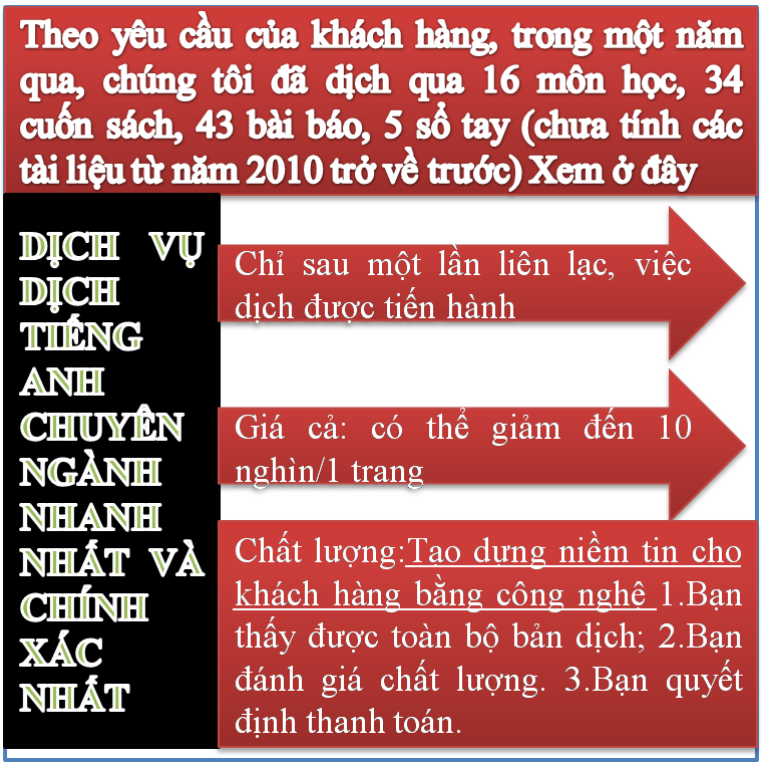

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The oceans cover large parts of the earth's surface and play an important role in the cycling of elements. The large filamentous sulfide-oxidizing bacteria are capable of forming huge microbial mats at the oxic-anoxic interface of the sediment surface, where they oxidize sulfide using either oxygen or nitrate as electron acceptor. Thereby, they can strongly influence and connect the different nutrient cycles. The water column above is populated by planktonic bacteria, which account for a large fraction of biomass on earth. Consequently, these organisms also strongly influence the turnover of nutrients in the oceans.	Các đại dương bao phủ phần lớn diện tích bề mặt trái đất và đóng vai trò quan trọng trong chu trình của các nguyên tố. Vi khuẩn oxy hoá sunfua dạng sợi lớn có khả năng hình thành các thảm vi khuẩn lớn ở mặt phân cách oxy-thiếu ôxy của bề mặt trầm tích, ở đó, chúng oxy hoá sunfua dùng oxy hoặc nitrat như chất nhận điện tử. Do đó, chúng có tác động mạnh mẽ và kết nối các chu trình dưỡng chất khác nhau. Cột nước bên trên là nơi cư trú của vi khuẩn phù du, chúng chiếm phần lớn sinh khối trên trái đất. Do đó, những sinh vật này cũng ảnh hưởng mạnh mẽ đến sự luân chuyển dưỡng chất trong các đại dương.	
The first part of this thesis (Chapter 2) addresses the physiology and mat formation	Phần thứ nhất của luận văn này (Chương 2) đề cập đến đặc tính sinh lý học và các quá trình hình	

processes of the large sulfide-oxidizers belonging to the genus Beggiatoa. Until now, it was assumed that nitrate as an alternative electron acceptor is crucial for the migration of marine Beggiatoa spp. into deeper anoxic sediment layers. We found that a subpopulation of the investigated Beggiatoa filaments actively migrates into anoxic, sulfidic layers as a reaction to high sulfide fluxes without the presence of nitrate. Our experiments show that the reason for this so far unknown migration behavior seems to be excessive storage of elemental sulfur and organic carbon due to high sulfide fluxes, which leads to filaments extremely filled with storage compounds that tend to break easily at this stage. By moving into anoxic regions, aerobic sulfide oxidation is stopped and storage space is emptied by reducing the stored sulfur with carbon reserve compounds. The investigated sulfide-oxidizer (Beggiatoa sp.) depends on the presence of a small hetero-trophic bacterium (Pseudovibrio sp.). This association is investigated in the second part of this thesis (Chapter 3). The associated Pseudovibrio sp. mainly populates the oxic part of	thành thảm của các tác nhân oxy hoá sunfua lớn thuộc chi Beggiatoa. Đến nay, người ta giả sử rằng nitrat là một chất nhận electron khác rất quan trọng cho sự di cư của Beggiatoa spp. biển vào các lớp trầm tích thiếu oxy sâu hơn. Chúng tôi thấy rằng một quần thể sợi Beggiatoa đang nghiên cứu di cư vào các lớp sunfua, thiếu oxy như một phản ứng với các luồng sunfua cao mà không cần nitrat. Thí nghiệm của chúng tôi cho thấy rằng nguyên nhân của việc này xuất phát từ các hành vi di cư chưa được biết đến hiện nay có vẻ là một nguồn dự trữ quá nhiều lưu huỳnh nguyên tố và cacbon hữu cơ do luồng sunfua cao, dẫn đến các sợi được nạp đầy với các hợp chất dự trữ có khuynh hướng vỡ dễ dàng ở giai đoạn này. Khi di chuyển vào các vùng thiếu oxy, quá trình oxy hoá sunfua hiếu khí dừng lại và không gian lưu trữ được dọn sạch do sự khử lưu huỳnh dự trữ với các hợp chất lưu trữ các bon. Các tác nhân oxy hoá sunfua đang nghiên cứu (Beggiatoa sp.) phụ thuộc vào sự hiện diện của một loại vi khuẩn dị dưỡng nhỏ (Pseudovibrio sp.). Chúng tôi nghiên cứu sự kết hợp này trong phần thứ hai của luận án này (Chương 3). Pseudovibrio sp. kết hợp chủ yếu sống trong phần có	

the gradient co-culture. This suggests that these bacteria are mainly required for the oxic growth of the Beggiatoa sp. and might protect them from oxidative stress, as Beggiatoa spp. are typically known to lack the gene encoding for the enzyme catalase. Supporting this hypothesis, we found that the genome of the accompanying Pseudivibrio sp. possesses several genes for enzymes involved in the protection against reactive oxygen species.	oxy của môi trường nuôi cấy hai chất gradient. Điều này cho thấy rằng những vi khuẩn này cần thiết cho sự tăng trưởng có oxy của Beggiatoa sp. và có thể bảo vệ chúng khỏi stress oxy hoá (tình trạng kích phản ứng oxy hoá), vì Beggiatoa spp. thường được xem là thiếu các gen mã hóa enzyme catalase. Ủng hộ giả thuyết này, chúng tôi thấy rằng bộ gen của Pseudivibrio sp. đi kèm có một vài gen cho các enzyme tham gia vào quá trình bảo vệ chống lại các gốc oxy hoá hoạt động	
In contrast to the large Beggiatoa sp., the associated Pseudovibrio sp. is able to grow in pure culture. Besides heterotrophic growth on organic-rich media, the bacteria are also able to grow under extremely oligotrophic (nutrient-poor) conditions. A detailed analysis of the substrate use under oligotrophic conditions revealed that Pseudovibrio sp. grows on organic contaminations preferentially containing nitrogen (Chapter 4). Interestingly, we could isolate further facultatively oligotrophic bacteria from water overlaying Namibian sediments, which are known to inhabit many different large sulfide-oxidizers.	Trái với Beggiatoa sp. lớn, Pseudovibrio sp. liên kết có thể phát triển trong môi trường nuôi cấy thuần. Bên cạnh quá trình tăng trưởng dị dưỡng trên môi trường giàu hữu cơ, vi khuẩn cũng có thể phát triển trong điều kiện cực kỳ oligotrophic (nghèo dinh dưỡng). Phân tích chi tiết về việc sử dụng chất nền trong điều kiện nghèo dinh dưỡng cho thấy rằng Pseudovibrio sp. phát triển trên các chất nhiễm bẩn hữu cơ chứa nhiều nitơ (Chương 4). Điều thú vị là, chúng ta có thể phân lập thêm các vi khuẩn nghèo dinh dưỡng không bắt buộc từ trầm tích ở Namibia trong nước, từng là nơi sinh sống của nhiều tác nhân oxy hoá sunfua lớn khác nhau.	
„Science is built up of facts, as a house is built of stones; but an accumulation of facts is no more	Khoa học được xây dựng từ các sự kiện; nhưng nó không đơn thuần là sự tích luỹ các sự kiện	

a science than a heap of stones is a house.“ Chapter 1 General introduction Marine element cycles Nutrients are chemical compounds that are required for the metabolism of living organisms and have to be taken up from the environment. Bacterial nutrition includes both organic and inorganic molecules. The turnover of the individual elements in these nutrients is referred to as ‘element cycling’. The marine carbon cycle ~~Carbon is the major element of~~ cellular material (Battley, 1995). In the model organism Escherichia coli, for instance, the amount of cellular carbon accounts for 48 to 59% of the dry weight (Battley, 1995; Norland et al., 1995). The production of new organic material, also referred to as primary production, takes place in the ocean mainly via photosynthesis. In this process, Cacbon điôxít from the atmosphere is fixed to form new organic matter (Figure 1.1) using the energy from sunlight. Primary production is the main source of dissolved organic carbon (DOC) in the open ocean, which occurs within the euphotic zone (Hansell et al., 2009). An additional source of DOC is terrestrial organic carbon that is	cũng giống như một đóng gạch chưa hẳn là một ngôi nhà. [redacted] mẫu và các cộng sự	

transported into the ocean by rivers and serves likewise as a fixed carbon source for marine microorganisms (Schlunz and Schneider, 2000), but accounts for only a minor fraction. The rate of primary production in the ocean surface waters generally controls the flux of organic matter towards the sediment (Suess, 1980; J0rgensen, 1983). Sinking to the bottom of the ocean, the fixed organic material is degraded and transformed by microorganisms and chemical processes.

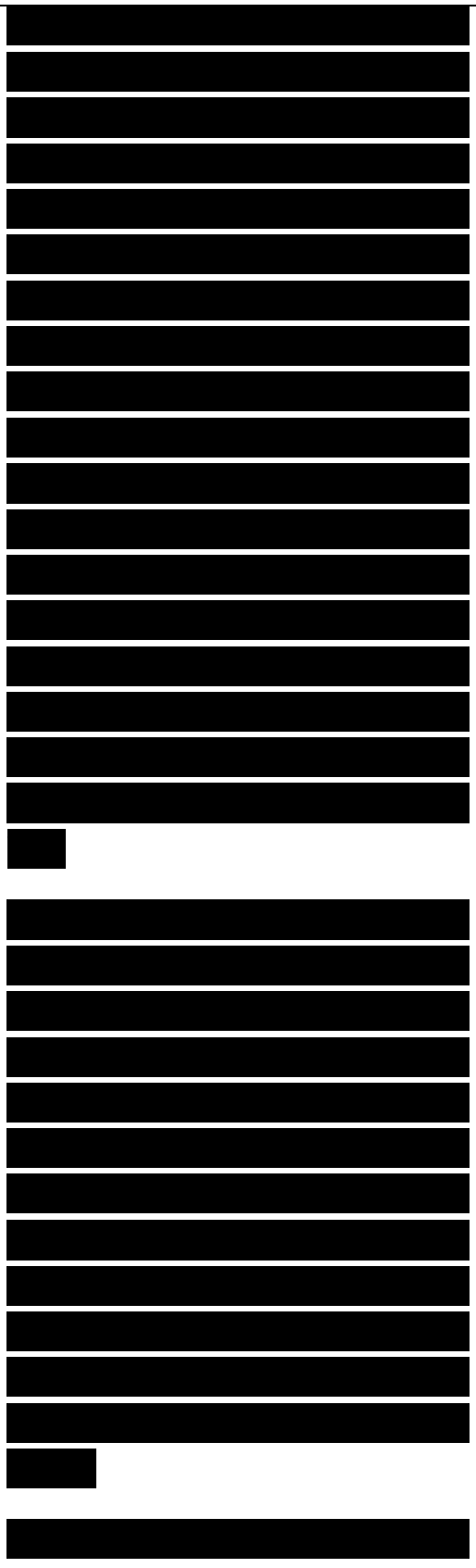


Figure 1.1: The oceanic carbon cycle. Cacbon điôxít from the atmosphere is fixed into organic carbon which can sink down to the seafloor as particulate organic matter (POM). The labile dissolved organic matter (LDOM) can be respired to CO2 and the recalcitrant dissolved organic matter (RDOM) is inert to bacterial breakdown. (Image redrawn from Jiao et al., 2010 and references therein)

The organic matter in the ocean

can be divided in particulate organic matter (POM) and dissolved organic matter (DOM). Part of the POM pool sinks down to the seafloor where it can be stored for long periods of time (Figure 1.1, Ducklow et al., 2001). The DOM pool consists of labile dissolved organic matter (LDOM) and recalcitrant dissolved organic matter (RDOM). The LDOM fraction can partly be transformed by microorganisms, thereby, LDOM is oxidized by heterotrophic microorganisms within days forming again Cacbon điôxít. Molecules like amino acids and monosaccharides as part of the LDOM fraction can easily be utilized by the marine bacterioplankton (Bauer et al., 1992; Cherrier et al., 1996; Kirchman et al., 2001) and make up 75% of the DOC that is consumed by marine microorganisms in the upper layers of the ocean (Cherrier and Bauer, 2004). The RDOM, on the other hand, is assumed to be resistant to biological degradation and can be stored in the ocean for millennia (Figure 1.1, Bauer et al., 1992; Kirchman et al., 2001; Hopkinson and Vallino, 2005; Jiao et al., 2010). The composition of dissolved organic matter in the ocean is highly diverse and DOM can

consist of thousands of different organic compounds of which only few (<10%) have yet been identified with specific molecular formulas (Koch et al., 2005; Hertkorn et al., 2006; Dittmar and Paeng, 2009).

The marine sulfur cycle

Sulfur makes up only about 1% of the cellular dry weight (Battley, 1995), however, it is essential for the formation of amino acids (cysteine, methionine) and vitamins (biotine). In most marine environments, sulfur is not a limiting factor due to the high sulfate concentration of 28 mmol L-1 in seawater (Volkov and Rozanov, 1983). In the marine environment, sulfur can be found in varying oxidation states ranging between [-2] and [+6] (Figure 1.2). The potential to transform between the different oxidation states represents the importance of this element as it can serve as an electron donor or acceptor in various key redox reactions.

Figure 1.2: Different oxidation states of the element sulfur ranging from [+6] to [-2]. (Image

adapted from Chameides and Perdue, 1997) In marine sediments, alternative electron acceptors, like sulfate, are present below the oxygen penetration depth. In anoxic layers, sulfate is used by microorganisms to oxidize organic and inorganic electron donors while reducing sulfate to sulfide. In coastal marine sediment from Aarhus Bay (Denmark) sulfate reduction takes place below 4 cm depth, which was concluded from hydrogensulfide (H2S) production (J0rgensen and Nelson, 2004). These anoxic sediment layers are, therefore, characterized by an upwards directed sulfide fulx. When sulfide reaches the oxic-anoxic interface and reacts with oxygen it gets oxidized back to sulfur or sulfate either chemically or biologically. The biological oxidation mediated by bacteria, for example of the genus Beggiatoa, was shown to be three times faster than the chemical oxidation (Nelson et al., 1986a). Due to the formation of large bacterial mats in certain habitats, the sulfide-oxidizing bacteria Beggiatoa spp. have a huge potential to oxidize large amounts of the upwards diffusing sulfide in these areas (J0rgensen, 1977), thereby strongly influencing the marine	[redacted]	

sulfur cycle.

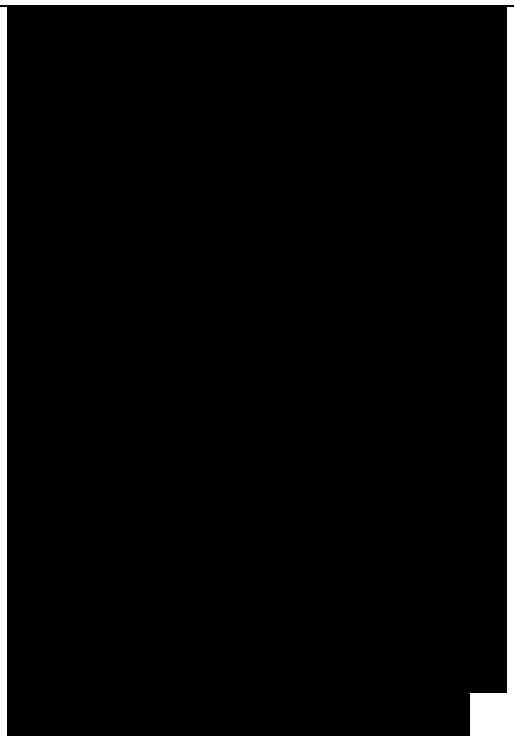
The marine nitrogen cycle

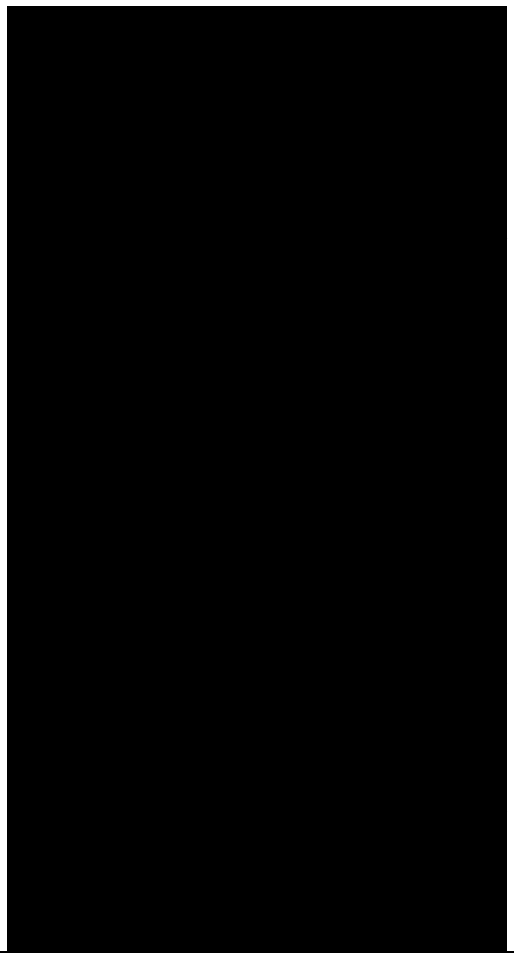
Nitrogen, as a component of proteins and nucleic acids, is a fundamental molecule of life and cellular material consists to about 15% of nitrogen (Battley, 1995). The major nitrogen reservoir is the atmosphere, consisting of 78% nitrogen in the form of N2 gas (Fiadeiro, 1983). Only few microorganisms have the ability to fix the atmospheric N2 and make it available also for other organisms. Nitrogen fixation is an energy consuming process since N2 is triplebonded and has to be cleaved during the fixation process. Thereby, nitrogen gets reduced and is present in organisms in the most reduced form, the particulate organic nitrogen (PON, Figure 1.3). The PON can be remineralized to ammonia. Nitrifying microorganisms are able to oxidize ammonia aerobically to nitrate over nitrite, which is a process mediated by two metabolically different groups of bacteria. The formed nitrate can be used as electon acceptor in anaerobic environments (Figure 1.3), for example by the large sulfur bacteria of the genus Beggiatoa. Thereby, nitrate is reduced back to ammonia (dissimilatory nitrate reduction to ammonia = DNRA)

or to gaseous nitrogen compounds (denitrification). Denitrification removes fixed nitrogen from the system, because the gaseous end-product N2 needs to be fixed again by microorganisms to make it biologically available. Besides denitrification, fixed nitrogen can also be removed from the system by anaerobic ammonium oxidation (anammox). During this process, ammonia is anaerobically oxidized to N2 using nitrite as electron acceptor (Strous et al., 1999). Figure 1.3: The marine nitrogen cycle. Nitrogen from the atmosphere is fixed into particulate organic nitrogen (PON) which can be remineralized to ammonia. Ammonia can be either oxidized aerobically to nitrate or anaerobically with nitrite (anammox) producing N2 and removing fixed nitrogen from the system. Nitrate can also be reduced to gaseous nitrogen compounds (denitrification) that leave the system. (Image based in part on Arrigo, 2005; and is reproduced from Francis et al., 2007) Connection of marine element cycles The cycling of the elements ranges from the turnover of single molecules to entire	[redacted]	

pathways occurring in living cells, thereby connecting all element cycles. The element cycling of individual cells does eventually influence the entire ecosystem on a broad scale (Bolin et al., 1983). Microorganisms are composed of many different elements, such as carbon, nitrogen, sulfur, phosphorus, oxygen, hydrogen and many microelements like iron or magnesium (Battley, 1995). As a consequence, the new production or decomposition of biomass will automatically connect the different element cycles.

The marine element cycles are, furthermore, connected by the diverse metabolisms of bacteria. Redox reactions always combine the reduction of an electron acceptor with the oxidation of an electron donor. In nearly all cases, electron acceptor and donor are composed of different elements. Denitrification, which is the reduction of nitrate (NO3-) to molecular nitrogen (N2, N-cycle), for example can be coupled to the oxidation of organic carbon compounds (C-cycle) or the oxidation of reduced inorganic sulfur compounds (S-cycle). Additionally, both organic carbon and inorganic reduced sulfur compounds can also be oxidized using oxygen (O-cycle)





as an electron acceptor. This is only an excerpt of many metabolic pathways connecting the cycling of the single elements, including different electron donors (e.g. sulfide, hydrogen, organic material) and electron acceptors (e.g. oxygen, nitrate, sulfate).

In marine habitats, the mineralization of organic matter, such as dead organic material consisting of many different elements, is an important process combining nutrient cycles. In pelagic regions, this mainly occurs in the water column by the metabolic activity of free-living bacteria (Azam and Hodson, 1977; Tabor and Neihof, 1982; Ishida et al., 1989). There, nutrient hotspots exist, such as marine snow particles that contain high amounts of organic matter. Bacteria densely aggregate on these particles (e.g. Smith et al., 1992; Azam and Malfatti, 2007 and references therein) and can achieve high growth rates (e.g. Alldredge et al., 1986; Ki0rboe and Jackson, 2001). In contrast, organic matter remineralization in shallow waters, such as fjords or continental shelfs, takes mainly place in the sediment. Thus, depending on the water depth, these are the substantial regions for nutrient cycling in the marine environment

(J0rgensen, 1983).

The connection of nutrient cycles in marine sediments (reviewed in J0rgensen, 1983) involves a cascade of transformation processes. Aerobic degradation of organic material in shallow marine sediments takes place within a thin layer at the sediment surface, where the oxidation of organic matter to Cacbon điôxít occurs. Below this oxic zone, anaerobic processes take place that successively oxidize the residual organic matter via different metabolic pathways by diverse microorganisms. From the top sediment layers to the deeper regions, the electron acceptor used is determined by its energy yield per mole carbon being oxidized. From top to bottom, the preferred electron acceptor gradually decreasing from oxygen to Cacbon điôxít via nitrate, iron, manganese and sulfate, combining the C-cycle to the N-, Fe-, Mn- and S-cycle (J0rgensen, 1983). Most importantly in the anoxic regions are, therefore, the highly abundant inorganic nitrogen and sulfur compounds, which are concomitantly reduced to N2 and H2S. Reduced substances, such as sulfide and methane that are

produced in deep sediment layers diffuse upwards and become oxidized to form sulfate and Cacbon điôxít, thereby closing the cycling of elements (J0rgensen, 1983).

Sulfide-oxidizing bacteria of the genus Beggiatoa

More than two centuries ago, bacteria of the genus Beggiatoa were discovered (Vaucher, 1803). They were originally described as Oscillatoria alba because they feature a similar filamentous morphology as the cyanobacteria of the genus Oscillatoria, but have a whitish appearance instead of the blue-green pigments (Figure 1.4). About 40 years later, these colorless sulfur bacteria were reclassified as Beggiatoa alba, named after the Italian scientist F. S. Beggiato (Trevisan, 1842). Based on their morphology, different filamentous sulfur bacteria were assigned to the genus Beggiatoa. Several species were differentiated on the basis of filament diameter size classes ranging between 1-55 |im (Vaucher, 1803; Trevisan, 1842; Hinze, 1901; Klas, 1937). However, only a small number of 16S rDNA sequences were available until recently, which made it difficult to phylogenetically classify the large sulfur bacteria. It was even found that filaments with a

similar morphology belong to phylo- genetically different genera (Ahmad et al., 1999; Ahmad et al., 2006). In a single-cell 16S rDNA gene sequencing approach of large sulfur bacteria, Salman et al. (2011) strongly extended the amount of available sequences and proposed based on phylogenetic analysis new candidatus genera names for the members of the family Beggiatoaceae. According to this reclassification, the genus Beggiatoa contains aerobic or microaerophilic filamentous bacteria with a diameter of 1-9 |im.

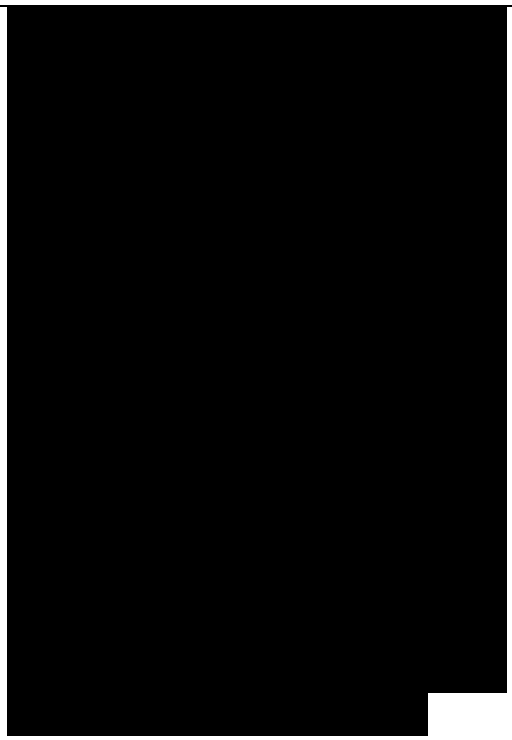


Figure 1.4: Bright field micrographs of filamentous bacteria of the genera (A) Oscillatoria and (B) Beggiatoa from a freshwater enrichment culture. (Image reproduced from Bondarev, 2007)

Mat-formation and physiology of Beggiatoa spp.

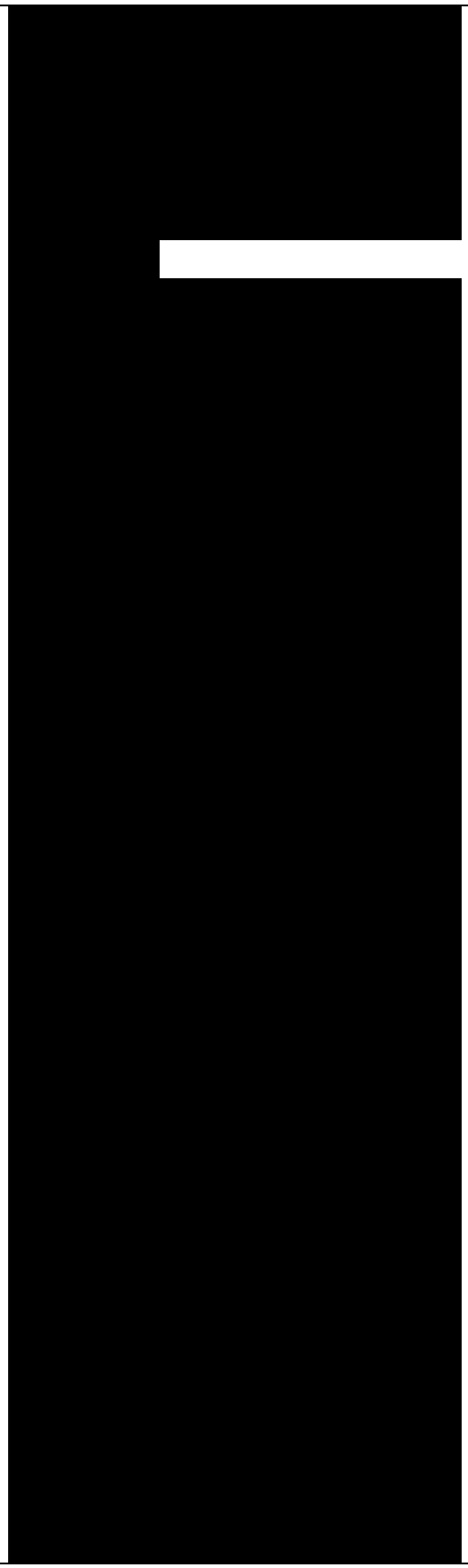
Filaments of the genus Beggiatoa can be several centimeters long and move by gliding. Pores on the surface of Beggiatoa filaments are arranged as spirals and are assumed to be involved in the gliding motility by the excretion of slime (Larkin and Henk, 1996). This spatial flexibility allows the Beggiatoa filaments to position themselves in the chemical microenvironment of sediments.

As a consequence, Beggiatoa are able to form mats in different habitats, such as sulfidic marine and freshwater sediments (Winogradsky, 1887; J0rgensen, 1977; Nelson and Castenholz, 1982; McHatton et al., 1996), activated sludge (Farquhar and Boyle, 1971), hot vents (Nelson et al., 1989), cold seeps (Barry et al., 1996) and in hypersaline lakes (Hinck et al., 2007).

Beggiatoa filaments usually form a distinct mat in the transition zone of oxygen and sulfide (Winogradsky, 1887; Keil, 1912; J0rgensen, 1977). Beggiatoa spp. oxidize the upwards diffusing sulfide, via elemental sulfur to sulfate using oxygen as electron acceptor (Winogradsky, 1887; Nelson and Castenholz, 1981). The consumption of oxygen and sulfide by the bacteria steepens the gradients of oxygen and sulfide and narrows the transition zone to a few micrometer (Figure 1.5, Nelson et al., 1986a).

Figure 1.5: H2S and O2 microprofiles in (A) an uninoculated control medium and (B) an inoculated Beggiatoa culture. In the uninoculated medium, O2 and H2S gradients overlap, whereas in the culture the bacteria form a mat between the opposing gradients (shaded area) and steepen the gradients

by aerobic sulfide oxidation and raise the overlapping zone to 2.5 mm. (Image reproduced from Nelson et al., 1986a)



Simulating the natural habitat of the Beggiatoa, agar-based oxygen-sulfide gradient media are used to cultivate these large sulfide-oxidizers (Nelson et al., 1982; Nelson and Jannasch, 1983). The formation of a distinct mat of Beggiatoa filaments between their electron acceptor and donor depends on different parameters. Besides the fact that both oxygen and sulfide are essential for the growth of the bacteria, each of these substances is also toxic if present in higher concentrations. Exceeding 5% air saturation, oxygen induces a phobic reaction of Beggiatoa filaments (M0ller et al., 1985). In contrast, long-lasting depletion of oxygen causes filaments to move into the direction of the oxygen source (Winogradsky, 1887; M0ller et al., 1985). The concentration of oxygen, therefore, defines the upper border of the Beggiatoa mat. The lower border of the Beggiatoa layer is defined by the sulfide flux from below. With increasing sulfide flux the Beggiatoa filaments position themselves at higher layers in the

agar-based gradient culture tubes (Figure 1.6, Nelson and Jannasch, 1983) and if sulfide exceeds a critical concentration, the filaments die (Winogradsky, 1887). Additionally, a phobic reaction of Beggiatoa filaments towards light was observed and thus light might also influence the gliding direction and consequently the position of the Beggiatoa mat (Winogradsky, 1887; Nelson and Castenholz, 1982; M0ller et al., 1985).

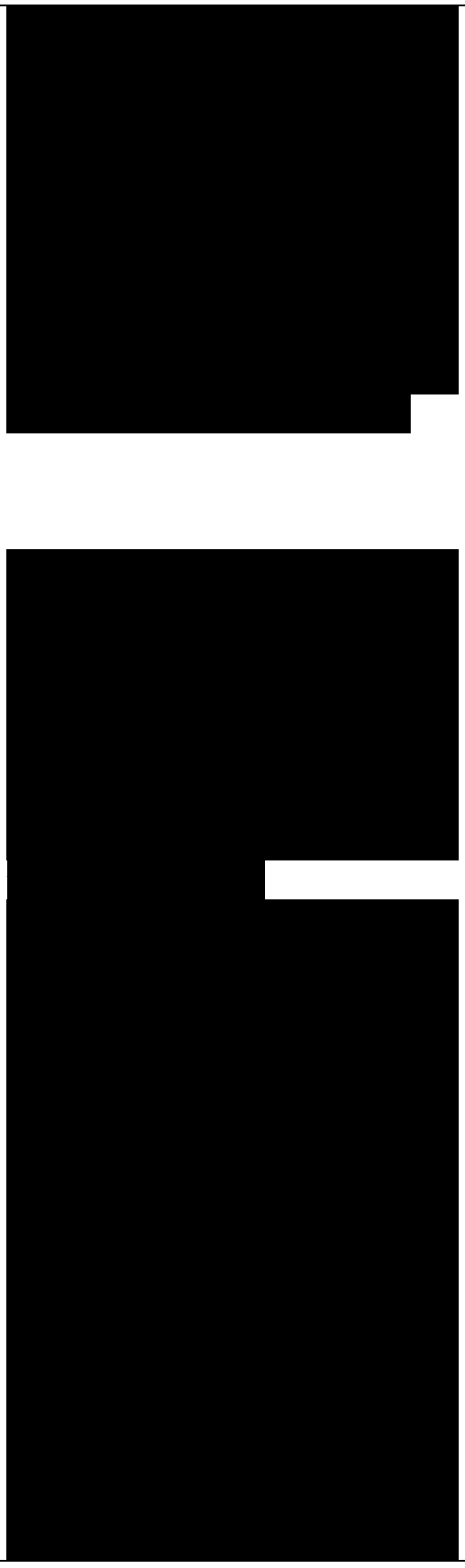


Figure 1.6: Position of Beggiatoa cell layers (mats) in culture tubes with different sulfide concentrations in the bottom agar plug. With in-creasing sulfide, the filaments form a mat located higher in the culture tube. (Image reproduced from Nelson and Jannasch, 1983)

[NogS] in agar plug (mM

Elemental sulfur, which is the intermediate of sulfide oxidation, can be stored inside the Beggiatoa cells (Winogradsky, 1887) and leads to the whitish appearance of the filaments. Using electron microscopy, it was shown that the sulfur globules in the cells are surrounded by the cytoplasmic membrane and are located in the periplasm (Figure 1.7 A, Strohl et al., 1982). The intracellular sulfur can serve as an electron donor and be further oxidized to sulfate when sulfide gets limited

in the environment (Winogradsky, 1887). In addition to the storage of sulfur, Beggiatoa have the ability to store polyhydroxyalkanoates (PHA, sometimes specifically denoted as poly-P-hydroxybutyric acid [PHB]) in the cytoplasm of the cell (Figure 1.7 A, Pringsheim, 1964; Strohl and Larkin, 1978; Strohl et al., 1982). The amount of PHA in the cell can account for up to 50% of the dry weight of the cell (Gude et al., 1981). Furthermore, an accumulation of polyphosphate in Beggiatoa cells was shown by transmission electron microscopy and different staining methods (Figure 1.7 C, Maier and Murray, 1965; Strohl and Larkin, 1978; de Albuquerque et al., 2010; Brock and Schulz-Vogt, 2011).

About two decades ago, extremely large marine filamentous sulfur bacteria (116-122 |im in diameter) containing a central vacuole were found and identified as Beggiatoa spp. based on morphological similarities to these organisms (Figure 1.7 B, Nelson et al., 1989). Few years later, the storage of nitrate, an alternative electron acceptor, was detected within the vacuoles of these large filaments (McHatton et al., 1996). It was proposed that the

oxidation of sulfide can be coupled to either DNRA (Sayama, 2001; Sayama et al., 2005) or denitrification (Sweerts et al., 1990). The storage of nitrate allows the filaments to inhabit deeper anoxic sediment layers. Carrying nitrate down into anoxic sediment layers and use it for sulfide oxidation can lead to the separation of oxygen and sulfide gradients over several centimeters (MuBmann et al., 2003; Sayama et al., 2005; Kamp et al., 2006).

This life strategy enables large, vacuolated sulfur bacteria like Beggiatoa spp. to outcompete non-vacuolated, non-motile sulfide-oxidizers in anaerobic environments. Close relatives of Beggiatoa, like bacteria belonging to the candidate genus "candidatus Marithioploca", also use and store nitrate and even show a positive chemotactic response towards nitrate (Huettel et al., 1996; reclassified by Salman et al., 2011).

Thus, the orientation and mat formation of the vacuolated nitrate- storing sulfur bacteria may also be influenced by the nitrate flux.

Studying the physiology of Beggiatoa, Winogradsky (1887) developed the concept of chemolithotrophy. He observed

that the growth of Beggiatoa was dependent on reduced inorganic sulfur compounds but not on the presence of organic compounds. The utilization of CO2 as a sole carbon source was later confirmed by isotope-labeling studies (Nelson and Jannasch, 1983). Besides these chemolithoautotrophic strains, many chemoorganoheterotrophic Beggiatoa strains were isolated (Strohl and Larkin, 1978; Gude et al., 1981; Strohl et al., 1981), which are able to oxidize sulfide only in the presence of organic compounds. Furthermore, also mixotrophic Beggiatoa strains were isolated (Pringsheim, 1967; Gude et al., 1981) thus reflecting the diverse metabolisms present within the genus Beggiatoa.

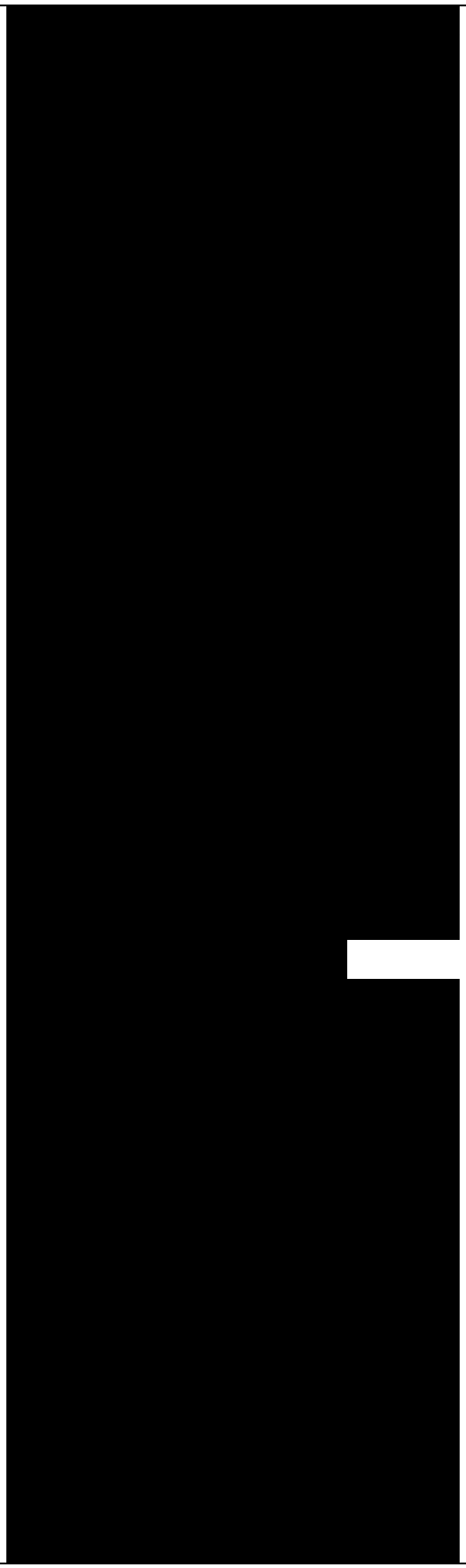


Figure 1.7: Cell structures of Beggiatoa filaments. (A) Schematic representation of Beggiatoa alba strain B15LD indicating the location of sulfur globules [S] in the periplasm and poly-P-hydroxybutyrate [PHB] in the cytoplasm. (B) Transmission electron micrograph of a Beggiatoa sp. cross section. The cytoplasm of this large Beggiatoa filament is restricted to the edge of the cell and the interior mainly consists of a large central vacuole. (C)

Transmission electron micrograph showing electron-dense inclusion bodies in the cytoplasm of Beggiatoa filaments probably consisting of polyphosphate [P]. (Images adapted and reproduced from Strohl et al., 1982 [A]; Nelson et al., 1989 [B]; de Albuquerque et al., 2010 [C])

The investigated Beggiatoa sp. co-culture

The marine Beggiatoa sp. strain 35Flor investigated in this thesis was isolated in 2002 from a microbial community associated with scleractinian corals suffering from black band disease off the coast of Florida. This Beggiatoa sp. strain grows under chemolithoautotrophic conditions in an agar-stabilized oxygen-sulfide gradient medium gaining energy from the aerobic oxidation of sulfide. Both, a fixed carbon and a fixed nitrogen source are absent from the medium and nitrogen fixation in the investigated Beggiatoa sp. was determined earlier (Henze, 2005). Typical storage compounds of the genus Beggiatoa, such as sulfur, PHA and polyphosphate were found in the investigated filaments (Schwedt, unpublished data, Brock and Schulz-Vogt, 2011). A central vacuole is present (Kamp et al., 2008; Brock and

Schulz- Vogt, 2011), but the storage of nitrate could not be detected (Schwedt et al., unpublished data).

The Beggiatoa sp. strain 35Flor is accompanied by only one type of organism (Bachmann, 2007), the Pseudovibrio sp. strain FO-BEG1. Unlike the Beggiatoa sp., the associated bacteria are able to grow in pure culture and could be isolated in artificial seawater medium. The investigated Pseudovibrio sp. is able to grow in pure artificial seawater medium under extreme nutrient-poor conditions (Bachmann, 2007) and thus belongs to the few so far cultured extremely oligotrophic organisms.

Bacterial growth under nutrient deficiency

The term ‘oligotroph’ was introduced by Weber (1907) to describe an organism growing under nutrient deficiency as opposed to that, bacteria growing under nutrient affluence are called ‘eutrophs’ (organisms living in nutrient-rich environments are sometimes also referred to as ‘copiotrophs’). Over time, several definitions of oligotrophy arose and today it is generally accepted that bacteria are referred to as oligotrophic

when they are able to grow in medium containing less than 0.5 mg C L-1 (e.g. Ishida et al., 1989). When their growth is inhibited by high substrate concentrations, the bacteria are considered to be obligately oligotrophic, which is in contrast to facultatively oligotrophic bacteria, which are able to grow under both nutrient-poor and nutrient-rich conditions (Ishida et al., 1989). Facultative oligotrophs are, therefore, successful in environments with changing nutrient conditions.

The open ocean, covering large parts of the earth's surface, is low in nutrients and contains less than 1 mg DOC in 1 L seawater (Schut et al., 1997; Hansell et al., 2009). Thus, it is denoted as an oligotrophic environment. 75% of the carbon consumed by the bacteria in the ocean can be composed of dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA) and monosaccharides. The utilization of these substances can cover 5 to 93% of the carbon demand of the bacteria and 9 to 100% of the nitrogen demand (Fuhrman, 1987; J0rgensen, 1987; Stanley et al., 1987; Keil and Kirchman, 1999; Cherrier and Bauer, 2004).
Attached and free-living marine

bacteria The particulate organic matter (POM) is an important part of the organic matter in the ocean. Particles larger than half a millimeter are so-called marine snow particles (Suzuki and Kato, 1953; Silver et al., 1978). Besides the larger marine snow particles, there are also smaller microaggregates (Figure 1.8 A and B) and both consist of detrital organic and inorganic matter (Azam and Long, 2001), thereby representing hotspots of high nutrient concentration. The aggregates can be colonized by metazoans (e.g. Shanks and Edmondson, 1990; Ki0rboe, 2000), protozoans (e. g. Silver et al., 1978) and prokaryotes (e. g. Alldredge et al., 1986; Smith et al., 1992; Azam and Malfatti, 2007 and references therein), whereas only the latter was found on all types of aggregates studied so far. Extracellular hydrolytic enzymes produced by aggregate-associated bacteria can convert the POM of the sinking aggregates into cell biomass and non-sinking dissolved organic matter (DOM) (Smith et al., 1992; Grossart et al., 2007). While sinking down the particles leave behind a DOM plume that is composed mainly of carbon and nitrogen. The DOM plume is colonized by some of the attached bacteria but	[redacted]	

also by free-living bacteria from the surrounding water (Figure 1.8 C, Azam and Long, 2001; Ki0rboe and Jackson, 2001). Compared to the surrounding water, bacterial cell densities on aggregates are typically >100 times higher (e. g. Smith et al., 1992; Turley and Mackie, 1994). Nevertheless, the particle-associated bacteria account only for <5% of the total bacterial numbers in seawater (e.g. Alldredge et al., 1986; Alldredge and Gotschalk, 1990; Turley and Stutt, 2000) and contribute to only 3 to 12% of the total bacterial production (Alldredge et al., 1986; Turley and Stutt, 2000). Even though the total activity is low, the per cell activity of the attached bacteria is higher compared to free-living bacteria, as demonstrated by higher incorporation rates and shorter doubling times (Alldredge et al., 1986; Alldredge and Gotschalk, 1990; Smith et al., 1992; Azam and Long, 2001; Ki0rboe and Jackson, 2001). Furthermore, some studies have shown that the free-living bacteria may either starve and not be active (Boylen and Ensign, 1970; Novitsky and Morita, 1976; 1977), while other studies show that they may be	[redacted]	

metabolically active (Azam and Hodson, 1977; Tabor and Neihof, 1982; Ishida et al., 1989).

