Unraveling predatory-prey interactions between bacteria

Dissertation

To Fulfill the

Requirements for the Degree of

„Doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty of Biology and Pharmacy of the Friedrich Schiller University Jena

By

Ivana Seccareccia (M.Sc. in Biology)

born on 8th April 1986 in Rijeka, Croatia

Die Forschungsarbeit im Rahmen dieser Dissertation wurde am Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie e.V. – Hans-Knöll–Institut in der Nachwuchsgruppe Sekundärmetabolismus räuberischer Bakterien unter der Betreuung von Dr. habil. Markus Nett von Oktober 2011 bis Oktober 2015 in Jena durchgeführt.

Gutachter: ……………………………………………….
……………………………………………….
……………………………………………….

Tag der öffentlichen Verteidigung:

We make our world significant by the courage of our questions

and by the depth of our answers.

Carl Sagan

Table of Contents

1 Introduction 6
1.1 Predation in the microbial community 6
1.2 Bacterial predators 7
1.3 Phases of predation 8
1.3.1 Seeking prey 9
1.3.2 Prey recognition 10
1.3.3 Prey killing and consumption 10
1.4 Bacterial defense against predators 11
1.4.1 Production of secondary metabolites 11
1.4.2 Morphological adaptations, multicellular structures and high motility 12
1.4.3 Induction of bacterial defense mechanisms 13
1.5 Predation strategies 13
1.5.1 Individual predation 14
1.5.2 Group predation 14
1.6 The genus *Lysobacter* 15
1.6.1 Predatory behavior of *Lysobacter* spp. 15
1.7 The genus *Cupriavidus* 16
1.7.1 Predatory behavior of *Cupriavidus necator* N-1 17
2 Scope of the thesis 19
3 Overview of Manuscripts 20
3.1 Manuscripts resulting from the main research project 20
3.1.1 Manuscript A 20
3.1.2 Manuscript B 20
3.1.3 Manuscript C 21
3.2 Manuscripts from side projects 21
3.2.1 Manuscript D 21
3.2.2 Manuscript E 22
4 Published Results 23
4.1 Manuscript A: Quantitative analysis of *Lysobacter* predation 23
4.2 Manuscript B: Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis* 41
4.3 Manuscript C: Identification of new metallothioneins in the bacterium *Cupriavidus necator* N-1 68
5 Unpublished Results 93
5.1 Evaluation of predatory potential in *Cupriavidus* spp. 93
5.2 Antimicrobial activity of recombinant peptides from *C. necator* N-1 97
6 Discussion 99

6.1 Analysis of bacterial predation in the laboratory 99
6.2 Comparative analysis of predation behavior among bacteria.................................... 101
6.2.1 Bacterial predation efficiency across different assays 102
6.2.2 Predator-prey ratio and cell contact 104
6.3 Secondary metabolites of *C. necator*.. 105
6.4 Prey-specific factors that influence predation 106
6.5 Distinguishing predation from competition... 107
7 Summary 109
8 Zusammenfassung.. 111
9 Manuscripts from side projects 113
9.1 Manuscript D: *Micromonospora schwarzwaldensis* sp. nov., a producer of telomycin, isolated from soil. 113
9.2 Manuscript E: Variochelins, lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining. 131
10 List of abbreviations 174
11 References 175
12 Acknowledgement.. 182
13 Eigenständigkeitserklärung 184
14 Curriculum Vitae... 185

1 Introduction

1.1 Predation in the microbial community

Microorganisms are living in dynamic and complex communities, communicating and interacting with each other. Microbial interactions can be neutral, beneficial or harmful for the partners involved. Although extensive research is conducted in the field of microbial relationships, it was focused only on few of many different modes of interactions described [1]. When it comes to predation among bacteria, our knowledge is limited and many questions remain to be answered. Predation is considered an important force that shapes microbial communities [2] by simultaneously enforcing selection escape strategies in prey and killing efficiency of predators [3, 4]. Additionally, the ecological role of predators is reflected on the determination of the structure and dynamics in the microbial community. Predation can maintain diversity in the community, as abundant species are statistically more likely to be attacked by predators [5, 6]. Predation is a significant force in trophic interactions between microorganisms that promotes primary production of organic compounds through the process of photosynthesis and chemosynthesis [7]. In aquatic environments, predation pressure leads to more efficient nutrient regeneration and cycling of organic compounds and other nutrients in particular nitrogen and phosphorus [8, 9]. Additionally, predators facilitate release of photosynthetically fixed organic carbon by preying on phytoplankton [10]. Despite the fact that the highest predation pressure comes from microfaunal predators consisting of bacteriovorous protozoa and nematodes [10] bacterial predators have an important role in the microbial food chain, and thus are in the focus of this study. Because of their production of extracellular lytic enzymes, predatory bacteria were already used as biocontrol agents to prevent cyanobacterial blooms in lakes, e.g. *Bdellovibrio*-like bacteria [11] and *Myxococcus* sp. [12, 13]. Additionally, the powerful lytic activity of *Streptomyces exfoliates*, was found to cause 50% mortality of the cyanobacteria *Anabaena*, *Microcystis* and *Oscillatoria* [14]. A recent study suggests that the genus entire *Streptomyces* has predatory potential [15], although it is not clear yet if this bacterial group is truly capable to use lysed microorganisms as a nutrient source. Nevertheless, the genus *Streptomyces* possesses a wide range of antimicrobial activity and, therefore, has great potential in biocontrol applications.

1.2 Bacterial predators

Predatory bacteria are distributed over many bacterial phyla, and they appear to be rather common in the environment. A number of predatory bacteria have been isolated from different habitats and they vary greatly in prey range and modes of predation (Table 1). Predatory bacteria show great diversity of feeding strategies. However, it is hard to discriminate bacteria based upon their modes of predation, since clear distinctions between the hunted strategies are often not possible. The main reason is that predator can use more than one hunting strategy, depending on different factors. Therefore, the main division of predatory bacteria is on obligate and facultative predators.

Table 1. Predatory bacteria

Taxonomy	Predation strategy	Prey	Habitats	References
α-Proteobacteria				
Ensifer adhaerens	epibiotic facultative	Gram+ bacteria	soil	[16]
Micavibrio admirantus	epibiotic obligate	Gram- bacteria	wastewater	[17]
β-Proteobacteria				
Cupriavidus necator N-1	unknown facultative	Gram+/- bacteria	soil	[18]
γ-Proteobacteria				
Lysobacter spp.	epibiotic wolfpack facultative	cyanobacteria, Gram+/- bacteria, fungi	soil, freshwater	[19]
δ-Proteobacteria				
Myxobacteria	cell contact wolfpack facultative	bacteria, fungi, nematodes	soil	[20]
Bdellovibrio spp.	epibiotic obligate	Gram- bacteria	freshwater, soil	[21]
Actinobacteria				
Agromyces ramosus	unknown facultative	Gram+/-bacteria yeast	soil	[22]
Streptomyces spp.	unknown facultative	Gram+/- bacteria	soil	[15]
Bacteroidetes				
Saprospira grandis	ixotrophy, cell contact wolfpack facultative	cyanobacteria, diatoms, bacteria	seawater, sediments	[23]
Chloroflexi				
Herpetosiphon aurantiacus	cell contact wolfpack facultative	Gram+/-bacteria yeast	freshwater, lakes	[24]

Obligate predators can only survive by hunting other organisms. Furthermore, in most cases, predator multiplication is occurring inside prey cells, obscuring the distinction between

predatory and parasitic lifestyle [25]. A "model" organism for obligate predators is *Bdellovibrio bacteriovorus*, which has been extensively studied for decades [26]. On the other hand, non-obligate or facultative predators may survive without prey, and they often only prey on other organisms under conditions of low substrate availability [27, 28]. Myxobacteria are facultative predators that have been thoroughly studied in the past [20, 29] and many available predation assays were established for investigating their predatory behavior. Even though predation between bacteria was first noted more than 75 years ago [30], research in this area has focused on few taxonomic groups and there are still many gaps in our knowledge about their ecology and interactions with prey organisms. Furthermore, the specific predation strategies are often not resolved.

1.3 Phases of predation

Predation is a complex process, consisting of several phases [31]. Predators need to find their prey, attack, kill and finally consume it (Fig. 1). For both facultative and obligate predators, each of these phases involves unique adaptations and some of which will be discussed in the following.

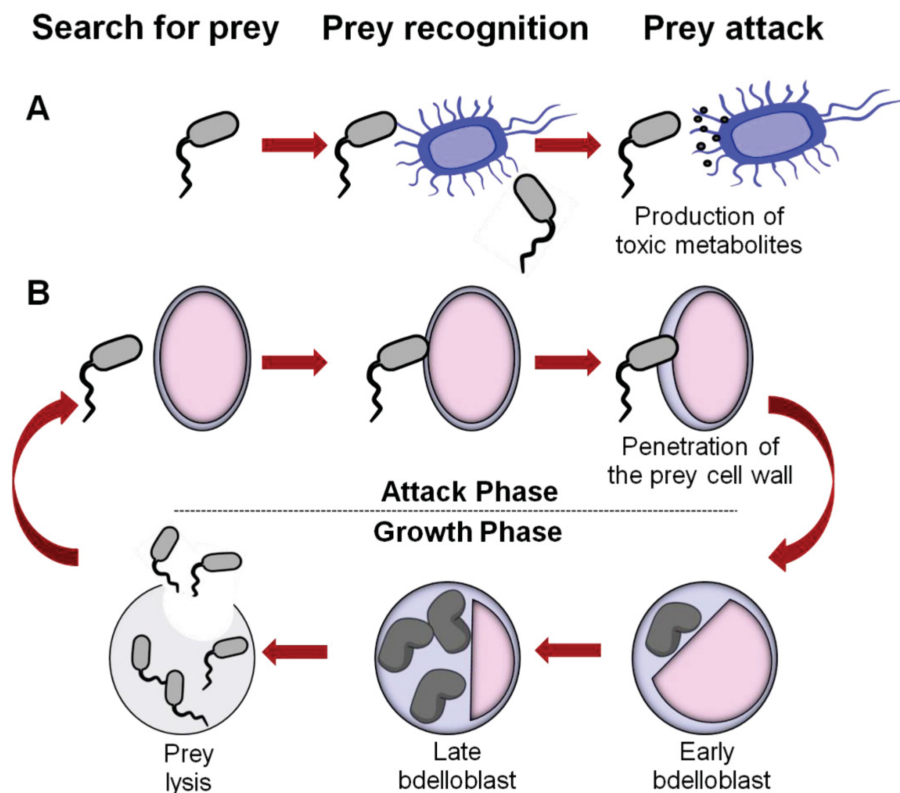


Figure 1. Phases of predation for facultative predators (A) and the life cycle of obligate predator *Bdellovibrio* (B). Predator uses chemotaxis to find its prey and afterwards, carry out the attack on the prey. Typically, after finding its prey facultative predators produce toxic compounds or lytic enzymes which kill and lyse the prey cells. However, following attachment to prey *Bdellovibrio* is penetrating to intraperiplasmic space and it forms bdelloblast. After the growth in side prey, *Bdellovibrio* lyse the prey envelope and the progeny cells are released [32].

