 145
gi|495863164|ref|WP_008587743.1|QVIIEKFNVAKAFIASKPFLNRLNLSDESLLRALGKVTAYFPGLINSVAE 80
gi|312131790|ref|YP_003999130.1|QVIIEKYNAKDFLATKPIILNRLNLSDDVALGALSKVTAYFPGLINSVAE 150
gi|489091523|ref|WP_003001417.1|DSIMQKFNQVKEYMSKPVLENIDLSDEALDFLQSLTKYLPRLINSVAE 150
NDIIRKFNISKDYIATVPLKKNVDLSEELNLNLIKQLTRYLPILNSIAE 150
: : : * : :

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Supplementary Data

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gi | 374298562 | ref | YP_005050201. -----GEDKASVGDPLEIALLQAGKTKGINRKEELLEAKPE 362
gi | 492828908 | ref | WP_005982862. -----GEDKASVGDPLEIALLQAGKTKGINRKEELLEAKPE 362
gi | 428203951 | ref | YP_007082540. -----ESSNDNAIGDPMVEALLVAGAKAGIRHEALLNQFPE 358
gi | 517207403 | ref | WP_018396221. -----EGEGDETIIGDPMVEALLVAGAKAGIRHEALLNQFPE 441
gi | 527031551 | ref | WP_020880290. GGADAQDGEDGDAGDGGGSRGTGDPLEVALLAAGALGGFTREGLAATLPE 372
gi | 451946897 | ref | YP_007467492. -----EGVIVKSVGDPLEVALLAIAVAKAGMTRDLAVQFPE 370
gi | 523631912 | ref | WP_020769201. -----SGSEKEIGDPLETGLLKFARLRSDDPTKIRKMPK 336
gi | 516341118 | ref | WP_017731151. -----GQDQSGKGGEGDPLEIALLHFSKFFNSEAYQYRQLER 479
gi | 496585888 | ref | WP_009285602. -----DTDTKVEVGDPEVEALLKFAAAGQDDPIIREQYPR 358
gi | 522086765 | ref | WP_020597974. -----DGNFREVGDPEVDVSLKFAFARVGLNPDAINLEYAR 433
mutant -----
gi | 319954445 | ref | YP_004165712. -----ENKKKYLGDPIETALLQFVNTSKVNVEAINATYPR 431

gi | 374298562 | ref | YP_005050201. VREDAFDPPERALMATWHKEGDGFLVAVKGTPEVLEASTRVLGPDGEQDL 412
gi | 492828908 | ref | WP_005982862. VREDAFDPPERALMATWHREGDGLVAVKGTPEVLEASTRVLGPDGEQEL 412
gi | 428203951 | ref | YP_007082540. VREVAFSETKMMATIKHKGADGYHFVAVKGAESVLPACTHKLTPGSGQPM 408
gi | 517207403 | ref | WP_018396221. QREVAFDTEKMMATYHESDNGIRVAVKGAPEAVFEVCSHYLGTGDKQEM 491
gi | 527031551 | ref | WP_020880290. VREEAFDTEKMMATFHEQDDGLVAVKGAPEAVLAACSRVVDGGGETPL 422
gi | 451946897 | ref | YP_007467492. GI451946897ref|WP_007467492. IGEIPFDSDTKIMLTLHRNEEGNRLAKGAAEVMIIQKSKMSLSSSGEKEM 386
gi | 523631912 | ref | WP_020769201. RLHDPFDSSETMVMGTAYEEERGYYIAGKGAADALLSRCTHILVKGVEVRE 529
gi | 516341118 | ref | WP_017731151. EAERKPFSSDTRMMGTLRHTDNRVYVAVKGAEEIMD-----RCPDM 399
gi | 496585888 | ref | WP_009285602. RAEKAFSSDTRMMGTLHEHEDRYIVAVKGAEEVMN-----RSVSL 474
mutant -----
gi | 522086765 | ref | WP_020597974. IAEEFPNSETKLMATLHKNNAGNFVAAKGAVEKLIDTCSFYSAGDRVIPL 481
gi | 319954445 | ref | YP_004165712. IAEEFPNSETKLMATLHKNNAGNFVAAKGAVEKLIDTCSFYSAGDRVIPL 481