Figure 1.8: In situ photographs of (A) a marine snow aggregate in a pelagic environment and (B) micro-aggregates in a shallow environment (photos M. Lunau). (C) Scheme of a marine snow particle colonized by bacteria which excrete hydrolytic enzymes converting marine snow into DOM forming a plume behind the sinking aggregate that is also colonized by attached and free-living bacteria. The DOM consists mainly of carbon [C] and nitrogen [N]. (Images adapted and redrawn from Azam and Long, 2001 [C]; and reproduced from Simon et al., 2002 [A and B])

The majority of the free-living bacteria in the open ocean is exposed to extremely low nutrient concentrations and many different survival strategies have evolved to cope with this nutrient limitation. These strategies include concentration-independent enzyme production (cells are considered to be prepared and have enzymes ready for substrates becoming available), derepression of substrates (the use of one substrate is not repressed by another more efficient one) and

the use of multiple substrates simultaneously (use different substrates at the same time, independent of their efficiency) (Egli, 2010 and references therein). Substrate tests on organisms grown under carbon limitation revealed that these cells can oxidize a much broader spectrum of organic compounds than cells that were pre-grown under carbon excess (Upton and Nedwell, 1989; Ihssen and Egli, 2005). The use of multiple carbon sources enables growth on extremely low concentrations of each individual compound (Lendenmann et al., 1996; Kovárová-Kovar and Egli, 1998) and is thus beneficial in an oligotrophic environment with a frequently changing supply of nutrients.

Cultivation of marine bacteria

So far, only about half of the known bacterial phyla have cultivable representatives (Hugenholtz, 2002), even though pure cultures are essential to study metabolic pathways of the different bacteria in detail. Possible reasons for the yet inability to cultivate many bacteria maybe unsuited growth conditions and could include a lack of nutrients or growth factors, inappropriate pH, pressure or temperature conditions or unsuitable levels of

oxygen (reviewed in Vartoukian et al., 2010). Furthermore, many of the used media contain very high amounts of nutrients, compared to most marine environments, and thus favor fast-growing bacteria rather than slow-growing ones. In turn, such conditions might even inhibit the growth of some oligotrophic bacteria (Ishida et al., 1989; Koch, 1997; Connon and Giovannoni, 2002). Consequently, new strategies for the isolation of marine bacteria have to be developed to understand the different metabolic pathways of marine bacteria and their ecology and evolution (Grossart, 2010).

One approach to prevent overgrowth of slow-growing bacteria is the dilution-to-extinction method, that reduces the number of cells per sample until ideally solely single cells are left for cultivation (e. g. Button et al., 1993; Connon and Giovannoni, 2002). Additionally, the use of low-nutrient natural seawater for isolation and in vitro simulation of the natural environment using diffusion chambers placed in natural seawater provoked isolation of new, so far uncultured bacteria (Connon and Giovannoni, 2002; Kaeberlein et al., 2002; Rappe et

al., 2002; Zengler et al., 2002; Bollmann et al., 2007).

However, the utilization of natural seawater always implies undefined conditions because merely a few percent of the highly diverse organic compounds in natural seawater is already characterized (Dittmar and Paeng, 2009). Hence, in order to study bacterial metabolism at the lower border of bacterial growth in detail and to identify the essential substances for growth, a defined artificial seawater medium is crucial. Those approaches so far reported to isolate and cultivate marine bacteria using artificial seawater contained either agar or vitamins, both of which represent a fixed carbon source, or were supplemented with at least 0.1 to 3 mg C L-1 of organic substrates to support growth (Van der Kooij et al., 1980; Ishida et al., 1982; Schut et al., 1993; Azam and Long, 2001; Vancanneyt etal., 2001).

Aims of this study

This work was initiated by the question of how marine Beggiatoa spp. form mats and succeed in anoxic habitats. Until today, it was believed that only

the presence of nitrate as alternative electron acceptor allows the population of anoxic environments by the large sulfide-oxidizing bacteria of the family Beggiatoaceae.

Recently, I found that nitrate is not essential for the thriving of Beggiatoa filaments in anoxic parts. For these experiments, I used the marine Beggiatoa sp. 35Flor that is cultivated in gradient culture tubes. It was observed that filaments moved below the oxygen-sulfide interface without the presence of nitrate and aggregated in anoxic parts of the culture tube. Therefore, the aim of the first part of this thesis (Chapter 2) was to study this behavior and to reveal how the filaments can survive in the anoxic layers and why they leave the overlapping zone of oxygen and sulfide, where both electron acceptor and donor are present.

[redacted]

Already during my diploma thesis (Bachmann, 2007) I was able to show that the investigated Beggiatoa culture is not a pure culture. Instead, the Beggiatoa sp. 35Flor is in co-culture with a single accompanying organism, Pseudovibrio sp. FO-BEG1. Accordingly, the second

objective of my PhD thesis (Chapter 3) was to examine whether the growth of the sulfide- oxidizer is dependent on the presence of the accompanying Pseudovibrio sp. and, if so, whether the Pseudovibrio denitrificans type strain (DSM number 17465) can also provoke growth of the Beggiatoa sp. 35Flor.

The accompanying Pseudovibrio sp. FO-BEG1 is able to grow in pure culture without the Beggiatoa sp. under extreme nutrient deficiency in artificial seawater medium (Bachmann, 2007). The physiology of the Pseudovibrio sp. should now be subject to a detailed physiological analysis. Despite omitting the addition of an energy source, DOC was detected in the range of 5 |imol C L-1 (0.06 mg C L-1), which is 1 to 2 orders of magnitude below natural oligotrophic seawater (Schut et al., 1997; Hansell et al., 2009). This contamination could have potentially been used as an energy source. To address this question, the third objective of this thesis (Chapter 4) was to analyze the artificial medium used for cultivation, before and after growth of the Pseudovibrio strain, in order to find out which compounds were used by the bacteria. Eventually, other heterotrophic bacterial strains

were isolated in the course of this thesis under nutrient limitation to estimate how common the ability among heterotrophic bacteria (associated with large sulfide-oxidizers) is to grow under nutrient limitation.

Chapter 2 Physiology and mat formation of a marine Beggiatoa culture

This second chapter of my PhD thesis deals with the physiology of the large, sulfide- oxidizing Beggiatoa sp. strain 35Flor. The focus is laid on mat formation processes and was motivated by a new observation that filaments migrate into deeper anoxic regions without the presence of nitrate (Figure 2). In the first part of this chapter, the physiology behind the observed migration event is discussed in detail in form of a manuscript. The second part of this chapter deals with the inducibility of this migration process by blue/green light and the influence of chemical substances on the mat. This part of the chapter is presented in form of a short communication.

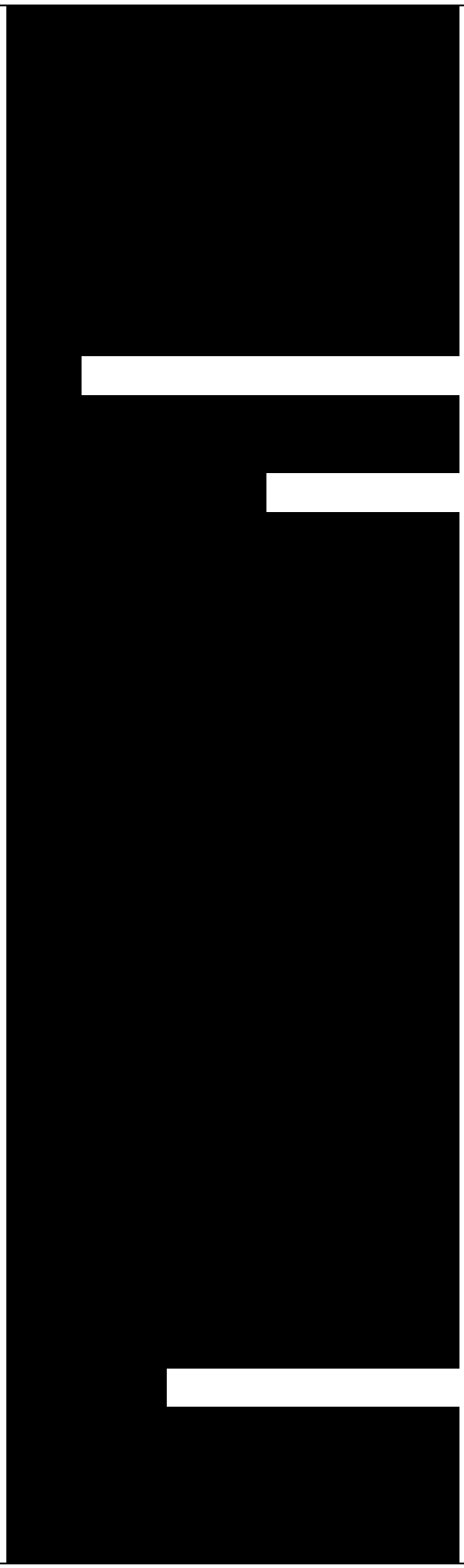


Figure 2: Image of marine Beggiatoa cultivated under chemolithotrophic conditions without nitrate at a high sulfide

flux (43.1 mmol m-2 d-1) after two weeks. A subpopulation of filaments migrated downwards into deeper layers. Contributions: 2.1 Sulfur respiration in a chemolithoautotrophic marine Beggiatoa strain A. Schwedt, The concept of the study was developed by me and H. N. Schulz-Vogt. All initial experiments were performed by me. The final experiments and data analysis were performed by me, A.-C. Kreutzmann and L. Polerecky. The manuscript was written together with A.-C. Kreutzmann and with the help of the other two co-authors. 2.2 Coordinated movement of Beggiatoa filaments in oxygen/sulfide gradients and the effect of blue/green light The concept of the study was developed by H. N. Schulz-Vogt and T. Hohmann. Major experiments were performed by H. N. Schulz-Vogt and T. Hohmann with the help of L. Polerecky during data analysis. I assisted during migration experiments and filming and performed c-di-GMP experiments. 2.1 Sulfur respiration in a marine chemolithoautotrophic Beggiatoa strain Abstract The chemolithoautotrophic strain	[redacted]	

Beggiatoa sp. 35Flor shows an unusual migration behavior when cultivated in a gradient medium under high sulfide fluxes. As common for Beggiatoa spp., the filaments form a mat at the oxygen-sulfide interface. However, upon prolonged incubation, a subpopulation migrates actively downwards into the anoxic and sulfidic section of the medium, where the filaments become gradually depleted in their sulfur and polyhydroxyalkanoates (PHA) inclusions. This depletion is correlated with the production of hydrogen sulfide. The sulfur- and PHA-depleted filaments return to the oxygen-sulfide interface, where they switch back to depositing sulfur and PHA by aerobic sulfide oxidation. Based on these observations we conclude that internally stored elemental sulfur is respired at the expense of stored PHA under anoxic conditions. Until now, nitrate has always been assumed as the alternative electron acceptor in lithotrophic Beggiatoa spp. under anoxic conditions. As our medium and the filaments were free of oxidized nitrogen compounds we can exclude this metabolism. Furthermore, sulfur respiration with PHA under anoxic conditions has so far only been described for heterotrophic

Beggiatoa spp., but our medium did not contain accessible organic carbon. Hence the PHA inclusions must originate from atmospheric CO2 fixed by the filaments while at the oxygen-sulfide interface.

We propose that the directed migration of filaments into the anoxic section of an oxygen-sulfide gradient system is a strategy to preserve cell integrity, which could be compromised by excessive sulfur deposition occurring in the presence of oxygen and high sulfide fluxes. The regulating mechanism of this migration is hitherto unknown.

Introduction

The genus Beggiatoa comprises large, filamentous bacteria that inhabit diverse sulfidic environments, such as sediments (Winogradsky, 1887; J0rgensen, 1977; Nelson and Castenholz, 1982; McHatton et al., 1996), springs (Winogradsky, 1887; Macalady et al., 2006) and activated sludge (Farquhar and Boyle, 1971). The motile filaments typically aggregate in a narrow overlapping zone of opposed oxygen and sulfide diffusion gradients where they form a sharply bounded mat (Faust and Wolfe, 1961; Nelson and Jannasch, 1983; Nelson et al., 1986a).

Within this mat, Beggiatoa spp. oxidize sulfide with oxygen and deplete both compounds (Nelson et al., 1986a). This process is accompanied by deposition of elemental sulfur inside the filaments. Several filamentous and non-filamentous members of the Beggiatoaceae (Salman et al., 2011) are moreover capable of anaerobic sulfide oxidation with nitrate as an alternative electron acceptor (Fossing et al., 1995; McHatton et al., 1996; Schulz et al., 1999). Dissimilatory nitrate reduction enables these organisms to colonize anoxic environments such as deeper layers in sediments, microbial mats or gradient cultures (Sweerts et al., 1990; MuBmann et al., 2003; Sayama et al., 2005; Kamp et al., 2006; Hinck et al., 2007). Nitrate-based sulfide oxidation seems to have been of great importance for some members of the family Beggiatoaceae, as suggested by their ability to store nitrate within intracellular vacuoles at concentrations up to 104 fold higher than in the ambient water (Fossing et al., 1995; McHatton et al., 1996; Schulz et al., 1999; Sayama, 2001; MuBmann et al., 2003; Kalanetra et al., 2004; Kalanetra et al., 2005; Hinck et al., 2007).	[redacted]	

However, also non-vacuolated strains were shown to use externally provided nitrate as a terminal electron acceptor (Sweerts et al., 1990; Kamp et al., 2006).

We cultivated the chemolithoautotrophic, marine strain Beggiatoa sp. 35Flor in an agar- stabilized oxygen-sulfide gradient medium. Upon prolonged incubation in the presence of medium to high sulfide fluxes, we observed an unusual migration behavior, where a subpopulation of filaments migrated downwards from the oxygen-sulfide interface. These filaments were able to survive although sulfide concentrations were high and terminal electron acceptors that are known to be utilized by Beggiatoa spp., i.e., oxygen and nitrate, were not detectable in medium nor filaments. In this study, we investigated the possibility of an alternative metabolism of Beggiatoa sp. 35Flor under anoxic, nitrate-free and sulfidic conditions, and discuss its possible ecological significance and link to the peculiar migration behavior.

Material and methods Strain and cultivation The strain Beggiatoa sp. 35Flor was originally enriched from a black band disease of scleractinian corals from the coast of Florida, and can so far only be cultivated in the presence of the Pseudovibrio denitrificans strain FO-BEG1 ~~(Schwedt et al., unpublished). Filaments of the strain 35Flor are about 6 |im wide, and the cells contain polyphosphate inclusions and a central vacuole filled with polyphosphate (Kamp et al., 2008; Brock and Schulz-Vogt, 2011).~~	Vật liệu và phương pháp Chủng và nuôi cấy Chủng Beggiatoa sp. 35Flor ban đầu được làm giàu từ một khu vực nhiễm bệnh sọc đen của các san hô scleractinian từ bờ biển Florida, và đến thời điểm hiện tại, chỉ có thể được nuôi cấy khi có chủng Pseudovibrio denitrificans FO-BEG1 (Schwedt và các cộng sự., Chưa xuất bản). Các sợi của chủng 35Flor rộng khoảng 6 | im, và các tế bào chứa các thể vùi polyphosphate và một không bào trung tâm được nạp polyphosphate (Kamp và cộng sự, 2008; Brock và Schulz-Vogt, 2011.). checked	

Cultivation was performed in tubes with an agar-based mineral gradient medium modified after Nelson et al. (1982) and Nelson and Jannasch (1983) using artificial seawater (Kamp et al., 2008). The medium was composed of a sulfidic bottom agar plug (1.5% w/v agar) covered with a sulfide-free, semisolid top agar layer (0.25% w/v agar) of 5 cm height (Tables 2.1.1 and 2.1.2). The medium was prepared free of nitrate, nitrite and nitric oxide, as verified by measurements with an NOx analyzer (CLD 66, Eco Physics, Rosrath, Germany). Gas exchange between headspace and the atmosphere was possible, and opposing gradients of oxygen and sulfide were allowed to form for one day before inoculation. The cultures were inoculated about 1 cm below the air-agar interface using 100 |iL filament suspension from an established mat. Incubations were performed at room temperature in the dark.	điều chỉnh từ môi trường được sử dụng bởi sunfua sunfua phía trên huyền phù dạng sợi Quá trình ủ	
During incubations, the distribution of filaments in the tube was measured simultaneously with the vertical profiles of H2S and pH.	ủ biên dạng thẳng đứng ở các ống nuôi cấy	

Filaments from parallel culture tubes were subsampled and used for microscopic determination of their sulfur and PHA inclusions. Oxygen profiles were measured in parallel tubes.	song song được lấy mẫu phụ xác định các của chúng dưới kính hiển vi Biên dạng (hình dáng, dạng, mô hình)	
Table 2.1.1 Solutions for the preparation of agar-stabilized gradient media. c All vitamins were dissolved separately and then combined in a final stock solution (1 mL of each vitamin solution in a final volume of 100 mL distilled water) that was filtersterilized twice.	để điều chế gradient được ổn định hóa bằng agar gốc cùng mỗi trong cuối cùng bằng cách lọc	
Table 2.1.2 Composition of gradient medium. a All components were sterilized separately before combination. b The agar was washed two times in distilled water before use.	của biệt	
Microsensor measurements		
Microsensors for O2 (OX-10 standard), H2S (H2S-10), and pH (PH-10) were purchased from Unisense A/S (Aarhus, Denmark). The external reference for the pH electrode was manufactured and connected in-house. Calibration of the H2S sensor was performed in anoxic, acidified artificial seawater (Table 2.1.1, pH<2) to which an	sau đó, gốc	

anoxic Na2S stock solution was added stepwise. The exact sulfide concentration of the Na2S stock solution was determined by iodometric titration. Total sulfide (Stot) profiles were calculated from measured H2S and pH profiles using equation Stot = H2S X [1 + K1 / H3O+], with pK1 = 6.569 at 21°C and 39%0 salinity (Millero et al., 1988). The oxygen sensor was two-point calibrated in a calibration chamber filled with artificial seawater. Signal readings were taken in water saturated with N2 and ambient air. Oxygen concentrations at the respective salinity and temperature were calculated according to Weiss (1970). The pH electrode was calibrated using buffer solutions of pH 4.01, pH 7.00, and pH 9.21 (Mettler-Toledo, Giessen, Germany). All sensors were calibrated immediately before the measurement. In case of long time series measurements the sensor calibration was checked afterwards and a possible drift was corrected for.	thêm vào đó sunfua Biên dạng sunfua toàn phần C buồng hiệu chuẩn việc hiệu chỉnh	
Vertical profiling in 250 |im steps was performed with sensors mounted on a motorized linear positioner (VT-80, Pollux motor, Micos, Eschbach, Germany) controlled by a computer using a software for	tính	

automated microsensor measurements (^-Profiler, L. Polerecky, http://www.microsen-wiki.net). The sensors were aligned by manually adjusting their tips to the air-agar interface using a dissecting microscope (Stemi 2000-C, Zeiss, Jena, Germany).	biến http://www.microsen-wiki.net canh chỉnh mũi của chúng	
Filament imaging The distribution of sulfur-containing Beggiatoa sp. 35Flor filaments in the gradient cultures was monitored using time-lapse photography. An amber light-emitting diode (LXHL-NM98, Luxeon, Philips, San Jose, CA, USA) was positioned below the culture tube and switched on for one second when an image was taken with a cooled CCD camera (Sensicam, PCO, Kelheim, Germany). Illumination and image acquisition in 10 min intervals were controlled by a computer using a custom-written program (Look@Molli, B. Grunwald, http://www.microsen-wiki.net).	kỹ thuật chụp ảnh tua nhanh thời gian nuôi cấy được viết tùy chỉnh http://www.microsen-wiki.net	
Intensities of the recorded images were horizontally averaged over an area with visible filaments (~5 mm wide, ~2 cm high), and the resulting vertical profiles were assembled	được lấy các biên dạng thẳng đứng	

into a 2D map with the x-axis representing incubation time and the y-axis corresponding to depth. Since the average image intensity was proportional to the density of sulfur globules, which were present exclusively inside filaments, vertical movement of sulfur-rich filaments was detected as a change in the shape of the vertical intensity profile. In contrast, an increase and decrease in the profile intensity that was not accompanied with the change in the profile shape indicated accumulation and depletion of sulfur inside the filaments, respectively. Because this method relied on light scattering from sulfur inclusions, it did not allow visualization of sulfur-free filaments. Staining of internal PHA Staining with Nile Red was used to visualize PHA inclusions in the filaments. A subsample from the culture tube (volume 90 |iL) was incubated for 5 minutes with 10 |iL of a Nile Red (Sigma-Aldrich, Steinheim, Germany) staining solution (25 mg L-1 in dimethyl sulfoxide). The filament suspension was transferred onto a poly-L-lysine (Sigma-Aldrich) coated microscope slide for immobilization of the filaments. Fluorescence of Nile Red was	cuối cùng được gộp các hạt chỉ hiện diện bên trong các sợi của biên dạng cường độ biên dạng biên dạng các nó không cho phép các sợi không lưu huỳnh hiển thị	

excited with a laser at 546 nm and emission was recorded above 590 nm (filter set 15, Zeiss, Jena, Germany) using an epifluorescence microscope (Axiophot equipped with AxioCam MRm, Zeiss, Jena, Germany).	[redacted]	
Transfer experiment with sulfur-free filaments To verify that sulfur-free filaments from the anoxic subpopulation of an aged culture (cultivated at high sulfide flux conditions) were alive, able to migrate back to the oxygen-sulfide interface and re-establish their sulfide-oxidizing metabolism, they were transferred into the anoxic section of a fresh gradient medium (cultivated at low sulfide flux conditions). All cultivation media were prepared in plexiglass tubes (2x12 cm in size) with lateral holes (Brock and Schulz-Vogt, 2011). Fresh medium for inoculation with sulfur-free filaments was pre-incubated with the accompanying Pseudovibrio strain. This was done to ensure a sufficient cell density of Pseudovibrio sp. irrespective of the inoculum as the Pseudovibrio sp. is required for growth of Beggiatoa sp. 35Flor, but its abundance is negligible in the anoxic part of the gradient	[redacted]	

(Schwedt et al., unpublished). Subsequently, sulfur-free filaments were removed laterally from the aged culture and injected laterally into the fresh medium at a depth of about 1 cm below the oxygen-sulfide interface. Lateral removal ensured that no sulfur-containing filaments from the oxygen-sulfide interface of the aged culture were transferred, whereas lateral injection prevented inoculation of the transferred sulfur-free filaments to the oxygen-sulfide interface of the fresh gradient media. The development of a mat at the oxygen- sulfide interface was inspected visually.

[redacted]

Results

Migration of Beggiatoa sp. 35Flor in gradient cultures

[redacted]

Beggiatoa sp. 35Flor filaments aggregated and formed a dense mat at the oxygen-sulfide interface within the gradient medium. In cultures with medium to high sulfide fluxes (Table 2.1.3 A) a subpopulation of filaments began a downward migration to the anoxic zone about 3-4 days after establishment of the mat. For medium sulfide fluxes, this migration resulted in a layer with a homogenous filament density extending up to 2-3 mm below

the mat (Figure 2.1.1). In contrast, for high sulfide fluxes the migrating filaments were not homogenously distributed, but progressively aggregated in a region distinctly separated from the mat at the oxygen-sulfide interface (Figure 2.1.1 and 2.1.2 B). Because the aggregation of filaments in the anoxic part increased the chance of detecting metabolic products all further experiments were conducted with cultures growing under a high sulfide flux.

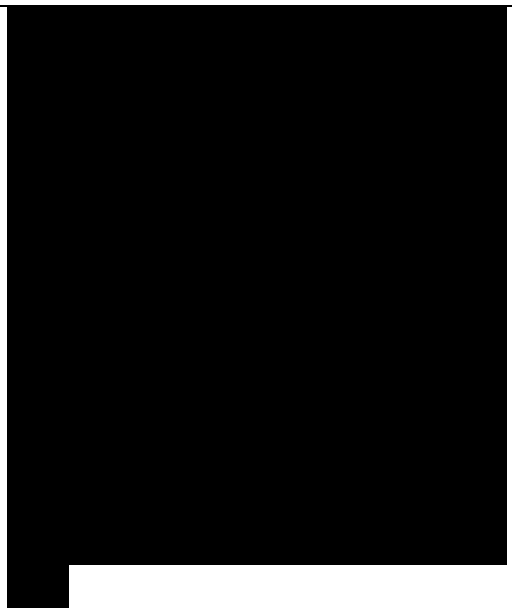
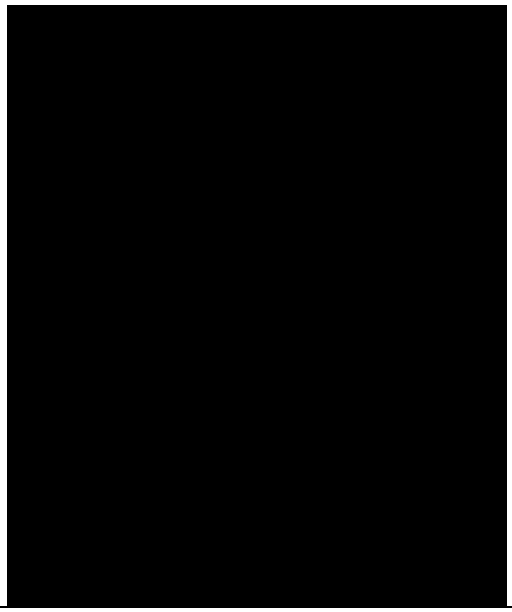


Figure 2.1.1: Distribution of Beggiatoa sp. 35Flor filaments over depth in gradient cultures after 6 (open symbols) and 12 (closed symbols) days in the presence of different sulfide fluxes. The flux values represent theoretical maxima under the given cultivation conditions (Table 2.1.3 A).

Migration of filaments in cultures with a high sulfide flux followed a general pattern (Figure 2.1.2 E). During the initial 3-4 days of incubation, the mat at the oxygen-sulfide interface gradually formed. After about 6-7 days, the sulfur globule density in the mat decreased moderately, followed by a more pronounced decrease after 8-9 days. These decreases were correlated with two pronounced events of downward



migration at days 5-6 and 7-8, respectively (arrows 1 and 2 in Figure 2.1.2 E). After reaching a depth of around 10 mm, the migrating filaments formed a layer of increased filament density. These filaments slowly disappeared from view due to a gradual loss of their internal sulfur granules. The disappearance of filaments was accompanied by a parallel increase in the sulfur globule density in the mat at the oxygen-sulfide interface (arrow 3 in Figure 2.1.2 E), suggesting that the filaments returned to this zone and switched back to sulfide oxidation, thereby depositing sulfur. This was confirmed by the transfer experiment, which showed that sulfur-free filaments transferred from the anoxic subpopulation of an aged culture into the anoxic section of a fresh gradient medium formed, within 12 days, a new mat of sulfur-containing filaments at the oxygen-sulfide interface.

Table 2.1.3 A Diffusive sulfide fluxes in gradient cultures from this study.
a The initial theoretical maximum of the sulfide flux in the gradient cultures, calculated using Fick's first law of diffusion (J = -D Ac/Ax). The diffusion coefficient D for HS-

was corrected for temperature (21°C) according to J0rgensen and Revsbech (1983), resulting in a value of 1.56 X 10-9 m2 s-1. The concentration gradient was calculated from the height of the top agar (Ax = 5 cm) and the initial sulfide concentration in the bottom agar, assuming that sulfide was depleted at the agar surface, i.e. Ac = c (Na2S).

Table 2.1.3 B Diffusive sulfide fluxes in natural Beggiatoa spp. mats.

Sulfide production by filaments in the anoxic section

Throughout the incubation, sulfide oxidation in the mat at the oxygen-sulfide interface was confirmed by pronounced acidification and steep gradients of total sulfide (Figure 2.1.2 C and D). A small but detectable peak in the H2S profile was observed at a depth of ~10 mm when the anoxic subpopulation was present (Figure 2.1.2 D). As pH varied only smoothly with depth in this region, the H2S peak was not linked to pH variation, but indicated a true production of sulfide at and around this depth. This production was strongly spatially and temporally correlated with the presence of the anoxic subpopulation (Figure 2.1.2 F), suggesting that it was linked to the metabolic activity of the

filaments from this subpopulation. Figure 2.1.2: Relationship between the migration of Beggiatoa sp. 35Flor filaments and the dynamics of O2, pH, H2S and Stot in the gradient culture tube. (A-B) Images of culture tubes showing the filament distribution after 2 and 11 days, respectively. (C-D) Examples of pH, H2S and total sulfide profiles in the gradient culture incubated for 8 and 13 days. Shaded areas mark the oxic zone. (E) Average sulfur globule density as a function of time and depth, showing the dynamics of the filament distribution and their sulfur content. Arrows 1 and 2 indicate the onset of major downward migration events, arrow 3 indicates the onset of an increase in the filament density in the mat at the oxygen-sulfide interface. Although the timing of these events varied amongst experimental runs, the general pattern was reproducible. (F) H2S excess as a function of time and depth, calculated by subtracting the measured H2S profile from the background trend. The trend was derived from the H2S concentrations measured above and below the peak (line indicated by arrow in panel D). Contour lines of the sulfur globule density from panel	[redacted]	

E are overlaid. Data shown in panels A, B, E and F are from the same culture tube, profiles in panels C and D are from a parallel culture tube.

[redacted]

Sulfur and PHA content in single filaments

Beggiatoa sp. strain 35Flor filaments accumulated elemental sulfur and PHA during growth at the oxic-anoxic interface. Sulfur inclusions were visible as dark, highly refractory globules in bright-field micrographs, while PHA inclusions appeared as strongly fluorescent globules in the images of Nile Red stained samples (Figure 2.1.3).

When grown under high sulfide fluxes, most filaments from the mat at the oxygen-sulfide interface were densely filled with sulfur and PHA inclusions (Figure 2.1.3 B and 2.1.3 E). With increasing sulfide fluxes the amount of internal sulfur strongly increased (compare Figure 2.1.3 A and 2.1.3 B), whereas PHA inclusions were equally abundant in all treatments (data not shown). In contrast, filaments from the anoxic subpopulation were heterogeneous with respect to their inclusion density; while some were densely filled with

sulfur and PHA, others lacked both (Figure 2.1.3 D and 2.1.3 F). At high sulfide fluxes, filaments were frequently observed to burst, particularly in older cultures (Figure 2.1.3 C).

[redacted]

Figure 2.1.3: Bright-field and fluorescence micrographs illustrating the typical appearance of Beggiatoa sp. 35Flor filaments cultivated under different conditions. (A-B) Filaments from the mat at the oxygen-sulfide interface of 6 days old cultures growing under low (A) and high (B) sulfide flux conditions. (C-D) Filaments from cultures grown under high sulfide flux conditions, collected from the mat at the oxygen-sulfide interface after 27 days (C) and from the anoxic subpopulation after 12 days (D). (E-F) Nile Red-stained filaments from a 14 days old culture, collected from the mat at the oxygen-sulfide interface (E) and from the anoxic subpopulation (F). Bright fluorescence in panel E originates from PHA inclusions, whereas faded fluorescence in panel F is due to staining of the cell membrane.

[redacted]

Discussion

Sulfide production by members of the genus Beggiatoa is known from chemoheterotrophic strains that were cultivated in liquid

[redacted]

medium and artificially exposed to short-term anoxic conditions (Schmidt et al., 1987). Based on those experiments it was hypothesized that sulfur respiration may provide Beggiatoa spp. in gradient systems with energy for return from the anoxic zone to the oxygen-sulfide interface under changing environmental conditions. In this study, we cultivated the chemolithoautotropic strain Beggiatoa sp. 35Flor in an oxygen- sulfide gradient medium, and we observed a directed migration of the filaments from the oxygen-sulfide interface into the anoxic and sulfidic zone where they reduced internal sulfur to sulfide. This suggested an alternative or additional function of sulfur respiration in Beggiatoa filaments. We propose that the observed behavior is a survival strategy of Beggiatoa sp. 35Flor at prolonged incubation under high sulfide fluxes. Under this condition the filaments become densely filled with sulfur and were often observed to burst. By moving to the anoxic zone of the gradient system, the filaments can prevent further deposition of sulfur through aerobic sulfide oxidation and may even reduce the amount of	[redacted]	

storage compounds by sulfur respiration with PHA. We observed that filaments can migrate back to the oxygen-sulfide interface, where they resume aerobic sulfide oxidation and accumulate new sulfur globules.	[redacted]	
Sulfur respiration for regulation of the amount of stored sulfur The alteration between sulfide oxidation and sulfur reduction in spatially separated environments seems to allow Beggiatoa sp. 35Flor to control the amount of stored sulfur beyond the scope of enzymatic regulation. Sulfide is oxidized by Beggiatoa spp. in a two-step process via internally stored sulfur (2 H2S + O2 2 S0 + 2 H2O) and further to sulfate (2 S0 + 3 O2 + 2 H2O -> 2 SO42- + 4 H+). The regulation of these reactions is unknown in Beggiatoa spp., but the presence of internal sulfur globules demonstrates that the rates of the two reactions are not always well balanced. In principle, a balanced sulfur content can be achieved by either down-regulating sulfide oxidation or up-regulating sulfur oxidation. It is likely that sulfide oxidation is controlled kinetically and cannot be regulated by the cell,	[redacted]	

because both O2 and H2S are freely diffusing into the cytoplasm. This is supported by observations on two related genera Thiomargarita and Marithioploca, which both immediately increased their respiration rate upon addition of sulfide to the medium (Schulz and de Beer, 2002; H0gslund et al., 2009). Therefore, up-regulation of the sulfur oxidation seems to be the more likely mechanism for balancing the internal sulfur content. However, at high sulfide fluxes bursting of Beggiatoa sp. 35Flor filaments densely filled with sulfur globules indicates that further up-regulation of sulfur oxidation was not possible, e.g. due to enzymatic rate limitation.

As an alternative to enzymatic regulation, the filaments may leave the overlapping zone of oxygen and sulfide in order to starve themselves of electron donor or acceptor, thereby interrupting sulfur deposition. A negative chemotactic response to oxygen (M0ller et al., 1985) presumably prevented the filaments from moving upwards into the oxic section of the gradient system. Instead, they migrated downwards into the anoxic and sulfidic section, where sulfide could no longer be

oxidized to sulfur due to the lack of an electron acceptor. These filaments moved into the sulfidic zone, which is surprising, because elevated sulfide concentrations have previously been reported to be toxic for Beggiatoa spp. (Winogradsky, 1887; Keil, 1912; Nelson et al., 1986a). However, all earlier studies were done under oxic conditions. Our study indicates that Beggiatoa can tolerate higher sulfide concentrations under anoxic conditions, whereas under oxic conditions high sulfide concentrations can cause cell death indirectly by inducing excessive sulfur accumulation.

Metabolism of Beggiatoa in the anoxic zone of gradient systems

The depletion of sulfur and polyhydroxyalkanoate inclusions together with the production of sulfide suggests that Beggiatoa sp. 35Flor reduced internal sulfur by oxidizing stored carbon in the anoxic part of the gradient system. It is not known which type of PHA was synthesized by Beggiatoa sp. 35Flor, but for the most frequent PHA, poly (3-hydroxybutyrate) (PHB), the reaction ([C4O2H6] + n • 9 S0 + n • 6 H2O ^ n • 4 CO2 + n • 9 H2S), which is pH-neutral, would be in agreement with the observed pH profiles. By

reducing stored sulfur with stored PHA that derived from previously fixed CO2, the filaments do not exploit an additional energy source in the anoxic environment. Instead, they use this process as the only possibility to empty storage space.

Presence of filamentous Beggiatoaceae in the anoxic section of oxygen-sulfide gradient systems has so far been shown in multiple laboratory and field studies (J0rgensen, 1977; Sweerts et al., 1990; MuBmann et al., 2003; Kamp et al., 2006; Hinck et al., 2007; J0rgensen et al., 2010). However, in these systems either external or internal nitrate was present and could have been used for oxidizing sulfide in the anoxic zone of the sediment. Nitrate respiration could, however, be excluded in our experiments as NOx compounds were absent from the medium and filaments.

The role of sulfur reduction by Beggiatoa spp. in the environment

The migration behavior and sulfur reduction by Beggiatoa filaments described in our study may occur and play the same role also in natural habitats. In the environment, filaments could respond to high sulfide fluxes

either by moving laterally to an adjacent region with a lower sulfide flux or, if this is not possible, by migrating vertically to the sulfidic and anoxic sediment section below, where they respire sulfur (Figure 2.1.4). This is supported by the fact that sulfide fluxes in our culture tubes (Table 2.1.3 A) were in the range of fluxes measured in different natural Beggiatoa mats (Table 2.1.3 B), and that similar heterogeneity in internal sulfur content of Beggiatoa filaments was also observed for filaments collected from natural mats (Sassen et al., 1993; Bernard and Fenchel, 1995). However, the conditions at which these phenomena occur will depend on the possible maximum oxidation rates of sulfide and ultimately sulfur, which likely define the tolerance of different Beggiatoa species towards high sulfide fluxes.

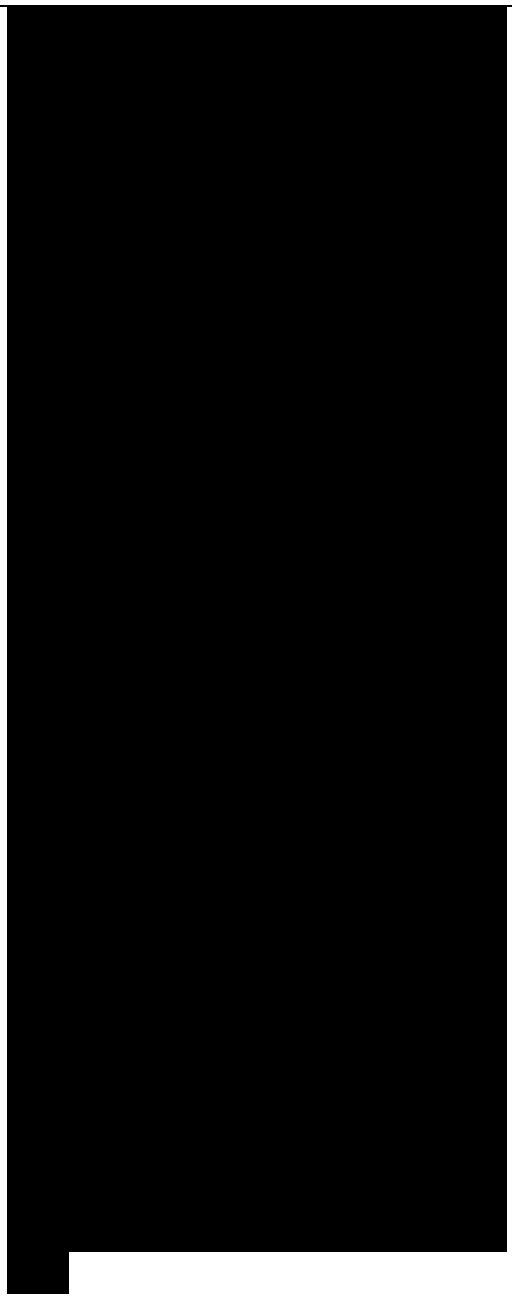


Figure 2.1.4: Proposed function of sulfur reduction as a survival strategy of Beggiatoa sp. 35Flor under high sulfide fluxes. In locations with high sulfide fluxes (right side) Beggiatoa filaments become excessively filled with sulfur (black dots inside the filaments), because the two

oxidation rates of sulfide to sulfur (K1) and sulfur to sulfate (K2) are not well balanced (K1>K2). To prevent bursting, filaments can move into a region with a lower sulfide flux (black arrow) where these two reactions may proceed in a balanced way. If this is not possible, filaments leave the oxygen-sulfide interface and move down into an anoxic region to reduce their internal sulfur deposits and thus prevent bursting (white arrow). They do so by using internally stored PHA as an electron donor to reduce S0 to H2S. After emptying the storage space, the filaments return to the oxygen-sulfide interface and continue with sulfide oxidation.

2.2 Coordinated movement of Beggiatoa filaments in oxygen-sulfide gradients and the effect of blue/green light

Abstract

Filamentous sulfide-oxidizing bacteria of the genus Beggiatoa are gradient organisms. When grown autotrophically in opposing gradients of oxygen and sulfide, the filaments establish a thin and well-defined mat in the overlapping zone of oxygen and sulfide. We found that cyclic Adenosine-mono-phosphate (cAMP) and cyclic diguanylate (c-di-GMP) or blue

light can modify or disturb this typical behavior.

Introduction

Large filamentous sulfur bacteria of the genus Beggiatoa form a thin mat in the overlapping zone of oxygen and sulfide (Nelson et al., 1986a), where they oxidize the upwards diffusing sulfide with oxygen (e.g. Winogradsky, 1887; Keil, 1912; J0rgensen, 1977; Nelson and Castenholz, 1981). The formation of a thin mat between steep opposing gradients of oxygen and sulfide is thought to be the result of toxicity of both compounds for these bacteria (Winogradsky, 1887; Keil, 1912; M0ller et al., 1985). Furthermore, a migrational reaction was also observed when filaments were illuminated with light (Winogradsky, 1887; Nelson and Castenholz, 1982; M0ller et al., 1985), suggesting that light may also play a role in the migration of Beggiatoa during mat formation. In this study, we found that, in addition to light, chemical substances such as cyclic Adenosine-mono-phosphate (cAMP) or cyclic diguanylate (c-di-GMP) also influence the migrational behavior and mat formation by Beggiatoa. Our results suggest that light might directly lead to a coordinated migration of a

subpopulation of filaments and that cAMP and c-di-GMP induce a dispersion of the mat.	[redacted]	
Results and discussion Beggiatoa filaments of strain 35Flor were cultivated in oxygen-sulfide gradient media (chapter 2.1) where they typically form a distinct mat in the overlaying zone of oxygen and sulfide. We found that several factors can modify or disturb the typical mat formation behavior of Beggiatoa filaments. The previously formed mat is disturbed and filaments distribute randomly throughout the gradient medium when cAMP and c-di-GMP are added to the culture (Figure 2.2.1). When grown under high sulfide fluxes (> 40 mmol m-2 d-1), a part of the population from the mat in the oxygen-sulfide overlapping zone migrates down into the anoxic and sulfidic zone after an incubation time of 10-14 days. These filaments were shown to use sulfur as an electron acceptor for the oxidation of PHA, both stored internally during the time spent in the oxygen-sulfide overlapping zone (Chapter 2.1). The downward migration was proposed as the last resort	[redacted]	

strategy for the filaments to prevent cell disruption, which would otherwise occur due to excessive accumulation of internal sulfur by sulfide oxidation. A very similar migration behavior was observed when the previously formed Beggiatoa mat was illuminated by light in the blue to green region (Fig. 2.2.2). When cyan light is applied to a four days old culture incubated at a high sulfide flux (40 mmol m-2 d-1) a downward migration of a subpopulation of filaments can be observed immediately (Figure 2.2.2). In contrast to the migration in the dark, that occurs after 10-14 days, the migration can be induced with light even after incubation times of a few days.