1.3.1 Seeking prey

Finding prey is an essential step to secure predation success. Predators can encounter their prey randomly or track them in a specific manner using chemotaxis and quorum sensing signals [3]. It has been estimated that free-living predators belonging to *Bdellovibrio* have only a few hours to find their prey before they are no longer capable to maintain metabolic processes [33]. Chemotaxis by wild-type *B. bacteriovorus* has been demonstrated towards amino acids [34], and high concentrations of prey cells [35]. In addition, recent study confirmed that bdellovibrios use chemotaxis to navigate themselves towards natural habitats of their potential prey bacteria [36]. *Bdellovibrio* contains 20 chemotaxis receptor genes and it was reported that a mutation in a receptor gene encoding a methyl-accepting chemotaxis protein causes *Bdellovibrio* to be a less efficient predator [36]. The authors suggested that the methyl-accepting chemotaxis system is used for directing *Bdellovibrio* in the direction of its prey. The flagellated *Bdellovibrio* is capable of rapid movement in liquid environments. Furthermore, a recent study also indicated that the bacterium can glide similar to facultative myxobacterial predators [36]. It is possible that *Bdellovibrio* use this kind of slow motility to search for prey bacteria on solid surfaces.

Although facultative predators are not exposed to such a strong pressure to quickly find their prey, there have been some investigations of the role of chemotaxis in these organisms. For example, the predatory genus *Myxococcus* harbors gliding bacteria that are able to move in different directions and it was initially proposed that these predators employ chemotaxis to surround prey cells, indicating a wolfpack predation strategy [28]. However, the fact that *M. xanthus* cells move slowly at 0.02 µm/s, compared to 50 µm/s for a swimming *E. coli* cell, rendered it difficult to measure the response of this bacterium towards a chemotaxis attractant [29]. Chemotaxis is not just important for finding prey, but also to organize and coordinate individual predator cells. *M. xanthus* cells were found to migrate in a very unique manner, forming wave patterns. This so-called rippling phenomenon [37], is controlled by the chemotaxis (Che) signal transduction pathway [38]. Further analyses of these formed cell structures indicated that the rippling patterns depend on prey availability. It has been hypothesized that this behavior enables the predator to enlarge contact with its prey in order to facilitate predation [29]. *M. xanthus* has eight gene clusters encoding Che-like components. The importance of the Frz chemotaxis system for the predatory behavior was verified [39]. The Frz system is involved in controlling individual cell movements [40, 41]. Mutants with impaired Frz system exhibited significantly reduced predation efficiency. However, it remains

unclear if *M. xanthus* uses a chemotactic signal to locate its prey under natural circumstances [29].

1.3.2 Prey recognition

Prey recognition is mediated by specific recognition mechanisms. In protozoa, this phase is well-studied and prey phenotypic properties significantly affect the predation success [42]. The respective prey is targeted based on its surface characteristics like flagella, cell wall components [43] or lipopolysaccharide envelope compositions [44]. However, little data are available on prey recognition by bacterial predators. While *Bdellovibrio* and *Bdellovibrio*-like organisms (BALOs) are known to preferentially feed on certain bacterial species, receptor sites on prey cell surfaces have not been identified to date [8]. Given that many bacteria have developed defense strategies such as the production of secondary metabolites, one of the possible ways for facultative predators to discriminate between mixed bacteria is partially based on prey toxicity [45, 46]. A recently published study revealed important aspects of predator-prey recognition and binding in *Bdellovibrio* [47]. The authors developed a novel predator cultivation system composed of emptied prey cells which context can be easily manipulated. Apparently, for prey recognition and binding there are two signals: one which is an early recognition signal and it is situated in the prey envelope and a late signal which is found within the prey soluble fraction. Both signals trigger regulatory factors for differential transcription of the predator genes involved in transitory phase between prey recognition and attack till the formation of the bdelloplast in the prey cell (Fig. 1B) [47].

1.3.3 Prey killing and consumption

Many bacterial predators are assumed to kill their prey using antibiotics and hydrolytic enzymes that might also contribute to extracellular digestion, facilitating subsequent consumption (Fig. 1A) [48]. For example, myxobacteria mediate killing by secreting diffusible factors in direct cell-to-cell contact [37, 49, 50]. The antibiotic myxovirescin (Fig. 2A), isolated from *M. xanthus* [51], is an inhibitor of lipoprotein production and it is effective against Gram-negative prey bacteria [52-54]. Notably, a myxovirescin-deficient *M. xanthus* mutant loses its ability to feed on *E. coli* [53]. A similar observation was made with the myxobacterial antibiotic corallopyronin (Fig. 2B), which is a RNA polymerase inhibitor isolated from *Corallococcus coralloides* [55]. This myxobacterium is restricted to preying on corallopyronin-sensitive prey cells [53]. Given examples suggest an important role of antibiotics in the predation strategy of myxobacteria. Other predatory bacteria, such as *Lysobacter* spp., *Aristabacter necator* and *Streptomyces* spp. produce antibiotics, but a

contribution to predation has not been confirmed [48, 56]. It is possible that the produced antibiotic spectrum of a predator determines the prey range.

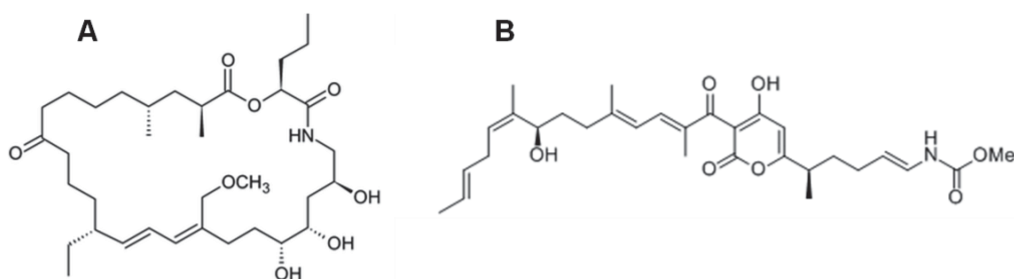


Figure 2. Structures of myxovirescin (A) and corallopyronin (B)

In addition, myxobacteria produce various proteases and lytic enzymes capable of destroying the prey cell wall [57]. The genome of *M. xanthus* DK1622, features about 150 genes that are annotated to code for hydrolytic enzymes [58]. However, further research is necessary to determine which of these enzymes have a specific role in predation. Many predators release their membrane vesicles with hydrolytic enzymes that can fuse with other bacteria and lyse them [59], simultaneously attacking their prey and facilitating consumption.

1.4 Bacterial defense against predators

Predation is a major cause of bacterial mortality [10] and, therefore, it is not surprising that anti-predator defense mechanisms have evolved to enhance bacterial fitness under predation pressure. Bacteria have developed a variety of defensive traits and the following examples illustrate highly specific responses to prevent predators attack.

1.4.1 Production of secondary metabolites

Many bacterial strains produce toxic secondary metabolites that can have a role in inhibiting predators. A recent study compared the resistance of two *Bacillus subtilis* strains against myxobacterial predation [60]. The ancestral *B. subtilis* strain NCIB3610 was found to be resistant to myxobacterial predation in stark contrast to the laboratory strain 168. Comparative gene analysis revealed that both strains possess a polyketide synthase (*pks*) gene cluster involved in bacillaene biosynthesis (Fig. 3). In *B. subtilis* 168, this cluster was not functional, probably due to SNPs mutations. Furthermore, addition of exogenous bacillaene to domesticated strains resulted in resistance to predation [60]. Moreover, while the NCIB3610 wild type was resistant, NCIB3610 *pksL* mutant was susceptible to predation. From given experiments a conclusion has been made that bacillaene is the major factor allowing *B.*

subtilis NCIB3610 cells to evade *M. xanthus* predation (Fig. 3). A similar observation was made with another facultative predator *S. coelicolor* [15, 62].

Figure 3. Structures of bacillaene [61]

Secondary metabolites can be even more effective after ingestion of the prey, since diffusion into the environment is avoided [63, 64]. *Burkholderia cepacia* and *Pseudomonas aeruginosa* were demonstrated to kill the bacteriovorus nematode *Caenorhabditis elegans* after ingestion, most likely by the production of an extracellular toxin which has not been identified yet [65]. Furthermore, Shiga toxin-producing strains of *Escherichia coli* can sense phagosome-like conditions and kill ciliate predators by expressing the toxin after ingestion [66]. Besides directly producing toxic compounds targeting the predator, another defensive strategy is to produce repellents that may be used to deter predators [67, 68]. For instance, *Enterobacter intermedium* repels possible predators by acidifying its environment [69].

1.4.2 Morphological adaptations, multicellular structures and high motility

Morphological adaptations, such as sporulation, filament formation, clumping [70, 71], or the production of exopolysaccharide (EPS) capsules can reduce predator ingestion either by masking the prey surface or by reducing the effect of toxic compounds produced by predators [70]. Recent evidence suggests that prey bacteria can avoid predation by sporulation; for example, *B. subtilis* spores are resistant to predation by the predatory bacterium *Myxococcus xanthus* [60] as well as the protozoan *Tetrahymena thermophile* [72], and the nematodes *C. elegans* [73]. The protein coat of *Bacillus* spores was identified as the main carrier of the spore resistance against predators [60, 72], indicating the evolutionary benefit of sporulation as a survival strategy. Another morphological defense is the formation of biofilms [4]. The cumulative production of protective metabolites in a biofilm has a stronger defensive effect against predators than the release of such compounds by a single prey cell [74]. The general belief is that during environmental challenges like competition for nutrients, biofilms arise as a survival mechanism. Recent evidence further suggests that biofilm formation facilitates the escape from predators [75]. Exposing *B. subtilis* to *M. xanthus* induces the formation of so

called 'megastructures' which are filled with *B. subtilis* spores. Furthermore, it was shown that a *B. subtilis* incapable of producing the defensive secondary metabolite bacillaene formed megastructures more rapidly than the wild type strain [75]. Interestingly, *B. subtilis* mutants defective in spore generation were still able to form megastructures. However, it seems as if the matrix alone is not sufficient to prevent predation, because *M. xanthus* was capable of penetrating the megastructures. Therefore, it is more likely that the megastructure is another mechanism of *B. subtilis* to protect cells during an escape to dormancy via sporulation. Another means by which bacteria avoid predators is through high motility. In general, bacterial motility has been observed primarily as an adaptive feature that allows bacteria to reach a nutrient rich environment in a heterogeneous habitat. However, it was demonstrated that high motility of planktonic bacteria may help to evade encounters with grazing protozoan predators and subsequent their capture [4].

1.4.3 Induction of bacterial defense mechanisms

Bacteria employ a number of mechanisms to weaken or avoid predator attack to secure their survival. The induction of bacterial defense mechanisms can be predator- mediated or density-dependent [3]. In most cases, it is more beneficial for the prey to only express defense traits when a predator is nearby since the preparation of these factors is demanding for the cell [76, 77]. Therefore, it is not unexpected that many bacteria are able to adjust their resistance strategies in the presence of predators. A bacterium that reacts to predator presence is *Pseudomonas fluorescens,* which starts to produce toxic compounds like biosurfactants [78] or mitochondrial inhibitors against the free-living amoeba *Acanthamoeba castellanii* [46, 79]. Another strategy for activating predator defense is density-dependent regulation. Most bacteria can produce and sense autoinducers, i.e. small diffusible molecules [80]. Once a certain population density is achieved, all cells activate social behaviors, a process called quorum sensing. In this way, multiple defense mechanisms can be activated. As an example, the production of the toxic pigment violacein by *Chromobacter violaceum* is regulated by quorum sensing [64]. In addition, biofilm formation against predation is also mediated by quorum sensing in *P. aeruginosa* [81] and *Serratia marcescens* [74].

1.5 Predation strategies

Due to the phylogenetic diversity of predatory bacteria, it is conceivable that these organisms evolved several different feeding strategies, ranging from prey cell invasion, as exemplified by *Bdellovibrio* and its relatives (Fig. 1B) [21], to the production of lytic exoenzymes by myxobacteria [82, 83] and some members of the genus *Lysobacter* [84]. Predator attacks can

be made by individuals or by swarms. At present, four basic predation strategies are distinguished, including "wolfpack" or group predation, epibiotic attachment, direct cytoplasmic invasion, and periplasmic invasion [28].