gi | 374298562 | ref | YP_005050201. TDDARDEWRRKNSEMAAQGLRVLGSAAMK-----TGDEHDDSYEDLTFGL 457
gi | 492828908 | ref | WP_005982862. TDDARDEWRRKNSEMAAQGLRVLGSAAMK-----TGNEHDDSYEDLTLGL 457
gi | 428203951 | ref | YP_007082540. SQQDRDYQERGDRMARDGLRILALAEKT-----VDNPDAPAYEQTLTFGL 453
gi | 517207403 | ref | WP_018396221. GPSEQ--WQERSTQLAQEGLRILALAEKQ-----VDNPDTEPPYKNTLVG 534
gi | 527031551 | ref | WP_020880290. SAQAREEWNVRKRLAGQGLRVLALAEHT-----VADRSAPPYEDLALLG 467
gi | 451946897 | ref | YP_007467492. TAEADRKQWSQLNEEMAAKGLRVLALAEKQ-----SNNLEEEPTAKLQLVG 465
gi | 523631912 | ref | WP_020769201. ANSDSKSWLDRADEMASSGLKVIIFGAYS-----TKRSEIDPSSSEFVFG 431
gi | 516341118 | ref | WP_017731151. TEKDKEWLRKNDMSANDLRLVLFAYQPPDELPSGEAADDPLQLNLTfVG 579
gi | 496585888 | ref | WP_009285602. DEALQKQKQRMSEQMAADGLRITLAFAYREIDE---KPGDDFADSNLTfYG 446
gi | 522086765 | ref | WP_020597974. DEKARKAQYEIAEKMAADGLRITLAFAYREIDKT--TRPGDDFADRLTfYG 523
mutant -----
gi | 319954445 | ref | YP_004165712. TEAAKDFLNRAETIEAQGSKVLAFAPKEAAN---IPTTNFLSG-LTLVG 527

gi | 374298562 | ref | YP_005050201. LYGLMDPPRKGVDKAEIKLRRAGIKVVMVTDGQPMATAKSIGETLKLNS-G 506
gi | 492828908 | ref | WP_005982862. LYGLMDPPREGVDKAEIKLRRAGIKVVMVTDGQPMATAKSIGETLKLNS-D 506
gi | 428203951 | ref | YP_007082540. ILGLLDPFRQDAKAAIEKCCQAGIRVIMLTGDPQITARQIGLAVGLTS-E 502
gi | 517207403 | ref | WP_018396221. IVGLLDPREDVKPAIKDCHDAGIRVIMVTDGQPVARNIGLAVGLTN-D 583
gi | 527031551 | ref | WP_020880290. LVGLLDPFRAQVREAEIATRAAGVRVVMVTDGQITLAKNIAKAVGIAH-D 516
gi | 451946897 | ref | YP_007467492. LVGLMDPPREDVKEALAQCREAGIRVIMATGQAVTAQAIGRAVGLVA-E 514
gi | 523631912 | ref | WP_020769201. LVGLKDPPEPEVYSALAECKTAGIKVIMITGDHPPTAKNIALELGTDPDS 481
gi | 516341118 | ref | WP_017731151. LIGFLDPPHKEVASAIEHTCRHAGIKVVMVTDGHPGTAVNIGRQVAVLVTAE 629
gi | 496585888 | ref | WP_009285602. LIGFLDPPRTEVTPALESCREAGIKVIMVTDGHPATALTARQVHLIEFG 496
gi | 522086765 | ref | WP_020597974. LIGFLDPPQTEVTPALEACRRAGIKVIMVTDGHPATALTIAKVKVLEIEFG 573
mutant -----
gi | 319954445 | ref | YP_004165712. LIGFLDPPRMEVVEALQSCRNAGIKVIMITGDHPATALNIAEKIKLSEK- 576