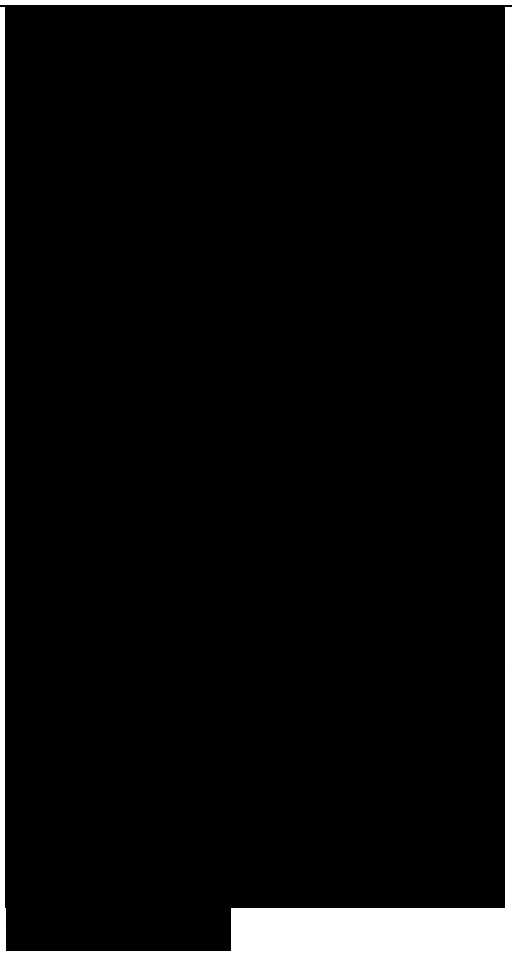


Figure 2.2.1: Beggiatoa sp. 35Flor filament distribution at a low sulfide flux (10.8 mmol m-2d_1) is shown in A. The addition of 20 of c-di-GMP (0.01 ^mol L-1; B) or 10 cAMP (100 mM; C) leads to the disruption of the mat starting after 2 hours (here shown after 8 hours). When Phosphate buffer or water was added there was no migrational reaction of the Beggiatoa filaments (data not shown).

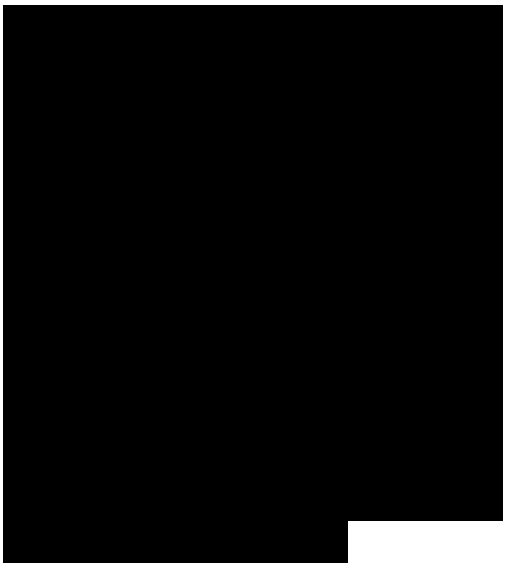


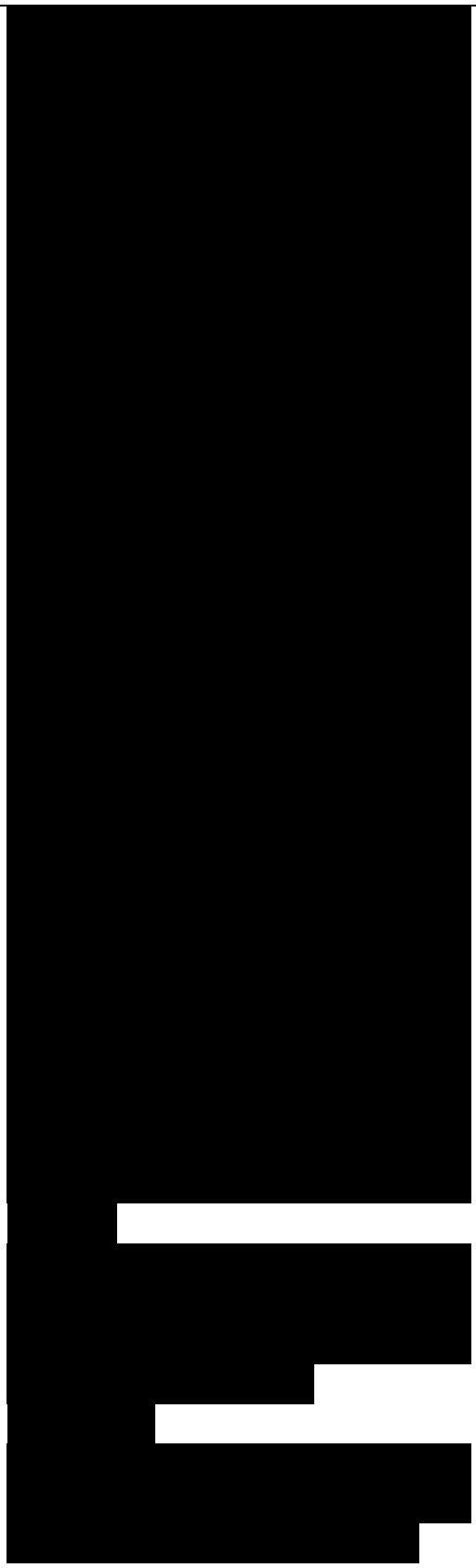
Figure 2.2.2: Average sulfur globule density of Beggiatoa filaments at a high sulfide flux as

a function of time and depth (calculation see Chapter 2.1). The culture was pre-incubated for 4 days in the dark where the mat at the oxygen sulfide interface established. The imaging started in the dark (black bar). After 4 hours the culture was illuminated with cyan light (cyan bar) and a subpopulation of filaments immediately migrated downwards.

The data presented here show that the known signalling molecules also have an effect on an established Beggiatoa mat and can lead to migrational reactions of the filaments. The mat is disturbed and the filaments distribute randomly in the culture tube. Therefore, it is possible that these substances might be used as signalling molecules among Beggiatoa filaments. In contrast to this, a directed migration known from cultures with a high sulfide flux (Chapter 2.1) can be induced by applying blue/green light to the culture, indicating that light might be involved in the migration of Beggiatoa filaments.

Chapter 3 Co-cultivation of a marine Beggiatoa strain and Pseudovibrio sp.

In this chapter, the association between the large Beggiatoa sp.

35Flor and the accompanying Pseudovibrio sp. FO-BEG1 (Figure 3) is investigated. The first part of this chapter is a study on the co-culture of the two organisms written as a short communication. In order to study the association from the side of the Pseudovibrio sp, the genome of this bacterium was analyzed in detail. This second part of this chapter comprises an abstract about the genomic analysis and comparison of the isolated Pseudovibrio sp. and another Pseudovibrio sp. strain associated with a sponge. Both strains are able to grow in pure culture and both genomes reveal a versatile metabolism of the bacteria. During the genome analysis of Pseudovibrio sp. strain FO-BEG1 we found several genes encoding for more than 20 superoxide dismutases, catalases and peroxidases additionally to genes for the interaction with other cells. The manuscript to this second part of this chapter is added as an appendix to the thesis. Figure 3: Differential interference contrast micrograph of a Beggiatoa sp. and Pseudovibrio sp. co-culture. Contributions: 3.1 A chemolithoautotrophic Beggiatoa strain requiring the presence of a Pseudovibrio sp.		

for cultivation The concept of this study was developed by both authors. I performed all experiments. The manuscript was written by me with the help of H. N. Schulz-Vogt. 3.2 The Pseudovibrio genus contains metabolically versatile and symbiotically interacting bacteria The study was initiated by V. Bondarev, H. N. Schulz-Vogt and me. Cultivation and DNA extraction for sequencing of the genome was performed by me. I coordinated the sequencing of the genome. Genome analysis was performed by V. Bondarev with assistance of M. Richter. J. Piel did NRPS and PKS cluster analysis. Cultivation experiments were performed by V. Bondarev. The manuscript was written by V. Bondarev with assistance of all co-authors. 3.1 A chemolithoautotrophic Beggiatoa strain requiring the presence of a Pseudovibrio sp. for cultivation Abstract Numerous attempts to isolate larger chemolithoautotrophic members of the family Beggiatoaceae into pure culture have so far met little success. Here, we report the successful cultivation of a marine Beggiatoa sp. strain of 6 |im in diameter, which exclusively	[redacted]	

grows in the presence of a Pseudovibrio sp. strain.

Introduction

Beggiatoa spp. are colorless sulfur bacteria forming mats at the oxic-anoxic interface where they oxidize sulfide with oxygen (e. g. Winogradsky, 1887; Keil, 1912; J0rgensen, 1977; Nelson and Castenholz, 1981). Smaller chemolithoautotrophic strains (diameter < 5 |im) have been isolated into pure culture by using an agar based medium with opposing gradients of sulfide and oxygen (Nelson and Jannasch, 1983; Nelson et al., 1986a). Besides these strains, only heterotrophic freshwater Beggiatoa spp. strains were obtained in pure culture (Strohl and Larkin, 1978).

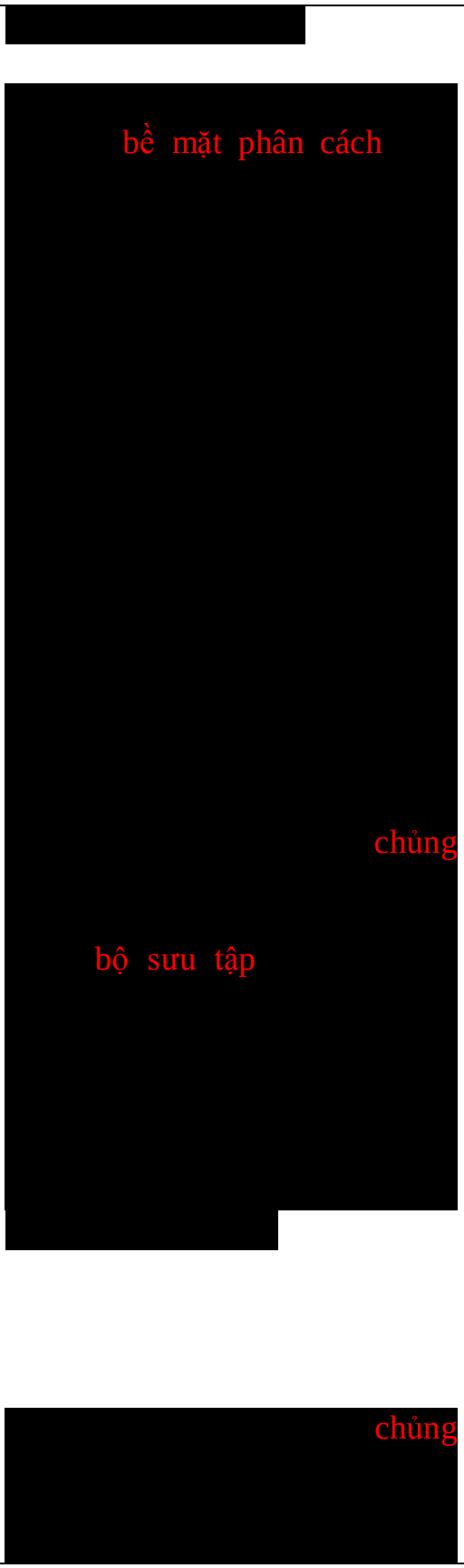
Thanh Lâm bắt đầu The reason for the general difficulty to isolate larger chemolithoautotrophic members of the family Beggiatoaceae in pure culture is still unknown. We obtained a marine Beggiatoa sp. strain of 6 |im in diameter into a stable culture, but did not succeed in growing it axenically. Apart from all attempts to clean the Beggiatoa sp. 35Flor a single type of organism, which was identified as Pseudovibrio sp. FO-BEG1 remained. Here, we present experiments indicating that the presence of this accompanying organism is required for the growth of the Beggiatoa sp. strain.	Nguyên nhân khiến việc tách các thành viên hóa tự dưỡng vô cơ thuộc họ Beggiatoaceae trong môi trường nuôi cấy thuần gặp khó khăn vẫn còn là ẩn số. Ở môi trường nuôi cấy ổn định, chúng tôi đã thu được một chủng Beggiatoa sp. biển có đường kính 6 |im nhưng khi nuôi nó trong môi trường không có ngoại vật thì lại thất bại. Bất chấp mọi cố gắng nhằm tách lọc Beggiatoa sp. 35Fl, vẫn luôn còn một loại sinh vật khác tồn tại song hành - nó được xác định là Pseudovibrio sp. FO-BEG1 Ở đây, chúng tôi trình bày thí nghiệm chứng minh rằng sự hiện diện của sinh vật đi kèm này là cần thiết cho sự tăng trưởng của chủng Beggiatoa sp.	
Results and discussion Under higher sulfide fluxes (> 40 mmol m-2 d-1) a subpopulation of the Beggiatoa filaments actively migrated into lower and anoxic parts of the culture tube (Figure 3.1) where they reduced the amount of internal sulfur by sulfur reduction (Chapter 2.2). As the accompanying Pseudovibrio sp. FO-BEG1 is predominantly found in the oxic part of the culture tube (Figure 3.1), these lower filaments were used to inoculate fresh culture tubes with only Beggiatoa filaments and relatively free of	Trong điều kiện luồng sunfua cao hơn đã khử Vì chủng chủng	

the accompanying Pseudovibrio sp. strain.

When Beggiatoa filaments from the mat at the oxic-anoxic interface were transferred into fresh medium a new mat developed in 4 out of 5 tubes. However, if filaments from the anoxic subpopulation with no or very few Pseudovibrio cells were transferred into fresh medium, only in 1 out of 7 tubes a mat developed. Examining this single mat-containing tube under the microscope, besides Beggiatoa filaments also Pseudovibrio cells were observed. When filaments from the anoxic part were transferred into tubes containing already a Pseudovibrio population, most of the tubes were positive for growth of Beggiatoa sp. (5 positive tubes out of 7 tubes). Even another Pseudovibrio strain from the culture collection (Pseudovibrio denitrificans DSM number 17465) had a similar effect on growth (4 positive tubes out of 7). Therefore, we conclude that the growth of the Beggiatoa sp. depends on the presence of an accompanying bacterium of the genus Pseudovibrio.

Pseudovibrio sp. strain FO-BEG1 (cells mL~1 ẻ106)

Figure 3.1: Distribution of the accompanying Pseudovibrio sp. strain FO-BEG1 (red squares) in co-culture with the marine



Beggiatoa sp. strain 35Flor (photograph, white filaments). The Beggiatoa filaments form a mat at the oxic-anoxic interface and a subpopulation migrates into the anoxic part of the tube. The accompanying Pseudovibrio strain is mainly present above the upper Beggiatoa mat in the oxic part of the culture tube. Thanh Lâm kết thúc Thu Yên bắt đầu There are a few indications about the possible reasons for a positive effect of the accompanying Pseudovibrio sp. on the growth of the large sulfide-oxidizer. Pseudovibrio cells populate mainly the oxic part of the culture tube, but Beggiatoa filaments also thrive well in the anoxic zone (Figure 3.1). Thus, it seems reasonable to assume that Pseudovibrio sp. is only required during the oxic metabolism of the Beggiatoa filaments. It may be suggested that Pseudovibrio sp. helps the Beggiatoa sp. in detoxifying oxygen radicals, as Beggiatoa typically do not possess the ability to produce the enzyme catalase (Larkin and Strohl, 1983). Moreover, earlier studies have shown a positive effect of catalase on the growth of Beggiatoa spp. (Burton and Morita, 1964; Strohl and Larkin, 1978; Gude et al., 1981; Nelson et al., 1986b). In contrast, the	[redacted] vài đến kích thước lớn các tốt cần thiết Chúng ta có thể thấy rằng hỗ trợ vì thường	

accompanying Pseudovibrio strain is catalase-positive and the genome suggests further potential to detoxify oxygen radicals (Chapter 3.2). Therefore, we propose that the Beggiatoa sp. benefits from the oxygen protection system of the accompanying Pseudovibrio sp. strain and is consequently mandatory for the growth of the large sulfide-oxidizer.	thêm kích thước lớn	
3.2 The Pseudovibrio genus contains metabolically versatile and symbiotically interacting bacteria Beggiatoa 53 Abstract The majority of strains belonging to the genus Pseudovibrio have been isolated from marine invertebrates like tunicates, corals and especially sponges, but the physiology of these bacteria is poorly understood. In this study, we analyze the genomes of two Pseudovibrio strains. One is a required symbiont of a cultivated Beggiatoa strain, a sulfide oxidizing, autotrophic bacterium. The other one was isolated from a sponge (Enticknap et al., 2006). The data show that both strains are generalistic bacteria capable of importing and oxidizing a wide range of organic and inorganic	có thể được phân lập từ	

compounds to meet their carbon, nitrogen, phosphorous and energy requirements under oxic and anoxic conditions. Several physiological traits encoded in the genome were verified in laboratory experiments with a pure culture of the Pseudovibrio strain originally associated with Beggiatoa. Besides the versatile metabolic abilities of both Pseudovibrio strains, our study reveals a number of open reading frames and gene clusters in the genomes that seem to be involved in symbiont-host interactions. Pseudovibrio has the genomic potential to attach to host cells, might be capable of interacting with the eukaryotic cell machinery, produce secondary metabolites and may supply the host with cofactors.

Chapter 4 Isolation and cultivation of Pseudovibrio sp. and other facultatively oligotrophic bacteria
Thu Yên kết thúc
The fourth chapter includes a detailed physiological investigation of the growth and substrate use of the isolated Pseudovibrio sp. FO-BEG1 under oligotrophic conditions. These results are presented in the first manuscript of this chapter. The investigated Pseudovibrio strain is on the one hand able to

thể

grow under extremely oligotrophic conditions and on the other hand it can grow on organic-rich media (Figure 4). Eventually, we were able to isolate further facultatively oligotrophic bacteria from Namibian sediments, which are highly populated by large sulfur bacteria. The isolation and cultivation of these bacteria belonging to different phylogenetic groups is presented in the second manuscript of this chapter.

Figure 4: Growth of Pseudovibrio sp. FO-BEG1 on organic-rich agar plates at day 2 forming colonies (left) and in pure artificial seawater (right).

Contributions:

4.1 Substrate use of Pseudovibrio sp. growing in extremely oligotrophic seawater

I developed the concept of this study with assistance of H. N. Schulz-Vogt, M. Seidel and T. Dittmar. Furthermore, I performed the medium preparation and sampling with the help of M. Seidel. FT-ICR-MS measurements were performed together with M. Seidel. HPLC analysis was performed by the group of M. Simon. G. Lavik assisted during the measurement and analysis of the isotope-labelling

experiments. V. Bondarev performed glucose and ammonia addition experiments and S. Romano the biolog experiment. I wrote the manuscript with assistance of the co-authors.	[redacted]	
4.2 Facultatively oligotrophic bacteria isolated from the habitat of large sulfide-oxidizers A. Schwedt, V. Bondarev, M. Seidel, T. Dittmar, H. N. Schulz-Vogt	[redacted]	
This study was initiated by me with the help of H. N. Schulz-Vogt. I performed all isolations and cultivations. V. Bondarev assisted me with counting of the cells, M. Seidel and T. Dittmar measured DOC concentrations. The manuscript was written by me including comments of all co-authors.	[redacted]	
4.1 Substrate use of Pseudovibrio sp. growing in extremely oligotrophic seawater Abstract Marine planktonic bacteria often live in habitats with extremely low concentrations of dissolved organic matter (DOM). To study the use of trace amounts of DOM by the facultatively oligotrophic bacterium Pseudovibrio sp. strain FO-BEG1, we investigated the composition of artificial and natural seawater before and after	[redacted]	

growth. For this, we determined dissolved organic carbon (DOC), total dissolved nitrogen (TDN), composition of DOM by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS) and amino acids by high performance liquid chromatography (HPLC). The DOC concentration of artificial seawater was 0.06 mg C L-1 (5 ^mol C L-1), which was an order of magnitude below the concentration in the natural seawater (0.9 mg C L-1 or 75 |imol C L-1). DOC concentrations did not decrease measurably during growth. Cell numbers increased from about 20 cells mL-1 to 20,000 cells mL-1 in artificial and to 800,000 cells mL-1 in natural seawater. No nitrogen fixation and minor CO2 fixation (< 1%) was observed. In both media, amino acids were not the major nutrient. Instead, we observed a decrease of compounds resembling detergents in artificial seawater, which also contained nitrogen. In natural seawater, we detected a decrease of two groups of compounds, one resembling amino- and thiosugars, which are also rich in nitrogen, and another group containing condensed hydrocarbons. The present study shows that heterotrophic bacteria	máy quang phổ khối cộng hưởng cyclotron ion sử dụng phép biến đổi Fourier	

can grow with even lower DOC concentrations than available in natural oligotrophic seawater and may use unexpected organic compounds to fuel their energy, carbon and nitrogen requirements.

Introduction

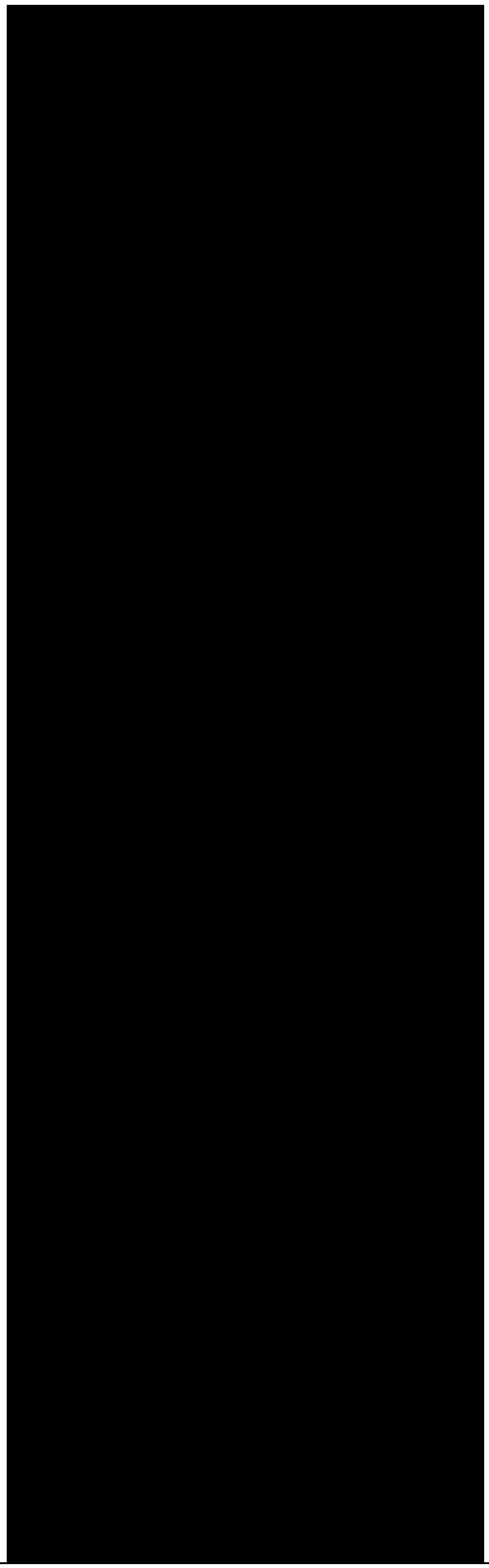
In open oceans, the amount of dissolved organic matter (DOM) is typically indicated as the concentration of dissolved organic carbon (DOC) and is generally below 1 mg C L-1 (Schut et al., 1997; Hansell et al., 2009). Consequently, marine bacteria are commonly exposed to very low concentrations of organic material. Marine DOM is an extremely diverse pool of different compounds, consisting of more than ten thousand types of molecules with different reactivities (Dittmar and Paeng, 2009). Previous studies demonstrated that most of the DOM in the ocean is inert to bacterial break-down and cannot be utilized by marine microorganisms (Bada and Lee, 1977; Ammerman et al., 1984; Zweifel et al., 1993). Measurements of the consumption of specific substrates revealed that a large fraction of the labile organic material that is used in the upper ocean can consist of dissolved free amino acids (DFAA), dissolved combined amino acids

(DCAA) and monosaccharides. These substances can account for 5 to 100% of the bacterial carbon and nitrogen demand (Fuhrman, 1987; J0rgensen, 1987; Stanley et al., 1987; Cowie and Hedges, 1992; Keil and Kirchman, 1999; Cherrier and Bauer, 2004). In many natural systems, amino acids are only present in nanomolar concentrations even though the flux of amino acids is comparably high due to a close coupling of their release and uptake by planktonic microorganisms (Fuhrman, 1987; Fuhrman, 1990; Keil and Kirchman, 1999). Still, amino acids and monosaccharides alone cannot fully explain the growth of the bacteria in all cases, where they represent only a small fraction of the C- and N-requirements (Fuhrman, 1987; Stanley et al., 1987). The remaining demand must be covered by different DOM compounds, which might be present at only very low concentrations. Therefore, to specifically investigate the compounds consumed by the bacteria during growth under oligotrophic conditions, a method detecting a broad spectrum of different organic compounds with a low detection limit, such as electrospray ionization Fourier transform ion cyclotron resonance mass	[redacted] [redacted] quang phổ khối cộng hưởng cyclotron ion sử dụng phép biến đổi Fourier ion hóa tia điện	

spectrometry (ESI FT-ICR-MS), is necessary.

In contrast to natural systems where several processes supply nutrients simultaneously, a batch culture containing a single strain of bacteria is a closed system with defined amounts of nutrients. In such a system, it is possible to analyze compounds that are utilized during the growth of this strain. For the cultivation of marine bacteria, defined artificial seawater or natural seawater containing a diverse DOM pool can serve as a medium. In both seawater media, the risk of contamination especially with amino acids is a well known problem (Dittmar et al., 2009).

In the present study, we chose to investigate the substrate use of the marine Pseudovibrio sp. strain FO-BEG1, because it is capable to grow under oligotrophic conditions in extremely pure artificial seawater. In spite of special purification of the artificial seawater medium, a DOC concentration of 0.06 mg C L-1 (5 |imol C L-1) was measured, which is, nevertheless, low compared to the natural oligotrophic seawater with a DOC concentration of 0.9 mg C L-1 (75 |imol C L-1). We



compared the substrate use in artificial seawater with natural oligotrophic seawater. In both media, DOC concentrations did not decrease measurably during growth of the bacteria. The composition of the DOM before and after growth in artificial and natural seawater was analyzed by high performance liquid chromatography (HPLC) measuring amino acids and by ESI FT-ICR-MS revealing changes on the level of individual intact molecules indicated by molecular formulas. The combination of these methods with isotope-labeling techniques to study incorporation of N2 and CO2 provides information of compounds used by the bacteria during growth under these extremely oligotrophic conditions.

[redacted]

Material and methods

Medium preparation and bacterial strain

The basal artificial oligotrophic seawater medium is composed of 30.3 g NaCl, 3.3 g MgCl2 • 6 H2O, 2.8 g MgSO4 • 2 H2O, 0.44 g CaCl2 • 2 H2O, 0.7 g KCl, 50 |il 2 mol L-1 NaOH, 2 mL 1 mol L-1 NaHCO3, 1 mL 1 mmol L-1 K2HPO4 and 1 mL trace elements solution in 1 L MembraPure water (Optilab-Standard Water System,

[redacted]

MembraPure, Bodenheim, Germany). The trace elements solution was composed of 2.1 mg L-1 $FeSO_4 \cdot 7 H_2O$, 13 mL L-1 HCl (25%), 60 mg L-1 H_3BO_3, 100 mg L-1 $MnCl_2 \cdot 4 H_2O$, 190 mg L-1 $CoCl_2 \cdot 6 H_2O$, 24 mg L-1 $NiCl_2 \cdot 6 H_2O$, 2 mg L-1 $CuCl_2 \cdot 2 H_2O$, 144 mg L-1 $ZnSO_4 \cdot 7 H_2O$ and 36 mg L-1 $Na_2MoO_4 \cdot 2 H_2O$. All glassware, Teflon stoppers, screw caps and tubes were acid washed (0.1 mol L-1 HCl, Merck, Darmstadt, Germany) and rinsed with MembraPure water (Optilab-Standard Water System, MembraPure, Bodenheim, Germany). Glassware, aluminium foil and NaCl were combusted at 480°C for at least 3 hours. Serum bottles (Wheaton 125 mL serum bottles clear, Wheaton, Millville, NJ, USA) were used for cultivation, filled completely with medium and closed with Teflon stoppers. Before bottling of the medium, the artificial seawater was cleaned by solid phase extraction of dissolved organic matter (SPE-DOM, Dittmar et al., 2008), i.e. organic contaminations. Incubations were performed without shaking at 28°C in the dark. For glucose and ammonium addition experiments the oligotrophic artificial seawater medium was not specifically purified and	[redacted]	

glucose and ammonium were added at a final concentration of 30 |imol L-1. These cultures were incubated gently shaken (shaker GFL-3013, Gesellschaft fur Labortechnik mbH, Burgwedel, Germany) at 28°C in the dark.

Natural surface seawater was collected in the South Pacific during Integrated Ocean Drilling (IODP) Expedition 329 from IODP Site U1368 (27.9°S ; 137.9°W) with a bucket and stored at 4°C. Before incubation, seawater was filtered through washed Acrodisc 25 mm syringe filters with a 0.2 |im GHP membrane (Pall Life Sciences, Ann Arbor, MI, USA) and filled into pre-combusted serum bottles that were closed with Teflon stoppers. Inoculated cultures were incubated without shaking at 28°C in the dark.

Media were inoculated with 100 |iL of an oligotrophically pre-grown culture (about 2 X 104 cells mL-1) of Pseudovibrio sp. FO-BEG1. Thus, the initial cell number after inoculation accounts to 4 to 20 cells mL-1

Cell counts

Cells were stained with 1:5000 SYBR Green (SYBR Green I 10,000x, Sigma, Taufkirchen, Germany) for 20 minutes. The stained cells were filtered onto black filters (GTTP Isopore Membrane Filters 0.22 |im,

Millipore, Schwalbach/Ts., Germany) using a Bio-Dot apparatus (Bio-Rad, Munchen, Germany). Counting of cells was performed using a fluorescence microscope (Axiophot, Zeiss, Jena, Germany) at 450-490 nm excitation and 515-565 nm emission (filter set 10, Zeiss, Jena, Germany).

Sampling and solid phase extraction of dissolved organic matter (SPE-DOM)

About 900 mL medium (six serum bottles, 156 mL each) were pooled at four different time points (blank, to, t1, t2). The blank was taken directly after medium preparation without inoculation and inoculated samples were taken before (t0), after 1 week (t1) and after 3 weeks (t2) of incubation. Samples were filtered into 1 L glass bottles using Acrodisc 25 mm syringe filters with a 0.2 |im GHP membrane (Pall Life Sciences, Ann Arbor, MI, USA) and acidified to pH 2 with 25% HCl. Samples for DOC measurements and amino acid analysis were taken after pooling the six serum bottles into a 1 L glass bottle. DOC samples were measured directly and samples for amino acid analysis were stored at -20°C. All samples were stored at 4°C until further analysis. From all four time points, DOM was extracted

using the solid phase extraction of dissolved organic matter (SPE-DOM) method (Dittmar et al., 2008). The extracts of dissolved organic matter were analyzed using ESI FT-ICR-MS (see below).

Measurement of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN)

Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were analyzed before, during and after growth of the bacteria using a Shimadzu TOC-VCPH total organic carbon analyzer (Shimadzu, Kyoto, Japan) equipped with a TNM-1 total nitrogen measuring unit and an ASI-V autosampler. To remove cells prior to analysis, samples were filtered through Acrodisc 25 mm syringe filters with a 0.2 |im GHP membrane (Pall Life Sciences, Ann Arbor, MI, USA). Within the instrument, samples were acidified with 1% v/v 2 mol L-1 HCl and sparged with synthetic air for 2 minutes to remove inorganic carbon. Detection limits were 0.5 |imol L-1 for DOC and TDN (0.006 mg C L-1 and 0.007 mg N L-1, respectively). Analytical errors based on the standard deviations for replicated measurements (at least three measurements per sample) were within 5% for DOC and TDN. Analytical

precision and accuracy was tested in each run against deep Atlantic seawater reference material and low carbon water provided by the consensus reference materials program (D.A. Hansell, University of Miami, Coral Gables, FL, USA). Procedural blanks, including the filtration step, were obtained with ultrapure water.

Amino acids analysis

The concentrations of dissolved free amino acids (DFAA) and total hydrolysable dissolved amino acids (THDAA) were analyzed using high performance liquid chromatography (HPLC) as described earlier (Lunau et al., 2006). Derivatization was performed using an ortho-phthaldiadehyde precolumn (Lindroth and Mopper, 1979). All samples were filtered through prewashed Acrodisc 25 mm syringe filters with a 0.2 |im GHP membrane (Pall Life Sciences, Ann Arbor, MI, USA). Prior to the analysis of THDAA, HCl was added to a final concentration of 1.7 mol L-1 and incubated for 1 hour at 155°C under nitrogen atmosphere. The concentration of dissolved combined amino acids (DCAA) was calculated by subtracting the concentration of DFAA from the concentration of THDAA.

H13CO3 and 15N2 labeling experiments

For labeling experiments, oligotrophic artificial seawater medium was prepared as described above, however, DOM was not extracted prior to use and it was prepared under synthetic air atmosphere (20% O2 in N2; H2O < 3 ppm-mol, CnHm < 0.1 ppm-mol, CO < 1 ppm-mol, CO2 < 1 ppm-mol; Air Liquide, Krefeld, Germany). From each 156 mL serum bottle, which was completely filled with medium, 5 mL medium were exchanged with 5 mL 15N2 gas and 300 |iL of 1 mol L-1 NaH13CO3 were added. After inoculation with Pseudovibrio sp. FO- BEG1 (100 |iL of a pre-culture containing about 2 X 104 cells mL-1), samples were incubated for 12 hours, 3 and 5 days. Then the bacteria were filtered onto pre-combusted Glass Microfibre Filters (GF/F, Whatman, GE Healthcare, Dassel, Germany), frozen for 12 hours and dehydrated in an HCl desiccator. Filters were folded into tin cups and flash combusted (1050°C) within the mass spectrometer to release N2 and CO2. For analysis, we used an elemental analyzer (with autosampler) coupled to a Delta Plus Advantage mass spectrometer isotope-ratio monitoring mass spectrometer (ThermoFinnigan, Bremen,	[redacted]	

Germany). To calculate the amount of label in the cells the percentage of label added was taken into account. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) SPE-DOM extracts were diluted 1:1 with ultrapure water to yield DOC concentrations of 10 ppm for natural seawater and about 2 ppm for artificial seawater incubations, respectively. Diluted samples were analyzed on a solariX Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS; Bruker Daltonik GmbH, Bremen, Germany) connected to a 15 Tesla superconducting magnet (Bruker Biospin, Wissembourg, France). Samples were infused at a rate of 2 |iL min-1 into an electrospray source (ESI; Apollo II ion source, Bruker Daltonik GmbH, Bremen, Germany) with the capillary voltage set to 4 kV in negative mode and 4.5 kV in positive mode. Ions were accumulated in the hexapole for 0.3 seconds prior to transfer into the ICR cell. Data acquisition was done in broadband mode using 4 megaword data sets and a scanning range of 150-2000 Da. The instrument was externally calibrated with an in-house marine deep sea DOM standard (mass accuracy of less	[redacted] Máy quang phổ khối cộng hưởng cyclotron ion sử dụng phép biến đổi Fourier [redacted] máy quang phổ khối cộng hưởng cyclotron ion sử dụng phép biến đổi Fourier [redacted]	

than 0.1 ppm). Before each sample set, blank checks with methanol/ultrapure water 1:1 were measured. For one mass spectrum, 500 scans were added and internally calibrated with the in-house marine deep sea DOM standard to yield a mass accuracy of less than 0.1 ppm for the internal calibrants (series of CxHyOz compounds from 281 to 621 Da). The molecular formulas were calculated in the mass range between 150 and 850 Da by applying the following restrictions: 12C0-1301H0-200O1-5014N0-4S0-2P0-2 (considering also 23Na clusters in ESI positive mode). Assignment of molecular formulas was done with the Bruker software DataAnalysis 4.0 SP 4 using the criteria described by Koch et al. (2005). Molecular formulas were considered valid when the mass error was below 0.5 ppm. Only compounds with a signal-to-noise ratio of 3 and higher were used for further analysis. The achieved resolving power (full width half maximum) was on average 480,000 at m/z 400.

Relative peak heights were calculated by normalization to the ten most abundant peaks in each spectrum (triplicate measurements). These peak heights were then used to semi-quantitatively assess changes in

DOM composition in natural oligotrophic seawater experiments. FT-ICR-MS is not a quantitative tool for DOM analysis due to the lack of standards and differences in ionization efficiencies for different compounds. However, this method yields reproducible results because the variability of relative peak heights is low under similar analytical conditions (Kido Soule et al., 2010). Sequential standard addition was applied to quantify the amount of polyethylene glycol (PEG) contamination in the artificial seawater medium using an authentic PEG standard (octaethylene glycol, C16H34O9, > 99% oligomer purity, 370 g mol-1, Sigma, Taufkirchen, Germany). PEG was added in concentrations of 1 to 3.6 nmol L-1 and FT-ICR mass spectra were recorded in ESI positive mode. To yield a linear response range in the MS, ion accumulation time was lowered to 0.1 seconds and 50 scans were added for one mass spectrum. The amount of PEG in the samples was calculated from a linear calibration derived from peak heights in FT-ICR mass spectra. To investigate the suppression effect of the inorganic contamination on organic peaks during FT-ICR-	[redacted]	

MS, the in-house natural deep sea DOM standard was added in the range of 0.04 to 4 mg C L-1 to a sample contaminated with the inorganic substances and measured in ESI negative mode.

Biolog experiment

A substrate respiration test was performed using a biolog GN2 plate (Hayward, CA, USA) with 95 different substrates. The bacteria were pre-grown in artificial seawater medium in 1 L bottles (Schott, Mainz, Germany). After 6 days, cells were concentrated by centrifugation for 2 hours at 11,000 X g and 15°C using a J-26XP Beckman centrifuge (Beckman Coulter GmbH, Krefeld, Germany). All centrifugation tubes were acid washed (0.1 mol L-1 HCl) and rinsed with water. The obtained pellet was suspended in 20 mL of sterile saline solution (23.5 g L-1 NaCl and 10.6 g L-1 MgCl2 • 6 H2O). To verify the viability of the cells, 50 |iL of the cell suspension was spread on organic-rich medium containing 2 g polypeptone, 0.5 g Bacto yeast extract (BD Diagnostics, Heidelberg, Germany), 30 g NaCl, 5 g MgCl2 • 6 H2O, 0.005 g CaCl2 • 2 H2O, 0.005 g Na2MoO4 • 7 H2O, 0.004 g CuCl2 • 2 H2O, 0.006 g FeCl3 • 6 H2O, 15 g bactoagar in 1 L MembraPure water and the pH

was adjusted to 8 with 1 mol L-1 NaOH. The biolog plate was inoculated with 150 |iL of the concentrated cells and incubated at 28oC in the dark in a humidity chamber to prevent excess evaporation. Activity of cells was checked visually each day for a total of 14 days.

Results

Growth under oligotrophic conditions

The investigated Pseudovibrio sp. strain FO-BEG1 was able to multiply in both artificial and natural seawater. The initial cell number was about 20 cells mL-1 and in artificial seawater cell numbers increased to 2 X 104 cells mL-1 at time points t1 and t2 (one and three weeks after inoculation, respectively). Natural seawater medium contained about 8 X 105 cell mL-1 at both time points. In both media, the increase in cell numbers was detected during the first week of incubation (to to t1) and cell numbers did not change in the following two weeks (t1 to t2).

Dissolved organic carbon (DOC), total dissolved nitrogen (TDN) and dissolved free and combined amino acids (DFAA and DCAA)

In pure artificial seawater medium before bottling, the DOC contamination from salts was only 1 ụrnol C L-1. During

the bottling of the medium and with the addition of NaHCO3, K2HPO4, NaOH and trace elements, the concentration of DOC increased to about 4.5 |imol C L-1 (Table 4.1.1). The TDN was below limit of detection (0.5 |imol N L-1) in the sterile artificial medium before and after bottling (Table 4.1.1). Inoculation did not introduce further measurable DOC or TDN to the medium (t0). After one (t1) and three weeks (t2) of incubation, the concentrations of DOC and TDN did not change significantly in the medium (Table 4.1.1). In comparison to the artificial seawater medium, the DOC concentration in natural seawater was about 16 times higher (71 |imol L-1; Table 4.1.1). However, no significant change in DOC concentration was detectable during growth, as DOC concentrations one and three weeks after inoculation were 78.5 and 75 |imol C L-1, respectively. A similar pattern was observed for TDN concentrations, which were around 12 |imol N L-1 at all time points (Table 4.1.1).

In contrast to DOC and TDN, the amount of DFAA decreased in the artificial seawater cultures within the first week of growth (from t0 to t1). This decrease represents a DFAA uptake of 0.06 |imol C L-1 and 0.02 |imol

N L-1 (Table 4.1.1). However, the concentrations of DFAA remained constant until t2. The most abundant amino acids were serine, glycine and alanine, which together accounted for 0.05 |imol C L-1. The decrease in DFAA (from t0 to t1) was concurrently observed with an increase of DCAA. During the stationary growth phase (from t1 to t2), the DCAA concentrations decreased again (Table 4.1.1). Opposed to the artificial seawater incubations, the amino acids concentrations (THDAA) in natural seawater were very low and no change during incubation could be observed (Table 4.1.1).

Nitrogen and Cacbon điôxít fixation

The incorporation of labeled nitrogen (15N2) and carbon (NaH13CO3) into Pseudovibrio sp. FO-BEG1 growing in pure artificial seawater medium was examined using isotope-ratio monitoring mass spectrometry. Less than 1% of cellular carbon originated from Cacbon điôxít fixation under the oligotrophic growth conditions in artificial seawater. Nitrogen fixation was not detectable.

Table 4.1.1: Artificial and natural seawater medium: Amounts of dissolved organic carbon (DOC) and total

dissolved nitrogen (TDN), dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA) or total hydrolysable dissolved amino acids (THDAA) at the different time points (before bottling: only medium; blank: medium poured into serum bottles before inoculation; t0: directly after inoculation; tí 1 week after inoculation; t2: 3 weeks after inoculation). (n.d. = not determined)

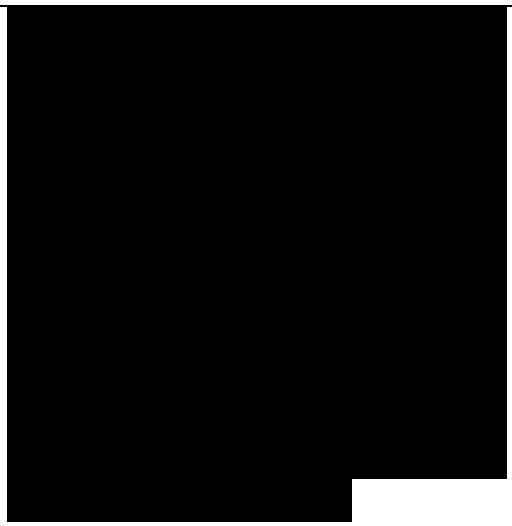
Composition of the dissolved organic matter

The analysis of extracted DOM from artificial and natural seawater using FT-ICR-MS was performed in the negative and positive electrospray ionization (ESI) mode. In ESI negative mode, 690 of 7347 peaks (10%) in artificial and 3685 of 14873 peaks (25%) in natural seawater were assigned with molecular formulas (Figure 4.1.2). However, in negative ESI mode inorganic contaminants (as indicated by a negative mass defect) suppressed ionization of organic molecules. To estimate this suppression effect, natural deep sea SPE-DOM was added to the artificial seawater extracts in the same, tenfold and hundredfold concentration (0.04, 0.4 and 4 mg C L-1). The DOM signal was completely suppressed after addition of 0.04

mg C L-1 (same range as the measured DOC contamination in the artificial seawater). In contrast, the typical distribution of natural DOM peaks in the mass spectrum was detected after addition of 0.4 and 4 mg C L-1 natural DOM (ten- and hundredfold the DOC concentration in artificial seawater). As a consequence, FT-ICR-MS spectra of the artificial seawater medium had to be analyzed in ESI positive mode, in which the inorganic contaminants were not detected.

The mass spectra obtained after positive ionization revealed two homologous series of high peaks with distances of 44.02567 Da ($C_2H_4O_1$) corresponding to a homologous series of polyethylene glycol (PEG) oligomers (Figure 4.1.1 A). The PEG peaks were present in the mass spectra of all sampled time points. Therefore, the mean intensity of five PEG peaks (m/z 305.157061, 349.183268, 393.209484. 437.235707, 481.261932) was used to normalize all other peaks in the mass spectra (Figure 4.1.1 A, arrows). The amount of PEG contamination was quantified by standard addition to the contaminated SPE-DOM blanks from artificial seawater using an authentic PEG standard (Figure 4.1.1 B and C). The total amount

of PEG in the sample was then calculated to account for only 3 nmol C L-1, which is three orders of magnitude below the measured concentration of the DOC contamination in the artificial seawater medium (5 |imol C L-1). Thus, the PEG contamination was extremely low despite the very high peaks in the ESI FT-ICR mass spectra (Figure 4.1.1). A screening for potential PEG contaminations indicated NaOH as a likely source (data not shown).