1.5.1 Individual predation

Some individual predators kill their prey directly after cell contact. Epibiotic predators such as *Vampirovibrio* species secrete hydrolytic enzymes right into their prey (e.g., *Chromatium* spp.) and then assimilate nutrients from the interior of the cell [85]. Another epibiotic predator is *Micavibrio*, which preys on Gram-negative bacteria like *Pseudomonas aeruginosa* [86]. Additionally, it possesses flagella and swims at high speeds [17]. Besides their solitary hunting habit, these epibiotic swimming predators have all been found to be obligate predators, incapable of growth in the absence of living prey cells. In the contrast, endobiotic predators enter prey cells to feed and, subsequently, to replicate within the cytoplasm or periplasm of the prey [25]. Predators belonging to this group include bacteria of the genus *Bdellovibrio*, which have been thoughtfully studied. *Bdellovibrio bacteriovorus* was originally discovered while analyzing bacteriophages. *B. bacteriovorus* is a small and highly motile predator. After attaching to the surface of Gram-negative bacteria, it penetrates the cell wall and enters the periplasmic space, where it multiplies, eventually leading to lysis of the prey cell [21]. Obligate bacterial predators, such as *B. bacteriovorus*, typically engage in contact-mediated predation and tend to be small in size relative to their prey [25].

1.5.2 Group predation

Social predation is manifested when a large number of predatory bacteria jointly attack and decompose the prey [25]. Myxobacteria are among the best characterized bacterial predators, and together with *Lysobacter* spp., they are generally assumed to practice group predation [19, 25, 28]. Their approach of hunting requires a quorum of cells as well as gliding motility, which allows them to actively seek for prey [87, 88]. Another essential feature of group predation is the release of cell wall degrading enzymes and antibiotics in order to kill the prey organisms [53, 89]. Predation factors like lytic exoenzymes may be attached to the predator cell surface or fixed in the extracellular polysaccharide matrix of the predator pack, allowing their release when prey cells are close to the predator cells [90]. However, some studies suggest that *M. xanthus* is also capable of individual prey hunting [29, 50]. In this case, however, cell-to-cell contact is required for prey cell lysis and not the high number of the predator [29].

1.6 The genus *Lysobacter*

Christensen and Cook introduced the genus *Lysobacter* in 1978. Until then, strains later classified as *Lysobacter* spp. were falsely assigned to the taxonomically distinct myxobacteria [19]. This confusion is conceivable, as both bacterial groups share several distinctive traits, including high G+C content in their DNA, the ability to glide on solid surfaces, and the secretion of lytic enzymes [19, 91, 92]. 16S rDNA analysis revealed that the genus *Lysobacter* belongs to the γ-proteobacteria and is most closely related to bacteria within the family *Xanthomonadaceae*. After the original description of four *Lysobacter* species [91], the genus has been significantly expanded and, at the time of writing, it includes 31 species (http://www.bacterio.net/lysobacter.html). In recent years, *Lysobacter* spp. have been recognized as potent biocontrol agents, which are capable of suppressing the growth of phytopathogenic bacteria and fungi alike [93-97]. In the context of bacterial plant diseases, *L. antibioticus* is a particularly promising biocontrol agent, inhibiting the growth of numerous bacterial phytopathogens, including *Ralstonia solanacearum*, *Pseudomonas syringae*, *Xanthomonas axonopodis* and *Xanthomonas campestris* [98].

1.6.1 Predatory behavior of *Lysobacter* spp.

In most literature reviews on predatory bacteria, *Lysobacter* spp. are often placed next to myxobacteria, as they are assumed to pursue a similar predation strategy. Analogous to myxobacteria, *Lysobacter* spp. can prey on Gram-negative and Gram-positive bacteria using a wolfpack strategy, although there was also evidence for an epibiotic feeding mechanism [99]. Surprisingly, there is a strong discrepancy between the number of reviews on *Lysobacter* predation [19, 25, 26, 28, 29] and original research articles. Literature search revealed only three articles dealing with the predatory behavior of these bacteria. An early report described the lytic effect of *Lysobacter* sp. (originally assigned as *Myxobacter* FP-1) on blue-green algae [99]. In this study, a concentrated suspension of the predatory bacterium was added to a cyanobacterial culture. After incubation, samples were examined under the microscope and cyanobacterial lysis was observed. Predation was confirmed using a plaque formation technique for the estimation of cyanophages [100]. In the second study, the incorporation of biomass carbon into a soil microbial food web was investigated. For this purpose, agricultural soil was inoculated with ^{13}C-labeled *E. coli*, which was supposed to serve as prey for naturally present micropredators. Preliminary data revealed that soil micropredators were able to utilize ^{13}C-labeled *E. coli*, with 16S rRNA sequencing revealing members of the *Lysobacter* genus among the predators [101]. The most recently published study observed lytic effect of *Lysobacter* sp. SB-K88 against cystospores of the plant pathogen *A. cochlioides* [102]. A

mixture of bacterial cells and fungal cytospores was monitored under a microscope, which showed that after a few hours the cytospores stopped moving and *Lysobacter* cells aggregated around the dead or the dead cystospores or lysed residues. In this study, the observed behavior of *Lysobacter* sp. was attributed to wolfpack predation.

In stark contrast, many studies are focused on evaluating the capacity of this genus to produce antimicrobial natural products by measuring inhibition zones of different bacterial strains and fungi overlaid with cell-free *Lysobacter* culture supernatants [103] or killed *Lysobacter* cells [104]. Indeed, *Lysobacter* strains were found to be a rich source of antibiotics [92]. Moreover, the lytic effect of extracellular *Lysobacter* enzymes on Gram-negative bacteria was investigated [105]. However, none of these studies confirmed that the lytic enzymes and/or toxic compounds from *Lysobacter* spp. are linked to the predatory activity. In summary, there is little experimental data on the predatory activity of *Lysobacter* bacteria or evidence for a predatory strategy that is similar to that of myxobacteria [39, 50, 106]. The hunting behavior of *Lysobacter* is poorly understood and further exploration is required in order to verify a possible correlation between secondary metabolism and predation.

1.7 The genus *Cupriavidus*

The genus *Cupriavidus* was introduced in 1987 by Makkar and Casida [18], after discovering of a soil non-obligate predator *Cupriavidus necator* N-1. At that time *C. necator* has cell morphology similar to already established the genus *Alcaligenes*, both belonging to β-proteobacteria. However, since N-1 exhibits predatory activity, it was into a novel genus and species. Today, the genus *Cupriavidus* belongs to the family *Burkholderiaceae* and after 16S rRNA gene sequences analysis many already described species were reclassified [107] and, at the moment the genus *Cupriavidus* accommodates 15 bacterial species (http://www.bacterio.net/cupriavidus.html). It includes Gram-negative, peritrichously flagellated rod-shaped bacteria which are obligate aerobic organisms, chemoheterotrophic or chemolithotrophic [107]. Many microbiologists know the genus *Cupriavidus* for its resistance to various heavy metals and its metabolic adaptiveness.

The first representative of this genus was the bacterium *C. necator* N-1. The species epithet means “slayer” and is referring to the predatory behavior of this strain and until now it is the only described predatory species in the genus. *C. necator* N-1 is a gram-negative, short rod equipped with 2-10 peritrichous flagella (Fig. 4) [18]. It reproduces by binary fission, with the rods decreasing in size and becoming more coccoid in older cultures. In soil, these rounded forms appeared to be dormant [22] and they elongated only when they started to multiply.

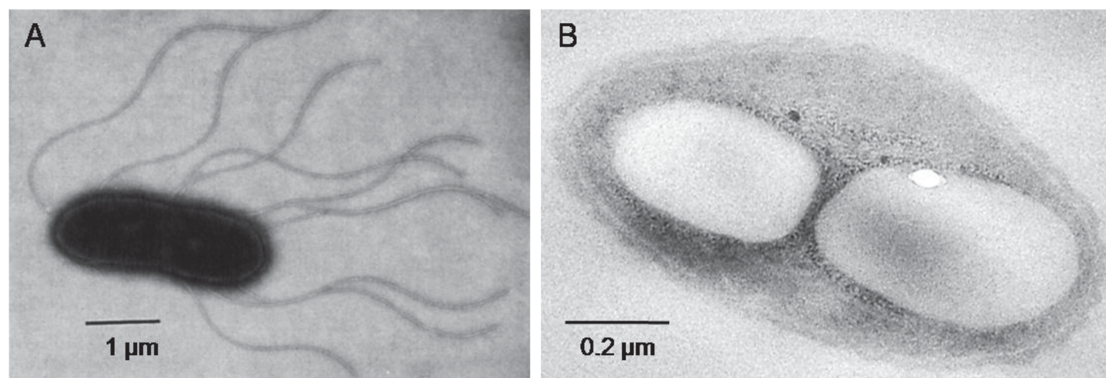


Figure 4. *Cupriavidus necator* N-1 observed by electron microscopy (bar = 1 µm). Photo reprinted from Makkar and Casida, 1987 (A). Transmission electron micrograph of *C. necator* N-1 cultivated at 28°C on H-3 mineral medium for 4 days (B) (photo made by Dr. Nietzsche S. "Jena University Hospital". The lighter grey surfaces are accumulated polyhydroxyalkanoates (PHA) granules).

C. necator N-1 grows aerobically and only limited growth was observed in an anaerobic environment [18]. The organism usually thrives in soil habitats, where it metabolizes organic substances as sources of carbon and energy. The additional specific characteristics of the genus *Cupriavidus* are high copper resistance and initial growth is highly stimulated in the presence of copper [108]. It was speculated that this organism secretes a growth initiation factor (GIF) to sequester copper from the environment. After the initial growth phase, the production of this GIF decreases and its isolation becomes very challenging [108]. It was speculated that the compound is a peptide with a mass larger than 1,500 Da. However, all attempts to fully characterize this molecule failed. Since the copper resistance of tested prey organisms was much weaker compared to *C. necator,* it was proposed that besides promoting growth, this metal chelating small protein may also play a role in predation, however, experimental proof is still missing [108].

1.7.1 Predatory behavior of *Cupriavidus necator* N-1

C. necator N-1 was isolated from soil and found to lyse various Gram-negative and Gram-positive bacteria [18, 109]. N-1 is considered a powerful predator that might be at the top of the hierarchy of bacterial soil predators, capable of preying on a wide range of bacteria including other non-obligate predators such as *Agromyces ramosus* and *Ensifer adhaerens* [22]. Predatory activity of *C. necator* N-1 against other soil bacteria was observed by indirect phage analysis [16, 18]. In this method, the soil from which N-1 was originally isolated was used and it was inoculated with different prey bacteria. The consumption of the latter and the concomitant multiplication of *C. necator* were indirectly assessed via the number of soil bacteriophages specifically attacking the predator that could be retrieved from the fluid percolating the soil. However, in this predatory assay, predator cells were not counted

directly, and they were neither plated nor isolated. Thus, the number of phages might not necessarily reflect the exact number of their host bacteria so the data obtained might not be precise [16]. Nevertheless, using this approach, the authors proposed that N-1 preys on *Agromyces ramosus*, *Arthrohacter globiformis*, *Azotobacter vinelandii*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Ensifer adhaerens*, *Escherichia coli*, *Micrococcus luteus* and *Staphylococcus aureus*. Examples of bacteria not attacked by N-1 in soil are *Agrobacterium tumefaciens*, *Nocardia salmonicolor* and *Salmonella typhosa* [18]. Byrd and coworkers investigated the interaction between *A. ramosus*, a predatory actinomycete, and N-1 in more detail. In case of *A. ramosus* it was postulated that mycelial contact with prey cells induces the release of lytic enzymes. An interaction experiment was set up in a soil column where these two predators were incubated together and monitored over a week. Soil samples were taken every day and stained with crystal violet so that each predator could be counted under the microscope. In the first period of cocultivation, *A. ramosus* mycelium was spread across soil sample and it appeared to deliberately seek out N-1 cells and *C. necator* was lysed at the interaction zone. Around 30% of N-1 population was killed in the first three days of the experiment. However, after the fourth day, the situation dramatically changed and *A. ramosus* was successively destroyed by the surviving *C. necator* cells. Additionally, N-1 proliferated again, which suggested that it was hunting and feeding on *A. ramosus*. Therefore, it seemed that after the attack of *A. ramosus*, N-1 returns the attack in what is known as a counter-attack phenomenon.