gi | 374298562 | ref | YP_005050201. QRPME--GKELGDPDKLSESKRRELDRDTTIFSRVTPKQKLNLIALLYQKGT 554
gi | 492828908 | ref | WP_005982862. QRPME--GKELGDPDKLSESKRRELDRDTTIFSRVTPKQKLNLIALLYQKGT 554
gi | 428203951 | ref | YP_007082540. QEAEARLGKELKNPDELSDQEKLRQVPIFARVSPKQKLNLIALLYHQAG 552
gi | 517207403 | ref | WP_018396221. QEAEARKGSDLKFLADLSSQEQNLQVPIFARVSPKQKLNLIALLYHQANN 633
gi | 527031551 | ref | WP_020880290. GVRTAE--GRELKAPEDLSDEERELLDVSVFARVSPKQKLDLIALHQAG 565
gi | 451946897 | ref | YP_007467492. DNAQVIHGLDLGRLVDLSEAEKDRITASAQLFARVSPKQKLDLIALFHQERH 564
gi | 523631912 | ref | WP_020769201. SSSKVIILGKMDPMLKELTQEQKQDWISGNIIFARVSPKQKLDLIVTLQENK 531
gi | 516341118 | ref | WP_017731151. NESEHTVLHGKLNQAEALDKNDNEKLVSTPVFSRVDPGQKLSLKHQYQQAG 679
gi | 496585888 | ref | WP_009285602. EDIALT--GRDLKPLDQLTADADRELMACRFRVAVSPTQKLDMLDIYQQQG 545
gi | 522086765 | ref | WP_020597974. EEIVLT--GKDLKPIDQLRVDEKQVNLNCRVAVVSPKQKLDMLIYQHEG 622
mutant -----
gi | 319954445 | ref | YP_004165712. DNVVIN--GKELD-----SKLSDKLFATIFARVTPKQKLVAMVSFYQKHG 620

gi | 374298562 | ref | YP_005050201. AVVAMTGDGVNDAPALKKADIGVAMGLRGTQVAREAADMVLKDDAFQTI 604
gi | 492828908 | ref | WP_005982862. AVVAMTGDGVNDAPALKKADIGVAMGLRGTQVAREAADMVLKDDAFQTI 604
gi | 428203951 | ref | YP_007082540. AVVAMTGDGVNDAPALKKADIGVAMGRGTQVAREAADMVLQDDAFSTIV 602
gi | 517207403 | ref | WP_018396221. AVVAMTGDGVNDAPALKKADIGVAMGRGTQVAREAADMVLQDDAFSTIV 683
gi | 527031551 | ref | WP_020880290. EVVAMTGDGVNDAPALKKADIGVAMGRGTQVAREAADMVLKDDAFSTIV 615
gi | 451946897 | ref | YP_007467492. AVVAMTGDGVNDAPALKKADIGVAMGLRGTQVAREASDMLKDDSFASIV 614
gi | 523631912 | ref | WP_020769201. FIVGMTGDGVNDAPALKKADIGVAMGRGTQVAREASDMLKDDSFASIV 581
gi | 516341118 | ref | WP_017731151. EIVGMTGDGVNDAPALKRANIGVAMGNVTQVAQEVSDMVLKDDAFSTIV 729
gi | 496585888 | ref | WP_009285602. DIVGMTGDGVNDAPALKKSDIGVAMGRGTQVAEAETAMVLKDDSFSTIV 595
gi | 522086765 | ref | WP_020597974. NIVGMTGDGVNDAPALKKSDIGVAMGLRGTQVAEAETADIVLKDDSFSTIV 672
mutant -----
gi | 319954445 | ref | YP_004165712. SIVAMTGDGINDAPALKKADIGVAMGRGTQVAKETAAMILKDDSFSTIV 670