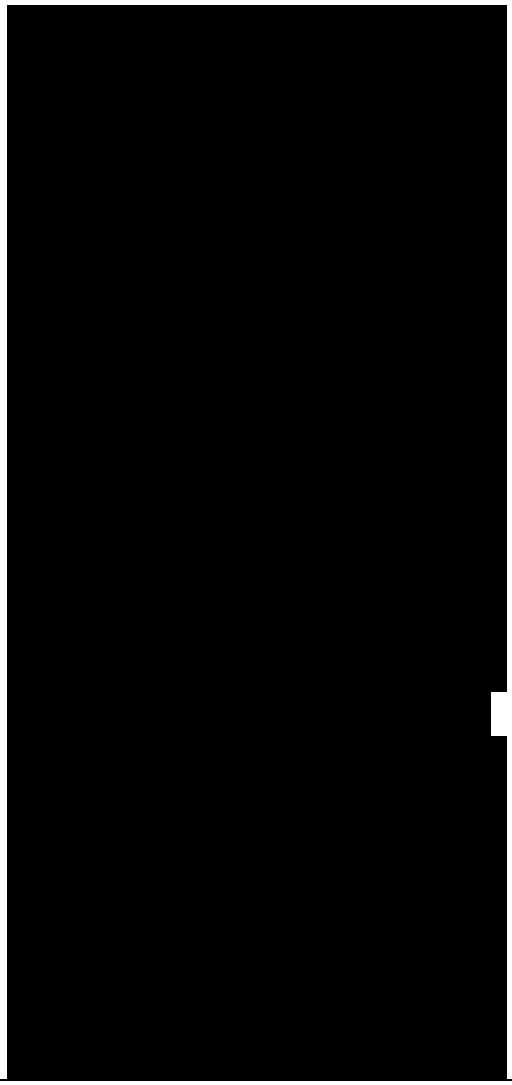


Figure 4.1.1: Polyethylene glycol (PEG) contamination in artificial seawater: (A) FT-ICR mass spectra measured from the artificial seawater extracts (ESI positive mode) showing high peaks of PEG contamination with intervals of 44.02567 Da. Vertical arrows indicate the peaks used for normalization. (B) FT-ICR mass spectra after standard addition of PEG in the range of 0 to 3.6 nmol L-1 to the extracted DOM of the blank sample. (C) Linear calibration curve of PEG standard after addition to blank sample.

Figure 4.1.2: FT-ICR mass spectra of the negative electrospray ionization at the time points to of (A) artificial seawater and (B) natural seawater. Asterisks indicate inorganic contaminations. Inserts show a zoom into the region of

mass 325.0 to 325.2. In the negative mode inorganic contaminations suppressed the ionization of organic substances present in low amounts in the artificial seawater. In van Krevelen diagrams, organic compounds are plotted based on their oxygen to carbon (O/C) and hydrogen to carbon (H/C) ratios. During the incubation in artificial seawater (from t0 to t2) the relative intensities of compounds with an O/C < 0.4 and H/C > 1.5 decreased strongly, while the relative intensity of only few compounds increased (Figure 4.1.3 A). After growth of the bacteria (from t0 to t2) in artificial seawater, many compounds with an O/C < 0.4 and H/C > 1.5 were not detected anymore. Most of the compounds that disappeared had molecular formulas containing nitrogen (Figure 4.1.3 B and C). No significant difference was found between the procedural blank of the artificial seawater and t0 or between t1 and t2 (blank and t0 before, t1 and t2 after growth). Major changes in incubations with natural seawater during growth (from t0 to t2) were found in two clusters of compounds: (i) compounds with O/C < 0.4 and H/C < 1.0, and (ii) compounds with O/C > 0.7 and	[redacted]	

HC > 1.25 (Figure 4.1.3 D, blue). Compounds of cluster (i) with low O/C and H/C ratios contained sulfur but no nitrogen (Figures 4.1.3 E and F, yellow). Compounds of cluster (ii) with high O/C and H/C ratios contained mainly nitrogen and sulfur (Figures 4.1.3 E and F, red).

Figure 4.1.3: Van Krevelen diagrams (O/C = oxygen to carbon ratio and H/C = hydrogen to carbon ratio of the molecular formulas of single molecules detected) showing the change in DOM composition from t0 to t2 in artificial (A-C) and natural seawater (D-F). (A+D). Changes in normalized relative intensities are color-coded showing a decrease in blue, an increase in red and no significant change during incubation in grey. (B+C, D+F): Compounds containing no nitrogen and sulfur (purple), sulfur but no nitrogen (yellow), nitrogen but no sulfur (blue) and both nitrogen and sulfur (red) at t0 (B+E) and t2 (C+F). In artificial seawater the most notable decrease is found in the fraction of compounds containing high H/C and low O/C ratios and nitrogen but no sulfur. These compounds may correspond to detergents. In natural seawater a group of substances with high ratios of H/C and O/C (probably thio- and

aminosugars) containing mostly nitrogen and sulfur decreased. In addition, compounds with low H/C and O/C ratios (probably condensed hydrocarbons) containing only sulfur were also observed to decrease.

Glucose and ammonium addition experiments

Cell numbers in artificial seawater medium without glucose and ammonium were about

3 X 105 cells mL-1. The separate addition of glucose and ammonium did not lead to a strong increase in cell numbers, whereas the simultaneous addition of glucose and ammonium increased cell numbers to about 107 cells mL-1 (Figure 4.1.4).

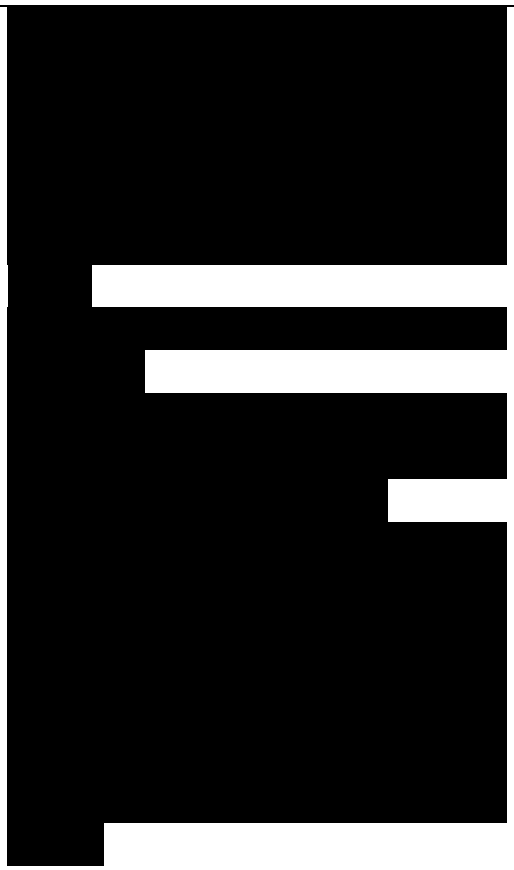


Figure 4.1.4: Growth curves of Pseudovibrio sp. strain FO-BEG1 incubated in artificial seawater without the addition of a fixed carbon or nitrogen source or with addition of ammonium or glucose. Addition of each substance alone did not lead to an increase in cell numbers. When both (ammonium and glucose) were added at the same time, cell numbers increased significantly.

Biolog experiment

Pseudovibrio sp. FO-BEG1 cells pre-grown in artificial seawater medium were used to inoculate a biolog GN2 microplate

containing 95 different growth substrates. The bacteria showed activity after 8 to 14 days on the following substrates: D-raffinose, D-trehalose, turanose, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, acetic acid, glucoronamide, L-glutamic acid, glycyl-L-glutamic acid, L-serine and D-glucose-6-phosphate. No activity was observed on the remaining 84 substrates.

Discussion

In the present study, the heterotrophic Pseudovibrio sp. strain FO-BEG1 was grown either in artificial seawater medium with extremely low DOC concentrations or in natural seawater from an oligotrophic ocean (south Pacific, surface water) representing typical marine DOM. The bacterial growth was sustained by very low amounts of DOC in both incubation setups. However, growth in artificial seawater with only 0.06 mg DOC per liter seawater shows that bacterial growth can be sustained by even lower concentration as present in most natural oligotrophic seawaters, which typically contain less than 1 mg C L-1 (Schut et al., 1997; Hansell et al., 2009).

Impurities in oligotrophic media

During the preparation of

oligotrophic medium, different potential contamination risks emerge. In our case, an inorganic contamination was introduced to the artificial seawater after the incubation during the solid phase extraction of DOM (SPE-DOM). This led to ion suppression of the organic compounds in the FT-ICR mass spectra in ESI negative mode. Consequently, it was not possible to analyze the DOM composition in that mode and we chose the positive mode instead. This problem does not occur in natural seawater samples, because the concentration of DOC is higher. We could show that the organic substances present in the artificial medium are detectable if the overall background of DOM is 10 to 100 times higher. This observation indicates that a specific concentration of organic matter has to be present to be detected by ESI FT-ICR-MS if inorganic substances are introduced in high amounts during extraction or ionize extremely well. In ESI positive mode, an organic contamination of the artificial seawater was observed. Two series of polyethylene glycol (PEG) oligomers were detected. As a potential source NaOH used for medium preparation was identified. Since PEGs ionize extremely well in ESI positive	[redacted]	

mode, the peaks are much higher compared to most other peaks in the sample. Using the standard addition method (described in material and methods), the total amount of PEG contaminations was estimated to be 1000fold less than the total DOC concentration in artificial seawater, and thus represented only a very small fraction of the entire DOC contamination. These impurities analyzed in the artificial seawater medium were not detected in the SPE-DOM of natural seawater samples and thus did not affect the FT-ICR-MS analysis of natural seawater incubations. Growth and substrate use in artificial seawater During growth in artificial seawater under extremely oligotrophic conditions, Pseudovibrio sp. strain FO-BEG1 multiplied from about 20 cells mL-1 to 2 X 104 cells mL-1, even though the overall amount of DOC did not measurably decrease. Thus, the amount of compounds that were consumed was probably below detection limit (0.5 |imol C L-1). Based on the increase in cell numbers when grown with glucose and ammonium (supplementary material Table S.4.1 and Figure S.4.1), it was calculated that about 1 to 3 |imol C L-1 is needed for the observed		

cell numbers as carbon and energy source. Apparently, the uptake of amino acids alone could not sustain bacterial growth because the initial amino acid concentration (0.13 |imol L-1) was already much lower than the required 1 to 3 |imol C L-1. During the initial growth phase, we found a decrease in dissolved free amino acids (DFAA) concentration concurrently with an increase in dissolved combined amino acids (DCAA), whereas the overall amino acid concentration (DFAA + DCAA) did not decrease (Table 4.1.1). Within the growth phase, the DFAA might have been used as precursors, e.g. for exo-enzymes, which lead in turn to an increase in the DCAA concentration, but no overall decrease in the amino acid concentration. During stationary phase, we found a slight decrease of total amino acids. This may suggest that amino acids were rather used as substrate for maintaining non-growing cells. The compositional analysis of DOM with ESI FT-ICR-MS revealed a decrease of nitrogen-containing compounds during the initial growth phase (Figure 4.1.3 B and C). Inferring from their molecular compositions (high H/C, low O/C, N-containing), these substances were most likely detergents, such		

as ampholytic amino oxides or betaines. Using this method we cannot quantify the amount of carbon corresponding to this decrease. In addition, a compound with a decreasing peak in the mass spectrum is not necessarily completely oxidized to CO2. Thus, we cannot ultimately clarify whether the use of these substrates alone explains the observed growth. Nevertheless, the preferential decrease of nitrogen-containing compounds suggests that these substances at least served as nitrogen source. This agrees with the observation that N2 fixation was not detectable. Growth and substrate use in natural seawater The overall concentration of amino acids in the natural seawater was already initially quite low and did not further decrease during the incubation. Thus, amino acids did not sustain growth or survival of cells. The compositional analysis of DOM showed a decrease of two groups of compounds. One of them was likely containing carbohydrate-like compounds with nitrogen and sulfur. These compounds are most likely thiosugars and/or aminosugars and may therefore have also served as nitrogen source. In addition, we observed a decrease in relative intensities of		

compounds with low O/C and H/C ratios containing sulfur but no nitrogen. Compounds with low amounts of oxygen and hydrogen in comparison to carbon are typically condensed or aromatic hydrocarbons. Since it cannot be excluded that these compounds were lost abiotically, e.g. by absorption to the glassware, it needs to be further investigated if the bacteria indeed metabolized these complex molecules during growth. Notably, during growth of Pseudovibrio sp. strain FO-BEG1 in natural seawater, we observed a decrease of different groups of substances compared to artificial seawater and preferentially substances containing sulfur decreased. The use of multiple substrates The potential use of different substrates under oligotrophic conditions was shown by the biolog experiment. The bacteria were able to metabolize different types of organic compounds, such as sugars (e.g. D-raffinose and D-trehalose), amino acids (L-serine, glutamic acid), carboxylic acids (glucuronic acid, acetic acid) and amide (glucuronamide). These compound classes were also present as contaminations in the artificial seawater medium and decreased during growth. This is in agreement with studies on E.		

coli showing a broad potential to use different substrates after adaptation to carbon starvation (Ihssen and Egli, 2005). Even though the substances decreasing during growth, as revealed by ESI FT-ICR-MS, tended to cluster into certain groups with a specific ratio of O/C and H/C, the overall pattern showed a broad simultaneous use of many substrates both in artificial and natural seawater. In contrast to the biolog experiment, this does not show the potential to use a substrate, but the actual decrease of compounds present in original concentrations. Using different compounds simultaneously may enable bacteria to grow on very low concentrations of each of the different substrates (Lendenmann et al., 1996; Kovárová-Kovar and Egli, 1998), if a substrate does not repress enzymes for another less efficient one (reviewed in Egli, 2010). This strategy, together with the potential to use substrates, which are not present but may become available, enables bacteria to survive in habitats with a low and fluctuating supply of nutrients, as it is found in the oceans. Conclusions The data presented in this study demonstrate that contaminations can arise from many different		

sources (e.g. chemicals, bottles, humans or plastics), which in turn might support growth of bacteria under oligotrophic conditions. Moreover, even under extremely oligotrophic conditions, the isolated bacteria were not in a resting state, but showed a moderate growth, even though nitrogen, carbon and energy sources were limiting factors at the same time. The investigated Pseudovibrio sp. FO-BEG1 uses many different types of substrates under nutrient-poor conditions as demonstrated by the FT-ICR-MS data (Figure 4.1.3). In our case, amino acids were not the primary substrate for growth, but rather a complex mixture of organic compounds, preferably containing nitrogen. Furthermore, we were able to show that ESI FT-ICR-MS is a strong tool to investigate bacterial growth under low-nutrient conditions. Table S.4.1: Cell numbers of isolate Pseudovibrio strain FO-BEG1 derived from different amounts of carbon added to the medium. Carbon in form of glucose was added to a Pseudovibrio strain FO-BEG1 culture in 2 different concentrations. As a negative control a culture without addition of carbon was used. To ensure carbon-dependent growth,		

ammonium was added to the medium (with and without carbon addition) as nitrogen source. Figure S.4.1: Amount of carbon needed for specific cell number. From additions of glucose to the medium (Table S.4.1) the amount of carbon needed for growth of isolate FO-BEG1 was calculated by subtracting the cell numbers without carbon addition (as negative control) from the cell numbers with carbon addition and plotting amount of carbon versus cell numbers produced. The amount of carbon needed for 105 cells mL-1 was calculated to be 3 ^mol C L-1 and for 104 cells 1 ^mol C L-1. 4.2 Facultatively oligotrophic bacteria isolated from the habitat of large sulfide-oxidizers Abstract Axenic cultivation of large chemolithoautotrophic bacteria belonging to the genus Beggiatoa succeed only rarely. Growth of the large sulfide-oxidizers often seems to be dependent on the presence of heterotrophic prokaryotes, similar to the often described associations of cyanobacteria and heterotrophs. Recently, we observed that the growth of the marine, chemolithoautotrophic Beggiatoa sp. strain 35Flor depends on the presence of the a-Proteobacterium Pseudovibrio		

sp. strain FO-BEG1. Furthermore, we found that this bacterium, besides heterotrophic growth on organic-rich medium, is capable of growth under extreme nutrient deficiency in artificial and natural seawater. This observation inspired us to investigate whether we could isolate other facultative oligotrophs from overlaying water of Namibian sediment, an environment known to contain a large number of different sulfide- oxidizers belonging to the family Beggiatoaceae. Indeed, we succeeded to obtain 14 new strains closely related to known marine bacteria, all of which were capable of growth under extreme nutrient deficiency. The potential of these isolates to support growth of the large sulfide-oxidizing bacteria can now be studied in culture-based experiments.

Introduction

Large bacteria, such as Beggiatoa spp. or filamentous cyanobacteria, often live together with heterotrophic prokaryotes and these associations seem to be the reason for the inability of axenic cultivation of the large bacteria (Burton and Morita, 1964; Cohen and Rosenberg, 1989; Palinska et al., 1999; Morris et al., 2008). Different reasons for these interactions have been

proposed, such as the recycling of Cacbon điôxít or the reduction of the oxygen concentration (Kuentzel, 1969; Paerl and Pinckney, 1996). It is known that Beggiatoa spp. typically lack the gene for catalase (Larkin and Strohl, 1983), but since aerobic respiration produces reactive oxygen species (ROS, Tapley et al., 1999) an efficient protection against such molecules is needed. Therefore, Beggiatoa spp. might depend on the enzyme catalase, which catalyzes the disproportionation of hydrogen peroxide to oxygen and water, or other protection systems of the associated heterotrophs against ROS. The positive effect of catalase and of accompanying heterotrophic bacteria on the growth of Beggiatoa filaments (Burton and Morita, 1964; Strohl and Larkin, 1978; Gude et al., 1981; Nelson et al., 1986b) and, furthermore, the accumulation of peroxides in cultures without catalase or accompanying bacteria was shown (Burton and Morita, 1964). Recently, we found that both Pseudovibrio sp. strain FO-BEG1 and Pseudovibrio denitrificans (DSM number 17465) support growth of the marine chemolithoautotrophic Beggiatoa sp. strain 35Flor (Chapter 3.1). Both bacterial strains are heterotrophic		

organisms (Chapter 3.2, Shieh et al., 2004). Additionally, we have shown that the newly isolated Pseudovibrio sp. strain FO-BEG1 can grow under extremely oligotrophic conditions and its substrate use in pure artificial and natural seawater was studied in detail (Chapter 4.1). The aim of the present study was to isolate heterotrophic, facultatively oligotrophic bacteria from Namibian sediments, the habitat of large sulfide-oxidizers, to investigate how common facultative oligotrophy is among bacteria associated with large sulfide-oxidizers. Here, we report the successful isolation of 14 facultatively oligotrophic bacteria from water overlaying Namibian sediments using a method relying on the change from oligotrophic to eutrophic growth conditions, called the CANgrow-method (changing availability of nutrients growth-method). In contrast to earlier methods for the isolation of marine bacteria, the artificial oligotrophic medium used here, is defined and contains much lower nutrient concentrations. Three initial transfers strongly pre-select for bacteria, which can grow under extreme nutrient deficiency. Subsequently, three transfers on nutrient-rich agar plates select for facultatively		

oligotrophic bacteria and are used to obtain pure cultures. Finally, the ability of the isolates to grow oligotrophically is ensured by at least seven transfers in pure artificial seawater. Material and methods Samples The new bacterial strains were isolated from oceanic bottom water overlaying Namibian sediments that harbor different large sulfur bacteria (sample acquisition described in Salman et al., 2011). All samples were stored at 4°C. In addition to the new isolates, the Pseudovibrio denitrificans type strain (DSM number 17465) was purchased from the German culture collection DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultivated under oligotrophic conditions. Growth media and cultivation conditions For cultivation and isolation, two different media were used, an oligotrophic and a eutrophic medium. The liquid, oligotrophic medium was composed as described above (Chapter 4.1), but prepared under synthetic air atmosphere (20% O2 in N2; H2O < 3 ppm-mol, CnHm < 0.1 ppm-mol, CO < 1 ppm-mol, CO2 < 1 ppm-mol).		

Furthermore, bottles were filled only with 50 mL medium and the medium was not cleaned using solid phase extraction. This medium was used for all oligotrophic cultivation experiments. The solid, eutrophic medium was composed as described above (Chapter 4.1, Methods section "Biolog experiment"). All incubations in oligotrophic and eutrophic media were performed without shaking at 28°C in the dark. CANgrow-method (changing availability of nutrients growth-method) For isolation, 50 mL of oligotrophic medium were inoculated with 100 |iL seawater sample (from off shore Namibia). The cultures were transferred (100 |iL enrichment in 50 mL fresh medium) at least three times in oligotrophic medium with incubation periods between the transfers of at least one week. Aliquots of the oligotrophic enrichments were then plated on eutrophic, solid medium and single colonies were transferred three times on eutrophic medium. Finally, at least another seven transfers (100 |iL culture in 50 mL fresh medium) were performed in liquid, oligotrophic medium (Figure 4.2.1). Sequencing of 16S rDNA genes		

and phylogenetic analysis Eutrophically grown colonies were picked and directly transferred to a polymerase chain reaction (PCR) mix containing 1x PCR MasterMix (Promega, Mannheim, Germany) and 1 |imol L-1 of each primer (GM3F and GM4R in Muyzer et al., 1995). The PCR program applied was as follows: initial denaturation at 95°C for 5 minutes, 32 cycles of 95°C for 1 minute, 50°C for 30 seconds and 72°C for 90 seconds followed by a final elongation at 72°C for 7 minutes. PCR products were cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Karlsruhe, Germany) according to manufacture's instructions. Sequencing of the cloned inserts was performed using the Big Dye Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and sequences were analyzed on an ABI Genetic Analyzer 3130x (Applied Biosystems, Carlsbad, CA, USA). Nearly full-length sequences were assembled with SeqMan (Lasergene software package, DNAStar, Madison, WI, USA) and deposited in the DDBJ/EMBL/GenBank databases under accession numbers FR716535 to FR716549. Phylogenetic analysis of the 16S rDNA		

sequences was performed using the ARB software package (Ludwig et al., 2004) and release 102 of the SILVA SSURef database (Pruesse et al., 2007). Tree reconstruction with maximum likelihood and neighbour joining methods was performed using 0, 30 and 50% positional conservatory filters that exclude highly variable regions. Finally, a consensus tree based on the different reconstruction methods was built. A total number of 102 nearly full-length sequences was used for initial calculation to stabilize tree topology. Displayed in the final tree (Figure 4.2.2) are the sequences of the 15 isolates grouped with their closest relatives. Cell counts Cell counts were performed as described in Chapter 4.1. Measurement of dissolved organic carbon (DOC) Dissolved organic carbon (DOC) was measured in the oligotrophic medium using a Shimadzu TOC-VCPH total organic carbon analyzer (Shimadzu, Kyoto, Japan). Acidification of samples was performed with 1% v/v 2 mol L-1 HCl followed by sparging with synthetic air in order to remove inorganic carbon. The detection limit of the method was 5 |imol C L-1 (0.06 mg C L-1). The analytical		

accuracy was confirmed with reference material (deep Atlantic seawater) and low carbon water from the consensus reference materials program (D.A. Hansell, University of Miami, Coral Gables, FL, USA). Results Isolation of facultatively oligotrophic bacteria Applying the CANgrow-method, which favors facultatively oligotrophic bacteria (Figure 4.2.1) , we obtained 14 isolates of marine bacteria that were able to adapt from oligotrophic to eutrophic growth conditions and vice versa within 3 to 5 days. We were able to obtain pure cultures of these strains by transferring single colonies from organic-rich agar plates and could show that these colonies were able to grow oligotrophically by at least seven transfers in pure artificial seawater. Changing availability of nutrients growth- method (CANgrow-method) Figure 4.2.1: Comparison of strategies for the isolation of oligotrophic bacteria. Three different methods for the isolation of obligately or facultatively oligotrophic bacteria are compared with the newly developed CANgrow-method (Changing availability of		

nutrients growth- method). Bacteria from three different phyla were isolated with the CANgrow-method. Phylogenetic relations are shown in a 16S rDNA sequence tree including the Pseudovibrio sp. strain FO-BEG1 showing > 99.5% sequence identity to the Pseudovibrio denitrificans type strain (Figure 4.2.2). Except for two isolates, which were closely related to Arthrobacter spp. (Actinobacteria) on 16S rDNA level (99.8 to 99.9% identity to closest related strain), all isolates were members of the Proteobacteria. The remaining 12 isolates were members of the Gammaproteobacteria, two of them grouping with Kangiella spp. (96.4% identity to closest related strain) and ten withMarinobacter spp. (98.7 to 100% identity to closest related strain). Figure 4.2.2: Phylogenetic trees based on a total number of 102 nearly full-length sequences were calculated with maximum likelihood and neighbor joining methods using different positional conservatory filters. The displayed tree is an excerpt from the consensus tree that was inferred based on the different reconstruction approaches. The 14 new isolates and strain FO-BEG1 are grouped with the most closely related type strains.		

Isolated strains listed in one line feature an identical 16S rDNA sequence, whereas isolated strains listed directly one below the other are 99.6 to 99.9% identical in their 16S rDNA sequence. The isolates FO-NAM13, 14 were only able to grow for six transfers under oligotrophic conditions and are therefore marked grey in the tree. Oligotrophic growth The artificial seawater contained a DOC concentration of 0.18 ± 0.06 mg C L-1 (15 ± 5 |imol C L-1). Growth curves in oligotrophic artificial seawater medium (Figure 4.2.3) were obtained for one isolate from each phylogenetic group (Actinobacteria, Alpha- and Gammaproteo- bacteria). We observed a clear increase in cell numbers starting from 4 to 20 cells mL-1 to a final density of 104 to 105 cells mL-1. The proteobacterial isolates showed growth after 2 days and reached a stationary phase after 5 to 7 days of incubation. Both actinobacterial isolates were characterized by delayed growth that was detectable after 12 days. Here, the stationary phase was reached after about 20 days of incubation. Moreover, all isolates except for the two isolated Kangiella strains were able to grow after at least seven		

transfers in the oligotrophic seawater medium. The two isolates closely related to Kangiella spp. were not able to grow after more than six transfers under oligotrophic conditions. The isolates closely related to Marinobacter spp. reached the highest final cell numbers (Figure 4.2.3 D), whereas the actinobacterial isolates showed lowest final cell densities (Figure 4.2.3 C). Furthermore, we observed growth under oligotrophic conditions for the type strain Pseudovibrio denitrificans (DSM number 17465). The growth curve of this strain (Figure 4.2.3 B) showed the same pattern as the Pseudovibrio sp. FO-BEG1, which is currently growing in the 26th oligotrophic transfer in highly purified artificial seawater.

Figure 4.2.3: Oligotrophic growth curves of (A) strain FO-BEG1 (Alphaprobteobacteria, related to Pseudovibrio spp.), (B) Pseudovibrio denitrificans type strain (Alphaproteobacteria), (C) isolate FO-NAM2 (Actinobacteria, related to Arthrobacter spp.) and (D) isolate FO-NAM6 (Gammaproteobacteria, related to Marinobacter spp.).

Discussion

Isolation of facultatively

oligotrophic bacteria with the CANgrow-method Each strategy that is applied for the isolation of bacteria selects for a specific physiology and metabolism. Most approaches used recently for the isolation of oligotrophic bacteria are based on the dilution to extinction method (Button et al., 1993; Connon and Giovannoni, 2002) and thereby select for the most abundant microorganisms. In contrast, the CANgrow-method favors bacteria, which might not have been particularly abundant in the original inoculum, but can adapt fast to changes in nutrient availability. Previous studies have shown that many bacteria isolated under oligotrophic conditions can adapt to nutrient-rich media (Yanagita et al., 1977; MacDonell and Hood, 1982; Carlucci et al., 1986). Also, the single-cell encapsulation method (Zengler et al., 2002) is based on oligotrophic growth followed by eutrophic growth conditions. This cultivation approach is similar to ours (Figure 4.2.1), but we used artificial seawater of very low DOC concentration rather than natural seawater. The measured DOC concentration of 0.18 mg C L-1 is two to five times lower than in natural seawater (Schut et al., 1997; Hansell et al., 2009).		

Nevertheless, we are certain to observe true growth under these extremely oligotrophic conditions, since we performed at least seven transfers in purified artificial seawater with each of the isolated strains and 26 transfers for Pseudovibrio sp. FO-BEG1, for which we also identified growth substrates under oligotrophic conditions (Chapter 4.1). The initial cell number after each transfer was 4 to 20 cells mL-1. Thus, 9 to 15 divisions must have occurred between two consecutive transfers to account for a final cell number of 104 to 105 cells mL-1 as observed at the end of the growth phase. This accounts for 60 to 100 divisions during a total of 7 incubations. Therefore, we conclude that all isolates are viable under oligotrophic conditions by the definition of Button et al. (1993), who characterize organisms as viable after having performed 13 divisions which we observe already after 1 to 2 transfers. Diverse phylogeny of facultatively oligotrophic bacteria The isolated bacterial strains belong to different phylogenetic groups, namely Alphaproteo-bacteria, Gammaproteobacteria and Actinobacteria. Growth under oligotrophic conditions as observed for the isolates FO-		

NAM13 and FO-NAM14 (related to Kangiella spp.), has so far never been described for any member of the genus Kangiella. Long-term starvation and survival but not growth in the absence of external nutrients has previously been reported for Arthrobacter spp. and was proposed to be fueled by internally stored reserve material (Zevenhuizen, 1966). In our study, isolates FO-NAM1, FO-NAM2 (related to Arthrobacter spp.) were transferred in oligotrophic medium more than seven times and active growth was always observed. Hence, we assume that the cells gained energy and produced biomass from an external source, since we determined growth and not only survival. Bacteria belonging to the genus Marinobacter are known to be diverse in physiology (e.g. Gauthier et al., 1992; Huu et al., 1999). Substrate uptake under low nutrient conditions was shown for Marino- bacter arcticus (Button et al., 2004), but oligotrophic growth was not studied in detail. In contrast, the isolates FO-NAM3 to FO-NAM12 (related to Marinobacter spp.) actively grow under nutrient deficiency. Cell numbers of the isolated strains growing under oligotrophic conditions differed		

between the phylogenetic groups. The cell numbers of isolates related to Marinobacter spp. were higher than cell numbers of the other isolates, whereas the isolates related to Kangiella spp. did not grow for more than six transfers. This suggests that the different bacteria vary in their capabilities of adapting to oligotrophic growth conditions or that the present organic and inorganic material can be used differently among the isolates. This might be due to the presence of different metabolic pathways and a different number and type of high affinity transporters necessary for scavenging nutrients at such low concentrations. Taken together, our data support earlier findings (Yanagita et al., 1977; MacDonell and Hood, 1982; Carlucci et al., 1986) that the ability to switch between extreme nutrient deficiency and affluence of substrate is not unusual or restricted to a certain phylogenetic group, even if the level of adaptation might differ. Therefore, we propose that the ability to grow under extreme substrate limitation is much more widespread among known heterotrophic bacteria than currently recognized.

As expected, the heterotrophic bacteria isolated from the habitat

of large sulfide-oxidizers are phylogenetically diverse. It was possible to isolate bacteria following a similar metabolic strategy - facultative oligotrophy. If these bacteria are associated with sulfide-oxidizers, if their metabolic activity is somehow related to the presence or absence of the lithothrophs or whether they can support growth of the large sulfur bacteria by scavenging ROS needs to be further investigated. ~Sir William Lawrence Bragg (1890-1971) Chapter 5 Concluding remarks and outlook The findings of this thesis contribute to different topics ranging from migration behavior of mat-forming sulfur bacteria via associations between different bacteria to the lower limits of bacterial growth. Nevertheless, all these different aspects are linked to each other. The growth of Beggiatoa sp. filaments, for which we discovered an unusual migration behavior (Chapter 2), depends on the presence of Pseudovibrio sp. (Chapter 3), which is capable of growth under extreme nutrient deficiency (Chapter 4). This chapter (Chapter 5) connects all these different aspects, including preliminary data, which are not presented in the previous chapters and considers the		

obtained results in a broader context. Furthermore, a detailed discussion of special proceedings and precautions during performed experiments is given. Finally, this chapter ends with an outlook for future research concerning the discussed topics. Associations between small heterotrophic and larger bacteria Axenic cultivation of large marine Beggiatoa spp. under chemolithoautotrophic conditions is difficult and only rarely successful (Nelson and Jannasch, 1983; Nelson et al., 1986a). In this thesis, a successful and stable cultivation of the marine chemolithoautotrophic Beggiatoa sp. strain 35Flor (6 |im in diameter) is described. This Beggiatoa strain grows solely in the presence of an accompanying organism, the heterotrophic Pseudovibrio sp. strain FO-BEG1 (Chapters 2 and 3). We propose that the accompanying bacterium protects the Beggiatoa sp. from oxidative stress because we have shown that the genome of the accompanying organism possesses more than 20 genes for the enzymes catalase, superoxide dismutase and peroxidase (Chapter 3.2), whereas sulfide-oxidizing bacteria of the genus Beggiatoa typically do not possess the gene for the enzyme catalase (reviewed in Larkin and		

Strohl, 1983). Similar to large sulfide-oxidizers, also large marine cyanobacteria can often be found associated with small heterotrophic bacteria and it is difficult to sustain axenic cultures (Palinska et al., 1999; Morris et al., 2008). The association of small heterotrophic bacteria with larger bacteria, such as Beggiatoa spp. or cyanobacteria in microbial mats is a common observation (Cohen and Rosenberg, 1989; van Gemerden, 1993). The complex interactions between cyanobacteria and heterotrophic bacteria have been studied and different reasons for their interactions have been proposed. These reasons include recycling of Cacbon điôxít needed by the cyanobacteria (Kuentzel, 1969), production of growth factors and formation of anoxic microniches due to aerobic respiration (Paerl and Pinckney, 1996). Interestingly, for cyanobacteria of the genus Prochlorococcus, it was also proposed that the function of the heterotrophs is to scavenge reactive oxygen species (ROS) because the Prochlorococcus spp. themselves have no gene encoding for a catalase or peroxidase (Morris et al., 2008). Furthermore, a mutant of the accompanying heterotroph lacking the gene for catalase was		

found to not support growth of the cyanobacteria, whereas the addition of catalase had a positive effect on their growth (Morris et al., 2008). These observations concerning the association between cyanobacteria and accompanying heterotrophic bacteria point in the same direction as the observations presented here on the association between Beggiatoa sp. and Pseudovibrio sp. (Chapter 3). It suggests that the protection system of the large bacteria from ROS might be less efficient than the ones of smaller heterotrophic bacteria. Cyanobacteria and Beggiatoa spp. are often found to share one habitat as for example in microbial mats (van Gemerden, 1993) or in biofilms associated with the black band disease of scleractinian corals (Richardson, 1996). Therefore, in these common habitats they might also live together with similar types of heterotrophic bacteria. The bacteria associated with cyanobacteria belong to a diverse range of phylogenetic groups, including Actinobacteria, Bacteroidetes, Alpha-, Beta-, Gamma- and Deltaproteobacteria (Salomon et al., 2003; Kolmonen et al., 2004; Hube et al., 2009). The isolation of phylogenetically different bacteria from the habitat of large		

sulfide-oxidizers (Chapter 4.2) indicates that also the large sulfide- oxidizers are not restricted to the association with bacteria of one phylogenetic group. It seems more likely that the large bacteria depend on specialized functions performed by certain types of the associated bacteria rather than the presence of a specific phylogenetic group. Very recently, a metagenomic sequence analysis of bacterial communities associated with the green macroalgae Ulva australis revealed that not the phylogeny of the associated bacteria but the function of their genes was correlated with the associations (Burke et al., 2011). Consequently, it was proposed that the functional genes rather than the 16S rDNA genes are more appropriate to investigate associations in microbial communities (Burke et al., 2011). Toxicity factors and migration behavior of Beggiatoa filaments The growth of Beggiatoa spp. depends on oxygen and sulfide, but both substances can also be harmful to the bacteria if concentrations exceed a critical threshold (Winogradsky, 1887; M0ller et al., 1985). Close relatives of Beggiatoa spp. belonging to the genus Thiomargarita can be found in sulfidic sediments with sulfide		

concentrations of 100 to 800 |imol L-1 (Schulz et al., 1999) or even up to 22 mmol L-1 (Bruchert et al., 2003). Moreover, these bacteria can also tolerate atmospheric oxygen levels while being exposed to lower sulfide concentrations (0 to 100 |imol L-1) at a pH of 7.3 (Schulz et al., 1999; Schulz and de Beer, 2002). However, Thiomargarita spp. cells are not as motile as Beggiatoa spp. and as a consequence they can not position themselves in the transition zone of oxygen and sulfide. Instead, the cells wait for resuspension of the sediment to get into contact with oxygenated, nitrate-containing seawater (Schulz et al., 1999). In contrast, Beggiatoa filaments are motile and built up mats between the opposing gradients of oxygen and sulfide (Winogradsky, 1887; Keil, 1912; J0rgensen, 1977; Nelson et al., 1982; Nelson and Jannasch, 1983). Consequently, Beggiatoa filaments usually get into contact with oxygen and sulfide at the same time, since they consume the two gases, they lower the concentrations and steepen the gradients (Nelson et al., 1986a). However, depending on the thickness of the Beggiatoa mat, some filaments might not be in direct contact with oxygen and sulfide at the same time (Nelson		

et al., 1986b). Taken this into account, the results presented in this thesis (Chapter 2.1) suggest that sulfide is probably more harmful to the cells under oxic conditions because the aerobic sulfide oxidation leads to a decrease in pH (Winogradsky, 1887) and as a consequence more sulfide outside the cells is present in form of H2S. In the presented experiments (Chapter 2.1) we measured a pH of 6.5 within the mat, leading to about 70% of the sulfide being present in form of H2S (compared to only about 30% present as H2S at pH 7.3). This uncharged, harmful gas can easily diffuse into the cells where it can act as a strong reductant and binds to iron in cytochromes, by this blocking the cellular respiration. Thus, the bacteria probably have to perform sulfide oxidation to detoxify the inside of the cells. At high sulfide fluxes the cells obviously accumulate large amounts of storage compounds during this process and might eventually burst. To prevent this, the bacteria have to dispose of the internal storage compounds. In the anoxic regions the pH is higher and sulfide predominantly occurs in form of HS- which can not diffuse into the cells and might only get inside via ion channels or transport systems.

In this thesis (Chapter 2.1), a

new strategy to cope with high sulfide fluxes is proposed. The bacteria actively migrate into anoxic regions with high sulfide concentrations. Here, the aerobic sulfide oxidation is stopped and with this also sulfur deposition. The bacteria can then reduce the intracellular sulfur with internal PHA to form sulfide. This strategy represents a novel explanation for the presence of Beggiatoa filaments in anaerobic habitats and shows that filaments actively migrate into anoxic, sulfidic regions. Previously, downwards migrations were typically observed in habitats containing nitrate and there the anaerobic sulfide oxidation with nitrate lowered the diffusion zone of sulfide and separated the oxygen and sulfide gradients over up to a few centimeters (MuBmann et al., 2003; Sayama et al., 2005; Hinck et al., 2007; Preisler et al., 2007). Although Beggiatoa spp. also require oxygen for growth (unless an alternative electron acceptor is present) they show phobic reactions to higher (above 5% air saturation) oxygen concentrations (Winogradsky, 1887; M0ller et al., 1985). During aerobic sulfide oxidation the production of oxygen radicals and, moreover, chemiluminescence was shown to occur (Tapley et al., 1999). As		

mentioned above, we propose that the Beggiatoa sp. 35Flor requires the accompanying organism to protect themselves against reactive oxygen species (Chapter 3.1). However, no correlation between the catalase activity of heterotrophic, associated cells and the beneficial effect of their presence on cyanobacteria (Prochlorococcus sp.) was found (Morris et al., 2008). In fact, the heterotroph with the lowest catalase activity was the only strain able to support growth of all studied cyanobacteria. Consequently, the dependency of the large bacteria on small heterotrophic bacteria can not be completely explained by their possession of a catalase enzyme. During our studies on the co-culture of Beggiatoa sp. and Pseudovibrio sp., the possibility that nitric oxide (NO) might be involved in the reaction counteracting oxidative stress arose. In oxygen-sulfide gradient co-cultures with high sulfide concentrations, the NO signal in Pseudovibrio cells, stained with a copper-based fluorescent probe (CuFL, Lim et al., 2006), was higher compared to cells exposed to a low sulfide flux (Figure 5.1). This increase in NO signal was also inducible when hydrogen peroxide (H2O2) was added to co-cultures with a low		

sulfide flux or to pure cultures of the isolated Pseudovibrio sp. (growing in seawater medium containing glucose and ammonia). Under both conditions, the enhanced signal was visible in the interior of the cells and not in the surrounding medium. The used dye is specific for NO and no interaction with H2O2 or other reactive oxygen and nitrogen species, such as HNO, NO2-, NO3- and ONOO-, could be detected (Lim et al., 2006). Figure 5.1: Nitric oxide staining (copper-based fluorescent probe) of Pseudovibrio sp. FO-BEG1 cells in coculture with Beggiatoa sp. 35Flor at high and low sulfide flux (12.6 and 50.4 mmol m 2 d 1, respectively) and in Pseudovibrio sp. FO-BEG1 pure cultures. White bars show measurements without the addition of hydrogen per-oxide and grey bars show those with 0.35% H2O2 added. (preliminary data, imaging performed together with M. Beutler) NO is known to protect against oxidative stress in other bacteria. In Bacillus subtilis, the addition of NO has been shown to lead to an increase in resistance against H2O2 by 100fold (Gusarov and Nudler, 2005). The enzymes of the group nitric oxide synthases (NOS) can produce NO and		

citrulline from L-arginine and oxygen and are typically known from eukaryotes (Alderton et al., 2001). However, recently genes encoding for NOS-like enzymes were found also in different gram-positive and some gram-negative bacteria, and also the NOS-dependent NO production could be shown (Adak et al., 2002a; Adak et al., 2002b; Gusarov and Nudler, 2005; Agapie et al., 2009; Schreiber et al., 2011). Interestingly, we found NOS-related domains (Filippovich, 2010) in the genome of the investigated Pseudovibrio sp. FO-BEG1 (Schwedt et al., unpublished data). A small domain of yet unknown function is followed by an amine oxidase domain (known to oxidize L-amino acids) and a flavodoxin/nitric oxide synthase domain in the genome sequence. This opens up the possibility that NO production might be involved in the reaction to ROS in the investigated Pseudovibrio sp. strain FO-BEG1. The production of NO as a protective measure against oxidative stress in Bacillus subtilis cells leads to an enhanced catalase activity, which was shown by comparison of wild type to Anos deletion mutants (Gusarov and Nudler, 2005). In that study, the bacteria were cultivated in a complex		

medium with yeast extract containing free amino acids. In the pure culture experiments presented here, the medium contained ammonia, as the sole nitrogen source, which could have potentially been used to produce NO. In our co-culture experiments together with Beggiatoa sp., no fixed nitrogen source was present, but the investigated Beggiatoa sp. are able to fix N2 (Henze, 2005). The genome of the accompanying organism does not contain any gene encoding for a nitrogenase enzyme (Schwedt et al, unpublished data). The transfer of fixed nitrogen sources from cyanobacteria to associated bacteria is a common observation (Paerl, 1984). It may well be that also the Beggiatoa sp. transfer fixed nitrogen to the accompanying bacteria. Nevertheless, taking into consideration that dyes can unspecifically bind to different compounds and that the production of NO in our study was not proven by direct measurements, these considerations remain speculative and await further investigation (see outlook). Apart from chemical substances, such as oxygen and sulfide, also light is known to be a potential cause of phobic migration		

reactions of Beggiatoa filaments (Winogradsky, 1887; Nelson and Castenholz, 1982; M0ller et al., 1985). The Beggiatoa sp. that we investigated showed an unusual reaction to blue/green light. Application of a blue or green light source (intensity: 67 |iE m-2 s-1) above or below the culture tube induced an immediate downwards movement of a subpopulation of filaments into the anoxic part of the culture tube when cultivated under a high sulfide flux (Chapter 2.2). The downward movement also occurred in the dark, but solely in cultures where a mat at the oxic-anoxic transition had already been established for about 10 days. The lower subpopulation was established 2 to 3 days after the downwards migration started (Chapter 2.1). Applying a blue or green light source in close proximity to the culture tube induced and also enhanced this filament movement and within a few hours the lower subpopulation was observed (Chapter 2.2). Because chemiluminescence occurs during chemical sulfide oxidation (Tapley et al., 1999) at the transition between oxygen and sulfide, it can be speculated that light at low intensities might be involved in the migration and/or mat formation processes of Beggiatoa filaments, but this		

remains to be studied in the future (see outlook). The lower limits of bacterial growth In contrast to the large Beggiatoa filaments, most bacterial cells are not visible with the naked eye. However, bacterial growth can lead to macroscopically visible colonies on nutrient-rich agar plates or cause turbidity in nutrient-rich liquid media due to high cell densities, whereas under nutrient limitation cell densities will be low and the small bacterial cells might not cause turbidity. Therefore, the build-up of bacterial biomass in liquid low-nutrient media has to be quantified in a different way than optical turbidity measurements. Although, there might be no measurable growth, the cells might still be metabolically active. This is due to the fact that there is a difference between biomass production and activity of bacterial cells. Although the consumption of substrate is essential for the cells to grow and build-up biomass, the opposite is not the case, as cells do not necessarily grow while utilizing substrate (del Giorgio and Cole, 1998). Therefore, the formation of biomass, called bacterial production (BP) has to be separated from the consumption of substrate, called		

bacterial respiration (BR). The bacterial growth efficiency (BGE) is defined as the quantity of bacterial biomass resulting from a certain amount of substrate respired (del Giorgio and Cole, 1998). The substrate is used by the bacteria for catabolic and anabolic purposes generating ATP (Adenosine-5'- triposphate) and cell biomass, respectively. The BGE can be determined by isotope-labeling techniques (BR and BP), rates of protein or DNA synthesis (BP), cell counts (BP), oxygen consumption (BR), Cacbon điôxít production (BR) and changes in DOC and POC (BR and BP) (del Giorgio and Cole, 1998 and references therein). Under oligotrophic conditions, the concentrations of substrates and cell numbers are extremely low. Therefore, most of these methods may not be sensitive enough and measured values are close to or below the limit of detection. Thus, new routines and methods have to be developed or methods have to be refined to be suitable for studying bacterial growth under extreme nutrient deficiency. Classical dissolved organic carbon (DOC) measurements, which are used as a parameter for the amount of dissolved organic matter (DOM) in environments with a low amount		

of nutrients, have a detection limit of about 0.5 |imol C L-1 (0.006 mg C L-1). However, the observed cell numbers in our medium (Chapter 4) are so extremely low that about 1 |imol L-1 of carbon would be enough to explain the observed growth. Consequently, the classical method might not provide sufficient sensitivity to detect potential small changes in DOC. The results presented in this thesis show that electospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS) provides sufficient resolution and can be used to study bacterial growth under oligotrophic conditions. Furthermore, this technique gives information on both potential fixed carbon and nitrogen sources of the bacteria. Combined with the measurement of amino acids by high performance liquid chromatography (HPLC) and isotope-labeling techniques, bacterial growth in oligotrophic seawater can be studied in detail. However, this technique does generally not give quantitative information and as a consequence respiration rates under oligotrophic conditions still remain unknown. The risk of impurities Even though no electron donor is		

added to an oligotrophic medium, there can still be impurities present that might support growth and lead to the formation of biomass and/or metabolic activity of the cells (Chapter 4). Studying bacterial growth under nutrient-poor conditions is challenging, since many different contaminations, such as macronutrients, trace elements or energy sources can occur. Accordingly, when studying the physiology of bacteria growing under oligotrophic conditions, it is crucial to keep the medium and equipment contamination-free for the mandatory substances for growth. Amino acids represent a serious contamination risk for seawater samples. Therefore, there are a number of precautions that have to be taken, such as working with gloves, combustion of glassware, avoidance of dust in the working room (working in laminar flow hood) and use of chemicals of highest available quality (Dittmar et al., 2009). Also, during the measurements of quantity and composition of DOM typically all used equipment is pre-combusted if possible. All these precautions were applied during the preparation of the oligotrophic seawater medium and all further analyses presented in Chapter		