2 Scope of the thesis

Predator-prey interactions have a significant impact on the diversity and dynamics of microbial communities which, in turn, affect the cycling of matter as well as nutrient and element fluxes in Earth's ecosystems. Although predation between prokaryotes has been known for decades, there are still many unresolved questions concerning the ecology of predatory bacteria and the mechanisms underlying predatory behavior. The main aim of this study was to unravel the predation strategies of the bacteria *Cupriavidus necator* and *Lysobacter* spp.

- For this purpose, a suitable predation assay had to be established that can be easily and reproducibly conducted under laboratory conditions. Furthermore, the bioassay should provide quantitative information on the killing and consumption of prey organisms.

- Using this assay, factors that trigger and influence the predatory activity of *C. necator* and *Lysobacter* spp. should be identified. This analysis was not confined to the testing of environmental conditions. Potential prey resistance mechanisms were also taken into account.

- Finally, based on the information obtained on factors that influence predation, the sequenced *C. necator* N-1 genome should be analyzed in order to identify genes that are likely to have a role in predation. This required an investigation of the secondary metabolism of *C. necator* N-1 with the focus on identification of molecules able to coordinate copper and analyzing whether elucidated compounds are involved in the predation mechanisms.

3 Overview of Manuscripts

3.1 Manuscripts resulting from the main research project

3.1.1 Manuscript A

Seccareccia Ivana, Kost Christian, Nett Markus. Quantitative analysis of *Lysobacter* predation. *Appl. Environ. Microbiol*. **2015**. 81:7098-7105.

Lysobacter are described as facultative predators that use a feeding strategy similar to that of myxobacteria. Therefore, novel quantitative CFU-based predation assay was established that allowed simultaneously quantification of both predator and prey population. All *Lysobacter* spp. tested were able to feed on other bacteria, although killing efficiencies varied across prey types. Additionally, obtained evidence revealed that *Lysobacter* bacteria hunt exclusively in groups, which is in stark contrast to myxobacterial predation.

Ivana Seccareccia performed the experiments and wrote the manuscript; Dr. Christian Kost assisted in statistical analysis, the design of the experiments and edited the final manuscript. Dr. Markus Nett designed the research project and edited the final manuscript.

3.1.2 Manuscript B

Seccareccia Ivana, Kovács Ákos T., Gallegos-Monterrosa Ramses and Nett Markus. Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*. Manuscript submitted to *Applied and Environmental Microbiol*ogy (manuscript ID: AEM04056-15).

Cupriavidus necator is a predatory soil bacterium that feeds on various Gram-negative and Gram-positive bacteria. However, the mechanisms by which *C. necator* seeks and kills other organisms have not been clarified to date. Therefore, the research aim was to unravel factors that induce and promote predatory behavior of this bacterium. We confirmed that the predatory performance of *C. necator* is correlated with elevated copper concentrations, and that the killing of other prey bacteria is mediated through extracellular factors.

Ivana Seccareccia performed predation assays, analyzed the coincubation of *C. necator* with different *B. subtilis* strains, designed experiments and wrote the manuscript. Dr. Ákos T. Kovács selected *B. subtilis* strains, provided advice on the design of the experiments and

edited the final manuscript. Ramses Gallegos-Monterrosa transformed *B. subtilis* strains and isolated spores from *B. subtilis* strains. Dr. Markus Nett designed the research project and edited the final manuscript.

3.1.3 Manuscript C

Seccareccia Ivana, Schmidt Andre, Hagen Matthias, Kothe Erika and Nett Markus. Identification of new metallothioneins in the bacterium *Cupriavidus necator* N-1. Manuscript in preparation.

Metallothioneins (MTs) are short peptides with high cysteine/histidine content that are able to coordinate metal ions. They play an essential role in detoxification and maintenance of optimal cell function. There are many challenges in the identification of new MTs, owing to their short amino acid sequences with little conservation. While bioinformatic tools can help to identify potential MT candidates, it is important to experimentally verify these predictions. In this study, the genome of *Cupriavidus necator* N-1 was bioinformatically screened for genes encoding ribosomally derived peptides with a high cysteine or histidine content. After these *in silico* analyses, all identified candidate peptides were heterologously produced and their capacity to bind different metals was evaluated.

Ivana Seccareccia performed all experiments and wrote the manuscript. Andre Schmidt advised on data analysis and edited the final manuscript. Matthias Hagen provided the software for selection of peptides with high cysteine and histidine content. Prof. Dr. Erika Kothe edited the final manuscript. Dr. Markus Nett designed the research project and edited the final manuscript.

3.2 Manuscripts from side projects

3.2.1 Manuscript D

Gurovic Maria Soledad Vela, Müller Sebastian, Domin Nicole, **Seccareccia Ivana**, Nietzsche Sandor, Martin Karin, Nett Markus. *Micromonospora schwarzwaldensis* sp. nov., a producer of telomycin, isolated from soil. **2013**. *Int. J. Syst. Evol. Microbiol*. 63:3812-3817.

A Gram-positive, spore-forming actinomycete strain (HKI0641T) was isolated from a soil sample collected in the Black Forest, Germany. During screening for antimicrobial natural products this bacterium was identified as a producer of the antibiotic telomycin. Morphological characteristics and chemotaxonomic data indicated that the strain belonged to the genus *Micromonospora*. To determine the taxonomic positioning of strain HKI0641T, we computed a binary tanglegram of two rooted phylogenetic trees that were based upon 16S

rRNA and *gyrB* gene sequences, respectively. A novel species of the genus *Micromonospora*, with the name *Micromonospora schwarzwaldensis* sp. nov., is proposed.

Dr. Maria Soledad Vela Gurovic performed 16S rRNA and *gyrB* gene sequence analyses, conducted the antimicrobial screening, isolated telomycin and wrote the manuscript. Dr. Sebastian Müller computed the phylogenetic trees. Nicole Domin and Ivana Seccareccia performed additional 16S rRNA and *gyrB* gene sequence analyses. Sandor Nietzsche prepared scanning electron micrographs of the bacterium. Martin Karin performed morphological and chemotaxonomic analyses and edited the final manuscript. Dr. Markus Nett analyzed NMR data, designed the research project and edited the final manuscript.

3.2.2 Manuscript E

Kurth Colette, Schieferdecker Sebastian, Athanasopoulou Kalliopi, **Seccareccia Ivana**, and Nett Markus. Variochelins, novel lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining. Manuscript submitted to *Journal of Natural Products* (manuscript ID: np-2015-009329).

In this paper, the genomics-driven discovery and characterization of variochelins, lipopeptide siderophores from the bacterium *Variovorax boronicumulans* was described. Variochelins are different from most other lipopeptide siderophores in that their biosynthesis involves a polyketide synthase. Additionally, it was demonstrated that the ferric iron complex of variochelin A possesses photoreactive properties and the MS-derived structure of a degradation product that emerges upon light exposure were presented.

Colette Kurth annotated the variochelin gene cluster, isolated the variochelins, conducted chemical analyses as well as photoreactivity experiments and wrote the manuscript. Sebastian Schieferdecker conducted the stereochemical analyses. Kalliopi Athanasopoulou contributed to the isolation of variochelins. Ivana Seccareccia performed siderophore screening studies. Dr. Markus Nett carried out bioinformatic analyses, conducted the structure elucidation, designed the research project and edited the final manuscript.

PD Dr. Markus Nett

4 Published Results

4.1 Manuscript A: Quantitative analysis of *Lysobacter* predation

Seccareccia Ivana, Kost Christian, Nett Markus. Quantitative analysis of *Lysobacter* predation. *Appl. Environ. Microbiol*. **2015**. 81:7098-7105

AEM
Journals.ASM.org

Quantitative Analysis of *Lysobacter* Predation

Ivana Seccareccia,[a] Christian Kost,[b] Markus Nett[a]

Secondary Metabolism of Predatory Bacteria Junior Research Group, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute, Jena, Germany[a]; Experimental Ecology and Evolution Research Group, Max Planck Institute for Chemical Ecology, Jena, Germany[b]

Bacteria of the genus *Lysobacter* are considered to be facultative predators that use a feeding strategy similar to that of myxobacteria. Experimental data supporting this assumption, however, are scarce. Therefore, the predatory activities of three *Lysobacter* species were tested in the prey spot plate assay and in the lawn predation assay, which are commonly used to analyze myxobacterial predation. Surprisingly, only one of the tested *Lysobacter* species showed predatory behavior in the two assays. This result suggested that not all *Lysobacter* strains are predatory or, alternatively, that the assays were not appropriate for determining the predatory potential of this bacterial group. To differentiate between the two scenarios, predation was tested in a CFU-based bioassay. For this purpose, defined numbers of *Lysobacter* cells were mixed together with potential prey bacteria featuring phenotypic markers, such as distinctive pigmentation or antibiotic resistance. After 24 h, cocultivated cells were streaked out on agar plates and sizes of bacterial populations were individually determined by counting the respective colonies. Using the CFU-based predation assay, we observed that *Lysobacter* spp. strongly antagonized other bacteria under nutrient-deficient conditions. Simultaneously, the *Lysobacter* population was increasing, which together with the killing of the cocultured bacteria indicated predation. Variation of the predator/prey ratio revealed that all three *Lysobacter* species tested needed to outnumber their prey for efficient predation, suggesting that they exclusively practiced group predation. In summary, the CFU-based predation assay not only enabled the quantification of prey killing and consumption by *Lysobacter* spp. but also provided insights into their mode of predation.

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

In nature, microorganisms do not occur as isolated living entities. Instead, they exist in complex communities of multiple species that interact with each other (1). While some of these interactions are beneficial for the partners involved, others tend to be parasitic or even competitive (2). A commonly encountered negative interaction among microorganisms is predation, which is considered an important evolutionary force that shapes microbial biodiversity (3). Predatory behavior can be observed in many taxonomically unrelated groups of bacteria, encompassing both obligate and facultative predators (4–6). The latter are capable of preying on other organisms but can also survive by utilizing nonliving nutrient sources (6). Predatory bacteria show an enormous diversity of feeding strategies (7). At present, four basic predatory lifestyles are known, i.e., "wolf pack" or group predation, epibiotic attachment, direct cytoplasmic invasion, and periplasmic invasion (8). It is, however, difficult to categorize predatory bacteria based on their hunting behaviors, since clear distinctions between the aforementioned strategies are often not possible.

Among the most thoroughly investigated facultative predators are myxobacteria. Although they are individually capable of penetrating and digesting prey microcolonies (9), myxobacteria are generally assumed to hunt collectively (7). Group predation requires a quorum of cells as well as gliding motility, which allows myxobacteria to actively seek their prey (10, 11). Another commonly observed feature is the concerted release of cell wall-degrading enzymes and antibiotics (12–15). Few bioassays are available for investigating predatory interactions among bacteria. Myxobacterial predation is typically analyzed on agar plates. For this purpose, myxobacteria are inoculated onto a spot or lawn of prey organisms in order to monitor the emergence of lysis or swarming (16–19). A variation of this methodology involves the recovery and enumeration of surviving prey cells after transferring to agar media, which exclusively suppress myxobacterial growth (13, 20).