gi | 374298562 | ref | YP_005050201. AAVEQGRVIFSNIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 654
gi | 492828908 | ref | WP_005982862. AAVEQGRVIFSNIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 654
gi | 428203951 | ref | YP_007082540. AAIEQGRAIFANIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 652
gi | 517207403 | ref | WP_018396221. AAVRQGRAIFANNIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 733
gi | 527031551 | ref | WP_020880290. EAVQGRVIFDNIIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 665
gi | 451946897 | ref | YP_007467492. HAVKQGRIFFNIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 664
gi | 523631912 | ref | WP_020769201. VAIRQGRAIFENIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 631
gi | 516341118 | ref | WP_017731151. KAIEQGRIFFNIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 779
gi | 496585888 | ref | WP_009285602. TAIQGRVIFENIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 645
gi | 522086765 | ref | WP_020597974. AAIAQGRVIFENIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 722
mutant -----
gi | 319954445 | ref | YP_004165712. TAIMQGRVIFFNIRKFLIYLLSGNIGGIIIVGTAILVGLPLPLPLQILF 720

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Supplementary Data

gi 374298562 ref YP_005050201.	INMLFDVFPALALGVGEGDPMVMDAPPRKPGHELVAGRHWALAGAYGLLI	704
gi 492828908 ref WP_005982862.	INMLFDVFPALALGVGEGDPMVMDAPPRKPGHELVGRHWALAGAYGLLI	704
gi 428203951 ref YP_007082540.	INAVNDVFPALALGVGEGNPQQMKHPPRPSSHEPILTRRHWGALAGYGLII	702
gi 517207403 ref WP_018396221.	LNAINDVFPALALGVGEGNPQQMHPPRDKSEAILTRSHWTAIGVYGVVI	783
gi 527031551 ref WP_020880290.	LNLVTDVFPALALGVGGQGGPIMRRPRPQGEAII TRRHWLAIGGYGLFI	715
gi 451946897 ref YP_007467492.	LNIVTDVFPALALAAGEENDNIMKNKPRDKREPIMSKIHWFVAVSYGAIM	714
gi 523631912 ref WP_020769201.	INLITDVLPALALGVTEAGPKIMDQPPRPSSEPIIDSKRWKTVFIYSFLI	681
gi 516341118 ref WP_017731151.	LNLSDVFPALALGIGPGSNIMTKPKDPREPLITRHNINIQVGVYGLLI	829
gi 496585888 ref WP_009285602.	INIITDVFPPALALGVGRENSELMKQPPRDPKQPLLSRNDWGVVYFAFVI	695
gi 522086765 ref WP_020597974.	INIVTDVFPALALGVGRENASLMERPPRDKPTIMDTRDQTVVLYALAM	772
mutant	-----	
gi 319954445 ref YP_004165712.	LNLVTDIFPALALGLGKGNLIMKIPPRNPQQPIVIKRDFWFTINVYAVLL	770
gi 374298562 ref YP_005050201.	AVCVLAAFVWALNRYGLDESEQ----AVTVSFLTLTFARMLHTFNMRDGGP	750
gi 492828908 ref WP_005982862.	AVCVLAAFVWALNRYGLDESEQ----AVTVSFLTLTFARMLHTFNMRDGGP	750