4.1 of this thesis. Furthermore, the medium might become contaminated with trace elements, which would probably not resemble a potential energy source but could be decisive for growth or no growth of microorganisms. To avoid the contamination with trace elements, equipment is usually washed with HNO3- (e.g. Fitzwater et al., 1982), which, however, represents an easily utilizable nitrogen source. For the experiments presented in this thesis (Chapter 4), a fixed nitrogen source was considered as worse contamination than trace elements, and the equipment was not washed with HNO3-. In spite of all precautions, it is still extremely difficult to prepare a seawater medium without any contaminations. However, if the contaminating substance is not used by the bacteria during growth, such as the PEG contamination in this study (Chapter 4.1), it might not disturb or influence further physiological analysis. The contamination can even be used to normalize other peaks in the mass spectra, if its peaks are high and consistent (as the PEG peaks in this study, Chapter 4.1). Facultative oligotrophy In addition to growth under oligotrophic conditions, the		

investigated Pseudovibrio sp. FO- BEG1 (Chapter 4) is capable of growing under eutrophic conditions as well and can switch within days from one trophic state to the other. Under oligotrophic conditions, the bacterial growth efficiency is typically very low and varies with the supply of nutrients (del Giorgio and Cole, 1998). Furthermore, the investigated bacteria are limited in carbon, nitrogen and energy at the same time (Chapter 4.1). Consequently, they are supposed to have high maintenance energy costs to maintain crucial transport systems and enzymes prepared (del Giorgio and Cole, 1998). With increasing nutrient supply, probably also the bacterial growth efficiency increases because the bacteria can exhibit higher growth rates and produce only enzymes necessary for the substrates available (reviewed in del Giorgio and Cole, 1998). Facultatively oligotrophic bacteria have to switch between oligotrophic and eutrophic growth conditions and as a consequence the cells have to switch also between both strategies for growth to adapt to the actual amount of substrates present. The variation in nutrient availability is common in natural		

environments such as the open ocean. Bacteria attached to marine snow particles can exhibit very high growth rates (Alldredge et al., 1986; Alldredge and Gotschalk, 1990; Smith et al., 1992; Azam and Long, 2001; Ki0rboe and Jackson, 2001), while free-living bacteria in the open ocean are restricted in nutrients and consequently their growth rates are low or they even starve (Boylen and Ensign, 1970; Novitsky and Morita, 1976; Azam and Hodson, 1977; Novitsky and Morita, 1977; Tabor and Neihof, 1982; Ishida et al., 1989). Facultatively oligotrophic bacteria can adapt fast to nutrient affluence or deficiency, while they have to up- or down-regulate internal pathways of anabolism and catabolism. The investigated facultatively oligotrophic Pseudovibrio sp. FO-BEG1 is highly versatile with respect to its energy gain (Chapter 3.2 and Chapter 4.1). This feature makes the bacteria flexible and they are, moreover, capable to interact with other prokaryotes or possibly even eukaryotes (Chapter 3.2). Already 30 years ago, it was suspected that two types of oligotrophic bacteria exist (Ishida and Kadota, 1981): 1. organisms, which disappear with		

increasing man-made eutrophication and 2. organisms, which can adapt fast to man-made eutrophication. In this thesis bacteria were not isolated from the oligotrophic open ocean, but from water directly overlaying marine sediments (Chapter 4.2). All of the isolated strains were able to adapt fast to the nutrient deficiency of the initial isolation medium. The observation that facultatively oligotrophic bacteria can also be isolated from non-oligotrophic water indicates that these bacteria might be more widespread and not limited to nutrient-poor environments. For example, they might live attached to marine snow particles (e. g. Alldredge et al., 1986; Smith et al., 1992; Azam and Malfatti, 2007 and references therein), which represent nutrient hotspots for heterotrophic bacteria, and possibly the bacteria sink down to the sediment with these particles. We assume that many facultatively oligotrophic bacteria have been overlooked so far because they were not searched for in non-oligotrophic environments. Likewise, many more already known heterotrophic bacteria may be capable of growing under much poorer nutrient conditions than currently assumed.		

Conclusions In conclusion, the results presented in this PhD thesis (summarized in Figure 5.2) show that filaments of Beggiatoa sp. strain 35Flor react to high sulfide fluxes by migration into anoxic regions, where they reduce the amount of internal storage compounds. This migration can be enhanced or induced by blue/green light for a yet unknown reason. We suggest that the accompanying Pseudovibrio sp. strain FO-BEG1 can detoxify reactive oxygen species (ROS), generally produced during sulfide oxidation (Tapley et al., 1999), and might be responsible for the protection of Beggiatoa filaments, which are known to lack the gene for catalase (Larkin and Strohl, 1983). Possibly, production of nitric oxide (NO) by the Pseudovibrio sp. might also be involved in the protection against ROS. However, the origin of NO is unknown because no fixed nitrogen source was present in the medium of the co-culture and the heterotroph does not posses a gene encoding for nitrogenase. On the other hand, the Beggiatoa sp. can fix N2 (Henze, 2005) and it might be that a fixed nitrogen source is transferred from the sulfide-oxidizer to the accompanying bacterium. The Pseudovibrio sp.		

examined in this thesis is a generalist able to gain energy in many different ways and can also grow under extremely nutrient-poor conditions. Furthermore, it possesses genes to interact with pro- and eukaryotes. We found that also other bacteria from the habitat of large sulfide- oxidizers can adapt fast from nutrient affluence to deficiency. Maybe these bacteria are able to also support growth of the large sulfide-oxidizers, but this needs to be investigated in the future. Figure 5.2: Illustration of the results obtained in this PhD thesis concerning the migration behavior of Beggiatoa sp. and the co-occuring Pseudovibrio strain. Some results were supported by our data (black) while others are hypothesis and remain to be proven (blue). ROS = reactive oxygen species, NO = nitric oxide, N = nitrogen Outlook The results of my PhD thesis provide new insights into the migration behavior of large sulfur bacteria, associations between bacteria, and growth under extreme nutrient deficiency. Nevertheless, the obtained results also raise new questions that need to be addressed in the future. We hypothesize that the Beggiatoa sp. filaments depend		

on the heterotrophic Pseudovibrio sp. because they do not themselves possess protective measure against oxidative stress (Chapter 3). However, direct evidence for this theory is still missing. To investigate the capability of other heterotrophic, catalase-positive bacteria or ROS scavenger to support growth of the Beggiatoa sp., filaments from the lower subpopulation should be transferred without (or with only few) accompanying bacteria into fresh media. If growth is positive in the presence of the other catalase-positive bacteria or ROS scavengers, the transfer of Pseudovibrio sp. from the inoculation source has to be excluded, which can be tested by Fluorescence in situ hybridization. The preliminary data on the staining of nitric oxide opens up the possibility that NO might be involved in the reaction with ROS (Chapter 5). However, to proof this, the production of NO has to be verified by direct measurement of NO with a NOx-analyzer or detection of NO by its molecular mass using mass spectrometry. The chemical reaction of NO with oxygen in water forming mainly nitrite (Ignarro et al., 1993) might complicate the direct measurement of nitric oxide. The		

sole nitrogen source in the co-culture is N2. Therefore, it can be speculated that the interaction between the two bacteria is not only one-sided because of the two partners only the Beggiatoa sp. are known to fix N2 (Chapter 5). To investigate if a direct transfer of fixed nitrogen in the co-culture occurs, labeled nitrogen (15N2) can be added and the label can be searched for in the Pseudovibrio sp. using mass spectrometry (e.g. nano secondary ion mass spectrometry). We have shown that the Pseudovibrio sp. FO-BEG1 growing in pure culture under nutrient- poor conditions can also use typical substrates known from heterotrophic growth (Chapter 4.1). It still remains unclear if the observed growth is the same as typical heterotrophic growth only with less nutrients or if special regulating enzymes are needed. A proteomic comparison between bacteria grown under nutrient deficiency and grown under nutrient affluence might provide more information on the growth and the regulation of enzymes under oligotrophic conditions. The detailed analysis of substrates used during oligotrophic growth shows that the bacteria use multiple substrates at the same time under		

oligotrophic conditions (Chapter 4.1). However, only amino acids (which account only for a small fraction) were measured quantitatively and the overall respiration rate remains unknown. Therefore, the measurement of oxygen consumption (e.g. using microelectrode respiration chambers) under nutrient-poor conditions could allow quantification and calculation of the respiration rate under oligotrophic conditions. Furthermore, the fact that we have both metabolites (detected by ESI FT-ICR-MS) and genes (closed genome of Pseudovibrio sp. strain FO-BEG1) may allow us to connect metabolites with genes present in the investigated organism in the future. Maybe, this will also help predicting new metabolic pathways. Eventually, several heterotrophic strains, which were able to grow under both nutrient deficiency and affluence, were isolated during our studies (Chapter 4.2). The possibility of heterotrophic bacteria to grow under nutrient-deficient conditions might be a more widespread feature. To test this hypothesis, common heterotrophic bacteria from bacterial strain collections can be ordered and their ability to grow under nutrient-poor conditions can be tested.		

Appendix The genus Pseudovibrio contains metabolically versatile and symbiotically interacting bacteria Abstract The majority of strains belonging to the genus Pseudovibrio have been isolated from marine invertebrates like tunicates, corals and especially sponges, but the physiology of these bacteria is poorly understood. In this study, we analyze the genomes of two Pseudovibrio strains. One is a required symbiont of a cultivated Beggiatoa strain, a sulfide oxidizing, autotrophic bacterium. The other one was isolated from a sponge (Enticknap et al., 2006). The data show that both strains are generalistic bacteria capable of importing and oxidizing a wide range of organic and inorganic compounds to meet their carbon, nitrogen, phosphorous and energy requirements under oxic and anoxic conditions. Several physiological traits encoded in the genome were verified in laboratory experiments with a pure culture of the Pseudovibrio strain originally associated with Beggiatoa. Besides the versatile metabolic abilities of both Pseudovibrio strains, our study reveals a number of open reading frames and gene clusters in the		

genomes that seem to be involved in symbiont-host interactions. Pseudovibrio has the genomic potential to attach to host cells, might be capable of interacting with the eukaryotic cell machinery, produce secondary metabolites and may supply the host with cofactors.

Introduction

The first strain of the genus Pseudovibrio has been isolated from coastal seawater in 2004 and was described as Pseudovibrio denitrificans - a marine, heterotrophic, facultatively anaerobic bacterium capable of denitrification and fermentation (Shieh et al., 2004). Two further type strains, P. ascidiaceicola (Fukunaga et al., 2006) and P. japonicus (Hosoya and Yokota, 2007), were isolated from a tunicate and coastal seawater, respectively. Physiologically, these isolates were not notably different from P. denitrificans. Besides the three type strains, Pseudovibrio spp.-related bacteria have been found in various studies throughout the world either by 16S rRNA gene analysis or direct isolation methods (Hentschel et al., 2001; Webster and Hill, 2001; Olson et al., 2002; Thakur et al., 2003; Thiel and Imhoff, 2003; Thoms et al., 2003; Agogué et al., 2005; Lafi et al., 2005; Enticknap et

al., 2006; Koren and Rosenberg, 2006; Sertan-de Guzman et al., 2007; Muscholl-Silberhorn et al., 2008; Riesenfeld et al., 2008; Kennedy et al., 2009; Rypien et al., 2010; Santos et al., 2010). Interestingly, besides P. denitrificans, P. japonicus and a Pseudovibrio spp.-related isolate from coastal, oligotrophic seawater (Agogué et al., 2005), all other strains belonging to this genus discovered until today have been found closely associated with marine invertebrates like tunicates, corals, and sponges. Especially Porifera seem to harbor Pseudovibrio populations, e.g., as the dominating species of the culturable bacterial community (Webster and Hill, 2001; Muscholl-Silberhorn et al., 2008). Additionally, Pseudovibrio has been found in sponge larvae as the most abundant prokaryote, indicating vertical transmission of these bacteria in their hosts (Enticknap et al., 2006). Such a consistent pattern of Pseudovibrio spp. associated with sponges suggests that they are symbionts of those metazoa (Webster and Hill, 2001; Enticknap et al., 2006). Whether the nature of this symbiosis is mutualistic/ commensalistic or whether Pseudovibrio spp. rather represent pathogens/parasites is		

uncertain, but the fact that Pseudovibrio spp. have been isolated only from healthy sponges indicates that the bacteria do not harm the host and might be even required for its health (Webster and Hill, 2001). Another shared feature is the production of secondary metabolites by many of the cultured Pseudovibrio strains. For instance, heptylprodigiosin, a compound that exhibits antimicrobial activity, was isolated from a pure culture of P. denitrificans Z143-1 (Sertan-de Guzman et al., 2007) and the production of additional bioactive compounds could be shown in several other studies (Hentschel et al., 2001; Muscholl-Silberhorn et al., 2008; Kennedy et al., 2009; Santos et al., 2010). Despite the fact that members of the genus Pseudovibrio seem to be ubiquitous and important associates of marine invertebrates and are also found free-living, very little is known about the physiology and interactions with the host. In this study, we analyze the genomes of two Pseudovibrio strains. Pseudovibrio sp. FO-BEG1 has been isolated from an enrichment culture of a Beggiatoa strain, a filamentous, sulfide oxidizing bacterium (Brock and Schulz- Vogt, 2011;		

Chapters 2 and 3). Initially, this Beggiatoa strain was sampled from a coral suffering from the black band disease off the coast of Florida, which indicates that the strain Pseudovibrio FO-BEG1 could have been associated with the coral at the time of sampling - either in a commensalistic/mutualistic or pathogenic relationship - and is now available as an axenic culture in our lab. Intriguingly, strain FO-BEG1 is also maintained in a co-culture with a Beggiatoa sp., which seems to be unable to grow without Pseudovibrio and is therefore dependent on strain FO-BEG1. The second strain, Pseudovibrio sp. JE062, has been isolated in Florida from the sponge Mycale laxissima in the year 2006 and was described as a sponge symbiont by Enticknap et al. (2006). The analysis of these genomes gives us an insight into the physiological and symbiotic potential of both Pseudovibrio strains and reveals fascinating microorganisms that seem to be adapted to free-living and symbiotic life styles. Materials and Methods Growth conditions For aerobic growth CM medium modified after Shieh et al. (2004) was used. After autoclaving, the medium was supplemented with K2HPO4 (1.15 mmol L-1),		

glucose (10 mmol L-1 unless stated otherwise), 1 mL L-1 tungsten/selenium solution (Brysch et al., 1987), 1 mL L-1 trace elements (Widdel and Pfennig, 1984), and 1 mL L-1 of four vitamin solutions prepared according to Aeckersberg et al. (1991). For measurement of SO42- evolution during S2O32- oxidation, 10 mmol L-1 Na2S2O3 • 5 H2O and 5 mmol L-1 glucose were added and 2 g L-1 K2SO4 from the original recipe was replaced with 0.75 g L-1 KCl. To compare growth between a culture with and without Na2S2O3, K2SO4 was not omitted from the medium and glucose and Na2S2O3 • 5 H2O were used in the same concentrations as described above. To investigate the growth with 4-hydroxybenzoic acid and benzoate, both compounds were added in a concentration of 2 mmol L-1, respectively, without any other carbon source. Growth with phosphonoacetate (1 mmol L-1) as phosphorus source was tested by adding this compound as the only phosphorus source and all vitamins were omitted from the medium. For fermentation and denitrification experiments under anoxic conditions, aged North Sea water was buffered with 50 mmol L-1 TRIS, supplemented with NH4Cl (10 mmol L-1) and the		

pH adjusted to 8. Preparation of the medium was performed according to Widdel and Bak (1992) in order to prepare the medium anoxically. Cooling was performed under N2 atmosphere, except for experiments in which N2 production was measured, in which Ar was used as the atmosphere instead. After autoclavation, the medium was supplemented with 10 mmol L-1 glucose, 1 mL L-1 tungsten/selenium solution, 1 mL L-1 trace elements and 1 mL L-1 of four vitamin solutions prepared as described above. NaNO3 (10 mmol L-1) was added for experiments investigating denitrification. To test CO oxidation, CM medium was prepared as described above, containing 400 |imol L-1 glucose and supplied with 500 p.p.m. CO to the bottle headspace. For aerobic growth experiments, 250 mL Erlenmeyer flasks were filled with 100 mL medium. For anaerobic growth, 156 mL serum bottles (Wheaton, Millville, USA) were filled anoxically with 50 mL medium and closed with butyl rubber stoppers. For all experiments, 0.1% or 0.5% of a preculture grown aerobically in CM medium was used as inoculum. All growth experiments were performed with Pseudovibrio sp. FO-BEG1 in triplicates at 28°C in the dark		

with shaking at 110 rpm. Chemical analyses Bacterial growth was monitored as the optical density (OD600 nm) using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). SO42- was measured with a Metrohm 761 Compact IC with conductivity detector (Metrohm AG, Herisau, Switzerland) equipped with a Metrosep A Supp 5-100 column with a carbonate eluent (3.2 mmol L-1 Na2CO3/1 mmol L-1 NaHCO3 in deionised water) at a flow rate of 0.7 mL min-1. Tetrathionate was measured according to Kamyshny (2009). Glucose and organic acids were determined using a HPLC system (Sykam GmbH) equipped with an anion neutral pre-column (4x20 mm; Sykam GmbH) and an Aminex HPX-87H separation column (300x7.8 mm; Biorad, Munich, Germany) at a temperature of 60 °C. The eluent consisted of 5 mM H2SO4 in HPLC-grade water with a flow rate of 0.6 mL min-1. Quantification of glucose, succinate, lactate, formate, acetate, propionate and ethanol was performed with the 7515A RI detector (ERC, Riemerling, Germany); pyruvate was measured with the Sapphire UV-Vis detector at 210 nm (Ecom, Praha, Czech Republic). NO3" was quantified with a HPLC		

system (Sykam GmbH, Eresing, Germany) containing an anion neutral pre-column (4x20 mm; Sykam GmbH) and an IBJ A3 anion separation column (4x60 mm; Sykam GmbH) with a column temperature of 50 °C. The eluent consisted of 25 mmol L-1 NaCl and 45% ethanol in deionised water with a flow rate of 1 mL min-1. Detection of NO3- was conducted with Linear Uvis 200 (Thermo Fischer Scientific GmbH, Dreieich, Germany) at 220 nm. N2 was measured as described by Zedelius et al. (2011). CO determination was conducted with a Shimadzu GC-8A (Shimadzu, Duisburg, Germany) gas chromatograph with a Molecular Sieve 5A column (80 to 100; 0.125 in. by 2 m; Restek, Bellefonte, USA) at a flow of 20 mL of synthetic air per minute at 40°C and an RGD2 reduction gas detector (Trace Analytical, Menlo Park, USA). DNA extraction and sequencing DNA was extracted from strain FO-BEG1 using the Fast DNA SPIN Kit for Soil (MP Biomedicals LLC, Illkirch, France), according to manufacturers' instructions. 454 sequencing was conducted by LGC Genomics GmbH with a 454 GS FLX System. The Newbler 2.0.00.22 software was used for sequence assembly and		

quality assessment. Overall, 522919 sequenced reads with an average length of 336.30 bp lead to a 29-fold sequence coverage. In order to close the gaps, a fosmid library with a 1.5-fold physical coverage was created and used for direct sequencing of the fosmid clones. For the residual gaps, 96 specific primers were designed and used for combinatorial PCR on DNA level, the products of which were sequenced via the Sanger method. Gene prediction, annotation and data mining Gene prediction was carried out by using the software Glimmer3 (Delcher et al., 2007). Ribosomal RNA genes were detected by using the RNAmmer 1.2 software (Lagesen et al., 2007) and transfer RNAs by tRNAscan-SE (Lowe and Eddy, 1997). Annotation was performed by using the GenDB, version 2.2 system (Meyer et al., 2003), supplemented by the tool JCoast, version 1.6 (Richter et al., 2008). For each predicted open reading frame (ORF) observations have been collected from similarity searches against sequence databases NCBI- nr, Swiss-Prot, KEGG and genomesDB (Richter et al., 2008) and for protein family databases from Pfam (Bateman et al., 2004) and InterPro		

(Mulder et al., 2005). SignalP has been used for signal peptide predictions (Bendtsen et al., 2004) and TMHMM for transmembrane helix- analysis (Krogh et al., 2001). Predicted protein coding sequences were automatically annotated by the in-house software MicHanThi (Quast, 2006). The MicHanThi software predicts gene functions based on similarity searches using the NCBI-nr (including Swiss-Prot) and InterPro database. The annotation of proteins highlighted within the scope of this study was subject of manual inspection. For all observations regarding putative protein functions, an e-value cutoff of 10-4 was considered. Comparison of the shared gene content by reciprocal best matches (RBMs) and functional classification with Kyoto encyclopedia of genes and genomes (KEGG) Determination of the shared gene content has been performed by a BLAST all versus all search between FO-BEG1 and JE062. Reciprocal best matches were counted by a BLAST result with an E value <1e-5 each and a subject coverage of over 65%. For metabolic pathway identification, genes were searched for similarity against the KEGG database. A match was counted if the similarity		

search resulted in an expectation E value below 1e-5. All occurring KO (KEGG Orthology) numbers were mapped against KEGG pathway functional hierarchies and statistical analyzed. Functional classification with cluster of orthologous groups (COG) and calculation of the Average nucleotide identity (ANI) All predicted ORFs were also searched for similarity against the COG database (Tatusov et al., 2003). A match was counted if the similarity search resulted in an E value below 1e-5. ANI between the whole-genome sequences of strain FO-BEG1 and the draft genome sequences of strain JE062 was determined by using the in silico DNA-DNA hybridization method of the JSpecies (Richter and Rosselló-Móra, 2009) software with default parameters. Creation of circular genome maps and prediction of ABC and TRAP type transporters Comparative circular genome maps of the RBMs shared between JE062 and FO-BEG1 have been drawn by using JCoast's plugin for CGView (Stothard and Wishart, 2005). Circular GC- plot and GC-skew representation has been drawn by using DNAPlotter (Carver et		

al., 2009). As initial step for the identification of the ABC transporters, genes containing the Pfam domain ABC_tran (PF00005) were detected in the genome of strain FO-BEG1. For the identification of the permease and the periplasmic binding protein, the close proximity of genes containing the ABC_tran domain was searched. Only ABC systems with at least one ABC_tran domain, one permease and one periplasmic binding protein were regarded as functioning ABC transporters and substrate specificity was predicted from the annotations of the subunits. In several cases, one subunit (e.g. the permease) was missing in close proximity of genes with the ABC_tran domain. In this case, a single permease gene located on any place in the genome with the same substrate specificity prediction but not belonging to any complete ABC system, was used to complement the transporter system. TRAP transporters were regarded as complete when the subunits DctM, DctQ and DctP were present in close proximity. When two subunits were identified in close proximity and the third was missing, the single subunit located on any place in the genome not belonging to any complete TRAP system was		

used to complement the transporter system. In the case of fusion of the DctQ and M subunits in one gene, only the DctP subunit was required to complete the transporter.

Accession numbers

The genome shotgun project of strain FO-BEG1 has been deposited at DDBJ/EMBL/GenBank under the accession number CP003147 for the chromosome and CP003148 for the plasmid. The draft genome sequence of strain JE062 has the DDBJ/EMBL/GenBank accession number ABXL00000000.

Results and Discussion General genome characteristics

The genome size of strain FO-BEG1 is 5.9 Mbp, including a large plasmid of 0.4 Mbp (Figure S.1). The circular chromosome of 5.5 Mbp contains a large stretch of repeats at position 2,707,040. This area of unknown size could not be bridged with a direct sequencing approach despite the presence of this area on a fosmid, indicating strong secondary structures, and has been masked with the ambiguous nucleotide code 'N'. The G+C content is 52.5 mol% and is consistent with the known values of the described Pseudovibrio isolates (Shieh et al., 2004; Fukunaga et al., 2006; Hosoya

and Yokota, 2007). Altogether, we found 5,478 ORFs, 398 of which were located on the plasmid, which correspond to about 87% of encoding DNA. Six complete rRNA operons and 69 tRNA encoding regions were annotated, indicating the capability of a quick response to changing conditions and fast growth when nutrients are available. The genome of strain JE062 has not been closed, but there are 19 contigs available with an overall size of 5.7 Mbp, 5,225 ORFs and 52.4 mol% GC content, which is almost identical to the genome of strain FO-BEG1 (Figure S.1 A and B). It contains 72 tRNA genes and seven complete rRNA operons. Unfortunately, the repeat-rich area that could not be sequenced in the genome of strain FO-BEG1 shows an ambiguous sequence in strain JE062 as well, and could therefore not be used to close the gap in FO-BEG1. Figure S.1. Comparative circular map of Pseudovibrio sp. FO-BEG1 chromosome (A) and plasmid (B). Most outer lane represents the reciprocal best match (RBM)-shared gene content between FO-BEG1 and JE062. Lane two and three represent all predicted open reading frames (ORFs) on the lagging (red) and leading (green) strands. The two inner lanes		

display the GC-plot and the GC-skew. The red arrow indicates the area of unknown size that could not be closed during sequencing. The bar chart (C) express the amino acid percentage identity of each RBM shared gene-content between FO-BEG1 and JE062. The blue bar is representing the FO-BEG1 chromosome and orange the corresponding plasmid. Even though the genome of JE062 is not completely closed we assume that it also contains a plasmid with similar content, since most of the genes identified on the plasmid of FO-BEG1 were allocated in the genome of JE062 (Figure S.1 B). Table S.1 shows an overview of the genome characteristics of both strains as well as the assignment of the genes to COGs. The shared gene content between FO-BEG1 and the draft genome of JE062 comprises 84.4% (4,287 ORFs, Figure S.1 C). An ANI analysis conducted between strains FO-BEG1 and JE062 revealed a 94.5% ANIb (87% genome alignment) and 95.4% ANIm (86% genome alignment) value. The values are in the range of the proposed species definition boundary (Richter and Rosselló-Móra, 2009) indicating a species level degree of similarity. Table S.1. General genome		

features of Pseudovibrio sp. FO-BEG1 and JE062, including categorization of the genes into cluster of orthologous group (COG) categories. [J] Translation, ribosomal structure and biogenesis 196 190 [K] Transcription 387 367 [L] Replication, recombination and repair 135 125 [D] Cell cycle control, cell division, chromosome partitioning 21 20 [T] Signal transduction mechanism 138 140 [M] Cell wall/membrane/envelope biogenesis 190 176 [N] Cell motility 153 149 [O] Posttranslational modification, protein turnover, chaperones 135 127 [C] Energy production and conversion 245 245 [G] Carbohydrate transport and metabolism 323 311 [E] Amino acid transport and metabolism 507 492 [F] Nucleotide transport and metabolism 99 92 [H] Coenzyme transport and metabolism 185 181 [I] Lipid transport and metabolism 148 142 [P] Inorganic ion transport and metabolism 291 287 [Q] Secondary metabolites biosynthesis, transport and catabolism 210 204		

[R] General function prediction only 598 576 [S] Function unknown 281 272 Physiology In both genomes we found a number of genes that indicate high metabolic variety of Pseudovibrio FO-BEG1 and JE062. Degradation of carbohydrates is most likely performed via the Entner-Doudoroff pathway, which is present in both genomes, due to absence of the phosphofructokinase (PFK), a key enzyme of the glycolysis (Emden-Meyerhoff-Parnas), which is a regularly encountered phenomenon within marine a-Proteobacteria (Furch et al., 2009; Tang et al., 2009; Williams et al., 2009). Besides the PFK, all other enzymes involved in glycolysis can be identified in both genomes, including fructose-1,6-bisphosphatase I, the key enzyme for glyconeogenesis, indicating that the Emden-Meyerhoff-Parnas pathway can be used for anabolic purposes (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Genes encoding all enzymes of the citric acid cycle and pentose phosphate pathway are present. Additionally, both strains have		

the genetic potential to degrade aromatic compounds via the P-ketoadipate pathway, which we demonstrated by growing Pseudovibrio sp. FO-BEG1 with 4-hydroxybenzoate as the only carbon and energy source under aerobic conditions (Figure S 2.1 A). Benzoate, however, was not degraded, indicating that either the uptake of benzoate is detained or the hydroxylation of the aromatic ring structure cannot be performed by Pseudovibrio FO-BEG1. Under anoxic conditions without nitrate, strain FO-BEG1 metabolized glucose in mixed acid type fermentation, as suggested by the present genes in both strains (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000), resulting in acidification of the medium and formation of mainly formate, lactate, acetate, and ethanol. Ethanol production during fermentation has not been described for any Pseudovibrio strain yet. Additionally, pyruvate, propionate, and succinate have been formed, but to a lesser extent (Figure S 2.2 A). Production of trace amounts of fumarate was detected, but could not be quantified. As expected, we found the complete set of genes essential for		

denitrification, including a membrane-bound (nar) and a periplasmic nitrate reductase (nap). In agreement, we observed a complete denitrification to N2 in laboratory experiments with strain FO-BEG1 (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148 and Figure S 2.2 C). For the type strain P. denitrificans, simultaneous denitrification and fermentation was described by Shieh et al. (2004) and could be confirmed in our experiments for strain FO-BEG1 with acetate, formate, lactate, and ethanol as the main fermentation products (Figure S 2.2 B). No genes for assimilatory nitrate reduction could be identified in the genome. A set of sox genes suggests that both bacteria can use reduced inorganic sulfur compounds as a source of energy to complement heterotrophy. We could show experimentally that the addition of thiosulfate to the medium enhances the aerobic growth of the Pseudovibrio sp. FO-BEG1 culture and sulfate is produced over the incubation period (Figure S 2.1 B and C). No tetrathionate could be measured as an intermediate (results not shown). Therefore, we propose that thiosulfate is oxidized completely to sulfate without		

any intermediates, as it is known for the typical Sox pathway in a-Proteobacteria (for review, see Ghosh and Dam, 2009). We identified genes encoding a small (cutS), medium (cutM) and large (cutL) subunit of the aerobic form II carbon monoxide dehydrogenase (CODH) with the accessory gene coxG in the operon (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148), indicating the capability of CO oxidation. However, uptake of CO could not be demonstrated under tested conditions (results not shown). Interestingly, our results confirm the hypothesis from a recent publication testing CO oxidation in bacteria containing type II CODH genes (Cunliffe, 2011), in which none of the isolates containing only the type II variant was capable of CO oxidation. Only bacteria containing the form I CODH have been shown to effectively oxidize CO, thereby questioning whether form II CODH is involved in the process of carbon monoxide oxidation, or if it has another primary function not known until now, as suggested by King and Weber (2007). In both Pseudovibrio strains, we found genes for phosphonate import and degradation, which allows the bacteria to cleave the		

relatively stable C-P bonds of phosphonates (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Thereby, they can metabolize a less accessible phosphorous pool in times of phosphate limitation. We could demonstrate growth of Pseudovibrio sp. FO-BEG1 with phosphonoacetate as the only source of phosphorous (Figure S 2.3 A). Additionally, we could show adaptation of Pseudovibrio strain FO-BEG1 to oligotrophic conditions by culturing it with as little as 15 |imol C L-1 (0.18 mg C L-1) dissolved organic carbon in the medium (Chapter 4), which shows that Pseudovibrio FO-BEG1 is capable of growth under extreme nutrient depletion. The high metabolic variety of Pseudovibrio sp. FO-BEG1 and JE062 is also reflected in the analysis of encoded primary transporters. In the genome of strain FO- BEG1 we could identify 31 tripartite ATP-independent periplasmic (TRAP) type transporters (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148) that are required for import of dicarboxylic acids like malate, succinate and fumarate, one of the highest numbers of TRAP type transporters reported in a		

genome of a marine prokaryote so far (Wagner-Dobler et al., 2010). In strain JE062 we identified 27 TRAP transporters. Citric acid cycle intermediates seem therefore to be an important source of carbon and energy for the investigated Pseudovibrio strains. In addition, we reconstructed over 80 ATP-binding cassette (ABC) transporter systems with predicted substrate specificity from the genomic data of the strain FO-BEG1, including the plasmid, and over 70 ABC transporter systems for JE062 (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Sugars, oligopeptides and amino acids are the main substrates that are imported via the ABC systems. A large number of transporters for oligopeptides and amino acids in combination with over 85 genes encoding peptidases and proteases (over 75 genes in strain JE062, see DDBJ/EMBL/GenBank accession number ABXL00000000) could help Pseudovibrio to hydrolyze complex particulate nutrients into oligopeptides and amino acids, which could serve as nutrition for both, the prokaryote and the host, as has been suggested by Siegl et al. (2011).		

Also iron seems to be an important trace element, for which we identified eight transporters including three siderophores and three transporters for hemin (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Table S.2. Identified ATP-binding cassette (ABC) and tripartite ATP-independent periplasmic (TRAP) transporters in the genomes of both Pseudovibrio strains and their putative functions. Vitamin synthesis Growth of pro- and eukaryotes highly depends on their requirements for cofactors that the organism can or cannot synthesize on its own. Vitamins are important for many different enzymatic processes and the synthesis of some vitamins is mainly accomplished by bacteria, making the prokaryotes a necessary part of the eukaryotic diet or an important partner in symbiotic relationships. The genomes of Pseudovibrio sp. FO-BEG1 as well as JE062 contain genes encoding key enzymes of the biosynthesis pathways of biotin (H), thiamin (B1), pyridoxin (B6), cobalamin (B12), riboflavin (B2), folic acid (B9) and lipoic acid (see		

DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Independence of an external vitamin supply was confirmed during aerobic growth in the defined CM medium without the addition of any vitamins, which implies de novo synthesis of all required growth factors by strain FO-BEG1 under tested conditions (Figure S 2.3 B). Pseudovibrio spp. would therefore be beneficial companions for other prokaryotes or marine invertebrates, since the dependency on an external supply of those vitamins would be relieved. Bioactive compounds Symbiotic relationships between bacteria and marine invertebrates, especially sponges, are of special interest, because bacteria associated with sponges often produce novel bioactive compounds (Piel et al., 2004; Taylor et al., 2007; Fisch et al., 2009). In the chromosome of Pseudovibrio FO-BEG1 we identified a genomic island of more than 50 kb containing among others a gene cluster of 20 genes predicted to be involved in secondary metabolite production (see DDBJ/EMBL/GenBank accession number CP003147). The cluster exhibits high		

sequence similarity to an architecturally almost identical hybrid nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS) system previously reported from many pathogenic and commensal Escherichia coli strains (Figure S.2) (Nougayrède et al., 2006). The E. coli metabolite, termed colibactin, remains structurally uncharacterized. However, transposon mutagenesis of the gene cluster suggested that colibactin is a pathogenicity determinant that induces DNA double strand breaks in eukaryotic host cells, eventually resulting in cell death. The only significant difference between the gene clusters in Pseudovibrio FO-BEG1 and E. coli is an additional set of genes in the former, encoding putative transporters and the presence of a different phosphopantetheinyl transferase gene variant likely involved in generating holo-proteins from apo forms of PKSs and NRPSs (Lambalot et al., 1996). In addition, two E. coli genes are fused in the Pseudovibrio cluster. Despite these differences, the architecture strongly suggests that the product of the FO-BEG1 cluster is colibactin, providing new opportunities to unveil the identity of this elusive and biomedically relevant		

compound. Interestingly, we find this more than 50 kb NRPS/PKS fragment only in Pseudovibrio sp. FO-BEG1 but not in the genome of strain JE062, with flanking regions downstream and upstream of the inserted fragment highly conserved in synteny in strain JE062 (data not shown), indicating that it has been acquired via horizontal gene transfer. Additionally, the plasmid of strain FO-BEG1 contained an ORF encoding a type III PKS of a size of 7.4 kb, which could also be detected in strain JE062 (see DDBJ/EMBL/GenBank accession number CP003148). Figure S.2. Nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS) system in Pseudovibrio sp. FO- BEG1 and Escherichia coli strain IHE3034. White arrows represent the genes present in Enterobacteriaceae and strain FO-BEG1; black arrows represent the open reading frames (ORFs) present only in either Enterobacteriaceae or FO-BEG1 but presumably involved in the production of colibactin; the gray arrow shows a gene presumably not involved in the synthesis of colibactin. The symbol at ORF PSE_3331 represents a gene fusion of E. coli genes clbG and clbH in FO-BEG1; the symbol at PSE_3324-3321 represents gene insertion or		

deletion in strain FO-BEG1 or E. coli IHE3034, respectively. DNA exchange and horizontal gene transfer The genomes of both Pseudovibrio strains show a high metabolic variety. It is reasonable to assume that various genes were acquired via horizontal gene transfer from other microorganisms as is indicated e. g. by the presence of a 50 kb large NPRS-PKS island that can be found only in Pseudovibrio sp. FO-BEG1 but not in strain JE062, although both genomes are in general highly similar. In the genome of strain FO-BEG1 we identified a set of genes coding for a complete gene transfer agent (GTA) (in strain JE062 several genes were missing, see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000), a unit best described as a virus. It harbors small parts of the host DNA and capable of injecting it into appropriate cells, without having negative effects on the host cell (for reviews see Lang and Beatty, 2001; Lang and Beatty, 2007). By this process, Pseudovibrio could have taken up and dispersed DNA carried in virus-like particles, thereby gathering genes and establishing a diverse physiology for a		

symbiotic and a free-living lifestyle. Additionally, we found 14 integrase and 21 transposase elements in the genome of Pseudovibrio sp. FO-BEG1 (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148), 9 of which are located adjacent to the hybrid NRPS-PKS gene cluster, which verifies acquisition of this genomic island via horizontal gene transfer. Quorum sensing We could identify 15 genes in strain FO-BEG1 and 14 in strain JE062 containing the LuxR domain, which represents the transcriptional regulator of the acetylated homoserine lactone (AHL) type, allowing the bacterium to detect AHL quorum sensing molecules and to initiate a response (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Intriguingly, we could not find any luxI genes, which code for AHL quorum sensing molecules. This observation leads us to the hypothesis that both Pseudovibrio strains do not communicate via AHL within their own species, but seem to use the LuxR as receptors to react to quorum sensing molecules produced by other species and initiate a respective		

answer. Such a scenario has been described before by Case et al. (2008) and was called 'eavesdropping'. The response reaction could include the production of bioactive compounds to repel competing prokaryotes or to protect the host from pathogens or parasites. Alternatively, such LuxR-family 'solos' could participate in interkingdom signaling, as suggested by Subramoni and Venturi (2009), thereby facilitating prokaryote-host interactions of Pseudovibrio strains with marine invertebrates.