Bacteria of the genus *Lysobacter* share many properties with myxobacteria. Both groups feature a high G+C content in their DNA, the ability to glide on solid surfaces, and the secretion of lytic enzymes (10, 12, 21, 22). Prior to the introduction of phylogenetic markers, these commonalities caused some confusion concerning the taxonomic placement of isolates with the aforementioned features. As a consequence, many *Lysobacter* strains were originally falsely classified as myxobacteria (22). This also led to some ambiguities with regard to predatory behavior of the two bacterial groups. In general, *Lysobacter* spp. are assumed to practice group predation (4, 8), even though there was also evidence for epibiotic feeding (23, 24). In a study by Shilo (23), a concentrated suspension of a *Lysobacter* sp. (originally assigned as *Myxobacter* FP-1) was added to a cyanobacterial culture. After incubation, the mixed cultures were examined under the microscope, and lysis of cyanobacteria was observed after attachment of *Lysobacter*. In contrast, the hypothesis of wolf pack feeding was sup-

Received 28 May 2015 **Accepted** 28 July 2015
Accepted manuscript posted online 31 July 2015
Citation Seccareccia I, Kost C, Nett M. 2015. Quantitative analysis of *Lysobacter* predation. Appl Environ Microbiol 81:7098–7105. doi:10.1128/AEM.01781-15.
Editor: J. L. Schottel
Address correspondence to Markus Nett, markus.nett@hki-jena.de.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01781-15.
Copyright © 2015, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.01781-15

ported mainly by the observation that many *Lysobacter* strains are potent antibiotic producers (25).

Since its discovery by Christensen and Cook (21), the genus *Lysobacter* has been expanded from 4 to 30 species (www.bacterio.net/lysobacter.html). Several studies of the newly discovered species focused on the release of lytic enzymes and the production of antimicrobial compounds (see, e.g., references 26, 27, and 28) without providing direct evidence for their involvement in predatory interactions. To the best of our knowledge, there is only one recent study which investigated bacterial predation of *Lysobacter*. Lueders et al. quantified the incorporation of biomass carbon into a soil microbial food web (29). For this purpose, agricultural soil was inoculated with ^{13}C-labeled *Escherichia coli*, which was expected to serve as prey for predatory bacteria. Preliminary data confirmed this assumption, and 16S rRNA sequencing indicated that the predators included bacteria of the genus *Lysobacter* (29).

Taken together, experimental data on the predatory activity of *Lysobacter* spp. or evidence for their predatory strategy are scarce. To fill this gap, this study aimed at evaluating the predatory potentials of three different species from this genus. The CFU-based assay confirmed that all *Lysobacter* spp. tested were able to feed on other bacteria, although killing efficiencies varied across prey types. Additionally, we obtained evidence that *Lysobacter* bacteria hunt exclusively in groups, which is in stark contrast to myxobacterial predation.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Since predatory performance is known to be strongly affected by the type of prey (19), nine taxonomically distinct strains were chosen as prey bacteria. *Agrobacterium tumefaciens* and *Ralstonia solanacearum* are well-known soilborne plant pathogens (30), and their potential eradication by a predatory bacterium could be of agricultural interest. The actinobacterium *Rhodococcus rhodochrous* is frequently used as a soil inoculant (31). Moreover, its close relationship to *Rhodococcus fascians* made it an interesting model organism to evaluate potential effects of *Lysobacter* spp. against a Gram-positive plant pathogen (32). *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Chromobacterium pseudoviolaceum* represent widely distributed soil bacteria. *C. pseudoviolaceum* is also known to produce a violet pigment (33), which allows easy identification on agar plates. *Escherichia coli*, although not being a typical soil inhabitant, was selected because of its common use in predation assays (13, 16, 19). Likewise, *Micrococcus luteus* and *Lactococcus lactis* had been reported as suitable prey organisms before (6, 34). Strains used in this study were obtained from the German Strain Collection of Microorganisms and Cell Cultures (DSMZ) or the Jena Microbial Resource Collection (JMRC). *Ralstonia solanacearum* GMI1000 was kindly provided by C. Allen (Department of Plant Pathology, University of Wisconsin—Madison, USA). Strain numbers and cultivation conditions are provided in Table 1.

TABLE 1 Bacterial strains and cultivation conditions used

Species	Strain	Growth medium[a]	Growth temp (°C)
Predators			
Lysobacter capsici	DSM 19286	LB	30
Lysobacter enzymogenes	DSM 2043	LB	30
Lysobacter oryzae	DSM 21044	LB	30
Myxococcus fulvus	ST035975	MD1	30
Prey			
Agrobacterium tumefaciens	DSM 5172	LB	30
Bacillus subtilis	DSM 347	LB	30
Chromobacterium pseudoviolaceum	DSM 23279	LB	30
Escherichia coli	DSM 18039	LB	37
Lactococcus lactis	DSM 20069	SM17	30
Micrococcus luteus	DSM 14234	LB	30
Pseudomonas fluorescens	DSM 11532	LB	30
Ralstonia solanacearum	GMI1000	NB	30
Rhodococcus rhodochrous	DSM 43334	LB	30

[a] LB, Luria broth; NB, nutrient broth; SM17, M17 medium (Sigma) with 0.5% sucrose.

Correlation of optical densities with viable cell count data. For every bacterial strain, the statistical relationship between CFU and optical density at 600 nm (OD_{600}) was determined (35). For this, bacteria were grown in the appropriate growth medium until they reached early stationary phase. At this time, cells were harvested by centrifugation (2,400 × *g*, 5 min). The cell pellet was suspended in phosphate-buffered saline (PBS) buffer (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.6). Serial dilutions of these suspensions with defined OD_{600} values were streaked out on a suitable agar medium. Following 4 days of incubation at 30°C, the number of CFU were determined and plotted against the respective optical densities (see Fig. S1 to S3 in the supplemental material).

Generation of antibiotic-resistant prey bacteria. A pJET1.2-derived vector featuring a chloramphenicol resistance gene in its multiple-cloning site was introduced into *E. coli* by electroporation, yielding *E. coli*/pJET1.2-Cm. *A. tumefaciens*, *P. fluorescens*, and *R. solanacearum* were transformed with pBHR1 (Mobitec), generating the respective chloramphenicol-resistant strains. *B. subtilis* and *L. lactis* were transformed with pNZ8048 (36) to endow these bacteria with chloramphenicol resistance. Electrocompetent cells were prepared following previously described protocols (37–39).

Prey spot plate assay. Prey bacteria were grown on LB agar medium at 30°C for 2 days, except for *E. coli*, which was cultured for 1 day at 37°C. The resulting cell lawn was collected with a sterile spatula and suspended in PBS buffer to yield a final concentration of 10^7 cells ml^{-1}. Assay plates consisted of WAT agar (0.1% $CaCl_2 \cdot 2H_2O$, 1.5% agar, pH 7.2) which had been spotted with 150 μl of freshly prepared prey suspensions. Each prey spot was inoculated with a single predator colony (*Lysobacter capsici*, 4.5×10^7 cells ml^{-1}; *Lysobacter oryzae*, 6×10^7 cells ml^{-1}; *Lysobacter enzymogenes*, 1×10^7 cells ml^{-1}; and *Myxococcus fulvus*, 5×10^4 cells ml^{-1}). For this purpose, the predatory bacteria had previously been grown on LB or MD1 agar (0.3% Casitone, 0.7% $CaCl_2 \cdot 2H_2O$, 0.2% $MgSO_4 \cdot 7H_2O$, 1.5% agar) for 5 days at 30°C. The assay plates were incubated at 30°C for 10 days. Lysis of prey spots was monitored during the incubation period. A prey spot without any added predator colony served as a negative control. The experiment was conducted in three biological replicates. The diameter of the lysis area was measured on days 1 and 10.

Lawn predation assay. *R. rhodochrous*, *C. pseudoviolaceum*, *B. subtilis*, and *M. luteus* were cultivated in 5 ml LB medium for 2 days on a rotary shaker (220 rpm) at 30°C, whereas *E. coli* was incubated for 1 day at 37°C. After centrifugation (1,200 × *g*, 4°C, 5 min) and removal of the supernatant, the cell pellet was washed twice and suspended in TPM buffer (1 M 1.0% Tris-HCl, 0.1 M KH_2PO_4, 0.8 M 1.0% $MgSO_4$, pH 7.6) to yield a final concentration of 10^{10} cells ml^{-1}. Five hundred microliters of this suspension was evenly spread on a TPM agar plate (TPM buffer with 1.5% agar). Predatory bacteria were precultured in glass tubes containing 5 ml LB medium at 220 rpm for 2 days, except for *M. fulvus*, which was grown in 25 ml MD1 medium at 150 rpm for 5 days. The prey-covered TPM agar plates were individually spotted with 10 μl of *Lysobacter* and *M. fulvus* cell suspensions, which were adjusted to 5×10^7 cells ml^{-1}. In subsequent experiments, the concentrations of the *Lysobacter* suspensions were increased to 1.2×10^9 cells ml^{-1} for *L. capsici*, 1.6×10^9 cells ml^{-1} for *L. oryzae*, and 1×10^9 cells ml^{-1} for *L. enzymogenes*. As a control, predator suspensions were spotted on TPM agar plates without prey bacteria. The experiment was replicated three times. The diameter of the predator swarm was measured on days 1 and 10.

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

CFU-based predation assay. Glycerol stock cultures of the test bacteria were used to inoculate LB agar plates unless otherwise stated (Table 1). Agar cultures were incubated at 30°C until the appearance of first colonies. From every predator culture, six colonies were randomly selected and subcultured in glass tubes containing 5 ml LB medium at 220 rpm for 2 days, while *M. fulvus* was cultured in 25 ml MD1 medium at 150 rpm for 5 days. In parallel, a prey colony was selected and individually cultured in 10 ml of appropriate medium at 220 rpm for 2 days. After cultivation, 2 ml of each bacterial culture was harvested and centrifuged (1,200 $\times$ *g*, 4°C, 5 min). The supernatant was removed, and the cell pellet was washed three times with 2 ml of PBS buffer and then resuspended in 1.6 ml of nutrient-free PBS buffer. From these suspensions, 370-μl aliquots of prey (cell concentration adjusted to 1×10^6 cells ml^{-1}) and predator (adjusted to 1×10^8 cells ml^{-1}) were mixed in a 2-ml tube. The predator control sample contained 370 μl of predator cells and the same volume of PBS buffer, and the prey control sample contained 370 μl of prey mixed with 370 μl of PBS buffer. Control experiments included only monocultures of predator or prey. Every experiment was replicated six times. All cultures were incubated at 30°C and 220 rpm for 24 h. After cultivation, serial dilutions of cocultures and monocultures ranging from 10^{-3} to 10^{-5} were prepared by mixing with PBS buffer and were individually spread on nutrient-rich agar plates (Table 1). The CFU number was determined (see Fig. S4 in the supplemental material).

When using antibiotic resistance as a selection marker, prey bacteria harboring resistance plasmids were pregrown in medium supplemented with chloramphenicol (25 μg ml^{-1}). The antibiotic was removed by washing with PBS buffer prior to the addition of the predator suspension. Cocultivation of predator and prey cells was carried out without any antibiotics added. Control experiments confirmed that no significant loss of resistance plasmids occurred during this period (see Fig. S5 in the supplemental material). After 24 h, cocultures were spread either on nutrient-rich agar containing 25 μg ml^{-1} chloramphenicol for counting the prey population or on LB agar supplemented with 50 μg ml^{-1} kanamycin for quantifying the number of *Lysobacter* colonies. After incubation at 30°C for 3 to 5 days, prey and predatory colonies were counted and compared to the numbers of colonies in the control plates. Every experiment was repeated two times.