gi 428203951 ref YP_007082540.	AATILGVVIFSLRVLGLEERR----AVTISFLTLAFTRLWHVFNMRDSDS	748
gi 517207403 ref WP_018396221.	AAVVLGAFFLCLHVLGYEQNQ----AVTVSFLTLALGRLWHVFNMRDDDA	829
gi 527031551 ref WP_020880290.	TVAVLGSFAAAIHGVLGHGRE----AVSISFLTLAQLLWHVFNMRDSDS	761
gi 451946897 ref YP_007467492.	TCAVLGAIFYISLNTLNLPEKE----SVTISFLTLAQLLWHVFNMRERNS	760
gi 523631912 ref WP_020769201.	SLSLSVSVFISHYTVHRSEAWNFELCNINIFLTLIFSQTLLHAFNMGSGRS	731
gi 516341118 ref WP_017731151.	AICVSGAYYFAQEVWGAPEEII----TNNAFFSLAFAQLLHVLNMRDEAD	875
gi 496585888 ref WP_009285602.	TVAVLGVVLFGRQLGLSEGV----GNSITFYALSFAQLMHVFNLYSGKG	741
gi 522086765 ref WP_020597974.	TASVLGTYVIGTRLLGLSQDE----GNLTFYALSFTQLMHVFNLSER-	817
mutant	-----	
gi 319954445 ref YP_004165712.	ALPILLVTVWYCSHYLKYDAKL----CNNTFFSLALSQVHVLNLPFSRKI	816
gi 374298562 ref YP_005050201.	HVVVNEVTSNPFVWAALGCGALIAAALFVPSLAEILRVHEPGRGTWTLI	800
gi 492828908 ref WP_005982862.	HVVVNEVTSNPFVWAALGCGALIAAALFVPSLAEILRVHEPGRGTWTLI	800
gi 428203951 ref YP_007082540.	RLFRNEITQNRPIWALLIICITGLIILAVYVPIAQLVLLIDPPSLEQGWGLI	798
gi 517207403 ref WP_018396221.	PIFRNEVTTNPFVWVALVICTGLLLAVYIPGLAGVLIIVRPGQGWGII	879
gi 527031551 ref WP_020880290.	GLLRNDITRNPVWVALLLCLGLIAGAVFLPAAARVLGVVPTGNGWLLV	811
gi 451946897 ref YP_007467492.	GVLNNGIINKNPIWVALALCTVLLMLALYVPMANILRLANPGFGLALV	810
gi 523631912 ref WP_020769201.	GWRNSEILKNRYLWISSEFVICGLVLCIYIPVFKKALDIHEMTFFEDWSIS	925
gi 516341118 ref YP_017731151.	PVFNNQVTRNKYIWMALAFCAAAI LAAYFIPGLRSILLEQLLEPRVWGLI	925
gi 496585888 ref WP_009285602.	HFVKNEITRNRYYIWLALLTCAIMFLTYVYVFLREVLSIQPMQAAPLELI	791
gi 522086765 ref WP_020597974.	SFFINEITRNRYYVYAQMLCLALAFYFVPLHEVLMIQPMTTTEFGLI	867
mutant	-----	
gi 319954445 ref YP_004165712.	SFLNNEITRKNFTWAASLLCISIMAVFYFVSPLNYYIGLQKLSIDTWLII	866
gi 374298562 ref YP_005050201.	LAGSFTPFVVIQLGKALLGLRSREHRTQAA	830
gi 492828908 ref WP_005982862.	LAGSFTPFVVIQLGKALLGLRSREHRIQTA	830
gi 428203951 ref YP_007082540.	LGMSTIPLLIIQIWKLFKNRKFRRIAV---	825
gi 517207403 ref WP_018396221.	LGVSFVPIVFGQIWKAFKS-----	898
gi 527031551 ref WP_020880290.	LGASLFLPLAAGRIFFKRMARM-----	831
gi 451946897 ref YP_007467492.	LAMSLPLGGLLQFLHLLGRK-----	831
gi 523631912 ref WP_020769201.	IGFSLFSFCAIQIFKLLKLVTO-----	803
gi 516341118 ref YP_017731151.	VITSLLPILIIIVKIFIRKDF-----	946
gi 496585888 ref WP_009285602.	IAAGVVPVIVQVVRTLTGWGR-----	813
gi 522086765 ref WP_020597974.	LAAGFAPVLLIQVRLRLARMMK-----	889
mutant	-----	
gi 319954445 ref YP_004165712.	TFTSMTPVFLIQLFKRFKIID-----	888