Growth with Beggiatoa sp. 35Flor

Pseudovibrio sp. FO-BEG1 is the single accompanying organism of the Beggiatoa strain 35Flor, which is growing in a chemolithoautotrophic sulfide-oxygen-gradient medium (Brock and Schulz-Vogt, 2011; Chapters 2 and 3). All attempts to grow Beggiatoa without Pseudovibrio failed and so far we could not identify the factors required by the Beggiatoa strain for autonomous growth. It is known, however, that Beggiatoa spp. do not possess catalases (Larkin and Strohl, 1983) and therefore are susceptible to reactive oxygen molecules originating from respiration. Addition of catalase to the medium is known to increase the viability of

Beggiatoa sp. (Burton and Morita, 1964). We hence hypothesize that Beggiatoa sp. 35Flor depends on the radical protection system exhibited by Pseudovibrio sp. FO-BEG1 including genes coding for over 20 superoxide dismutases, catalases and peroxidases (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148). The role of heterotrophic bacteria as scavenger of reactive oxygen species has also been described by Morris et al. (2008), which could establish robust growth of cyanobacteria after addition of 'helper' heterotrophs. Secretion Systems In the genomes of FO-BEG1 and JE062 we could identify two loci that encode type VI secretion systems (T6SS) as well as one type III secretion system (T3SS) including effector molecules, which indicates the capability of specific interactions with eukaryotes and the possibility of influencing their cell machinery. The T6SS has been described as a major secretion system in the context of pathogenicity as a virulence factor in morbific bacteria (Mougous et al., 2006; Pukatzki et al., 2006) and a core of 13 highly conserved and essential subunits has been identified for this secretion system 129		

(Boyer et al., 2009). In both genomes of the Pseudovibrio strains, we found two gene clusters consisting of 12 (cluster I) and 20 (cluster II) genes that encode T6SSs. Cluster II contains the complete set of core subunits and therefore we assume that cluster II could, if expressed, produce a complete and functional type VI secretion system. In cluster I, two core genes are missing in the operon, hcpI and vgrG, which are main components of the injection apparatus with possible effector functions (Pukatzki et al., 2009; Bonemann et al., 2010). However, homologues of hcpI and vgrG could be identified in additional copies at other locations in the genomes of FO-BEG1 and JE062 (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000), which is a phenomenon regularly found in genomes containing T6SS (Pukatzki et al., 2009). The possible role for type VI secretion systems in bacteria has not been completely elucidated so far, but several functions have been attributed to it already. Mainly, T6SS is described as a virulence factor of pathogenic bacteria delivering effector proteins into host cells (Filloux et al., 2008). However, further		

studies reveal the involvement of T6SS in biofilm formation (Aschtgen et al., 2008), quorum sensing (Weber et al., 2009), interbacterial interactions (Hood et al., 2010) and antipathogenesis (Chow and Mazmanian, 2010; Jani and Cotter, 2010). In conclusion, it can be assumed that the T6SS of both strains are functional since the genomes contain the main structural components of the type VI secretion system. In addition to the T6SS, we identified a type III secretion system in the genomes of both Pseudovibrio strains, which is located in a genomic region encompassing around 35 ORFs with various highly conserved proteins known from T3S systems (Cornelis and Van Gijsegem, 2000) (Figure S.3 and see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Besides the secretion apparatus we also identified genes encoding homologues of three types of effector molecules in the genome of strain FO-BEG1 and two effector molecule types in strain JE062. Those effectors might be directly involved in the establishment of symbiosis between Pseudovibrio and its host. YpkA, IpgD (found in both genomes) and YopJ (only in		

strain FO-BEG1) are effector molecules that affect the cytoskeleton or the innate immune response of the host, respectively. YpkA is a serine/threonine kinase, which has negative effects on cytoskeletal dynamics due to its interaction with actin, thereby contributing to the resistance to phagocytosis (Cornelis, 2002). YpkA is present in three copies in both genomes. In Porifera, specialized amoeboid cells, the archaeocytes, resemble macrophages and eliminate non-self material via phagocytosis (Muller and Muller, 2003). Pseudovibrio, expressing and secreting the YpkA effector, could interfere with this process, preventing archaeocytes from digesting Pseudovibrio cells. A similar effect could be induced by a homologue of IpgD found in both genomes, a virulence factor that is responsible for morphological changes of a host cell by increasing membrane detachment from the cytoskeleton (Niebuhr et al., 2000; Niebuhr et al., 2002). Figure S.3. Operon coding for type III secretion system (T3SS) subunits and effector proteins. White arrows show annotated homologues of T3SS subunits including the gene name within the arrows; black arrows represent annotated effector		

homologues; dark gray arrows show annotated genes encoding proteins presumably not involved in T3SS; light gray arrows show hypothetical proteins with unknown function. The locus is indicated above and below some genes for orientation purposes. In FO-BEG1 we additionally identified a homologue of the YopJ effector exhibiting a serine/threonine acetyltransferase function. By acetylation of serine and threonine residues of mitogen-activated protein (MAP) kinases it prevents phosphorylation of those molecules and therefore inhibits the innate immune response of the organism (Mukherjee et al., 2006). Intriguingly, it has been shown that sponges possess a very efficient innate immune response system, using MAP kinases as the essential component of its response to bacterial endotoxin lipopolysaccharide (LPS) (Bohm et al., 2001; Muller and Muller, 2003). This indicates that homologues of the acetyltransferase YopJ effector in Pseudovibrio could prevent phosphorylation of MAP kinases via acetylation, thereby playing a role in the inactivation of the immune answer of the host organism, allowing Pseudovibrio to avoid phagocytosis, as		

described by Bartsev et al. (2004) for a Rhizobium strain, and to remain in the host for establishment of a symbiosis. This hypothesis is further supported by the fact that a homologue of YopJ (NopJ) was shown to be an effector in symbiotic rhizobia (Deakin and Broughton, 2009) and Lackner et al. (2011) demonstrated that T3SS is involved in maintenance of a symbiosis between bacteria and fungi by enhancement of intracellular survival of the prokaryote within the host. Adhesion In both genomes we found homologues of genes coding for proteins responsible for adhesion to surfaces or other cells. These proteins, belonging to the group of amyloids, are extracellular proteinaceous components and are known in Enterobacteriaceae as curli fibers. They are involved in adhesion to surfaces, cell aggregation, biofilm formation and mediate cell-cell adhesion and invasion of host cells (Barnhart and Chapman, 2006). The production of curli fibers in enteric bacteria is dependent on at least six proteins encoded by the operons csgAB and csgDEFG (agf in Salmonella) (Hammar et al., 1995), the latter of which is required for assembly, stability and secretion of the amyloids (Hammar et al.,		

1995). csgAB encodes the structural subunits of the curli fibers, both genes containing characteristic repeat motifs (Hammar et al., 1996). A gene cluster in the genome of Pseudovibrio sp. FO-BEG1 resembles the curli formation operon in enteric bacteria (Figure S.4). Figure S.4. Comparison of genes encoding amyloids in Enterobacteriaceae and the operon in Pseudovibrio sp. FO-BEG1. White arrows represent homologues of genes in enteric bacteria; gray arrows show genes present in Enterobacteriaceae only; black arrows show genes containing curli repeats, typical motifs of the amyloid structural subunits. The number within the black arrows shows the amount of curli repeats in the according gene. Homologues of csgF and csgG, required for stabilization and secretion of the amyloids are found in direct proximity to three genes containing curlin associated repeats as typical structural components of the curli fibers. We hypothesize that the identified operon might code for amyloid structures comparable to curli fibers due to the existence of characteristic curlin repeat motifs and genes involved in the assembly and secretion of such structures,		

therefore allowing Pseudovibrio to attach to other cells or form biofilms or aggregates. Additionally, we identified 35 genes in strain FO-BEG1 and 37 in JE062 containing domains mediating prokaryote-eukaryote interactions, supporting the proposed role of Pseudovibrio as a symbiont with possibilities to attach and interact with the host organism (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Conclusions In this study, we analyzed highly similar genomes of two Pseudovibrio strains that originate from the coast of Florida, the Pseudovibrio sp. FO-BEG1 sampled from a coral and maintained over 10 years in co-culture with Beggiatoa sp. and Pseudovibrio sp. JE062 sampled from a sponge in the same region (Enticknap et al., 2006). The physiology of both strains is extremely versatile and the metabolic traits found in the genome could be partially verified in experiments with strain FO-BEG1. Here, we describe for the first time a Pseudovibrio strain that uses aromatic compounds as a carbon and electron source, oxidizes thiosulfate under aerobic conditions and uses		

phosphonates as a phosphorous source. Notably, strain FO-BEG1 grows under extreme nutrient limitation, which emphasizes its adaptation to life in the open ocean. The metabolic variety is confirmed by the numerous transporter systems that are encoded in the genome. Compared with other marine bacteria, like the prominent Roseobacter clade, which is known to be ubiquitous, multitudinous and physiologically versatile (Newton et al., 2010), Pseudovibrio seems to be capable of a similarly generalistic life style, exploiting quite a number of sources for energy sources, nutrients and trace elements. Aside from metabolic variety, the genomic data of both strains also confirm close associations with marine invertebrates and indicate several potential mechanisms for establishing and maintaining a symbiosis. The most striking discovery is the presence of effector homologues secreted by type III secretion systems, which could affect sponges by interacting with their immune response system (YopJ) or the cytoskeleton (YpkA, IpgD) and thereby have a drastic impact on the cell machinery of the host. Another fascinating discovery is the presence of the		

hybrid NRPS-PKS system in strain FO-BEG1, which has so far only been described for members of the Enterobacteriaceae family (Putze et al., 2009), producing the bioactive compound colibactin with yet unknown in-vivo functions, but arresting eukaryotic cells in the G2 phase, eventually leading to cell death (Nougayrède et al., 2006). The presence of a gene cluster coding for a cytopathic compound in strain FO-BEG1 emphasizes the impact that Pseudovibrio cells might have on marine invertebrates. Intriguingly, strain FO-BEG1 seems to be a required partner in the Beggiatoa co-culture, indicating its important symbiotic role not only for marine invertebrates but also for prokaryotes. It is possible that Pseudovibrio has positive effects for certain bacteria under in-vivo conditions, e.g. by supplying vitamins or detoxifying metabolic intermediates or radical oxygen species. Figure S.5. Schematic overview of the possible life styles and the physiologic capabilities derived from genetic information of both Pseudovibrio genomes. On the left hand side, physiologic abilities are depicted that could be used in free-living, oxic and anoxic conditions. On the right hand side, the attached or		

associated life style is illustrated. The host organism for the associated life style can be represented by a sponge, coral or tunicate. Biofilm formation, aggregation and attachment to host cells could be performed via e. g. amyloid-like structures. The proposed secretion systems could be involved in prokaryote-eukaryote interactions, influencing the cell machinery of the host. Additionally, Pseudovibrio could supply the host with cofactors like vitamins or synthesize secondary metabolites as a defense mechanism against other prokaryotes or the host. The frequent identification and isolation of Pseudovibrio strains in many studies over the last years implies an important but rather unexplored role for this genus in marine habitats. According to the genomic and physiological data on Pseudovibrio spp., we propose a free- living and attached or associated life style model for this genus (Figure S.5). As a denitrifying heterotroph, Pseudovibrio has an obvious influence on the carbon and nitrogen cycles. Its ecological impact can now be extended to the sulfur and phosphorus cycles due to its ability to metabolize thiosulfate and phosphonates.		

Additionally, we hypothesize that, due to the predictions based on the genomic data, similar to E. coli in humans, Pseudovibrio is a commensalistic or even beneficial symbiont of marine invertebrates with a potential to become pathogenic.		

In our case, an inorganic contamination was introduced to the artificial seawater after the incubation during the solid phase extraction of DOM (SPE-DOM). This led to ion suppression of the organic compounds in the FT-ICR mass spectra in ESI negative mode. Consequently, it was not possible to analyze the DOM composition in that mode and we chose the positive mode instead. This problem does not occur in natural seawater samples, because the concentration of DOC is higher. We could show that the organic substances present in the artificial medium are detectable if the overall background of DOM is 10 to 100 times higher. This observation indicates that a specific concentration of organic matter has to be present to be detected by ESI FT-ICR-MS if inorganic substances are introduced in high amounts during extraction or ionize extremely well. In ESI positive mode, an organic contamination of the artificial seawater was observed. Two series of polyethylene glycol (PEG) oligomers were detected. As a potential source NaOH used for medium preparation was identified. Since PEGs ionize extremely well in ESI positive mode, the peaks are much higher compared to most other peaks in	[redacted]	Checked 17/8 lần cuối cùng

the sample. Using the standard addition method (described in material and methods), the total amount of PEG contaminations was estimated to be 1000fold less than the total DOC concentration in artificial seawater, and thus represented only a very small fraction of the entire DOC contamination. These impurities analyzed in the artificial seawater medium were not detected in the SPE-DOM of natural seawater samples and thus did not affect the FT-ICR-MS analysis of natural seawater incubations.

Growth and substrate use in artificial seawater

During growth in artificial seawater under extremely oligotrophic conditions, Pseudovibrio sp. strain FO-BEG1 multiplied from about 20 cells mL-1 to 2 X 104 cells mL-1, even though the overall amount of DOC did not measurably decrease. Thus, the amount of compounds that were consumed was probably below detection limit (0.5 |imol C L-1). Based on the increase in cell numbers when grown with glucose and ammonium (supplementary material Table S.4.1 and Figure S.4.1), it was calculated that about 1 to 3 |imol

C L-1 is needed for the observed cell numbers as carbon and energy source. Apparently, the uptake of amino acids alone could not sustain bacterial growth because the initial amino acid concentration (0.13 |imol L-1) was already much lower than the required 1 to 3 |imol C L-1. During the initial growth phase, we found a decrease in dissolved free amino acids (DFAA) concentration concurrently with an increase in dissolved combined amino acids (DCAA), whereas the overall amino acid concentration (DFAA + DCAA) did not decrease (Table 4.1.1). Within the growth phase, the DFAA might have been used as precursors, e.g. for exo-enzymes, which lead in turn to an increase in the DCAA concentration, but no overall decrease in the amino acid concentration. During stationary phase, we found a slight decrease of total amino acids. This may suggest that amino acids were rather used as substrate for maintaining non-growing cells.

The compositional analysis of DOM with ESI FT-ICR-MS revealed a decrease of nitrogen-containing compounds during the initial growth phase (Figure 4.1.3 B and C). Inferring from their molecular compositions (high H/C, low O/C, N-containing), these substances

were most likely detergents, such as ampholytic amino oxides or betaines. Using this method we cannot quantify the amount of carbon corresponding to this decrease. In addition, a compound with a decreasing peak in the mass spectrum is not necessarily completely oxidized to CO2. Thus, we cannot ultimately clarify whether the use of these substrates alone explains the observed growth. Nevertheless, the preferential decrease of nitrogen-containing compounds suggests that these substances at least served as nitrogen source. This agrees with the observation that N2 fixation was not detectable.

Growth and substrate use in natural seawater

The overall concentration of amino acids in the natural seawater was already initially quite low and did not further decrease during the incubation. Thus, amino acids did not sustain growth or survival of cells. The compositional analysis of DOM showed a decrease of two groups of compounds. One of them was likely containing carbohydrate-like compounds with nitrogen and sulfur. These compounds are most likely thiosugars and/or aminosugars and may therefore have also served as nitrogen source. In

addition, we observed a decrease in relative intensities of compounds with low O/C and H/C ratios containing sulfur but no nitrogen. Compounds with low amounts of oxygen and hydrogen in comparison to carbon are typically condensed or aromatic hydrocarbons. Since it cannot be excluded that these compounds were lost abiotically, e.g. by absorption to the glassware, it needs to be further investigated if the bacteria indeed metabolized these complex molecules during growth. Notably, during growth of Pseudovibrio sp. strain FO-BEG1 in natural seawater, we observed a decrease of different groups of substances compared to artificial seawater and preferentially substances containing sulfur decreased.

The use of multiple substrates

The potential use of different substrates under oligotrophic conditions was shown by the biolog experiment. The bacteria were able to metabolize different types of organic compounds, such as sugars (e.g. D-raffinose and D-trehalose), amino acids (L-serine, glutamic acid), carboxylic acids (glucuronic acid, acetic acid) and amide (glucuronamide). These compound classes were also

present as contaminations in the artificial seawater medium and decreased during growth. This is in agreement with studies on E. coli showing a broad potential to use different substrates after adaptation to carbon starvation (Ihssen and Egli, 2005).	[redacted]	
Even though the substances decreasing during growth, as revealed by ESI FT-ICR-MS, tended to cluster into certain groups with a specific ratio of O/C and H/C, the overall pattern showed a broad simultaneous use of many substrates both in artificial and natural seawater. In contrast to the biolog experiment, this does not show the potential to use a substrate, but the actual decrease of compounds present in original concentrations. Using different compounds simultaneously may enable bacteria to grow on very low concentrations of each of the different substrates (Lendenmann et al., 1996; Kovárová-Kovar and Egli, 1998), if a substrate does not repress enzymes for another less efficient one (reviewed in Egli, 2010). This strategy, together with the potential to use substrates, which are not present but may become available, enables bacteria to survive in habitats with a low and	[redacted]	

fluctuating supply of nutrients, as it is found in the oceans.

[redacted]

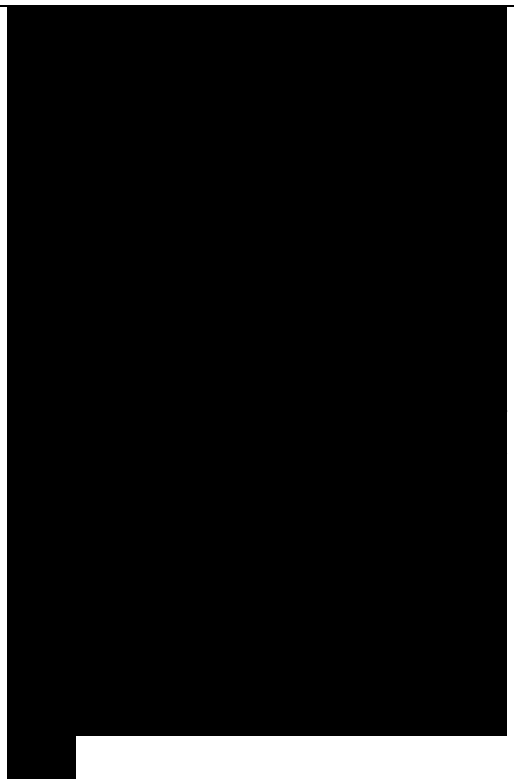
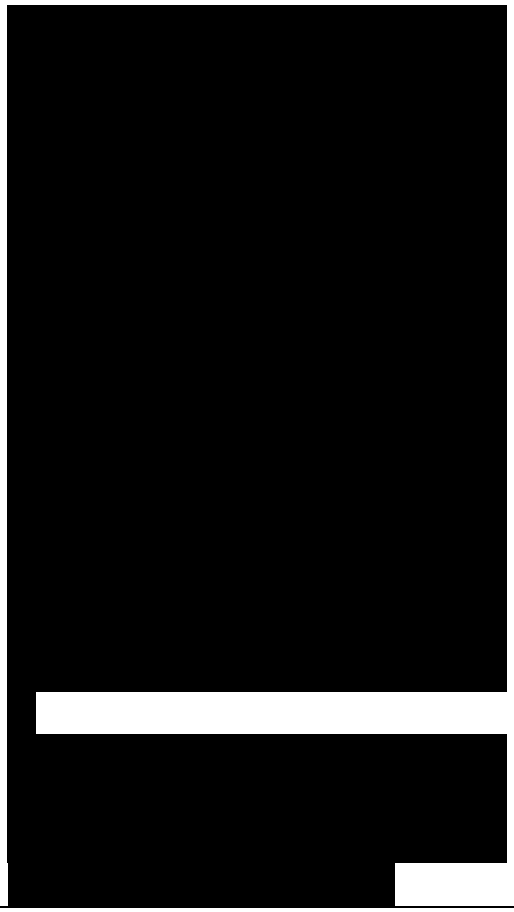
Conclusions

The data presented in this study demonstrate that contaminations can arise from many different sources (e.g. chemicals, bottles, humans or plastics), which in turn might support growth of bacteria under oligotrophic conditions. Moreover, even under extremely oligotrophic conditions, the isolated bacteria were not in a resting state, but showed a moderate growth, even though nitrogen, carbon and energy sources were limiting factors at the same time. The investigated Pseudovibrio sp. FO-BEG1 uses many different types of substrates under nutrient-poor conditions as demonstrated by the FT-ICR-MS data (Figure 4.1.3).

In our case, amino acids were not the primary substrate for growth, but rather a complex mixture of organic compounds, preferably containing nitrogen. Furthermore, we were able to show that ESI FT-ICR-MS is a strong tool to investigate bacterial growth under low-nutrient conditions.

[redacted]

Table S.4.1: Cell numbers of

isolate Pseudovibrio strain FO-BEG1 derived from different amounts of carbon added to the medium. Carbon in form of glucose was added to a Pseudovibrio strain FO-BEG1 culture in 2 different concentrations. As a negative control a culture without addition of carbon was used. To ensure carbon-dependent growth, ammonium was added to the medium (with and without carbon addition) as nitrogen source.		
Figure S.4.1: Amount of carbon needed for specific cell number. From additions of glucose to the medium (Table S.4.1) the amount of carbon needed for growth of isolate FO-BEG1 was calculated by subtracting the cell numbers without carbon addition (as negative control) from the cell numbers with carbon addition and plotting amount of carbon versus cell numbers produced. The amount of carbon needed for 105 cells mL-1 was calculated to be 3 ^mol C L-1 and for 104 cells 1 ^mol C L-1. 4.2 Facultatively oligotrophic bacteria isolated from the habitat of large sulfide-oxidizers		

Abstract
Axenic cultivation of large chemolithoautotrophic bacteria belonging to the genus Beggiatoa succeed only rarely. Growth of the large sulfide-oxidizers often seems to be dependent on the presence of heterotrophic prokaryotes, similar to the often described associations of cyanobacteria and heterotrophs. Recently, we observed that the growth of the marine, chemolithoautotrophic Beggiatoa sp. strain 35Flor depends on the presence of the a-Proteobacterium Pseudovibrio sp. strain FO-BEG1. Furthermore, we found that this bacterium, besides heterotrophic growth on organic-rich medium, is capable of growth under extreme nutrient deficiency in artificial and natural seawater. This observation inspired us to investigate whether we could isolate other facultative oligotrophs from overlaying water of Namibian sediment, an environment known to contain a large number of different sulfide- oxidizers belonging to the family Beggiatoaceae. Indeed, we succeeded to obtain 14 new strains closely related to known marine bacteria, all of which were capable of growth under extreme nutrient deficiency. The potential of these isolates to support growth of the

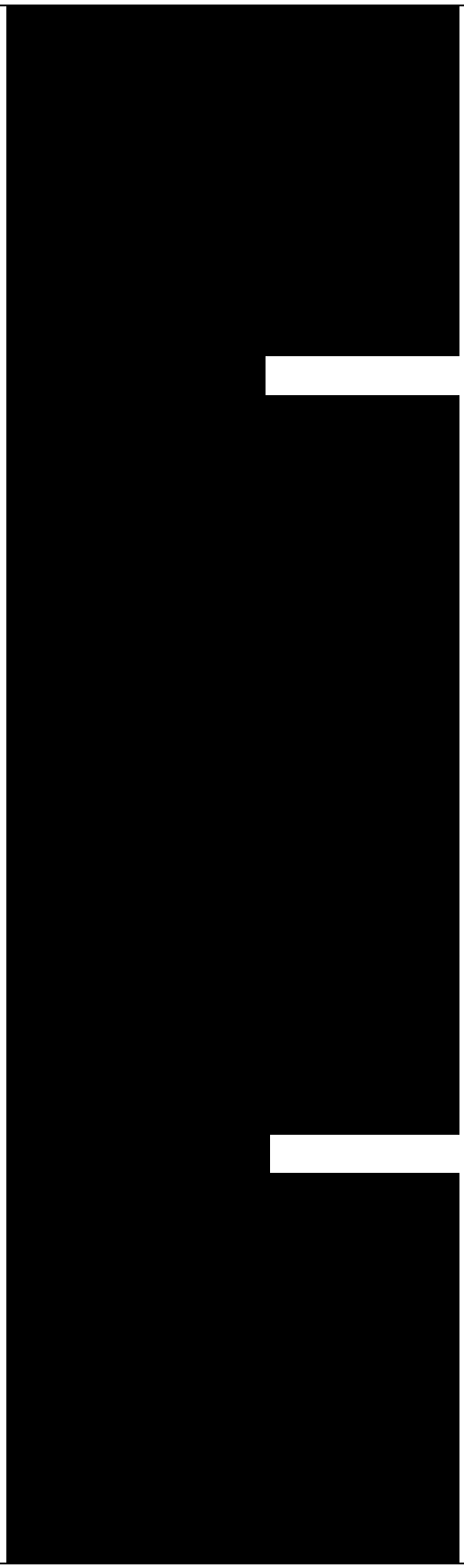
large sulfide-oxidizing bacteria can now be studied in culture-based experiments.

Introduction

Large bacteria, such as Beggiatoa spp. or filamentous cyanobacteria, often live together with heterotrophic prokaryotes and these associations seem to be the reason for the inability of axenic cultivation of the large bacteria (Burton and Morita, 1964; Cohen and Rosenberg, 1989; Palinska et al., 1999; Morris et al., 2008). Different reasons for these interactions have been proposed, such as the recycling of Cacbon điôxít or the reduction of the oxygen concentration (Kuentzel, 1969; Paerl and Pinckney, 1996). It is known that Beggiatoa spp. typically lack the gene for catalase (Larkin and Strohl, 1983), but since aerobic respiration produces reactive oxygen species (ROS, Tapley et al., 1999) an efficient protection against such molecules is needed. Therefore, Beggiatoa spp. might depend on the enzyme catalase, which catalyzes the disproportionation of hydrogen peroxide to oxygen and water, or other protection systems of the associated heterotrophs against ROS. The positive effect of catalase and of

accompanying heterotrophic bacteria on the growth of Beggiatoa filaments (Burton and Morita, 1964; Strohl and Larkin, 1978; Gude et al., 1981; Nelson et al., 1986b) and, furthermore, the accumulation of peroxides in cultures without catalase or accompanying bacteria was shown (Burton and Morita, 1964). Recently, we found that both Pseudovibrio sp. strain FO-BEG1 and Pseudovibrio denitrificans (DSM number 17465) support growth of the marine chemolithoautotrophic Beggiatoa sp. strain 35Flor (Chapter 3.1). Both bacterial strains are heterotrophic organisms (Chapter 3.2, Shieh et al., 2004). Additionally, we have shown that the newly isolated Pseudovibrio sp. strain FO-BEG1 can grow under extremely oligotrophic conditions and its substrate use in pure artificial and natural seawater was studied in detail (Chapter 4.1).

The aim of the present study was to isolate heterotrophic, facultatively oligotrophic bacteria from Namibian sediments, the habitat of large sulfide-oxidizers, to investigate how common facultative oligotrophy is among bacteria associated with large sulfide-oxidizers.



Here, we report the successful isolation of 14 facultatively oligotrophic bacteria from water overlaying Namibian sediments using a method relying on the change from oligotrophic to eutrophic growth conditions, called the CANgrow-method (changing availability of nutrients growth- method).

In contrast to earlier methods for the isolation of marine bacteria, the artificial oligotrophic medium used here, is defined and contains much lower nutrient concentrations. Three initial transfers strongly pre-select for bacteria, which can grow under extreme nutrient deficiency.

Subsequently, three transfers on nutrient-rich agar plates select for facultatively oligotrophic bacteria and are used to obtain pure cultures. Finally, the ability of the isolates to grow oligotrophically is ensured by at least seven transfers in pure artificial seawater.

Material and methods Samples

The new bacterial strains were isolated from oceanic bottom water overlaying Namibian sediments that harbor different large sulfur bacteria (sample

acquisition described in Salman et al., 2011). All samples were stored at 4°C. In addition to the new isolates, the Pseudovibrio denitrificans type strain (DSM number 17465) was purchased from the German culture collection DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultivated under oligotrophic conditions.

Growth media and cultivation conditions

For cultivation and isolation, two different media were used, an oligotrophic and a eutrophic medium. The liquid, oligotrophic medium was composed as described above (Chapter 4.1), but prepared under synthetic air atmosphere (20% O2 in N2; H2O < 3 ppm-mol, CnHm < 0.1 ppm-mol, CO < 1 ppm-mol, CO2 < 1 ppm-mol). Furthermore, bottles were filled only with 50 mL medium and the medium was not cleaned using solid phase extraction. This medium was used for all oligotrophic cultivation experiments. The solid, eutrophic medium was composed as described above (Chapter 4.1, Methods section "Biolog experiment"). All incubations in oligotrophic and eutrophic media were performed

without shaking at 28°C in the dark.	[redacted]	
CANgrow-method (changing availability of nutrients growth-method) For isolation, 50 mL of oligotrophic medium were inoculated with 100 |iL seawater sample (from off shore Namibia). The cultures were transferred (100 |iL enrichment in 50 mL fresh medium) at least three times in oligotrophic medium with incubation periods between the transfers of at least one week. Aliquots of the oligotrophic enrichments were then plated on eutrophic, solid medium and single colonies were transferred three times on eutrophic medium. Finally, at least another seven transfers (100 |iL culture in 50 mL fresh medium) were performed in liquid, oligotrophic medium (Figure 4.2.1).	[redacted]	
Sequencing of 16S rDNA genes and phylogenetic analysis Eutrophically grown colonies were picked and directly transferred to a polymerase chain reaction (PCR) mix containing 1x PCR MasterMix (Promega, Mannheim, Germany) and 1 |imol L-1 of each primer (GM3F	[redacted]	

and GM4R in Muyzer et al., 1995). The PCR program applied was as follows: initial denaturation at 95°C for 5 minutes, 32 cycles of 95°C for 1 minute, 50°C for 30 seconds and 72°C for 90 seconds followed by a final elongation at 72°C for 7 minutes. PCR products were cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Karlsruhe, Germany) according to manufacture's instructions. Sequencing of the cloned inserts was performed using the Big Dye Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and sequences were analyzed on an ABI Genetic Analyzer 3130x (Applied Biosystems, Carlsbad, CA, USA). Nearly full-length sequences were assembled with SeqMan (Lasergene software package, DNAStar, Madison, WI, USA) and deposited in the DDBJ/EMBL/GenBank databases under accession numbers FR716535 to FR716549. Phylogenetic analysis of the 16S rDNA sequences was performed using the ARB software package (Ludwig et al., 2004) and release 102 of the SILVA SSURef database (Pruesse et al., 2007). Tree reconstruction with	[redacted]	

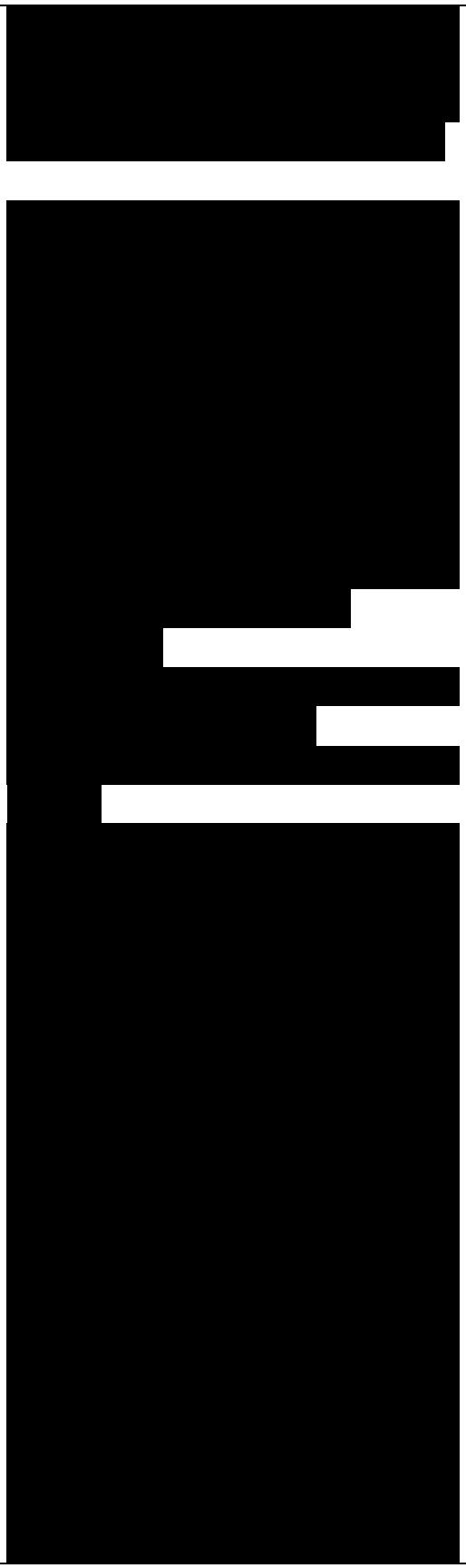
maximum likelihood and neighbour joining methods was performed using 0, 30 and 50% positional conservatory filters that exclude highly variable regions. Finally, a consensus tree based on the different reconstruction methods was built. A total number of 102 nearly full-length sequences was used for initial calculation to stabilize tree topology. Displayed in the final tree (Figure 4.2.2) are the sequences of the 15 isolates grouped with their closest relatives.

Cell counts

Cell counts were performed as described in Chapter 4.1.

Measurement of dissolved organic carbon (DOC)

Dissolved organic carbon (DOC) was measured in the oligotrophic medium using a Shimadzu TOC-VCPH total organic carbon analyzer (Shimadzu, Kyoto, Japan). Acidification of samples was performed with 1% v/v 2 mol L-1 HCl followed by sparging with synthetic air in order to remove inorganic carbon. The detection limit of the method was 5 |imol C L-1 (0.06 mg C L-1). The analytical accuracy was confirmed with reference material (deep Atlantic seawater) and low carbon water from the consensus reference materials program (D.A. Hansell, University of Miami,



Coral Gables, FL, USA).

Results

Isolation of facultatively oligotrophic bacteria

Applying the CANgrow-method, which favors facultatively oligotrophic bacteria (Figure 4.2.1) , we obtained 14 isolates of marine bacteria that were able to adapt from oligotrophic to eutrophic growth conditions and vice versa within 3 to 5 days.

We were able to obtain pure cultures of these strains by transferring single colonies from organic-rich agar plates and could show that these colonies were able to grow oligotrophically by at least seven transfers in pure artificial seawater.

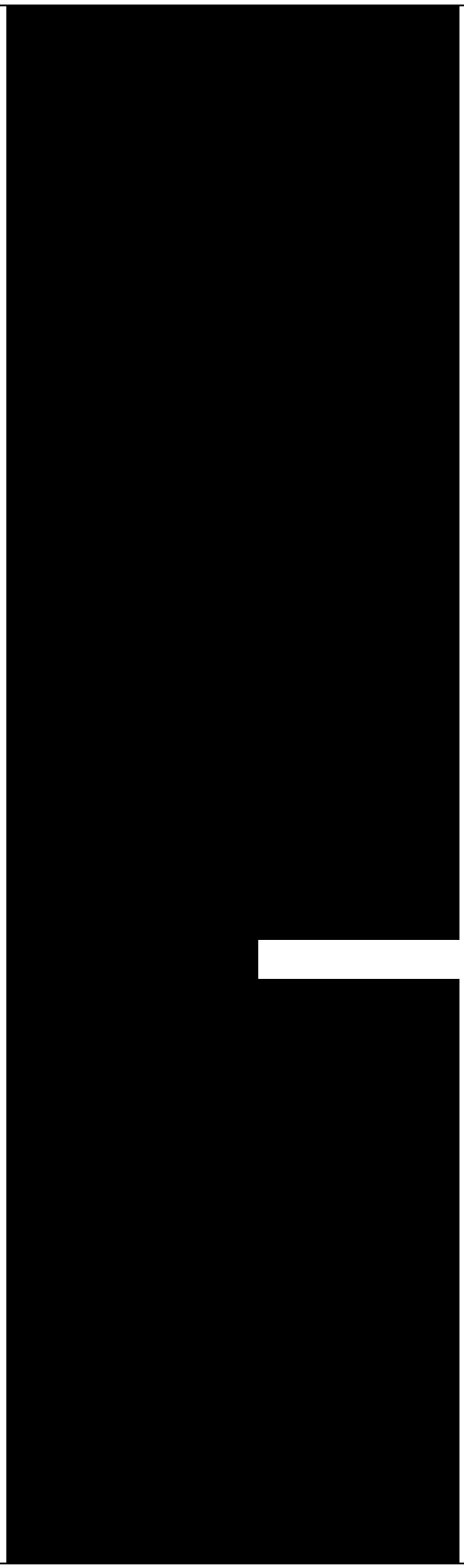
Changing availability of nutrients growth- method (CANgrow-method)

Figure 4.2.1: Comparison of strategies for the isolation of oligotrophic bacteria. Three different methods for the isolation of obligately or facultatively oligotrophic bacteria are compared with the newly developed CANgrow-method (Changing availability of nutrients growth- method).

Bacteria from three different

phyla were isolated with the CANgrow-method. Phylogenetic relations are shown in a 16S rDNA sequence tree including the Pseudovibrio sp. strain FO-BEG1 showing > 99.5% sequence identity to the Pseudovibrio denitrificans type strain (Figure 4.2.2). Except for two isolates, which were closely related to Arthrobacter spp. (Actinobacteria) on 16S rDNA level (99.8 to 99.9% identity to closest related strain), all isolates were members of the Proteobacteria. The remaining 12 isolates were members of the Gammaproteobacteria, two of them grouping with Kangiella spp. (96.4% identity to closest related strain) and ten withMarinobacter spp. (98.7 to 100% identity to closest related strain).

Figure 4.2.2: Phylogenetic trees based on a total number of 102 nearly full-length sequences were calculated with maximum likelihood and neighbor joining methods using different positional conservatory filters. The displayed tree is an excerpt from the consensus tree that was inferred based on the different reconstruction approaches. The 14 new isolates and strain FO-BEG1 are grouped with the most closely related type strains. Isolated strains listed in one line



feature an identical 16S rDNA sequence, whereas isolated strains listed directly one below the other are 99.6 to 99.9% identical in their 16S rDNA sequence. The isolates FO-NAM13, 14 were only able to grow for six transfers under oligotrophic conditions and are therefore marked grey in the tree.

Oligotrophic growth

The artificial seawater contained a DOC concentration of 0.18 ± 0.06 mg C L-1 (15 ± 5 |imol C L-1). Growth curves in oligotrophic artificial seawater medium (Figure 4.2.3) were obtained for one isolate from each phylogenetic group (Actinobacteria, Alpha- and Gammaproteo- bacteria). We observed a clear increase in cell numbers starting from 4 to 20 cells mL-1 to a final density of 104 to 105 cells mL-1. The proteobacterial isolates showed growth after 2 days and reached a stationary phase after 5 to 7 days of incubation. Both actinobacterial isolates were characterized by delayed growth that was detectable after 12 days. Here, the stationary phase was reached after about 20 days of incubation. Moreover, all isolates except for the two isolated Kangiella strains were

able to grow after at least seven transfers in the oligotrophic seawater medium. The two isolates closely related to Kangiella spp. were not able to grow after more than six transfers under oligotrophic conditions. The isolates closely related to Marinobacter spp. reached the highest final cell numbers (Figure 4.2.3 D), whereas the actinobacterial isolates showed lowest final cell densities (Figure 4.2.3 C). Furthermore, we observed growth under oligotrophic conditions for the type strain Pseudovibrio denitrificans (DSM number 17465). The growth curve of this strain (Figure 4.2.3 B) showed the same pattern as the Pseudovibrio sp. FO-BEG1, which is currently growing in the 26th oligotrophic transfer in highly purified artificial seawater.

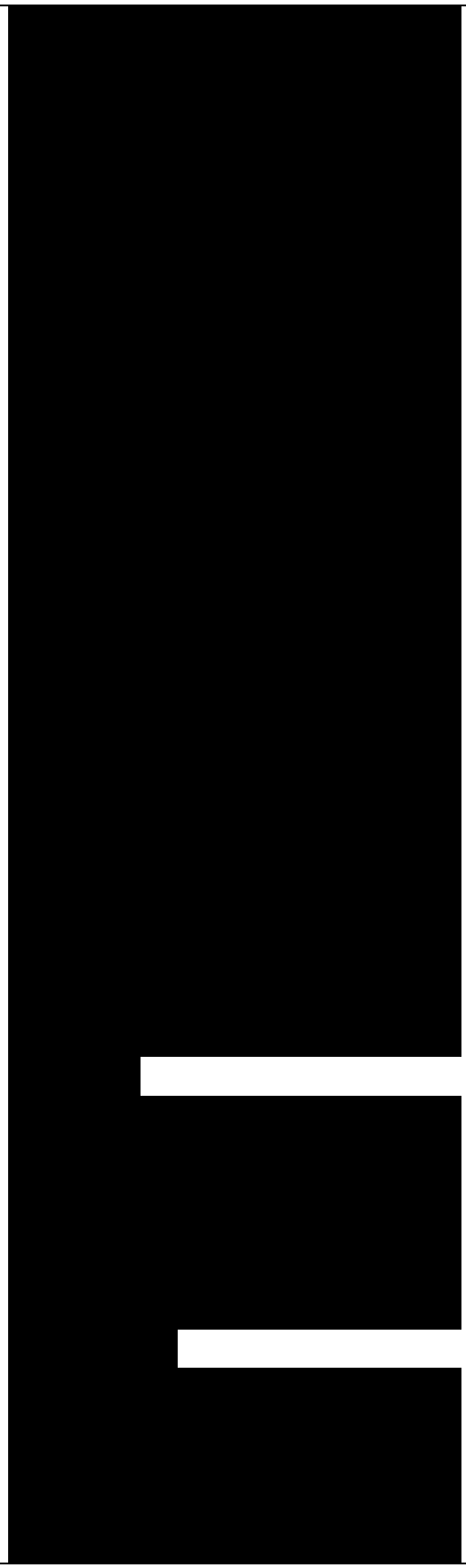


Figure 4.2.3: Oligotrophic growth curves of (A) strain FO-BEG1 (Alphaprobteobacteria, related to Pseudovibrio spp.), (B) Pseudovibrio denitrificans type strain (Alphaproteobacteria), (C) isolate FO-NAM2 (Actinobacteria, related to Arthrobacter spp.) and (D) isolate FO-NAM6 (Gammaproteobacteria, related

to Marinobacter spp.).

Discussion

Isolation of facultatively oligotrophic bacteria with the CANgrow-method

Each strategy that is applied for the isolation of bacteria selects for a specific physiology and metabolism. Most approaches used recently for the isolation of oligotrophic bacteria are based on the dilution to extinction method (Button et al., 1993; Connon and Giovannoni, 2002) and thereby select for the most abundant microorganisms. In contrast, the CANgrow-method favors bacteria, which might not have been particularly abundant in the original inoculum, but can adapt fast to changes in nutrient availability. Previous studies have shown that many bacteria isolated under oligotrophic conditions can adapt to nutrient-rich media (Yanagita et al., 1977; MacDonell and Hood, 1982; Carlucci et al., 1986).

Also, the single-cell encapsulation method (Zengler et al., 2002) is based on oligotrophic growth followed by eutrophic growth conditions. This cultivation approach is similar to ours (Figure 4.2.1), but we used artificial seawater of

very low DOC concentration rather than natural seawater. The measured DOC concentration of 0.18 mg C L-1 is two to five times lower than in natural seawater (Schut et al., 1997; Hansell et al., 2009). Nevertheless, we are certain to observe true growth under these extremely oligotrophic conditions, since we performed at least seven transfers in purified artificial seawater with each of the isolated strains and 26 transfers for Pseudovibrio sp. FO-BEG1, for which we also identified growth substrates under oligotrophic conditions (Chapter 4.1).

The initial cell number after each transfer was 4 to 20 cells mL-1. Thus, 9 to 15 divisions must have occurred between two consecutive transfers to account for a final cell number of 104 to 105 cells mL-1 as observed at the end of the growth phase. This accounts for 60 to 100 divisions during a total of 7 incubations. Therefore, we conclude that all isolates are viable under oligotrophic conditions by the definition of Button et al. (1993), who characterize organisms as viable after having performed 13 divisions which we observe already after 1 to 2 transfers.

Diverse phylogeny of facultatively oligotrophic bacteria

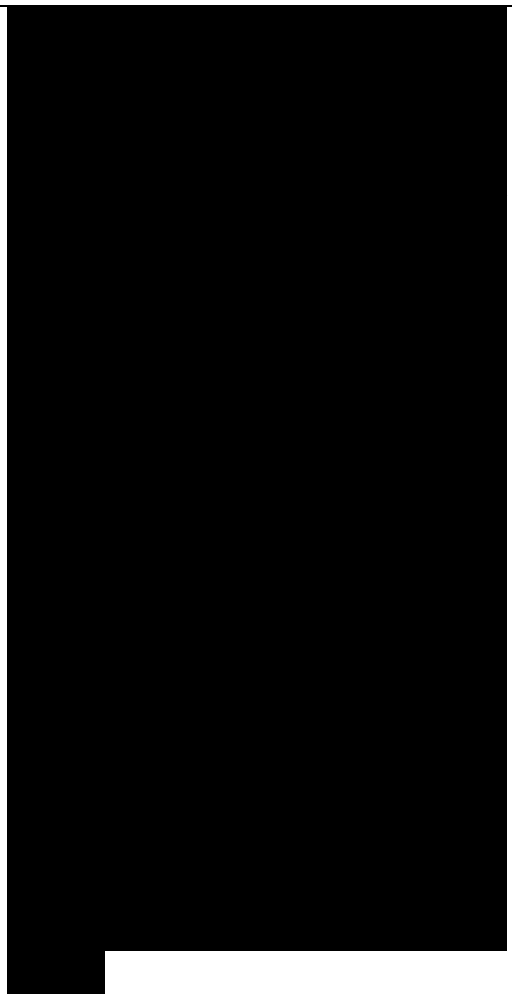
The isolated bacterial strains belong to different phylogenetic groups, namely Alphaproteo-bacteria, Gammaproteobacteria and Actinobacteria. Growth under oligotrophic conditions as observed for the isolates FO-NAM13 and FO-NAM14 (related to Kangiella spp.), has so far never been described for any member of the genus Kangiella.

Long-term starvation and survival but not growth in the absence of external nutrients has previously been reported for Arthrobacter spp. and was proposed to be fueled by internally stored reserve material (Zevenhuizen, 1966).

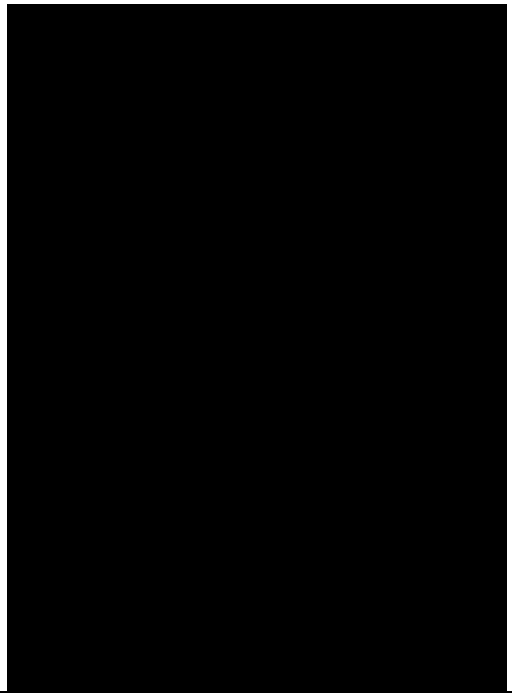
In our study, isolates FO-NAM1, FO-NAM2 (related to Arthrobacter spp.) were transferred in oligotrophic medium more than seven times and active growth was always observed. Hence, we assume that the cells gained energy and produced biomass from an external source, since we determined growth and not only survival. Bacteria belonging to

the genus Marinobacter are known to be diverse in physiology (e.g. Gauthier et al., 1992; Huu et al., 1999). Substrate uptake under low nutrient conditions was shown for Marino- bacter arcticus (Button et al., 2004), but oligotrophic growth was not studied in detail. In contrast, the isolates FO-NAM3 to FO-NAM12 (related to Marinobacter spp.) actively grow under nutrient deficiency.	[redacted]	
Cell numbers of the isolated strains growing under oligotrophic conditions differed between the phylogenetic groups. The cell numbers of isolates related to Marinobacter spp. were higher than cell numbers of the other isolates, whereas the isolates related to Kangiella spp. did not grow for more than six transfers. This suggests that the different bacteria vary in their capabilities of adapting to oligotrophic growth conditions or that the present organic and inorganic material can be used differently among the isolates. This might be due to the presence of different metabolic pathways and a different number and type of	[redacted]	

high affinity transporters necessary for scavenging nutrients at such low concentrations. Taken together, our data support earlier findings (Yanagita et al., 1977; MacDonell and Hood, 1982; Carlucci et al., 1986) that the ability to switch between extreme nutrient deficiency and affluence of substrate is not unusual or restricted to a certain phylogenetic group, even if the level of adaptation might differ. Therefore, we propose that the ability to grow under extreme substrate limitation is much more widespread among known heterotrophic bacteria than currently recognized.