Frequency dependence of predatory efficiency. To determine whether the ability of *Lysobacter* spp. to effectively lyse its prey depends on the predator/prey ratio (PPR), the CFU-based assay was conducted by varying the initial ratio between predator and prey. For this, the number of prey cells (i.e., *B. subtilis*) was held constant (2×10^7 cells ml^{-1}), while the number of predator cells (i.e., *Lysobacter* spp.) was varied ranging from 2×10^7 to 2×10^{10} cells ml^{-1}.

Contact dependence of predatory behavior. *Lysobacter* strains were grown in LB medium to an OD_{600} of 4. After centrifugation (2,400 $\times$ *g*, 5 min), the cell pellet was washed with PBS buffer and directly mixed with the prey bacterium *B. subtilis* as described previously. Alternatively, the recovered *Lysobacter* cells were propagated in PBS buffer for 24 h to mimic starvation conditions. Supernatants of starved (PBS) and nonstarved (LB) cultures were filter sterilized and diluted according to the predator cell suspension. Aliquots (370 μl) of these filtrates were mixed with 370 μl of prey suspension (adjusted to 1×10^6 cells ml^{-1}). Control experiments included *B. subtilis* suspensions treated with 370 μl of LB medium or PBS buffer. All cocultures and monocultures of *B. subtilis* were incubated at 30°C and 220 rpm for 24 h. After cultivation, serial dilutions ranging from 10^{-3} to 10^{-5} were prepared by mixing with PBS buffer and were individually spread on nutrient-rich agar plates. The CFU number was determined.

Evaluation of the predation efficiency. To quantify predatory activity, both the killing efficiency (*e*) and the utilization of prey (*u*) were determined for each experiment. The two parameters were calculated using the following formulas: e = (CFU of control prey − CFU of surviving prey)/CFU of control prey $\times$ 100 and u = (CFU of predator with prey − CFU of control predator)/CFU of control predator $\times$ 100.

Statistical analyses. The data obtained from the prey spot plate assay and lawn predation assay were analyzed using a paired-sample *t* test and nonparametric statistical tests. The Mann-Whitney U test and Wilcoxon median tests were applied to statistically analyze the CFU-based predation assay. All statistical analyses were performed using the SPSS software (version 22.0; IBM, USA).

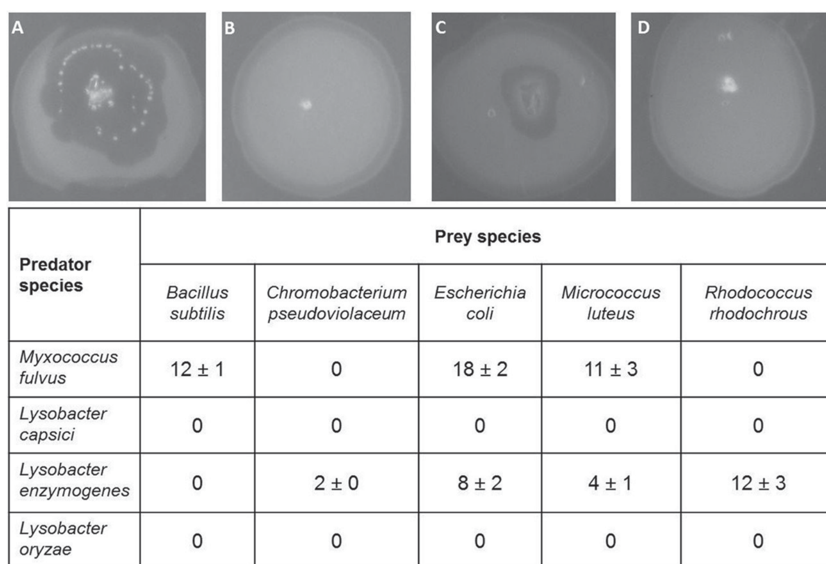
RESULTS

Prey spot plate assay. The predatory activity of three selected *Lysobacter* species was initially investigated using the prey spot plate assay. Since this bioassay was originally developed to isolate myxobacteria (40), *Myxococcus fulvus* was included as a positive control. After 10 days of incubation, the tested *M. fulvus* strain had produced lysis zones within spots of *E. coli*, *B. subtilis*, and *M. luteus*. However, no lysis was observed on plates covered with *C. pseudoviolaceum* and *R. rhodochrous* (Fig. 1). From the three *Lysobacter* strains tested, only *L. enzymogenes* exhibited lytic activity, whereas *L. capsici* and *L. oryzae* appeared to have no effect on any prey organism. *L. enzymogenes* was most active against *R. rhodochrous*. Moderate lytic activity could be observed against *E. coli* and *M. luteus*, while there was weak activity against *C. pseudoviolaceum* and no activity against *B. subtilis*. Although *L. enzymogenes* and *M. fulvus* were both found to attack *E. coli* and *M. luteus*, it appeared that the two prey strains were more susceptible to the myxobacterium. Thus, of the three *Lysobacter* strains tested, only one showed clear signs of predation using this assay.

Lawn predation assay. The predatory performance of myxobacteria is often correlated with their ability to swarm on prey-covered plates (10). Similar to the occurrence of lysis plaques in the prey spot plate assay, the swarming rate of a predator is prey specific (41). Subjecting the same four species of predators to this test indicated that *M. fulvus* exhibited the fastest swarm expansion on *E. coli*, while it was significantly lower on *M. luteus* and *B. subtilis* (Fig. 2). Interestingly, the myxobacterium failed completely to swarm on plates covered with *R. rhodochrous* and *C. pseudoviolaceum*, as indicated by the fact that no significant swarm expansion was observed after 10 days of incubation relative to the first day (paired-sample *t* test, $P < 0.05$ [$n = 3$]) (see Table S1 in the supplemental material). These results suggested that *C. pseudoviolaceum* and *R. rhodochrous* are unsuitable as prey for *M. fulvus*. Since bacteria belonging to the genus *Lysobacter* are considered to have gliding motility (21, 42), we expected them to display swarming behavior in the lawn predation assay, similar to that of *M. fulvus*. Again, however, only *L. enzymogenes* showed some moderate predatory activity. Significant predatory swarming was observed on *R. rhodochrous* and *E. coli* plates, although the effects were less pronounced than in case of *M. fulvus* (Fig. 2). In contrast, *L. capsici* and *L. oryzae* did not exhibit swarming behavior on the selected prey bacteria under the experimental conditions used. Thus, again, only one of the three *Lysobacter* species analyzed showed moderate signs of predation.

CFU-based predation assay. A prerequisite for the simultaneous determination of predator and prey populations from a mixed culture is the ability to phenotypically distinguish both partners. *C. pseudoviolaceum* and *R. rhodochrous* were initially selected as prey bacteria, because they form intensively colored colonies which can be easily differentiated from those of *Lysobacter* spp. To extend the application range of the assay, differences in antibiotic resistance also were used to discriminate predator and prey. Previous antibiotic susceptibility tests had revealed that all tested *Ly*-

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS



Predator species	Prey species				
	Bacillus subtilis	*Chromobacterium pseudoviolaceum*	*Escherichia coli*	*Micrococcus luteus*	*Rhodococcus rhodochrous*
Myxococcus fulvus	12 ± 1	0	18 ± 2	11 ± 3	0
Lysobacter capsici	0	0	0	0	0
Lysobacter enzymogenes	0	2 ± 0	8 ± 2	4 ± 1	12 ± 3
Lysobacter oryzae	0	0	0	0	0

FIG 1 Effect of predators on different prey bacteria as determined by the prey spot plate assay. The table shows the mean (± 95% confidence interval [$n = 3$]) diameter of the lysis zone (in millimeters). Images depict spots of *E. coli* that have been coinoculated with a single colony of *M. fulvus* (A), *L. capsici* (B), *L. enzymogenes* (C), or *L. oryzae* (D) after 10 days of incubation.

sobacter spp. were resistant to kanamycin (up to a concentration of 100 μg ml^{-1}), whereas they shared sensitivity to chloramphenicol (25 μg ml^{-1}). Therefore, chloramphenicol resistance genes were introduced into the kanamycin-sensitive prey bacteria *A. tumefaciens*, *B. subtilis*, *E. coli*, *L. lactis*, *P. fluorescens*, and *R. solanacearum*. Subsequently, the testing was carried out with the phenotypically labeled prey organisms. For evaluating the killing efficiency (e), the number of surviving prey bacteria from cocultures was compared with that from prey monocultures after incubation on LB-chloramphenicol plates. Likewise, LB-kanamycin plates were used to determine the prey utilization (u). The latter parameter quantified *Lysobacter*'s consumption of prey and was calculated by comparing the number of *Lysobacter* CFU when grown in monoculture to the number of *Lysobacter* CFU achieved in coculture with its prey.

In the CFU-based predation assay, *L. capsici* and *L. oryzae* preyed on all Gram-positive bacteria tested, namely, *B. subtilis*, *L. lactis*, and *R. rhodochrous* (Fig. 3A; see Table S2 in the supplemental material). *L. enzymogenes* was not active against *R. rhodochrous*, but it was found to negatively affect populations of *B. subtilis* and *L. lactis*. Besides the species-dependent prey utilization, we also observed quantitative differences in prey consumption. All three *Lysobacter* strains were found to significantly reduce the CFU number of *B. subtilis* and *L. lactis* (Fig. 3A; see Table S2 in the supplemental material). In contrast to *L. capsici* and *L. oryzae*, *L. enzymogenes* did not completely eradicate the *B. subtilis* population. *L. capsici* exhibited a comparatively reduced killing efficiency against *L. lactis* (Mann-Whitney U test, $P < 0.05$ [$n = 6$]) (Fig. 3A). Overall, Gram-negative bacteria appeared to be more resistant toward *Lysobacter* predation than Gram-positive bacteria. The only exception was *C. pseudoviolaceum*, which turned out to be a preferred prey organism for both *L. capsici* and *L. oryzae*. While some weak predatory activity was also observed against *E. coli* (at least in the case of *L. capsici* and *L. oryzae*), the growth of *A. tumefaciens*, *R. solanacearum*, or *P. fluorescens* remained unaffected (Mann-Whitney U test, $P < 0.05$ [$n = 6$]) (Fig. 3A). It is noteworthy that *L. enzymogenes* failed to prey on all tested Gram-negative bacteria. Despite the limited prey range, it became obvious that both Gram-positive and Gram-negative bacteria can be killed by *Lysobacter* spp., although e and u values differed significantly depending on the prey species tested.

Prey utilization was assessed for prey strains that were susceptible to *Lysobacter* predation. Consistent with the observed killing efficiencies against Gram-positive bacteria, the populations of *L. capsici* and *L. oryzae* increased significantly in the presence of *B. subtilis*, *R. rhodochrous*, and *L. lactis* (Fig. 3B; see Table S2 in the supplemental material). Growth of *L. enzymogenes*, however, increased only when *L. lactis* was provided as a food source. The prey utilization of *C. pseudoviolaceum* by *L. capsici* was 23.7% ± 0.2% (mean ± 95% confidence interval) and that by *L. oryzae* was 26.6% ± 0.1%, indicating that both species benefitted equally

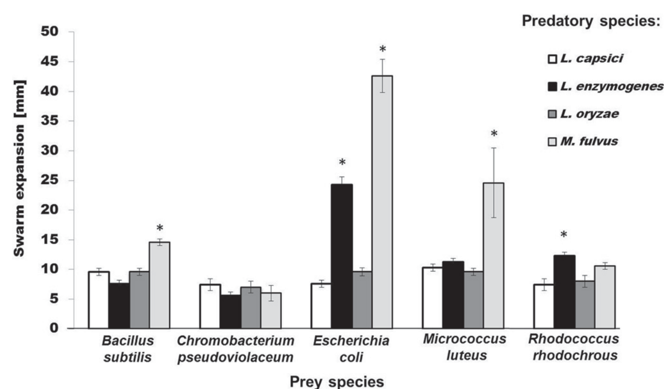


FIG 2 Effect of predators on different prey bacteria as determined by the lawn predation assay. Shown is the mean swarm expansion (± 95% confidence interval [$n = 3$]) (in millimeters) of four species of predatory bacteria. Paired t test: *, $P < 0.05$ between day 1 and day 10. All other comparisons were not significant ($P > 0.05$).