Figure S5.12: Alignment 31.90-ORF2. Alignment of ORF2 of mutant 31.90 with protein sequences of the NCBI database: Ca²⁺-transporting ATPase of *Fibrisoma limi* (Sequence ID: ref|WP_009285602.1; max. ident, 65%), hypothetical protein of *Spirosoma panaciterrae* (Sequence ID: ref|WP_020597974.1; max. ident. 63%), p-type ATPase (transporting), HAD superfamily, subfamily IC of *Desulfovibrio* sp. X2 (Sequence ID: ref|WP_020880290.1; 52%), p-type ATPase, translocating of *Pleurocapsa* sp. PCC 7327 (Sequence ID: ref|YP_007082540.1; max. ident. 50%), hypothetical protein of *Nafulsella turpanensis* (Sequence ID: ref|WP_017731151.1; max. ident. 50%), putative calcium-translocating P-type ATPase, PMCA-type of *Leptospira* sp. B5-022 (Sequence ID: ref|WP_020769201.1; max. ident 49%), hypothetical protein of filamentous cyanobacterium ESFC-1 (Sequence ID: ref|WP_018396221.1; max. ident. 49%), p-type ATPase (transporting), had superfamily, subfamily ic of *Cellulophaga algicola* (Sequence ID: ref|YP_004165712.1; max. ident 48%), p-type HAD superfamily ATPase of *Desulfovibrio africanus* str. Walvis Bay (Sequence ID: ref|YP_005050201.1; max. ident. 48%), p-type ATPase, translocating of *Desulfovibrio africanus* (Sequence ID: ref|WP_005982862.1; max. ident. 48%), p-type ATPase, translocating of *Desulfocapsa sulfexigens* (Sequence ID: ref|YP_007467492.1; max. ident. 48%). Other blast hits were below 48% maximum identity.

List of Contributions

The experiments of this thesis were designed, performed and analysed by myself and I wrote this thesis on my own unless described below. This thesis was created under the guidance of Prof. Peter G. Kroth.

Chapter 2: The Authors of this chapter proofread the manuscript and Dr. Ansgar Gruber helped me to edit the picture for publication.

Chapter 3: The Authors of this chapter proofread the manuscript. Dr. Ansgar Gruber provided advice with the design of the experiments and interpretation of the results and helped me to edit the pictures for publication. Dariia Bova and Dr. Anastasiia Kryvenda performed the morphological identification of the diatom isolates and wrote the corresponding section. Dr. Dietmar Straile wrote the computer program with the R software for data analysis and helped me with statistical evaluations. Dariia Bova contributed the last section of the discussion about the hypothetical role of bacteria in the cell wall synthesis of diatom frustules.

Chapter 4: The Authors of this chapter proofread the manuscript. Prof. Bodo Philipp provided advice with the design of the experiments and interpretation of the results. Katrin Leinweber reproduced several experiments for publication under my supervision.

Chapter 5: Prof. Peter G. Kroth and Dr. Nina Jagmann proofread the manuscript. Prof. Bodo Philipp and Dr. Nina Jagmann provided advice with the design of the experiments. Annette Ramsperger and Kirstin Meyer helped me with the screening of the transposon mutants under my supervision.

Chapter 6: Prof. Peter G. Kroth proofread the manuscript. Doris Ballert designed and tested the *Achnantheidium* Medium. She further performed the test for vitamin B₁₂ requirement of *Achnantheidium minutissimum*. Matthias Buhmann established the protocol for cryopreservation and Annette Ramsperger performed the laboratory work of this section.

Chapter 7: Prof. Peter G. Kroth and Dr. Bernard Lepetit proofread this chapter.

List of Publications

- 2012** Windler M, Gruber A, Kroth PG (2012) Purification of benthic diatoms from associated bacteria using the antibiotic imipenem. *Journal of Endocytobiosis and Cell Research* 22:62-65
- 2014** Windler M, Bova D, Kryvenda A, Straile D, Gruber A, Kroth PG (2014) Influence of bacteria on cell size development and morphology of cultivated diatoms. *Journal of Phycological Research* 62: 269-281
- 2015** Windler M, Leinweber K, Rio Bartulos C, Philipp B, Kroth PG (2015) Biofilm and capsule formation of the diatom *Achnantheidium minutissimum* are affected by a bacterium. *Journal of Phycology* 51: 343–355

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