As expected, the heterotrophic bacteria isolated from the habitat of large sulfide-oxidizers are phylogenetically diverse. It was possible to isolate bacteria following a similar metabolic strategy - facultative oligotrophy. If these bacteria are associated with sulfide-oxidizers, if their metabolic activity is somehow related to the presence or absence of the lithothrophs or whether they can support growth of the large sulfur bacteria by scavenging ROS needs to be further investigated.



Sir William Lawrence Bragg (1890-1971)

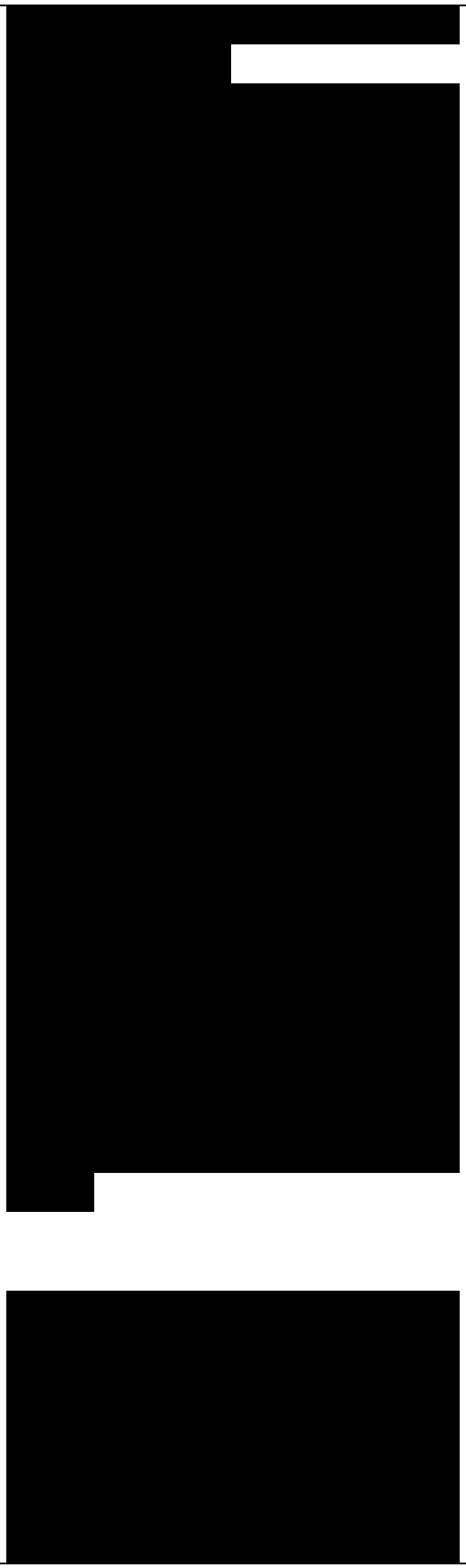
Chapter 5 Concluding remarks and outlook

The findings of this thesis contribute to different topics ranging from migration behavior of mat-forming sulfur bacteria via associations between different bacteria to the lower limits of bacterial growth. Nevertheless, all these different aspects are linked to each other. The growth of Beggiatoa sp. filaments, for which we discovered an unusual migration behavior (Chapter 2), depends on the presence of Pseudovibrio sp. (Chapter 3), which is capable of growth under extreme nutrient deficiency (Chapter 4). This chapter (Chapter 5) connects all these different aspects, including preliminary data, which are not presented in the previous chapters and considers the obtained results in a broader context. Furthermore, a detailed discussion of special proceedings and precautions during performed experiments is given. Finally, this chapter ends with an outlook for future research concerning the discussed topics.

Associations between small heterotrophic and larger bacteria

Axenic cultivation of large marine Beggiatoa spp. under chemolithoautotrophic conditions is difficult and only rarely successful (Nelson and Jannasch, 1983; Nelson et al., 1986a). In this thesis, a successful and stable cultivation of the marine chemolithoautotrophic Beggiatoa sp. strain 35Flor (6 |im in diameter) is described. This Beggiatoa strain grows solely in the presence of an accompanying organism, the heterotrophic Pseudovibrio sp. strain FO-BEG1 (Chapters 2 and 3). We propose that the accompanying bacterium protects the Beggiatoa sp. from oxidative stress because we have shown that the genome of the accompanying organism possesses more than 20 genes for the enzymes catalase, superoxide dismutase and peroxidase (Chapter 3.2), whereas sulfide-oxidizing bacteria of the genus Beggiatoa typically do not possess the gene for the enzyme catalase (reviewed in Larkin and Strohl, 1983).

Similar to large sulfide-oxidizers, also large marine cyanobacteria can often be found associated with small heterotrophic bacteria and it is difficult to sustain axenic cultures (Palinska et al., 1999;



Morris et al., 2008). The association of small heterotrophic bacteria with larger bacteria, such as Beggiatoa spp. or cyanobacteria in microbial mats is a common observation (Cohen and Rosenberg, 1989; van Gemerden, 1993). The complex interactions between cyanobacteria and heterotrophic bacteria have been studied and different reasons for their interactions have been proposed. These reasons include recycling of Cacbon điôxít needed by the cyanobacteria (Kuentzel, 1969), production of growth factors and formation of anoxic microniches due to aerobic respiration (Paerl and Pinckney, 1996). Interestingly, for cyanobacteria of the genus Prochlorococcus, it was also proposed that the function of the heterotrophs is to scavenge reactive oxygen species (ROS) because the Prochlorococcus spp. themselves have no gene encoding for a catalase or peroxidase (Morris et al., 2008). Furthermore, a mutant of the accompanying heterotroph lacking the gene for catalase was found to not support growth of the cyanobacteria, whereas the addition of catalase had a positive effect on their growth (Morris et al., 2008). These observations concerning the association between cyanobacteria and accompanying	[redacted]	

heterotrophic bacteria point in the same direction as the observations presented here on the association between Beggiatoa sp. and Pseudovibrio sp. (Chapter 3). It suggests that the protection system of the large bacteria from ROS might be less efficient than the ones of smaller heterotrophic bacteria.

Cyanobacteria and Beggiatoa spp. are often found to share one habitat as for example in microbial mats (van Gemerden, 1993) or in biofilms associated with the black band disease of scleractinian corals (Richardson, 1996). Therefore, in these common habitats they might also live together with similar types of heterotrophic bacteria. The bacteria associated with cyanobacteria belong to a diverse range of phylogenetic groups, including Actinobacteria, Bacteroidetes, Alpha-, Beta-, Gamma- and Deltaproteobacteria (Salomon et al., 2003; Kolmonen et al., 2004; Hube et al., 2009). The isolation of phylogenetically different bacteria from the habitat of large sulfide-oxidizers (Chapter 4.2) indicates that also the large sulfide- oxidizers are not restricted to the association with bacteria of one phylogenetic group. It seems more likely that the large bacteria depend on specialized functions performed

by certain types of the associated bacteria rather than the presence of a specific phylogenetic group. Very recently, a metagenomic sequence analysis of bacterial communities associated with the green macroalgae Ulva australis revealed that not the phylogeny of the associated bacteria but the function of their genes was correlated with the associations (Burke et al., 2011). Consequently, it was proposed that the functional genes rather than the 16S rDNA genes are more appropriate to investigate associations in microbial communities (Burke et al., 2011).

Toxicity factors and migration behavior of Beggiatoa filaments

The growth of Beggiatoa spp. depends on oxygen and sulfide, but both substances can also be harmful to the bacteria if concentrations exceed a critical threshold (Winogradsky, 1887; M0ller et al., 1985). Close relatives of Beggiatoa spp. belonging to the genus Thiomargarita can be found in sulfidic sediments with sulfide concentrations of 100 to 800 |imol L-1 (Schulz et al., 1999) or even up to 22 mmol L-1 (Bruchert et al., 2003). Moreover, these bacteria can also tolerate atmospheric oxygen levels while being exposed to

lower sulfide concentrations (0 to 100 |imol L-1) at a pH of 7.3 (Schulz et al., 1999; Schulz and de Beer, 2002). However, Thiomargarita spp. cells are not as motile as Beggiatoa spp. and as a consequence they can not position themselves in the transition zone of oxygen and sulfide. Instead, the cells wait for resuspension of the sediment to get into contact with oxygenated, nitrate-containing seawater (Schulz et al., 1999).

In contrast, Beggiatoa filaments are motile and built up mats between the opposing gradients of oxygen and sulfide (Winogradsky, 1887; Keil, 1912; J0rgensen, 1977; Nelson et al., 1982; Nelson and Jannasch, 1983). Consequently, Beggiatoa filaments usually get into contact with oxygen and sulfide at the same time, since they consume the two gases, they lower the concentrations and steepen the gradients (Nelson et al., 1986a). However, depending on the thickness of the Beggiatoa mat, some filaments might not be in direct contact with oxygen and sulfide at the same time (Nelson et al., 1986b). Taken this into account, the results presented in this thesis (Chapter 2.1) suggest that sulfide is probably more harmful to the cells under oxic conditions because the aerobic sulfide oxidation leads to a

decrease in pH (Winogradsky, 1887) and as a consequence more sulfide outside the cells is present in form of H2S. In the presented experiments (Chapter 2.1) we measured a pH of 6.5 within the mat, leading to about 70% of the sulfide being present in form of H2S (compared to only about 30% present as H2S at pH 7.3). This uncharged, harmful gas can easily diffuse into the cells where it can act as a strong reductant and binds to iron in cytochromes, by this blocking the cellular respiration. Thus, the bacteria probably have to perform sulfide oxidation to detoxify the inside of the cells. At high sulfide fluxes the cells obviously accumulate large amounts of storage compounds during this process and might eventually burst. To prevent this, the bacteria have to dispose of the internal storage compounds. In the anoxic regions the pH is higher and sulfide predominantly occurs in form of HS- which can not diffuse into the cells and might only get inside via ion channels or transport systems. In this thesis (Chapter 2.1), a new strategy to cope with high sulfide fluxes is proposed. The bacteria actively migrate into anoxic regions with high sulfide concentrations. Here, the aerobic	[redacted]	

sulfide oxidation is stopped and with this also sulfur deposition. The bacteria can then reduce the intracellular sulfur with internal PHA to form sulfide. This strategy represents a novel explanation for the presence of Beggiatoa filaments in anaerobic habitats and shows that filaments actively migrate into anoxic, sulfidic regions. Previously, downwards migrations were typically observed in habitats containing nitrate and there the anaerobic sulfide oxidation with nitrate lowered the diffusion zone of sulfide and separated the oxygen and sulfide gradients over up to a few centimeters (MuBmann et al., 2003; Sayama et al., 2005; Hinck et al., 2007; Preisler et al., 2007).

Although Beggiatoa spp. also require oxygen for growth (unless an alternative electron acceptor is present) they show phobic reactions to higher (above 5% air saturation) oxygen concentrations (Winogradsky, 1887; M0ller et al., 1985). During aerobic sulfide oxidation the production of oxygen radicals and, moreover, chemiluminescence was shown to occur (Tapley et al., 1999). As mentioned above, we propose that the Beggiatoa sp. 35Flor requires the accompanying

organism to protect themselves against reactive oxygen species (Chapter 3.1). However, no correlation between the catalase activity of heterotrophic, associated cells and the beneficial effect of their presence on cyanobacteria (Prochlorococcus sp.) was found (Morris et al., 2008). In fact, the heterotroph with the lowest catalase activity was the only strain able to support growth of all studied cyanobacteria. Consequently, the dependency of the large bacteria on small heterotrophic bacteria can not be completely explained by their possession of a catalase enzyme. During our studies on the co-culture of Beggiatoa sp. and Pseudovibrio sp., the possibility that nitric oxide (NO) might be involved in the reaction counteracting oxidative stress arose. In oxygen-sulfide gradient co-cultures with high sulfide concentrations, the NO signal in Pseudovibrio cells, stained with a copper-based fluorescent probe (CuFL, Lim et al., 2006), was higher compared to cells exposed to a low sulfide flux (Figure 5.1). This increase in NO signal was also inducible when hydrogen peroxide (H2O2) was added to co-cultures with a low sulfide flux or to pure cultures of the isolated Pseudovibrio sp. (growing in seawater medium

containing glucose and ammonia). Under both conditions, the enhanced signal was visible in the interior of the cells and not in the surrounding medium. The used dye is specific for NO and no interaction with H2O2 or other reactive oxygen and nitrogen species, such as HNO, NO2-, NO3- and ONOO-, could be detected (Lim et al., 2006).

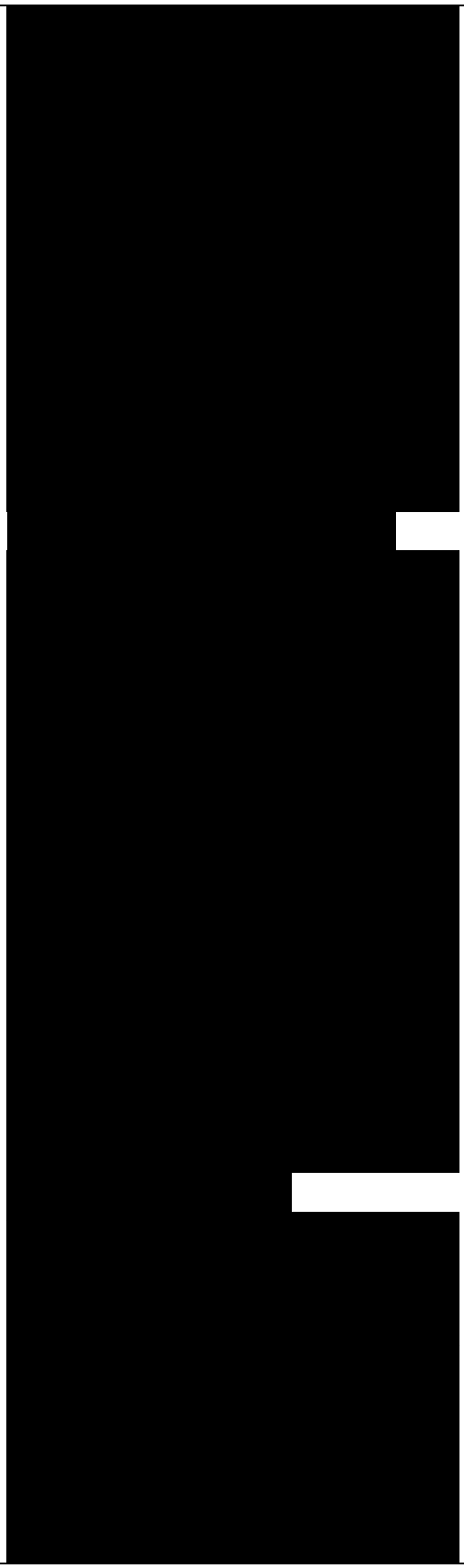


Figure 5.1: Nitric oxide staining (copper-based fluorescent probe) of Pseudovibrio sp. FO-BEG1 cells in coculture with Beggiatoa sp. 35Flor at high and low sulfide flux (12.6 and 50.4 mmol m 2 d 1, respectively) and in Pseudovibrio sp. FO-BEG1 pure cultures. White bars show measurements without the addition of hydrogen per-oxide and grey bars show those with 0.35% H2O2 added. (preliminary data, imaging performed together with M. Beutler)

NO is known to protect against oxidative stress in other bacteria. In Bacillus subtilis, the addition of NO has been shown to lead to an increase in resistance against H2O2 by 100fold (Gusarov and Nudler, 2005). The enzymes of the group nitric oxide synthases (NOS) can produce NO and

citrulline from L-arginine and oxygen and are typically known from eukaryotes (Alderton et al., 2001). However, recently genes encoding for NOS-like enzymes were found also in different gram-positive and some gram-negative bacteria, and also the NOS-dependent NO production could be shown (Adak et al., 2002a; Adak et al., 2002b; Gusarov and Nudler, 2005; Agapie et al., 2009; Schreiber et al., 2011).

Interestingly, we found NOS-related domains (Filippovich, 2010) in the genome of the investigated Pseudovibrio sp. FO-BEG1 (Schwedt et al., unpublished data). A small domain of yet unknown function is followed by an amine oxidase domain (known to oxidize L-amino acids) and a flavodoxin/nitric oxide synthase domain in the genome sequence. This opens up the possibility that NO production might be involved in the reaction to ROS in the investigated Pseudovibrio sp. strain FO-BEG1. The production of NO as a protective measure against oxidative stress in Bacillus subtilis cells leads to an enhanced catalase activity, which was shown by comparison of wild type to Anos deletion mutants (Gusarov and Nudler,

2005).

In that study, the bacteria were cultivated in a complex medium with yeast extract containing free amino acids. In the pure culture experiments presented here, the medium contained ammonia, as the sole nitrogen source, which could have potentially been used to produce NO.
In our co-culture experiments together with Beggiatoa sp., no fixed nitrogen source was present, but the investigated Beggiatoa sp. are able to fix N2 (Henze, 2005). The genome of the accompanying organism does not contain any gene encoding for a nitrogenase enzyme (Schwedt et al, unpublished data). The transfer of fixed nitrogen sources from cyanobacteria to associated bacteria is a common observation (Paerl, 1984). It may well be that also the Beggiatoa sp. transfer fixed nitrogen to the accompanying bacteria. Nevertheless, taking into consideration that dyes can unspecifically bind to different compounds and that the production of NO in our study was not proven by direct measurements, these considerations remain speculative and await further investigation (see outlook).
Apart from chemical substances,

such as oxygen and sulfide, also light is known to be a potential cause of phobic migration reactions of Beggiatoa filaments (Winogradsky, 1887; Nelson and Castenholz, 1982; M0ller et al., 1985). The Beggiatoa sp. that we investigated showed an unusual reaction to blue/green light. Application of a blue or green light source (intensity: 67 |iE m-2 s-1) above or below the culture tube induced an immediate downwards movement of a subpopulation of filaments into the anoxic part of the culture tube when cultivated under a high sulfide flux (Chapter 2.2). The downward movement also occurred in the dark, but solely in cultures where a mat at the oxic-anoxic transition had already been established for about 10 days. The lower subpopulation was established 2 to 3 days after the downwards migration started (Chapter 2.1). Applying a blue or green light source in close proximity to the culture tube induced and also enhanced this filament movement and within a few hours the lower subpopulation was observed (Chapter 2.2). Because chemiluminescence occurs during chemical sulfide oxidation (Tapley et al., 1999) at the transition between oxygen and sulfide, it can be speculated that light at low intensities might

be involved in the migration and/or mat formation processes of Beggiatoa filaments, but this remains to be studied in the future (see outlook). The lower limits of bacterial growth In contrast to the large Beggiatoa filaments, most bacterial cells are not visible with the naked eye. However, bacterial growth can lead to macroscopically visible colonies on nutrient-rich agar plates or cause turbidity in nutrient-rich liquid media due to high cell densities, whereas under nutrient limitation cell densities will be low and the small bacterial cells might not cause turbidity. Therefore, the build-up of bacterial biomass in liquid low-nutrient media has to be quantified in a different way than optical turbidity measurements. Although, there might be no measurable growth, the cells might still be metabolically active. This is due to the fact that there is a difference between biomass production and activity of bacterial cells. Although the consumption of substrate is essential for the cells to grow and build-up biomass, the opposite is not the case, as cells do not necessarily grow while	[redacted]	

utilizing substrate (del Giorgio and Cole, 1998). Therefore, the formation of biomass, called bacterial production (BP) has to be separated from the consumption of substrate, called bacterial respiration (BR). The bacterial growth efficiency (BGE) is defined as the quantity of bacterial biomass resulting from a certain amount of substrate respired (del Giorgio and Cole, 1998). The substrate is used by the bacteria for catabolic and anabolic purposes generating ATP (Adenosine-5'- triposphate) and cell biomass, respectively. The BGE can be determined by isotope-labeling techniques (BR and BP), rates of protein or DNA synthesis (BP), cell counts (BP), oxygen consumption (BR), Cacbon điôxít production (BR) and changes in DOC and POC (BR and BP) (del Giorgio and Cole, 1998 and references therein). Under oligotrophic conditions, the concentrations of substrates and cell numbers are extremely low. Therefore, most of these methods may not be sensitive enough and measured values are close to or below the limit of detection. Thus, new routines and methods have to be developed or methods have to be	[redacted]	

refined to be suitable for studying bacterial growth under extreme nutrient deficiency. Classical dissolved organic carbon (DOC) measurements, which are used as a parameter for the amount of dissolved organic matter (DOM) in environments with a low amount of nutrients, have a detection limit of about 0.5 |imol C L-1 (0.006 mg C L-1). However, the observed cell numbers in our medium (Chapter 4) are so extremely low that about 1 |imol L-1 of carbon would be enough to explain the observed growth. Consequently, the classical method might not provide sufficient sensitivity to detect potential small changes in DOC. The results presented in this thesis show that electospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS) provides sufficient resolution and can be used to study bacterial growth under oligotrophic conditions. Furthermore, this technique gives information on both potential fixed carbon and nitrogen sources of the bacteria. Combined with the measurement of amino acids by high performance liquid chromatography (HPLC) and isotope-labeling techniques,	quang phổ khối cộng hưởng cyclotron ion sử dụng phép biến đổi Fourier	

bacterial growth in oligotrophic seawater can be studied in detail. However, this technique does generally not give quantitative information and as a consequence respiration rates under oligotrophic conditions still remain unknown.

The risk of impurities

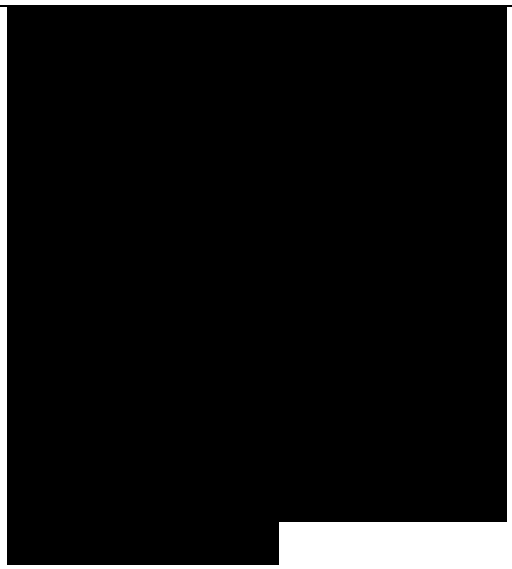
Even though no electron donor is added to an oligotrophic medium, there can still be impurities present that might support growth and lead to the formation of biomass and/or metabolic activity of the cells (Chapter 4). Studying bacterial growth under nutrient-poor conditions is challenging, since many different contaminations, such as macronutrients, trace elements or energy sources can occur.

Accordingly, when studying the physiology of bacteria growing under oligotrophic conditions, it is crucial to keep the medium and equipment contamination-free for the mandatory substances for growth. Amino acids represent a serious contamination risk for seawater samples. Therefore, there are a number of precautions that have to be taken, such as working with gloves, combustion of glassware, avoidance of dust in the working room (working in

laminar flow hood) and use of chemicals of highest available quality (Dittmar et al., 2009). Also, during the measurements of quantity and composition of DOM typically all used equipment is pre-combusted if possible. All these precautions were applied during the preparation of the oligotrophic seawater medium and all further analyses presented in Chapter 4.1 of this thesis.

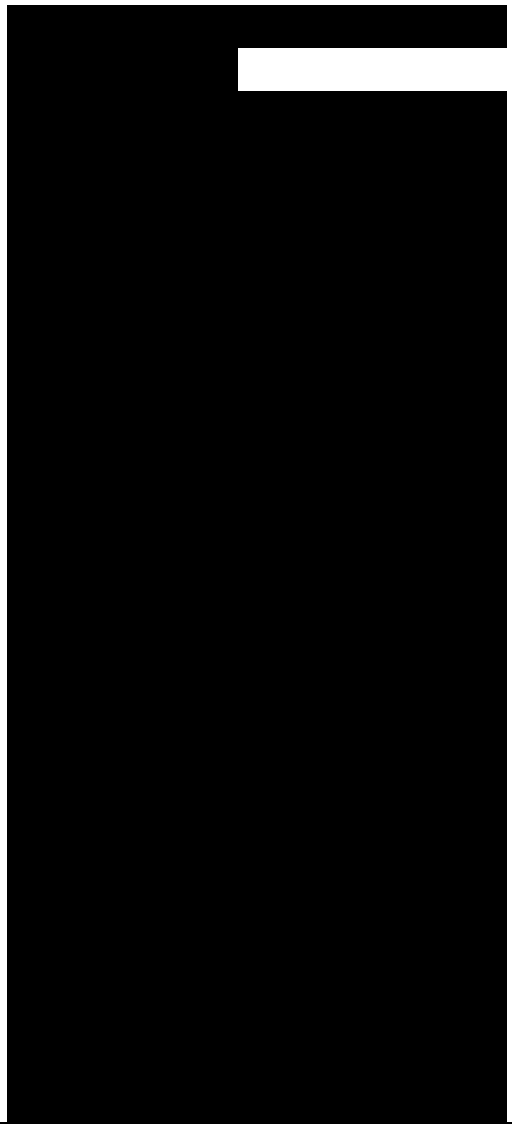
Furthermore, the medium might become contaminated with trace elements, which would probably not resemble a potential energy source but could be decisive for growth or no growth of microorganisms. To avoid the contamination with trace elements, equipment is usually washed with HNO3- (e.g. Fitzwater et al., 1982), which, however, represents an easily utilizable nitrogen source. For the experiments presented in this thesis (Chapter 4), a fixed nitrogen source was considered as worse contamination than trace elements, and the equipment was not washed with HNO3-.
In spite of all precautions, it is still extremely difficult to prepare a seawater medium without any contaminations.

However, if the contaminating substance is not used by the bacteria during growth, such as the PEG contamination in this study (Chapter 4.1), it might not disturb or influence further physiological analysis. The contamination can even be used to normalize other peaks in the mass spectra, if its peaks are high and consistent (as the PEG peaks in this study, Chapter 4.1).



Facultative oligotrophy

In addition to growth under oligotrophic conditions, the investigated Pseudovibrio sp. FO- BEG1 (Chapter 4) is capable of growing under eutrophic conditions as well and can switch within days from one trophic state to the other.



Under oligotrophic conditions, the bacterial growth efficiency is typically very low and varies with the supply of nutrients (del Giorgio and Cole, 1998). Furthermore, the investigated bacteria are limited in carbon, nitrogen and energy at the same time (Chapter 4.1). Consequently, they are supposed to have high maintenance energy costs to maintain crucial transport systems and enzymes prepared (del Giorgio and Cole, 1998). With increasing nutrient

supply, probably also the bacterial growth efficiency increases because the bacteria can exhibit higher growth rates and produce only enzymes necessary for the substrates available (reviewed in del Giorgio and Cole, 1998). Facultatively oligotrophic bacteria have to switch between oligotrophic and eutrophic growth conditions and as a consequence the cells have to switch also between both strategies for growth to adapt to the actual amount of substrates present.

The variation in nutrient availability is common in natural environments such as the open ocean. Bacteria attached to marine snow particles can exhibit very high growth rates (Alldredge et al., 1986; Alldredge and Gotschalk, 1990; Smith et al., 1992; Azam and Long, 2001; Ki0rboe and Jackson, 2001), while free-living bacteria in the open ocean are restricted in nutrients and consequently their growth rates are low or they even starve (Boylen and Ensign, 1970; Novitsky and Morita, 1976; Azam and Hodson, 1977; Novitsky and Morita, 1977; Tabor and Neihof, 1982; Ishida et al., 1989).

Facultatively oligotrophic bacteria can adapt fast to nutrient

affluence or deficiency, while they have to up- or down-regulate internal pathways of anabolism and catabolism. The investigated facultatively oligotrophic Pseudovibrio sp. FO-BEG1 is highly versatile with respect to its energy gain (Chapter 3.2 and Chapter 4.1). This feature makes the bacteria flexible and they are, moreover, capable to interact with other prokaryotes or possibly even eukaryotes (Chapter 3.2).

Already 30 years ago, it was suspected that two types of oligotrophic bacteria exist (Ishida and Kadota, 1981): 1. organisms, which disappear with increasing man-made eutrophication and 2. organisms, which can adapt fast to man-made eutrophication. In this thesis bacteria were not isolated from the oligotrophic open ocean, but from water directly overlaying marine sediments (Chapter 4.2). All of the isolated strains were able to adapt fast to the nutrient deficiency of the initial isolation medium. The observation that facultatively oligotrophic bacteria can also be isolated from non-oligotrophic water indicates that these bacteria might be more widespread and not limited to nutrient-poor environments. For

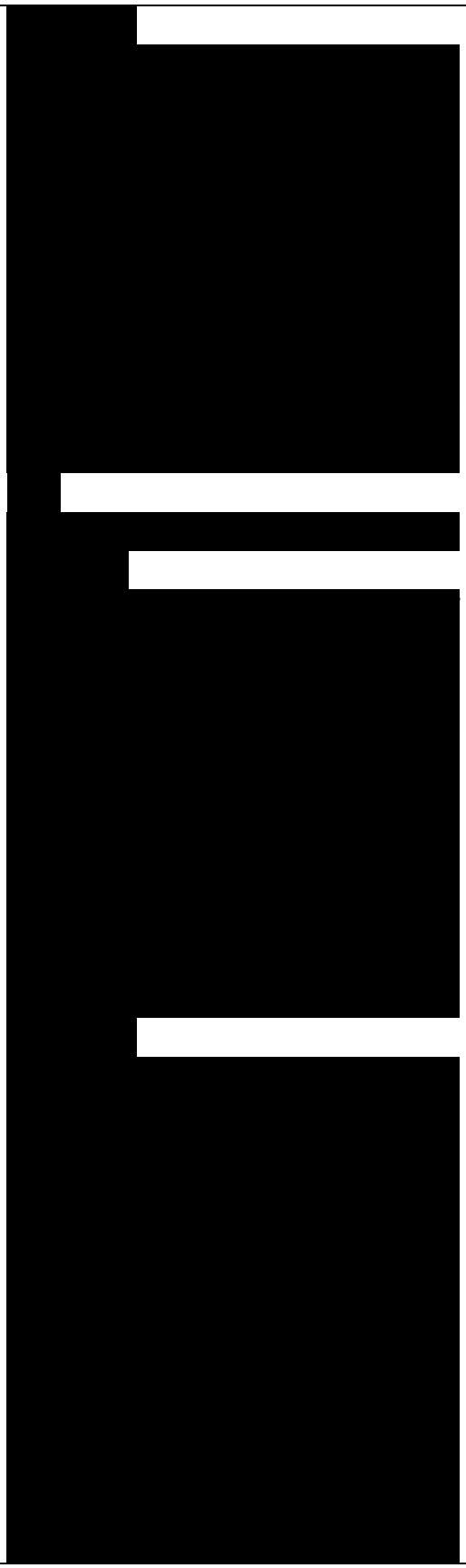
example, they might live attached to marine snow particles (e. g. Alldredge et al., 1986; Smith et al., 1992; Azam and Malfatti, 2007 and references therein), which represent nutrient hotspots for heterotrophic bacteria, and possibly the bacteria sink down to the sediment with these particles. We assume that many facultatively oligotrophic bacteria have been overlooked so far because they were not searched for in non-oligotrophic environments. Likewise, many more already known heterotrophic bacteria may be capable of growing under much poorer nutrient conditions than currently assumed.

Conclusions

In conclusion, the results presented in this PhD thesis (summarized in Figure 5.2) show that filaments of Beggiatoa sp. strain 35Flor react to high sulfide fluxes by migration into anoxic regions, where they reduce the amount of internal storage compounds. This migration can be enhanced or induced by blue/green light for a yet unknown reason. We suggest that the accompanying Pseudovibrio sp. strain FO-BEG1 can detoxify reactive oxygen species (ROS), generally produced during sulfide

oxidation (Tapley et al., 1999), and might be responsible for the protection of Beggiatoa filaments, which are known to lack the gene for catalase (Larkin and Strohl, 1983). Possibly, production of nitric oxide (NO) by the Pseudovibrio sp. might also be involved in the protection against ROS. However, the origin of NO is unknown because no fixed nitrogen source was present in the medium of the co-culture and the heterotroph does not posses a gene encoding for nitrogenase. On the other hand, the Beggiatoa sp. can fix N2 (Henze, 2005) and it might be that a fixed nitrogen source is transferred from the sulfide-oxidizer to the accompanying bacterium. The Pseudovibrio sp. examined in this thesis is a generalist able to gain energy in many different ways and can also grow under extremely nutrient-poor conditions. Furthermore, it possesses genes to interact with pro- and eukaryotes. We found that also other bacteria from the habitat of large sulfide- oxidizers can adapt fast from nutrient affluence to deficiency. Maybe these bacteria are able to also support growth of the large sulfide-oxidizers, but this needs to be investigated in the future.

Figure 5.2: Illustration of the results obtained in this PhD thesis concerning the migration behavior of Beggiatoa sp. and the co-occuring Pseudovibrio strain. Some results were supported by our data (black) while others are hypothesis and remain to be proven (blue). ROS = reactive oxygen species, NO = nitric oxide, N = nitrogen



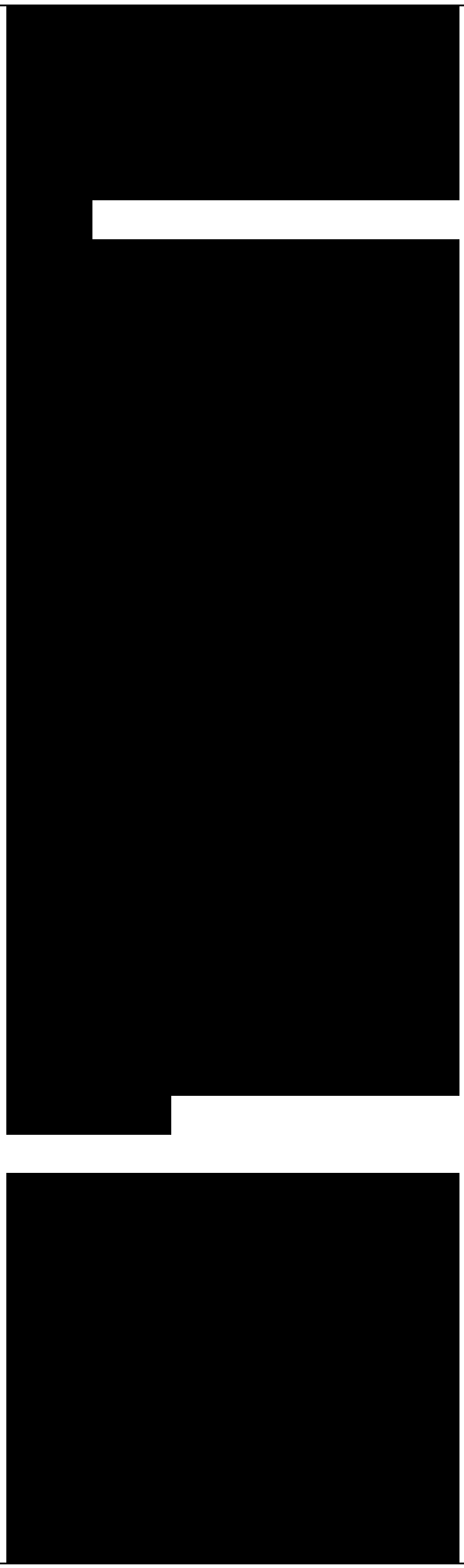
Outlook

The results of my PhD thesis provide new insights into the migration behavior of large sulfur bacteria, associations between bacteria, and growth under extreme nutrient deficiency. Nevertheless, the obtained results also raise new questions that need to be addressed in the future.

We hypothesize that the Beggiatoa sp. filaments depend on the heterotrophic Pseudovibrio sp. because they do not themselves possess protective measure against oxidative stress (Chapter 3). However, direct evidence for this theory is still missing. To investigate the capability of other heterotrophic, catalase-positive bacteria or ROS scavenger to support growth of

the Beggiatoa sp., filaments from the lower subpopulation should be transferred without (or with only few) accompanying bacteria into fresh media. If growth is positive in the presence of the other catalase-positive bacteria or ROS scavengers, the transfer of Pseudovibrio sp. from the inoculation source has to be excluded, which can be tested by Fluorescence in situ hybridization.

The preliminary data on the staining of nitric oxide opens up the possibility that NO might be involved in the reaction with ROS (Chapter 5). However, to proof this, the production of NO has to be verified by direct measurement of NO with a NOx-analyzer or detection of NO by its molecular mass using mass spectrometry. The chemical reaction of NO with oxygen in water forming mainly nitrite (Ignarro et al., 1993) might complicate the direct measurement of nitric oxide. The sole nitrogen source in the co-culture is N2. Therefore, it can be speculated that the interaction between the two bacteria is not only one-sided because of the two partners only the Beggiatoa sp. are known to fix N2 (Chapter 5). To investigate if a direct transfer of fixed nitrogen in the co-culture occurs, labeled nitrogen (15N2) can be added

and the label can be searched for in the Pseudovibrio sp. using mass spectrometry (e.g. nano secondary ion mass spectrometry). We have shown that the Pseudovibrio sp. FO-BEG1 growing in pure culture under nutrient- poor conditions can also use typical substrates known from heterotrophic growth (Chapter 4.1). It still remains unclear if the observed growth is the same as typical heterotrophic growth only with less nutrients or if special regulating enzymes are needed. A proteomic comparison between bacteria grown under nutrient deficiency and grown under nutrient affluence might provide more information on the growth and the regulation of enzymes under oligotrophic conditions. The detailed analysis of substrates used during oligotrophic growth shows that the bacteria use multiple substrates at the same time under oligotrophic conditions (Chapter 4.1). However, only amino acids (which account only for a small fraction) were measured quantitatively and the overall		

respiration rate remains unknown. Therefore, the measurement of oxygen consumption (e.g. using microelectrode respiration chambers) under nutrient-poor conditions could allow quantification and calculation of the respiration rate under oligotrophic conditions. Furthermore, the fact that we have both metabolites (detected by ESI FT-ICR-MS) and genes (closed genome of Pseudovibrio sp. strain FO-BEG1) may allow us to connect metabolites with genes present in the investigated organism in the future. Maybe, this will also help predicting new metabolic pathways. Eventually, several heterotrophic strains, which were able to grow under both nutrient deficiency and affluence, were isolated during our studies (Chapter 4.2). The possibility of heterotrophic bacteria to grow under nutrient-deficient conditions might be a more widespread feature. To test this hypothesis, common heterotrophic bacteria from bacterial strain collections can be ordered and their ability to grow under nutrient-poor conditions can be tested. Appendix The genus Pseudovibrio contains	[redacted]	

metabolically versatile and symbiotically interacting bacteria

Abstract

The majority of strains belonging to the genus Pseudovibrio have been isolated from marine invertebrates like tunicates, corals and especially sponges, but the physiology of these bacteria is poorly understood. In this study, we analyze the genomes of two Pseudovibrio strains. One is a required symbiont of a cultivated Beggiatoa strain, a sulfide oxidizing, autotrophic bacterium. The other one was isolated from a sponge (Enticknap et al., 2006). The data show that both strains are generalistic bacteria capable of importing and oxidizing a wide range of organic and inorganic compounds to meet their carbon, nitrogen, phosphorous and energy requirements under oxic and anoxic conditions. Several physiological traits encoded in the genome were verified in laboratory experiments with a pure culture of the Pseudovibrio strain originally associated with Beggiatoa. Besides the versatile metabolic abilities of both Pseudovibrio strains, our study reveals a number of open reading frames and gene clusters in the genomes that seem to be involved in symbiont-host

interactions. Pseudovibrio has the genomic potential to attach to host cells, might be capable of interacting with the eukaryotic cell machinery, produce secondary metabolites and may supply the host with cofactors.

Introduction

The first strain of the genus Pseudovibrio has been isolated from coastal seawater in 2004 and was described as Pseudovibrio denitrificans - a marine, heterotrophic, facultatively anaerobic bacterium capable of denitrification and fermentation (Shieh et al., 2004). Two further type strains, P. ascidiaceicola (Fukunaga et al., 2006) and P. japonicus (Hosoya and Yokota, 2007), were isolated from a tunicate and coastal seawater, respectively. Physiologically, these isolates were not notably different from P. denitrificans. Besides the three type strains, Pseudovibrio spp.-related bacteria have been found in various studies throughout the world either by 16S rRNA gene analysis or direct isolation methods (Hentschel et al., 2001; Webster and Hill, 2001; Olson et al., 2002; Thakur et al., 2003; Thiel and Imhoff, 2003; Thoms et al., 2003; Agogué et al., 2005; Lafi et al., 2005; Enticknap et

al., 2006; Koren and Rosenberg, 2006; Sertan-de Guzman et al., 2007; Muscholl-Silberhorn et al., 2008; Riesenfeld et al., 2008; Kennedy et al., 2009; Rypien et al., 2010; Santos et al., 2010).

Interestingly, besides P. denitrificans, P. japonicus and a Pseudovibrio spp.-related isolate from coastal, oligotrophic seawater (Agogué et al., 2005), all other strains belonging to this genus discovered until today have been found closely associated with marine invertebrates like tunicates, corals, and sponges.

Especially Porifera seem to harbor Pseudovibrio populations, e.g., as the dominating species of the culturable bacterial community (Webster and Hill, 2001; Muscholl-Silberhorn et al., 2008). Additionally, Pseudovibrio has been found in sponge larvae as the most abundant prokaryote, indicating vertical transmission of these bacteria in their hosts (Enticknap et al., 2006). Such a consistent pattern of Pseudovibrio spp. associated with sponges suggests that they are symbionts of those metazoa (Webster and Hill, 2001; Enticknap et al., 2006).

Whether the nature of this symbiosis is mutualistic/ commensalistic or whether Pseudovibrio spp. rather represent pathogens/parasites is uncertain, but the fact that Pseudovibrio spp. have been isolated only from healthy sponges indicates that the bacteria do not harm the host and might be even required for its health (Webster and Hill, 2001). Another shared feature is the production of secondary metabolites by many of the cultured Pseudovibrio strains. For instance, heptylprodigiosin, a compound that exhibits antimicrobial activity, was isolated from a pure culture of P. denitrificans Z143-1 (Sertan-de Guzman et al., 2007) and the production of additional bioactive compounds could be shown in several other studies (Hentschel et al., 2001; Muscholl-Silberhorn et al., 2008; Kennedy et al., 2009; Santos et al., 2010).

Despite the fact that members of the genus Pseudovibrio seem to be ubiquitous and important associates of marine invertebrates and are also found free-living, very little is known about the physiology and interactions with the host. In this study, we analyze the genomes of two Pseudovibrio strains. Pseudovibrio sp. FO-BEG1 has

been isolated from an enrichment culture of a Beggiatoa strain, a filamentous, sulfide oxidizing bacterium (Brock and Schulz- Vogt, 2011; Chapters 2 and 3). Initially, this Beggiatoa strain was sampled from a coral suffering from the black band disease off the coast of Florida, which indicates that the strain Pseudovibrio FO-BEG1 could have been associated with the coral at the time of sampling - either in a commensalistic/mutualistic or pathogenic relationship - and is now available as an axenic culture in our lab. Intriguingly, strain FO-BEG1 is also maintained in a co-culture with a Beggiatoa sp., which seems to be unable to grow without Pseudovibrio and is therefore dependent on strain FO-BEG1. The second strain, Pseudovibrio sp. JE062, has been isolated in Florida from the sponge Mycale laxissima in the year 2006 and was described as a sponge symbiont by Enticknap et al. (2006). The analysis of these genomes gives us an insight into the physiological and symbiotic potential of both Pseudovibrio strains and reveals fascinating microorganisms that seem to be adapted to free-living and symbiotic life styles.