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

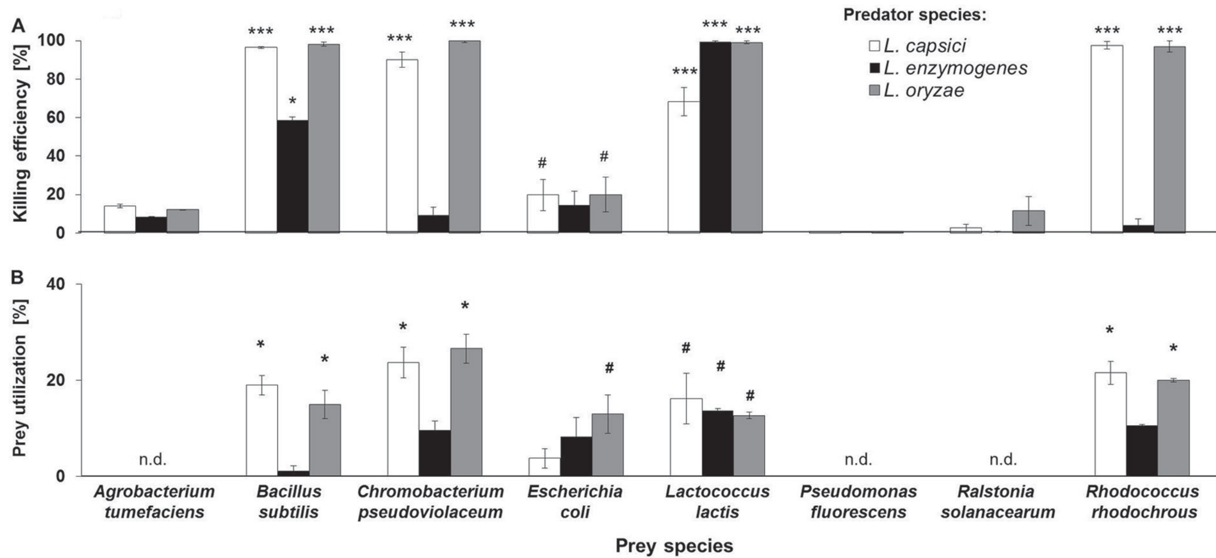


FIG 3 Effect of predators on different prey bacteria and *vice versa* as determined by the CFU-based predation assay. (A) Mean killing efficiency ($\bar{e}$; ± 95% confidence interval) of all three *Lysobacter* spp. tested against different species of prey bacteria (percent). Asterisks denote significant differences between the number of prey CFU of the control group (i.e., monocultures) and samples containing both predator and prey (i.e., cocultures) (Mann-Whitney U test: ***, $P < 0.001$ *, $P < 0.05$; #, $P < 0.07$; df = 2). (B) Mean prey utilization ($\bar{u}$; ± 95% confidence interval) of all three *Lysobacter* spp. tested against different species of prey bacteria (percent). Asterisks denote significant differences in the prey utilization when comparing control groups consisting exclusively of predators with samples containing both predators and prey (Wilcoxon test: *, $P < 0.05$; #, $P < 0.07$). n.d., prey species for which *u* was not determined.

from the presence of *C. pseudoviolaceum* (Wilcoxon test, $P < 0.05$ [$n = 6$]) (Fig. 3B). Similar observations were made when *E. coli* was used as prey. Under these conditions, only the growth of *L. oryzae* increased detectably (Fig. 3B).

For comparative purposes, *Myxococcus fulvus* also was included in this analysis and tested using the CFU-based predation assay. *B. subtilis* and *E. coli* were selected as prey, because they were susceptible to *M. fulvus* predation in both the prey spot predation assay and the lawn predation assay. In addition, *C. pseudoviolaceum* and *R. rhodochrous* were included as prey organisms, since the CFU-based predation assay had already revealed a larger prey spectrum for the tested *Lysobacter* spp. than was initially detected with the prey spot plate assay and the lawn predation assay. Since *M. fulvus* is a slow-growing bacterium, prey reduction was assessed after 24 h and 48 h of cocultivation. Surprisingly, *M. fulvus* completely failed to reduce the number of prey relative to that in control cultures (see Fig. S6 in the supplemental material).

Frequency dependence of predatory efficiency. Outnumbering prey is an important feature of wolf pack predation (8). To investigate whether the predatory performance of *Lysobacter* depended on the predator/prey ratio (PPR), the CFU-based predation assay was repeated, but this time the number of prey cells (i.e., *B. subtilis*) was held constant, while initial numbers of predator cells were varied. When predator populations outnumbered the prey by 1,000:1 or 100:1, killing efficiency was very high, and an almost complete eradication of prey populations was observed (Fig. 4). Lowering the PPR to 10:1, however, led to a loss of killing efficiency for *L. oryzae* and *L. enzymogenes*, whereas *L. capsici* retained effective predation ($e = 93.8\% \pm 4.0\%$). At a PPR of 1:1, no significant prey reduction was detectable for any of the three *Lysobacter* spp. tested. Thus, this experiment confirmed that the predatory success of the tested predators critically depended on their frequency relative to the number of prey bacteria.

Contact dependence of predatory behavior. Finally, we set out to clarify whether the predatory activity of *Lysobacter* depends on physical proximity to its prey or whether it is mediated exclusively by extracellular factors, such as lytic enzymes or antibiotics. For this purpose, the killing of *B. subtilis* by *Lysobacter* spp. was compared to the effect of cell-free *Lysobacter* culture supernatants. Surprisingly, none of the tested supernatants affected the growth of *B. subtilis* (Fig. 5). The outcome of this experiment was the same irrespective of whether the supernatants originated from *Lysobacter* cultures grown under nutrient-rich or nutrient-deficient conditions. This suggests that cell contact is likely important for the lysis of prey by *Lysobacter* spp., thus corroborating previous observations (23, 43). Nevertheless, this conclusion does not exclude an involvement of degradative enzymes or antibiotics, whose production might be induced by the presence of prey.

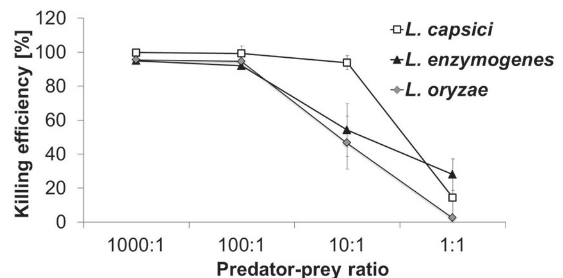


FIG 4 Frequency dependence of predatory efficiency. Shown are different predator/prey ratios versus the mean killing efficiency ($\bar{e}$; ± 95% confidence interval) of each predatory species. The number of *B. subtilis* CFU was held constant in all experiments.

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

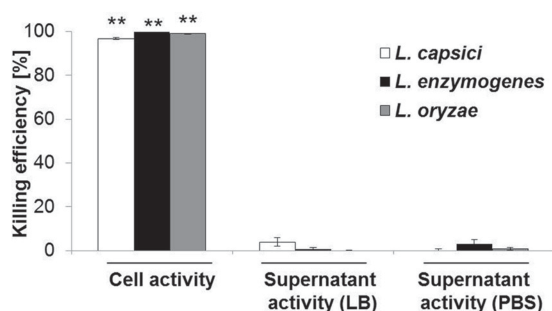


FIG 5 Contact dependence of predatory behavior. The killing of the prey bacterium *B. subtilis* by *Lysobacter* cells and culture supernatants was analyzed in the CFU-based predation assay. Asterisks denote significant differences between the number of prey CFU of the control group and samples containing both predator and prey (Mann-Whitney U test: **, $P < 0.01$; df = 2).

DISCUSSION

Although predatory behaviors pervade the entire bacterial realm, research in this area has focused on few taxonomic groups. In this context, especially facultative predators such as myxobacteria (7) as well as the obligate predator *Bdellovibrio bacteriovorus* (4, 5) have received the main attention. Assuming that *Lysobacter* spp. use a feeding strategy similar to that of myxobacteria (22), the predatory activity of three selected *Lysobacter* spp. was evaluated using two predation assays that had been previously established for myxobacteria (16, 17). In these assays, however, neither *L. capsici* nor *L. oryzae* displayed any lytic activity against the prey species tested. Also, *L. enzymogenes* showed only a relatively weak predatory activity compared to that of the myxobacterium *M. fulvus*. These results indicated that either (i) a different assay was needed for assessing their predatory activity or (ii) *L. capsici* and *L. oryzae* are not predatory bacteria.

To differentiate between these two possibilities, the selected *Lysobacter* strains were subjected to a CFU-based predation assay. Similar assays have been previously described to analyze the obligate bacterial predator *Bdellovibrio* by counting the numbers of plaques on plates after cocultivation with prey bacteria in liquid medium (44) as well as for quantifying the predatory efficiency of nonobligate myxobacterial predators (20). The initial setup of this assay required a cocultivation with prey bacteria that could be phenotypically distinguished from the predators using, for example, the pigmentation of their colonies. Subsequently, antibiotic resistance was used as an alternative labeling strategy, significantly extending the number of potential prey bacteria. Aside from measuring the killing of prey, the CFU-based predation assay enabled the simultaneous monitoring of growth of predators and prey. In this way, it was possible to exclude the possibility that the decline of prey resulted from competitive interactions (i.e., killing without feeding).

The CFU-based predation assay confirmed that the selected *Lysobacter* spp. were effectively feeding on *C. pseudoviolaceum*, *R. rhodochrous*, *B. subtilis*, and, to a reduced extent, also *E. coli* and *L. lactis* during 24 h of cocultivation. It is thus evident that *Lysobacter* can prey on both Gram-positive and Gram-negative bacteria. Since *Lysobacter* spp. often exhibit inhibitory effects against phytopathogenic fungi, they represent promising biocontrol agents (45, 46). It is noteworthy, however, that the three *Lysobacter* strains used in this study did not show any activity against the two phytopathogenic bacteria *A. tumefaciens* and *R. solanacearum* under the experimental conditions tested.

Further analyses revealed that to achieve high killing efficiencies, all *Lysobacter* strains required a numerical superiority over their prey, although they differed in their optimal PPRs. Overall, this suggested that the *Lysobacter* strains were restricted to group predation. This means that individual *Lysobacter* cells must work together to successfully kill their prey, which could be mediated, for instance, by the cooperative secretion of hydrolytic enzymes or antibiotics. Chemical analyses of *Lysobacter* spp. already have illuminated their huge potential for the production of antimicrobial agents (47). Among the antibiotics reported are inhibitors of cell wall biosynthesis, such as cephabacins (48) and tripropeptins (49), as well as a number of compounds which target the bacterial membrane (50–52). The strains used in this study are not yet known as antibiotic producers, although the biosynthesis of such compounds seems likely in light of previous investigations (26). In contrast to the case for myxobacteria (13), however, a clear causal link between antibiotic production and predation is still missing for *Lysobacter* spp.