Materials and Methods Growth

conditions For aerobic growth CM medium modified after Shieh et al. (2004) was used. After autoclaving, the medium was supplemented with K_2HPO_4 (1.15 mmol L-1), glucose (10 mmol L-1 unless stated otherwise), 1 mL L-1 tungsten/selenium solution (Brysch et al., 1987), 1 mL L-1 trace elements (Widdel and Pfennig, 1984), and 1 mL L-1 of four vitamin solutions prepared according to Aeckersberg et al. (1991). For measurement of SO_4^{2-} evolution during $S_2O_3^{2-}$ oxidation, 10 mmol L-1 $Na_2S_2O_3 \cdot 5 H_2O$ and 5 mmol L-1 glucose were added and 2 g L-1 K_2SO_4 from the original recipe was replaced with 0.75 g L-1 KCl. To compare growth between a culture with and without $Na_2S_2O_3$, K_2SO_4 was not omitted from the medium and glucose and $Na_2S_2O_3 \cdot 5 H_2O$ were used in the same concentrations as described above. To investigate the growth with 4-hydroxybenzoic acid and benzoate, both compounds were added in a concentration of 2 mmol L-1, respectively, without any other carbon source. Growth with phosphonoacetate (1 mmol L-1) as phosphorus source was	[redacted]	

tested by adding this compound as the only phosphorus source and all vitamins were omitted from the medium. For fermentation and denitrification experiments under anoxic conditions, aged North Sea water was buffered with 50 mmol L-1 TRIS, supplemented with NH4Cl (10 mmol L-1) and the pH adjusted to 8. Preparation of the medium was performed according to Widdel and Bak (1992) in order to prepare the medium anoxically. Cooling was performed under N2 atmosphere, except for experiments in which N2 production was measured, in which Ar was used as the atmosphere instead. After autoclavation, the medium was supplemented with 10 mmol L-1 glucose, 1 mL L-1 tungsten/selenium solution, 1 mL L-1 trace elements and 1 mL L-1 of four vitamin solutions prepared as described above. NaNO3 (10 mmol L-1) was added for experiments investigating denitrification. To test CO oxidation, CM medium was prepared as described above, containing 400 |imol L-1 glucose and supplied with 500 p.p.m. CO to the bottle headspace. For aerobic growth experiments, 250 mL Erlenmeyer flasks were filled with 100 mL medium. For anaerobic growth, 156 mL serum bottles (Wheaton, Millville,	[redacted]	

USA) were filled anoxically with 50 mL medium and closed with butyl rubber stoppers. For all experiments, 0.1% or 0.5% of a preculture grown aerobically in CM medium was used as inoculum. All growth experiments were performed with Pseudovibrio sp. FO-BEG1 in triplicates at 28°C in the dark with shaking at 110 rpm.

Chemical analyses

Bacterial growth was monitored as the optical density (OD600 nm) using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). SO42- was measured with a Metrohm 761 Compact IC with conductivity detector (Metrohm AG, Herisau, Switzerland) equipped with a Metrosep A Supp 5-100 column with a carbonate eluent (3.2 mmol L-1 Na2CO3/1 mmol L-1 NaHCO3 in deionised water) at a flow rate of 0.7 mL min-1. Tetrathionate was measured according to Kamyshny (2009). Glucose and organic acids were determined using a HPLC system (Sykam GmbH) equipped with an anion neutral pre-column (4x20 mm; Sykam GmbH) and an Aminex HPX-87H separation column (300x7.8 mm; Biorad, Munich, Germany) at a temperature of 60 °C. The eluent consisted of 5 mM H2SO4 in

HPLC-grade water with a flow rate of 0.6 mL min-1. Quantification of glucose, succinate, lactate, formate, acetate, propionate and ethanol was performed with the 7515A RI detector (ERC, Riemerling, Germany); pyruvate was measured with the Sapphire UV-Vis detector at 210 nm (Ecom, Praha, Czech Republic). NO3" was quantified with a HPLC system (Sykam GmbH, Eresing, Germany) containing an anion neutral pre-column (4x20 mm; Sykam GmbH) and an IBJ A3 anion separation column (4x60 mm; Sykam GmbH) with a column temperature of 50 °C. The eluent consisted of 25 mmol L-1 NaCl and 45% ethanol in deionised water with a flow rate of 1 mL min-1. Detection of NO3- was conducted with Linear Uvis 200 (Thermo Fischer Scientific GmbH, Dreieich, Germany) at 220 nm. N2 was measured as described by Zedelius et al. (2011). CO determination was conducted with a Shimadzu GC-8A (Shimadzu, Duisburg, Germany) gas chromatograph with a Molecular Sieve 5A column (80 to 100; 0.125 in. by 2 m; Restek, Bellefonte, USA) at a flow of 20 mL of synthetic air per minute at 40°C and an RGD2 reduction gas detector (Trace Analytical, Menlo Park, USA).	[redacted]	

DNA extraction and sequencing

DNA was extracted from strain FO-BEG1 using the Fast DNA SPIN Kit for Soil (MP Biomedicals LLC, Illkirch, France), according to manufacturers' instructions. 454 sequencing was conducted by LGC Genomics GmbH with a 454 GS FLX System. The Newbler 2.0.00.22 software was used for sequence assembly and quality assessment. Overall, 522919 sequenced reads with an average length of 336.30 bp lead to a 29-fold sequence coverage. In order to close the gaps, a fosmid library with a 1.5-fold physical coverage was created and used for direct sequencing of the fosmid clones. For the residual gaps, 96 specific primers were designed and used for combinatorial PCR on DNA level, the products of which were sequenced via the Sanger method.

Gene prediction, annotation and data mining

Gene prediction was carried out by using the software Glimmer3 (Delcher et al., 2007). Ribosomal RNA genes were detected by using the RNAmmer 1.2 software (Lagesen et al., 2007) and transfer RNAs by tRNAscan-SE (Lowe and Eddy, 1997). Annotation was

performed by using the GenDB, version 2.2 system (Meyer et al., 2003), supplemented by the tool JCoast, version 1.6 (Richter et al., 2008). For each predicted open reading frame (ORF) observations have been collected from similarity searches against sequence databases NCBI- nr, Swiss-Prot, KEGG and genomesDB (Richter et al., 2008) and for protein family databases from Pfam (Bateman et al., 2004) and InterPro (Mulder et al., 2005).

SignalP has been used for signal peptide predictions (Bendtsen et al., 2004) and TMHMM for transmembrane helix- analysis (Krogh et al., 2001). Predicted protein coding sequences were automatically annotated by the in-house software MicHanThi (Quast, 2006). The MicHanThi software predicts gene functions based on similarity searches using the NCBI-nr (including Swiss-Prot) and InterPro database. The annotation of proteins highlighted within the scope of this study was subject of manual inspection. For all observations regarding putative protein functions, an e-value cutoff of 10-4 was considered.

Comparison of the shared gene

content by reciprocal best matches (RBMs) and functional classification with Kyoto encyclopedia of genes and genomes (KEGG)

Determination of the shared gene content has been performed by a BLAST all versus all search between FO-BEG1 and JE062. Reciprocal best matches were counted by a BLAST result with an E value <1e-5 each and a subject coverage of over 65%. For metabolic pathway identification, genes were searched for similarity against the KEGG database. A match was counted if the similarity search resulted in an expectation E value below 1e-5. All occurring KO (KEGG Orthology) numbers were mapped against KEGG pathway functional hierarchies and statistical analyzed.

Functional classification with cluster of orthologous groups (COG) and calculation of the Average nucleotide identity (ANI)

All predicted ORFs were also searched for similarity against the COG database (Tatusov et al., 2003). A match was counted if the similarity search resulted in an E value below 1e-5. ANI between the whole-genome sequences of strain FO-BEG1 and the draft genome sequences of strain JE062 was determined

by using the in silico DNA-DNA hybridization method of the JSpecies (Richter and Rosselló-Móra, 2009) software with default parameters.

Creation of circular genome maps and prediction of ABC and TRAP type transporters

Comparative circular genome maps of the RBMs shared between JE062 and FO-BEG1 have been drawn by using JCoast's plugin for CGView (Stothard and Wishart, 2005). Circular GC- plot and GC-skew representation has been drawn by using DNAPlotter (Carver et al., 2009). As initial step for the identification of the ABC transporters, genes containing the Pfam domain ABC_tran (PF00005) were detected in the genome of strain FO-BEG1. For the identification of the permease and the periplasmic binding protein, the close proximity of genes containing the ABC_tran domain was searched. Only ABC systems with at least one ABC_tran domain, one permease and one periplasmic binding protein were regarded as functioning ABC transporters and substrate specificity was predicted from the annotations of the subunits. In several cases, one subunit (e.g. the permease) was missing in close proximity of genes with

the ABC_tran domain. In this case, a single permease gene located on any place in the genome with the same substrate specificity prediction but not belonging to any complete ABC system, was used to complement the transporter system. TRAP transporters were regarded as complete when the subunits DctM, DctQ and DctP were present in close proximity. When two subunits were identified in close proximity and the third was missing, the single subunit located on any place in the genome not belonging to any complete TRAP system was used to complement the transporter system. In the case of fusion of the DctQ and M subunits in one gene, only the DctP subunit was required to complete the transporter.

Accession numbers

The genome shotgun project of strain FO-BEG1 has been deposited at DDBJ/EMBL/GenBank under the accession number CP003147 for the chromosome and CP003148 for the plasmid. The draft genome sequence of strain JE062 has the DDBJ/EMBL/GenBank accession number ABXL00000000.

Results and Discussion General genome characteristics

The genome size of strain FO-

BEG1 is 5.9 Mbp, including a large plasmid of 0.4 Mbp (Figure S.1). The circular chromosome of 5.5 Mbp contains a large stretch of repeats at position 2,707,040. This area of unknown size could not be bridged with a direct sequencing approach despite the presence of this area on a fosmid, indicating strong secondary structures, and has been masked with the ambiguous nucleotide code 'N'. The G+C content is 52.5 mol% and is consistent with the known values of the described Pseudovibrio isolates (Shieh et al., 2004; Fukunaga et al., 2006; Hosoya and Yokota, 2007). Altogether, we found 5,478 ORFs, 398 of which were located on the plasmid, which correspond to about 87% of encoding DNA. Six complete rRNA operons and 69 tRNA encoding regions were annotated, indicating the capability of a quick response to changing conditions and fast growth when nutrients are available. The genome of strain JE062 has not been closed, but there are 19 contigs available with an overall size of 5.7 Mbp, 5,225 ORFs and 52.4 mol% GC content, which is almost identical to the genome of strain FO-BEG1 (Figure S.1 A and B). It contains 72 tRNA genes and seven complete rRNA operons. Unfortunately, the repeat-rich

area that could not be sequenced in the genome of strain FO-BEG1 shows an ambiguous sequence in strain JE062 as well, and could therefore not be used to close the gap in FO-BEG1.

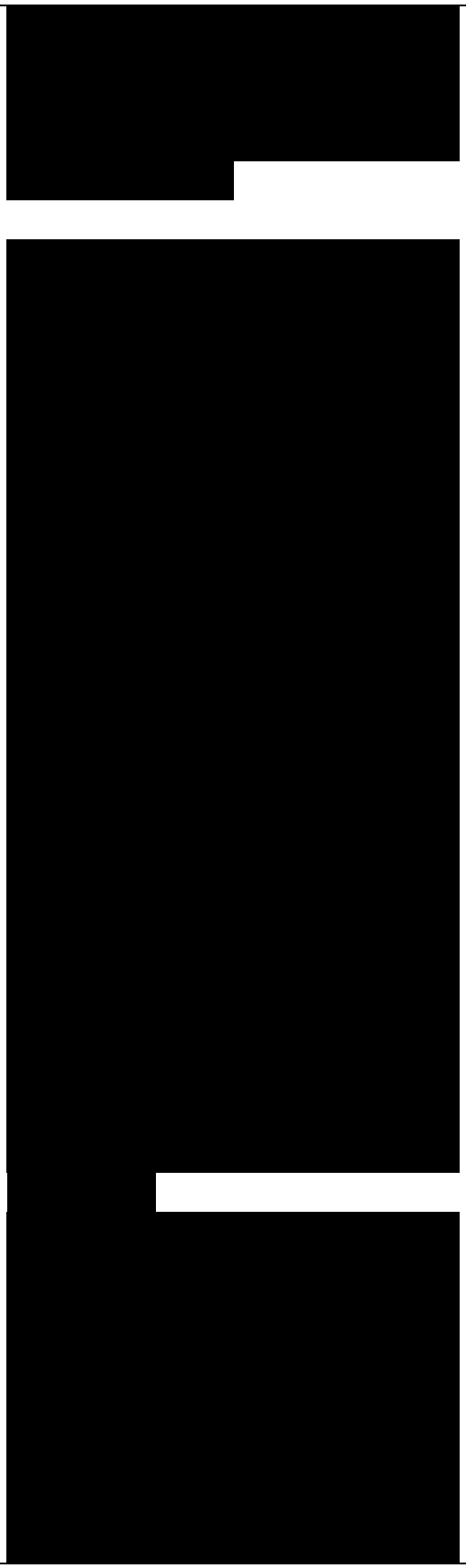


Figure S.1. Comparative circular map of Pseudovibrio sp. FO-BEG1 chromosome (A) and plasmid (B). Most outer lane represents the reciprocal best match (RBM)-shared gene content between FO-BEG1 and JE062. Lane two and three represent all predicted open reading frames (ORFs) on the lagging (red) and leading (green) strands. The two inner lanes display the GC-plot and the GC-skew. The red arrow indicates the area of unknown size that could not be closed during sequencing. The bar chart (C) express the amino acid percentage identity of each RBM shared gene-content between FO-BEG1 and JE062. The blue bar is representing the FO-BEG1 chromosome and orange the corresponding plasmid.

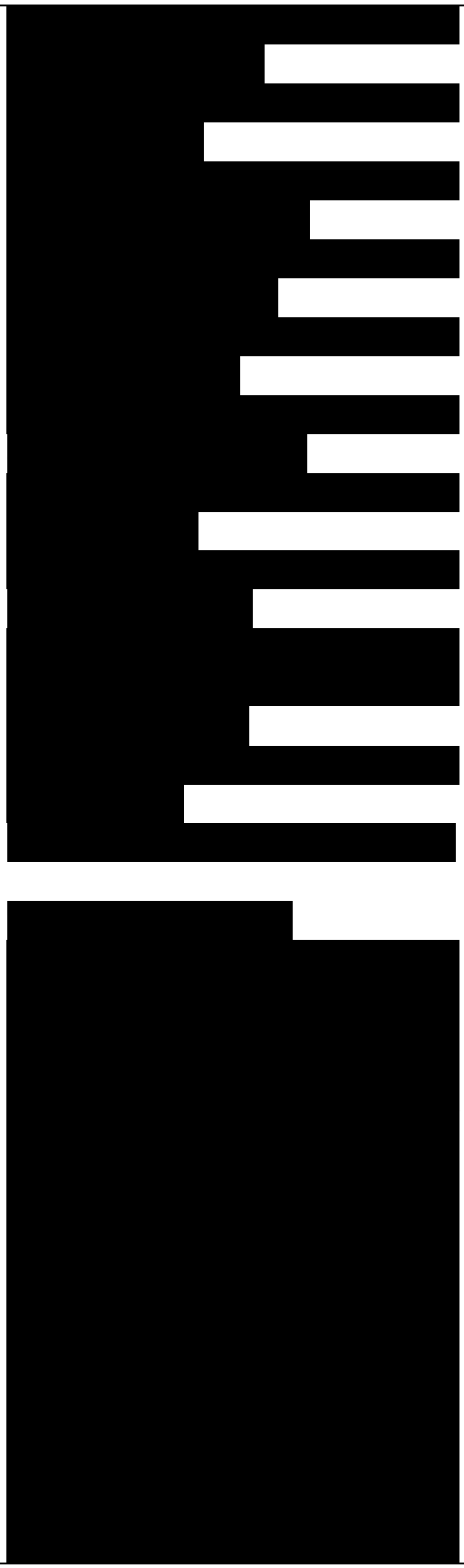
Even though the genome of JE062 is not completely closed we assume that it also contains a plasmid with similar content, since most of the genes identified on the plasmid of FO-BEG1 were allocated in the genome of JE062 (Figure S.1 B). Table S.1 shows an overview of

the genome characteristics of both strains as well as the assignment of the genes to COGs. The shared gene content between FO-BEG1 and the draft genome of JE062 comprises 84.4% (4,287 ORFs, Figure S.1 C). An ANI analysis conducted between strains FO-BEG1 and JE062 revealed a 94.5% ANIb (87% genome alignment) and 95.4% ANIm (86% genome alignment) value. The values are in the range of the proposed species definition boundary (Richter and Rosselló-Móra, 2009) indicating a species level degree of similarity.

Table S.1. General genome features of Pseudovibrio sp. FO-BEG1 and JE062, including categorization of the genes into cluster of orthologous group (COG) categories.

[J] Translation, ribosomal structure and biogenesis	196	190
[K] Transcription	387	367
[L] Replication, recombination and repair	135	125
[D] Cell cycle control, cell division, chromosome partitioning	21	20
[T] Signal transduction mechanism	138	140
[M] Cell wall/membrane/envelope biogenesis	190	176
[N] Cell motility	153	149
[O] Posttranslational		

modification, protein turnover, chaperones 135 127
[C] Energy production and conversion 245 245
[G] Carbohydrate transport and metabolism 323 311
[E] Amino acid transport and metabolism 507 492
[F] Nucleotide transport and metabolism 99 92
[H] Coenzyme transport and metabolism 185 181
[I] Lipid transport and metabolism 148 142
[P] Inorganic ion transport and metabolism 291 287
[Q] Secondary metabolites biosynthesis, transport and catabolism 210 204
[R] General function prediction only 598 576
[S] Function unknown 281 272



Physiology

In both genomes we found a number of genes that indicate high metabolic variety of Pseudovibrio FO-BEG1 and JE062. Degradation of carbohydrates is most likely performed via the Entner-Doudoroff pathway, which is present in both genomes, due to absence of the phosphofructokinase (PFK), a key enzyme of the glycolysis (Emden-Meyerhoff-Parnas), which is a regularly encountered phenomenon within marine a-Proteobacteria (Furch et al.,

2009; Tang et al., 2009; Williams et al., 2009). Besides the PFK, all other enzymes involved in glycolysis can be identified in both genomes, including fructose-1,6-bisphosphatase I, the key enzyme for glyconeogenesis, indicating that the Emden-Meyerhoff-Parnas pathway can be used for anabolic purposes (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Genes encoding all enzymes of the citric acid cycle and pentose phosphate pathway are present. Additionally, both strains have the genetic potential to degrade aromatic compounds via the P-ketoadipate pathway, which we demonstrated by growing Pseudovibrio sp. FO-BEG1 with 4-hydroxybenzoate as the only carbon and energy source under aerobic conditions (Figure S 2.1 A). Benzoate, however, was not degraded, indicating that either the uptake of benzoate is detained or the hydroxylation of the aromatic ring structure cannot be performed by Pseudovibrio FO-BEG1. Under anoxic conditions without nitrate, strain FO-BEG1 metabolized glucose in mixed acid type fermentation, as suggested by the present genes in both strains (see	[redacted]	

DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000), resulting in acidification of the medium and formation of mainly formate, lactate, acetate, and ethanol. Ethanol production during fermentation has not been described for any Pseudovibrio strain yet. Additionally, pyruvate, propionate, and succinate have been formed, but to a lesser extent (Figure S 2.2 A). Production of trace amounts of fumarate was detected, but could not be quantified. As expected, we found the complete set of genes essential for denitrification, including a membrane-bound (nar) and a periplasmic nitrate reductase (nap). In agreement, we observed a complete denitrification to N2 in laboratory experiments with strain FO-BEG1 (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148 and Figure S 2.2 C). For the type strain P. denitrificans, simultaneous denitrification and fermentation was described by Shieh et al. (2004) and could be confirmed in our experiments for strain FO-BEG1 with acetate, formate, lactate, and ethanol as the main fermentation products (Figure S 2.2 B). No genes for assimilatory	[redacted]	

nitrate reduction could be identified in the genome. A set of sox genes suggests that both bacteria can use reduced inorganic sulfur compounds as a source of energy to complement heterotrophy. We could show experimentally that the addition of thiosulfate to the medium enhances the aerobic growth of the Pseudovibrio sp. FO-BEG1 culture and sulfate is produced over the incubation period (Figure S 2.1 B and C). No tetrathionate could be measured as an intermediate (results not shown). Therefore, we propose that thiosulfate is oxidized completely to sulfate without any intermediates, as it is known for the typical Sox pathway in a-Proteobacteria (for review, see Ghosh and Dam, 2009).

We identified genes encoding a small (cutS), medium (cutM) and large (cutL) subunit of the aerobic form II carbon monoxide dehydrogenase (CODH) with the accessory gene coxG in the operon (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148), indicating the capability of CO oxidation. However, uptake of CO could not be demonstrated under tested conditions (results not shown). Interestingly, our results confirm the hypothesis from a recent

publication testing CO oxidation in bacteria containing type II CODH genes (Cunliffe, 2011), in which none of the isolates containing only the type II variant was capable of CO oxidation. Only bacteria containing the form I CODH have been shown to effectively oxidize CO, thereby questioning whether form II CODH is involved in the process of carbon monoxide oxidation, or if it has another primary function not known until now, as suggested by King and Weber (2007).

In both Pseudovibrio strains, we found genes for phosphonate import and degradation, which allows the bacteria to cleave the relatively stable C-P bonds of phosphonates (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Thereby, they can metabolize a less accessible phosphorous pool in times of phosphate limitation. We could demonstrate growth of Pseudovibrio sp. FO-BEG1 with phosphonoacetate as the only source of phosphorous (Figure S 2.3 A). Additionally, we could show adaptation of Pseudovibrio strain FO-BEG1 to oligotrophic conditions by culturing it with as little as 15 |imol C L-1 (0.18 mg C L-1) dissolved organic carbon

in the medium (Chapter 4), which shows that Pseudovibrio FO-BEG1 is capable of growth under extreme nutrient depletion. The high metabolic variety of Pseudovibrio sp. FO-BEG1 and JE062 is also reflected in the analysis of encoded primary transporters. In the genome of strain FO-BEG1 we could identify 31 tripartite ATP-independent periplasmic (TRAP) type transporters (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148) that are required for import of dicarboxylic acids like malate, succinate and fumarate, one of the highest numbers of TRAP type transporters reported in a genome of a marine prokaryote so far (Wagner-Dobler et al., 2010). In strain JE062 we identified 27 TRAP transporters. Citric acid cycle intermediates seem therefore to be an important source of carbon and energy for the investigated Pseudovibrio strains. In addition, we reconstructed over 80 ATP-binding cassette (ABC) transporter systems with predicted substrate specificity from the genomic data of the strain FO-BEG1, including the plasmid, and over 70 ABC transporter systems for JE062	[redacted]	

(see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Sugars, oligopeptides and amino acids are the main substrates that are imported via the ABC systems. A large number of transporters for oligopeptides and amino acids in combination with over 85 genes encoding peptidases and proteases (over 75 genes in strain JE062, see DDBJ/EMBL/GenBank accession number ABXL00000000) could help Pseudovibrio to hydrolyze complex particulate nutrients into oligopeptides and amino acids, which could serve as nutrition for both, the prokaryote and the host, as has been suggested by Siegl et al. (2011). Also iron seems to be an important trace element, for which we identified eight transporters including three siderophores and three transporters for hemin (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Table S.2. Identified ATP-binding cassette (ABC) and tripartite ATP-independent periplasmic (TRAP) transporters in the genomes of both Pseudovibrio strains and their putative functions.	[redacted]	

Vitamin synthesis Growth of pro- and eukaryotes highly depends on their requirements for cofactors that the organism can or cannot synthesize on its own. Vitamins are important for many different enzymatic processes and the synthesis of some vitamins is mainly accomplished by bacteria, making the prokaryotes a necessary part of the eukaryotic diet or an important partner in symbiotic relationships. The genomes of Pseudovibrio sp. FO-BEG1 as well as JE062 contain genes encoding key enzymes of the biosynthesis pathways of biotin (H), thiamin (B1), pyridoxin (B6), cobalamin (B12), riboflavin (B2), folic acid (B9) and lipoic acid (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Independence of an external vitamin supply was confirmed during aerobic growth in the defined CM medium without the addition of any vitamins, which implies de novo synthesis of all required growth factors by strain FO-BEG1 under tested conditions (Figure S 2.3 B). Pseudovibrio spp. would therefore be beneficial companions for other prokaryotes or marine invertebrates, since the	[redacted]	

dependency on an external supply of those vitamins would be relieved.

Bioactive compounds

Symbiotic relationships between bacteria and marine invertebrates, especially sponges, are of special interest, because bacteria associated with sponges often produce novel bioactive compounds (Piel et al., 2004; Taylor et al., 2007; Fisch et al., 2009). In the chromosome of Pseudovibrio FO-BEG1 we identified a genomic island of more than 50 kb containing among others a gene cluster of 20 genes predicted to be involved in secondary metabolite production (see DDBJ/EMBL/GenBank accession number CP003147). The cluster exhibits high sequence similarity to an architecturally almost identical hybrid nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS) system previously reported from many pathogenic and commensal Escherichia coli strains (Figure S.2) (Nougayrède et al., 2006). The E. coli metabolite, termed colibactin, remains structurally uncharacterized. However, transposon mutagenesis of the gene cluster suggested that colibactin is a pathogenicity

determinant that induces DNA double strand breaks in eukaryotic host cells, eventually resulting in cell death. The only significant difference between the gene clusters in Pseudovibrio FO-BEG1 and E. coli is an additional set of genes in the former, encoding putative transporters and the presence of a different phosphopantetheinyl transferase gene variant likely involved in generating holo-proteins from apo forms of PKSs and NRPSs (Lambalot et al., 1996). In addition, two E. coli genes are fused in the Pseudovibrio cluster. Despite these differences, the architecture strongly suggests that the product of the FO-BEG1 cluster is colibactin, providing new opportunities to unveil the identity of this elusive and biomedically relevant compound. Interestingly, we find this more than 50 kb NRPS/PKS fragment only in Pseudovibrio sp. FO-BEG1 but not in the genome of strain JE062, with flanking regions downstream and upstream of the inserted fragment highly conserved in synteny in strain JE062 (data not shown), indicating that it has been acquired via horizontal gene transfer. Additionally, the plasmid of strain FO-BEG1 contained an ORF encoding a type III PKS of a size of 7.4 kb,	[redacted]	

which could also be detected in strain JE062 (see DDBJ/EMBL/GenBank accession number CP003148).

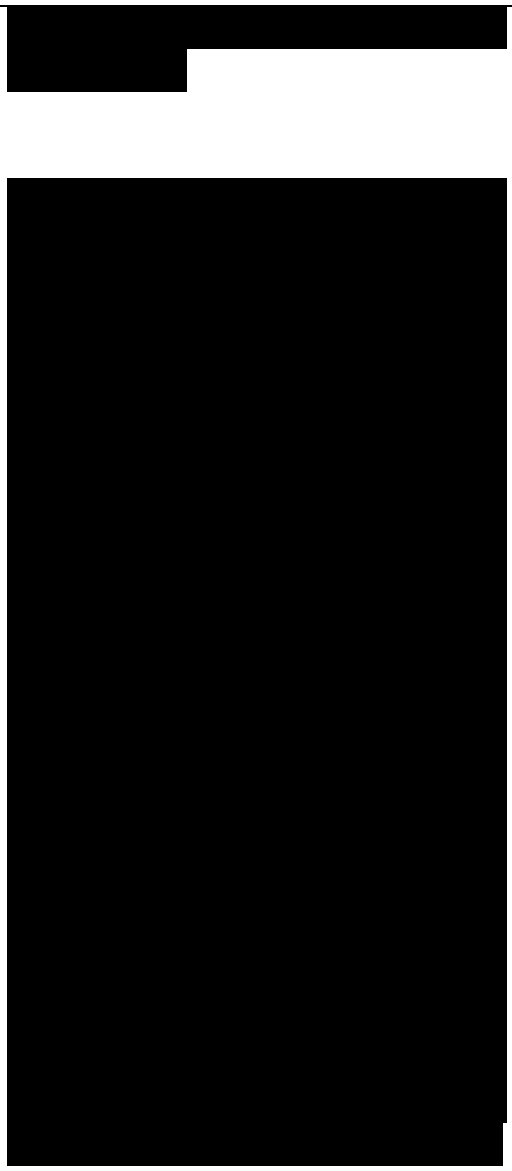


Figure S.2. Nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS) system in Pseudovibrio sp. FO- BEG1 and Escherichia coli strain IHE3034. White arrows represent the genes present in Enterobacteriaceae and strain FO-BEG1; black arrows represent the open reading frames (ORFs) present only in either Enterobacteriaceae or FO-BEG1 but presumably involved in the production of colibactin; the gray arrow shows a gene presumably not involved in the synthesis of colibactin. The symbol at ORF PSE_3331 represents a gene fusion of E. coli genes clbG and clbH in FO-BEG1; the symbol at PSE_3324-3321 represents gene insertion or deletion in strain FO-BEG1 or E. coli IHE3034, respectively.

DNA exchange and horizontal gene transfer

The genomes of both Pseudovibrio strains show a high metabolic variety. It is reasonable to assume that various genes were acquired via horizontal gene transfer from other microorganisms as is indicated e. g. by the presence of a 50 kb large NPRS-PKS island that can be found only in

Pseudovibrio sp. FO-BEG1 but not in strain JE062, although both genomes are in general highly similar. In the genome of strain FO-BEG1 we identified a set of genes coding for a complete gene transfer agent (GTA) (in strain JE062 several genes were missing, see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000), a unit best described as a virus. It harbors small parts of the host DNA and capable of injecting it into appropriate cells, without having negative effects on the host cell (for reviews see Lang and Beatty, 2001; Lang and Beatty, 2007). By this process, Pseudovibrio could have taken up and dispersed DNA carried in virus-like particles, thereby gathering genes and establishing a diverse physiology for a symbiotic and a free-living lifestyle. Additionally, we found 14 integrase and 21 transposase elements in the genome of Pseudovibrio sp. FO-BEG1 (see DDBJ/EMBL/GenBank accession numbers CP003147 and CP003148), 9 of which are located adjacent to the hybrid NRPS-PKS gene cluster, which verifies acquisition of this genomic island via horizontal gene transfer.	[redacted]	

Quorum sensing We could identify 15 genes in strain FO-BEG1 and 14 in strain JE062 containing the LuxR domain, which represents the transcriptional regulator of the acetylated homoserine lactone (AHL) type, allowing the bacterium to detect AHL quorum sensing molecules and to initiate a response (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Intriguingly, we could not find any luxI genes, which code for AHL quorum sensing molecules. This observation leads us to the hypothesis that both Pseudovibrio strains do not communicate via AHL within their own species, but seem to use the LuxR as receptors to react to quorum sensing molecules produced by other species and initiate a respective answer. Such a scenario has been described before by Case et al. (2008) and was called 'eavesdropping'. The response reaction could include the production of bioactive compounds to repel competing prokaryotes or to protect the host from pathogens or parasites. Alternatively, such LuxR-family 'solos' could participate in interkingdom signaling, as	[redacted]	

suggested by Subramoni and Venturi (2009), thereby facilitating prokaryote-host interactions of Pseudovibrio strains with marine invertebrates. Growth with Beggiatoa sp. 35Flor Pseudovibrio sp. FO-BEG1 is the single accompanying organism of the Beggiatoa strain 35Flor, which is growing in a chemolithoautotrophic sulfide-oxygen-gradient medium (Brock and Schulz-Vogt, 2011; Chapters 2 and 3). All attempts to grow Beggiatoa without Pseudovibrio failed and so far we could not identify the factors required by the Beggiatoa strain for autonomous growth. It is known, however, that Beggiatoa spp. do not possess catalases (Larkin and Strohl, 1983) and therefore are susceptible to reactive oxygen molecules originating from respiration. Addition of catalase to the medium is known to increase the viability of Beggiatoa sp. (Burton and Morita, 1964). We hence hypothesize that Beggiatoa sp. 35Flor depends on the radical protection system exhibited by Pseudovibrio sp. FO-BEG1 including genes coding for over 20 superoxide dismutases, catalases and peroxidases (see DDBJ/EMBL/GenBank	[redacted]	

accession numbers CP003147 and CP003148). The role of heterotrophic bacteria as scavenger of reactive oxygen species has also been described by Morris et al. (2008), which could establish robust growth of cyanobacteria after addition of 'helper' heterotrophs.

Secretion Systems

In the genomes of FO-BEG1 and JE062 we could identify two loci that encode type VI secretion systems (T6SS) as well as one type III secretion system (T3SS) including effector molecules, which indicates the capability of specific interactions with eukaryotes and the possibility of influencing their cell machinery.

The T6SS has been described as a major secretion system in the context of pathogenicity as a virulence factor in morbific bacteria (Mougous et al., 2006; Pukatzki et al., 2006) and a core of 13 highly conserved and essential subunits has been identified for this secretion system 129

(Boyer et al., 2009). In both genomes of the Pseudovibrio strains, we found two gene clusters consisting of 12 (cluster I) and 20 (cluster II) genes that encode T6SSs. Cluster II contains the complete set of core subunits and therefore we assume that cluster II could, if

expressed, produce a complete and functional type VI secretion system. In cluster I, two core genes are missing in the operon, hcpI and vgrG, which are main components of the injection apparatus with possible effector functions (Pukatzki et al., 2009; Bonemann et al., 2010). However, homologues of hcpI and vgrG could be identified in additional copies at other locations in the genomes of FO-BEG1 and JE062 (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000), which is a phenomenon regularly found in genomes containing T6SS (Pukatzki et al., 2009). The possible role for type VI secretion systems in bacteria has not been completely elucidated so far, but several functions have been attributed to it already. Mainly, T6SS is described as a virulence factor of pathogenic bacteria delivering effector proteins into host cells (Filloux et al., 2008). However, further studies reveal the involvement of T6SS in biofilm formation (Aschtgen et al., 2008), quorum sensing (Weber et al., 2009), interbacterial interactions (Hood et al., 2010) and antipathogenesis (Chow and Mazmanian, 2010; Jani and Cotter, 2010). In conclusion, it

can be assumed that the T6SS of both strains are functional since the genomes contain the main structural components of the type VI secretion system.

In addition to the T6SS, we identified a type III secretion system in the genomes of both Pseudovibrio strains, which is located in a genomic region encompassing around 35 ORFs with various highly conserved proteins known from T3S systems (Cornelis and Van Gijsegem, 2000) (Figure S.3 and see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000). Besides the secretion apparatus we also identified genes encoding homologues of three types of effector molecules in the genome of strain FO-BEG1 and two effector molecule types in strain JE062. Those effectors might be directly involved in the establishment of symbiosis between Pseudovibrio and its host. YpkA, IpgD (found in both genomes) and YopJ (only in strain FO-BEG1) are effector molecules that affect the cytoskeleton or the innate immune response of the host, respectively. YpkA is a serine/threonine kinase, which has negative effects on

cytoskeletal dynamics due to its interaction with actin, thereby contributing to the resistance to phagocytosis (Cornelis, 2002). YpkA is present in three copies in both genomes. In Porifera, specialized amoeboid cells, the archaeocytes, resemble macrophages and eliminate non-self material via phagocytosis (Muller and Muller, 2003). Pseudovibrio, expressing and secreting the YpkA effector, could interfere with this process, preventing archaeocytes from digesting Pseudovibrio cells. A similar effect could be induced by a homologue of IpgD found in both genomes, a virulence factor that is responsible for morphological changes of a host cell by increasing membrane detachment from the cytoskeleton (Niebuhr et al., 2000; Niebuhr et al., 2002).

Figure S.3. Operon coding for type III secretion system (T3SS) subunits and effector proteins. White arrows show annotated homologues of T3SS subunits including the gene name within the arrows; black arrows represent annotated effector homologues; dark gray arrows show annotated genes encoding proteins presumably not involved in T3SS; light gray arrows show hypothetical proteins with unknown function. The locus is indicated above and

below some genes for orientation purposes. In FO-BEG1 we additionally identified a homologue of the YopJ effector exhibiting a serine/threonine acetyltransferase function. By acetylation of serine and threonine residues of mitogen-activated protein (MAP) kinases it prevents phosphorylation of those molecules and therefore inhibits the innate immune response of the organism (Mukherjee et al., 2006). Intriguingly, it has been shown that sponges possess a very efficient innate immune response system, using MAP kinases as the essential component of its response to bacterial endotoxin lipopolysaccharide (LPS) (Bohm et al., 2001; Muller and Muller, 2003). This indicates that homologues of the acetyltransferase YopJ effector in Pseudovibrio could prevent phosphorylation of MAP kinases via acetylation, thereby playing a role in the inactivation of the immune answer of the host organism, allowing Pseudovibrio to avoid phagocytosis, as described by Bartsev et al. (2004) for a Rhizobium strain, and to remain in the host for establishment of a symbiosis. This hypothesis is further supported by the fact that a homologue of YopJ (NopJ) was	[redacted]	

shown to be an effector in symbiotic rhizobia (Deakin and Broughton, 2009) and Lackner et al. (2011) demonstrated that T3SS is involved in maintenance of a symbiosis between bacteria and fungi by enhancement of intracellular survival of the prokaryote within the host.

Adhesion

In both genomes we found homologues of genes coding for proteins responsible for adhesion to surfaces or other cells. These proteins, belonging to the group of amyloids, are extracellular proteinaceous components and are known in Enterobacteriaceae as curli fibers. They are involved in adhesion to surfaces, cell aggregation, biofilm formation and mediate cell-cell adhesion and invasion of host cells (Barnhart and Chapman, 2006).

The production of curli fibers in enteric bacteria is dependent on at least six proteins encoded by the operons csgAB and csgDEFG (agf in Salmonella) (Hammar et al., 1995), the latter of which is required for assembly, stability and secretion of the amyloids (Hammar et al., 1995). csgAB encodes the structural subunits of the curli fibers, both genes containing

characteristic repeat motifs (Hammar et al., 1996). A gene cluster in the genome of Pseudovibrio sp. FO-BEG1 resembles the curli formation operon in enteric bacteria (Figure S.4).

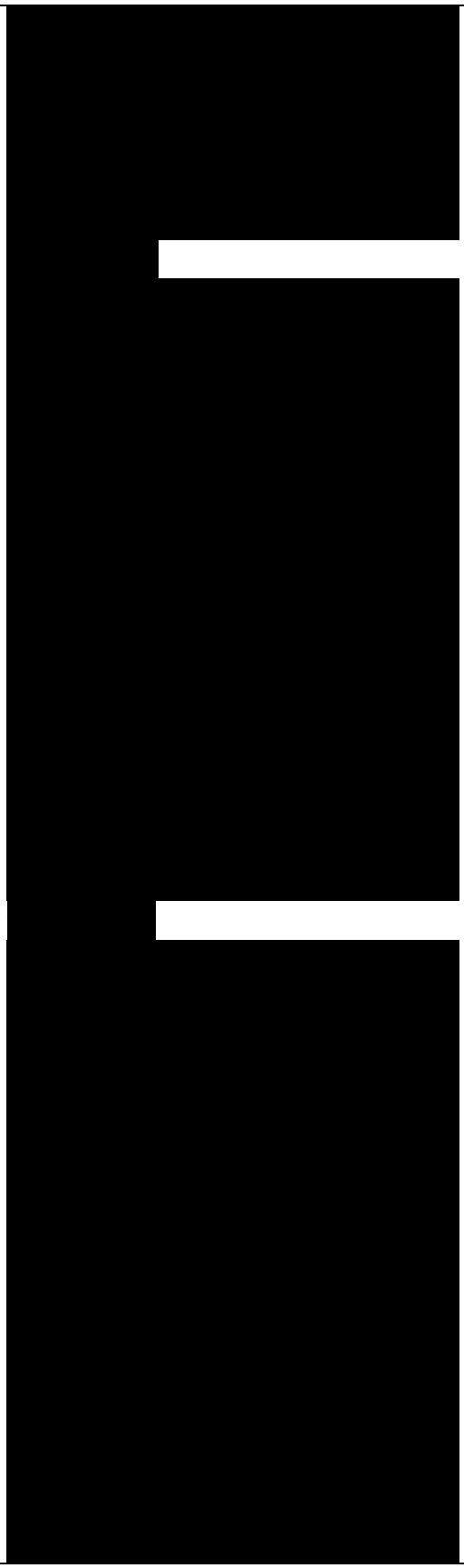


Figure S.4. Comparison of genes encoding amyloids in Enterobacteriaceae and the operon in Pseudovibrio sp. FO-BEG1. White arrows represent homologues of genes in enteric bacteria; gray arrows show genes present in Enterobacteriaceae only; black arrows show genes containing curli repeats, typical motifs of the amyloid structural subunits. The number within the black arrows shows the amount of curli repeats in the according gene.

Homologues of csgF and csgG, required for stabilization and secretion of the amyloids are found in direct proximity to three genes containing curlin associated repeats as typical structural components of the curli fibers. We hypothesize that the identified operon might code for amyloid structures comparable to curli fibers due to the existence of characteristic curlin repeat motifs and genes involved in the assembly and secretion of such structures, therefore allowing Pseudovibrio

to attach to other cells or form biofilms or aggregates. Additionally, we identified 35 genes in strain FO-BEG1 and 37 in JE062 containing domains mediating prokaryote-eukaryote interactions, supporting the proposed role of Pseudovibrio as a symbiont with possibilities to attach and interact with the host organism (see DDBJ/EMBL/GenBank accession numbers CP003147, CP003148 and ABXL00000000).

Conclusions

In this study, we analyzed highly similar genomes of two Pseudovibrio strains that originate from the coast of Florida, the Pseudovibrio sp. FO-BEG1 sampled from a coral and maintained over 10 years in co-culture with Beggiatoa sp. and Pseudovibrio sp. JE062 sampled from a sponge in the same region (Enticknap et al., 2006). The physiology of both strains is extremely versatile and the metabolic traits found in the genome could be partially verified in experiments with strain FO-BEG1. Here, we describe for the first time a Pseudovibrio strain that uses aromatic compounds as a carbon and electron source, oxidizes thiosulfate under aerobic

conditions and uses phosphonates as a phosphorous source. Notably, strain FO-BEG1 grows under extreme nutrient limitation, which emphasizes its adaptation to life in the open ocean. The metabolic variety is confirmed by the numerous transporter systems that are encoded in the genome. Compared with other marine bacteria, like the prominent Roseobacter clade, which is known to be ubiquitous, multitudinous and physiologically versatile (Newton et al., 2010), Pseudovibrio seems to be capable of a similarly generalistic life style, exploiting quite a number of sources for energy sources, nutrients and trace elements.

Aside from metabolic variety, the genomic data of both strains also confirm close associations with marine invertebrates and indicate several potential mechanisms for establishing and maintaining a symbiosis. The most striking discovery is the presence of effector homologues secreted by type III secretion systems, which could affect sponges by interacting with their immune response system (YopJ) or the cytoskeleton (YpkA, IpgD) and thereby have a drastic impact on the cell machinery of the host. Another fascinating

discovery is the presence of the hybrid NRPS-PKS system in strain FO-BEG1, which has so far only been described for members of the Enterobacteriaceae family (Putze et al., 2009), producing the bioactive compound colibactin with yet unknown in-vivo functions, but arresting eukaryotic cells in the G2 phase, eventually leading to cell death (Nougayrède et al., 2006). The presence of a gene cluster coding for a cytopathic compound in strain FO-BEG1 emphasizes the impact that Pseudovibrio cells might have on marine invertebrates. Intriguingly, strain FO-BEG1 seems to be a required partner in the Beggiatoa co-culture, indicating its important symbiotic role not only for marine invertebrates but also for prokaryotes. It is possible that Pseudovibrio has positive effects for certain bacteria under in-vivo conditions, e.g. by supplying vitamins or detoxifying metabolic intermediates or radical oxygen species.

Figure S.5. Schematic overview of the possible life styles and the physiologic capabilities derived from genetic information of both Pseudovibrio genomes. On the left hand side, physiologic abilities are depicted that could be used in free-living, oxic and

anoxic conditions. On the right hand side, the attached or associated life style is illustrated. The host organism for the associated life style can be represented by a sponge, coral or tunicate. Biofilm formation, aggregation and attachment to host cells could be performed via e. g. amyloid-like structures. The proposed secretion systems could be involved in prokaryote-eukaryote interactions, influencing the cell machinery of the host. Additionally, Pseudovibrio could supply the host with cofactors like vitamins or synthesize secondary metabolites as a defense mechanism against other prokaryotes or the host.

The frequent identification and isolation of Pseudovibrio strains in many studies over the last years implies an important but rather unexplored role for this genus in marine habitats. According to the genomic and physiological data on Pseudovibrio spp., we propose a free- living and attached or associated life style model for this genus (Figure S.5). As a denitrifying heterotroph, Pseudovibrio has an obvious influence on the carbon and nitrogen cycles. Its ecological impact can now be extended to the sulfur and phosphorus cycles

due to its ability to metabolize thiosulfate and phosphonates. Additionally, we hypothesize that, due to the predictions based on the genomic data, similar to E. coli in humans, Pseudovibrio is a commensalistic or even beneficial symbiont of marine invertebrates with a potential to become pathogenic.