In the CFU-based predation assay, the number of *Lysobacter* cells was more than 10 times higher than that of prey populations. Furthermore, both predator and prey were continuously mixed during the 24 h of cocultivation, contributing to a homogeneous distribution of diffusible lytic factors. On the basis of these findings, we hypothesize that the quorum of *Lysobacter* cells used was likely below the critical threshold in the prey spot plate and the lawn predation assays, and therefore predatory behavior was not observed. This result is consistent with earlier studies which showed that *L. enzymogenes* was unable to lyse cyanobacteria when the predator inoculum was less than 10^6 cells ml^{-1} (4). In case of *Myxococcus*, however, a much smaller predator concentration (i.e., a predator/prey ratio of 1:1) was sufficient to induce prey lysis (53). This finding is further corroborated by the results of the prey spot plate assay, in which *M. fulvus* was more efficient in lysing the prey organisms than *L. enzymogenes* despite a smaller initial inoculum (Fig. 1).

Some studies suggested that myxobacteria are single-cell hunters rather than wolf pack predators and that close proximity to the corresponding prey cells might be essential for them to penetrate and lyse prey colonies (7, 9). The *M. fulvus* strain tested here failed to exhibit predatory activity in the CFU-based predation assay. A possible explanation could be that myxobacterial cells do not just require close proximity to their prey but instead must establish physical contact with their prey for an extended period to promote lysis of prey cells. This condition seems to preclude effective lysis in the CFU-based predation assay, as the shaking of the liquid cocultures likely prevented effective predation.

L. capsici and *L. oryzae* exhibited no swarming behavior in this study, thereby limiting the use of the lawn predation and prey spot plate assays to analyze the predatory behavior of these bacteria. Obviously, *Lysobacter* spp. and myxobacteria do not show the same predation behavior. Nonetheless, further experiments are necessary to fully understand the predation mechanism used by these bacteria and to clarify the role of antibiotics in their predatory interactions. We expect the described CFU-based predation assay to facilitate studies on additional previously neglected predatory bacteria and assist in the quantitative evaluation of their predatory behavior.

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

ACKNOWLEDGMENTS

Research in the Secondary Metabolism of Predatory Bacteria and Experimental Ecology and Evolution research groups is supported by the DFG-funded graduate school Jena School for Microbial Communication (JSMC). Funding by the Volkswagen Foundation to Christian Kost is gratefully acknowledged.

We thank Franziska Müller von Klingspor for technical support, Ákos T. Kovács for providing plasmid pNZ8048, and Hirokazu Kage for providing *R. solanacearum*/pBHR1. Furthermore, we thank the three unnamed reviewers for their valuable input on improving the manuscript.

REFERENCES

1. **Faust K, Raes J.** 2012. Microbial interactions: from networks to models. Nat Rev Microbiol **10:**538–550. http://dx.doi.org/10.1038/nrmicro2832.
2. **Lidicker WZ, Jr.** 1979. A clarification of interactions in ecological systems. Bioscience **29:**475–477. http://dx.doi.org/10.2307/1307540.
3. **Abrams PA.** 2000. The evolution of predator-prey interactions: theory and evidence. Annu Rev Ecol Syst **31:**79–105. http://dx.doi.org/10.1146/annurev.ecolsys.31.1.79.
4. **Jurkevitch E, Davidov Y.** 2007. Phylogenetic diversity and evolution of predatory prokaryotes, p 1–56. *In* Jurkevitch E (ed), Predatory prokaryotes—biology, ecology, and evolution. Springer, Heidelberg, Germany.
5. **Davidov Y, Jurkevitch E.** 2004. Diversity and evolution of *Bdellovibrio*-and-like organisms (BALOs), reclassification of *Bacteriovorax starrii* as *Peredibacterstarrii* gen. nov., comb. nov., and description of the *Bacteriovorax-Peredibacter* clade as *Bacteriovoracaceae* fam. nov. Int J Syst Evol Microbiol **54:**1439–1452. http://dx.doi.org/10.1099/ijs.0.02978-0.
6. **Casida LE.** 1988. Nonobligate bacterial predation of bacteria in soil. Microb Ecol **15:**1–8. http://dx.doi.org/10.1007/BF02012948.
7. **Berleman JE, Kirby JR.** 2009. Deciphering the hunting strategy of a bacterial wolfpack. FEMS Microbiol Rev **33:**942–957. http://dx.doi.org/10.1111/j.1574-6976.2009.00185.x.
8. **Martin MO.** 2002. Predatory prokaryotes: an emerging research opportunity. J Mol Microbiol Biotechnol **4:**467–477.
9. **McBride MJ, Zusman DR.** 1996. Behavioral analysis of single cells of *Myxococcus xanthus* in response to prey cells of *Escherichia coli*. FEMS Microbiol Lett **137:**227–231. http://dx.doi.org/10.1111/j.1574-6968.1996.tb08110.x.
10. **Kaiser D.** 2003. Coupling cell movement to multicellular development in myxobacteria. Nat Rev Microbiol **1:**45–54. http://dx.doi.org/10.1038/nrmicro733.
11. **Hillesland KL, Lenski RE, Velicer GJ.** 2007. Ecological variables affecting predatory success in *Myxococcus xanthus*. Microb Ecol **53:**571–578. http://dx.doi.org/10.1007/s00248-006-9111-3.
12. **Rosenberg E, Varon M.** 1984. Antibiotics and lytic enzymes, p 109–125. *In* Rosenberg E (ed), Myxobacteria: development and cell interactions. Springer, New York, NY.
13. **Xiao Y, Wei X, Ebright R, Wall D.** 2011. Antibiotic production by *Myxobacteria* plays a role in predation. J Bacteriol **193:**4626–4633. http://dx.doi.org/10.1128/JB.05052-11.
14. **Reichenbach H, Höfle G.** 1993. Biologically active secondary metabolites from myxobacteria. Biotech Adv **11:**219–277. http://dx.doi.org/10.1016/0734-9750(93)90042-L.
15. **Schieferdecker S, König S, Weigel C, Dahse HM, Werz O, Nett M.** 2014. Structure and biosynthetic assembly of gulmirecins, macrolide antibiotics from the predatory bacterium *Pyxidicoccus fallax*. Chem Eur J **20:**15933–15940. http://dx.doi.org/10.1002/chem.201404291.
16. **Pham VD, Shebelut CW, Diodati ME, Bull CT, Singer M.** 2005. Mutations affecting predation ability of the soil bacterium *Myxococcus xanthus*. Microbiology **151:**1865–1874. http://dx.doi.org/10.1099/mic.0.27824-0.
17. **Berleman JE, Chumley T, Cheung P, Kirby JR.** 2006. Rippling is a predatory behavior in *Myxococcus xanthus*. J Bacteriol **188:**5888–5895. http://dx.doi.org/10.1128/JB.00559-06.
18. **Berleman JE, Kirby JR.** 2007. Multicellular development in *Myxococcus xanthus* is stimulated by predator-prey interactions. J Bacteriol **189:**5675–5682. http://dx.doi.org/10.1128/JB.00544-07.
19. **Morgan AD, MacLean RC, Hillesland KL, Velicer GJ.** 2010. Comparative analysis of *Myxococcus* predation on soil bacteria. Appl Environ Microbiol **76:**6920–6927. http://dx.doi.org/10.1128/AEM.00414-10.
20. **Müller S, Strack SN, Hoefler BC, Straight PD, Kearns DB, Kirby JR.** 2014. Bacillaene and sporulation protect *Bacillus subtilis* from predation by *Myxococcus xanthus*. Appl Environ Microbiol **80:**5603–5610. http://dx.doi.org/10.1128/AEM.01621-14.
21. **Christensen P, Cook FD.** 1978. *Lysobacter*, a new genus of nonfruiting, gliding bacteria with a high base ratio. Int J Syst Bacteriol **28:**367–393. http://dx.doi.org/10.1099/00207713-28-3-367.
22. **Reichenbach H.** 2006. The genus *Lysobacter*, p 939–957. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (ed), The prokaryotes: an evolving electronic resource for the microbiological community. Springer, Berlin, Germany.
23. **Shilo M.** 1970. Lysis of blue-green algae by myxobacter. J Bacteriol **104:**453–461.
24. **Daft MJ, Stewart WDP.** 1971. Bacterial pathogens of freshwater blue-green algae. New Phytol **70:**819–829. http://dx.doi.org/10.1111/j.1469-8137.1971.tb02582.x.
25. **Nett M, König GM.** 2007. The chemistry of gliding bacteria. Nat Prod Rep **24:**1245–1261. http://dx.doi.org/10.1039/b612668p.
26. **Park JH, Kim R, Aslam Z, Jeon CO, Chung YR.** 2008. *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. Int J Syst Evol Microbiol **58:**387–392. http://dx.doi.org/10.1099/ijs.0.65290-0.
27. **Puopolo G, Raio A, Zoina A.** 2010. Identification and characterization of *Lysobacter capsici* strain pg4: a new plant health-promoting rhizobacterium. J Plant Pathol **92:**157–164.
28. **Begunova EA, Stepnaya OA, Tsfasman IM, Kulaev IS.** 2004. The effect of the extracellular bacteriolytic enzymes of *Lysobacter* sp. on Gram-negative bacteria. Mikrobiologiia **73:**267–270.
29. **Lueders T, Kindler R, Miltner A, Friedrich MW, Kaestner M.** 2006. Identification of bacterial micropredators distinctively active in a soil microbial food web. Appl Environ Microbiol **72:**5342–5348. http://dx.doi.org/10.1128/AEM.00400-06.
30. **Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA, Toth I, Salmond G, Foster GD.** 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Mol Plant Pathol **13:**614–629. http://dx.doi.org/10.1111/j.1364-3703.2012.00804.x.
31. **Martínková L, Uhnáková B, Pátek M, Nešvera J, Kren V.** 2009. Biodegradation potential of the genus *Rhodococcus*. Environ Int **35:**162–177. http://dx.doi.org/10.1016/j.envint.2008.07.018.
32. **Goethals K, Vereecke D, Jaziri M, Montagu VM, Holsters M.** 2001. Leafy gall formation by *Rhodococcus fascians*. Annu Rev Phytopathol **39:**27–52. http://dx.doi.org/10.1146/annurev.phyto.39.1.27.
33. **Kämpfer P, Busse HJ, Scholz HC.** 2009. *Chromobacterium piscinae* sp. nov. and *Chromobacterium pseudoviolaceum* sp. nov., from environmental samples. Int J Syst Evol Microbiol **59:**2486–2490. http://dx.doi.org/10.1099/ijs.0.008888-0.
34. **Casida LE.** 1980. Bacterial predators of *Micrococcus luteus* in soil. Appl Environ Microbiol **39:**1035–1041.
35. **Madigan MT, Martinko JM.** 2006. Brock biology of microorganisms, 11th ed. Pearson Prentice Hall, Upper Saddle River, NJ.
36. **Kuipers OP, Ruyter PG, Kleerebezem M, Vos WM.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol **64:**15–21. http://dx.doi.org/10.1016/S0168-1656(98)00100-X.
37. **Dower WJ.** 1990. Electroporation of bacteria: a general approach to genetic transformation, p 275–296. *In* Setlow JK (ed), Genetic engineering—principles and methods, vol 12. Plenum Publishing Corp., New York, NY.
38. **Xue G-P, Johnson JS, Dalrymple BP.** 1999. High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Methods **34:**183–191. http://dx.doi.org/10.1016/S0167-7012(98)00087-6.
39. **Holo H, Nes IF.** 1989. High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl Environ Microbiol **55:**3119–3123.
40. **Singh BN.** 1947. Myxobacteria in soils and composts; their distribution, number and lytic action on bacteria. J Gen Microbiol **1:**1–10. http://dx.doi.org/10.1099/00221287-1-1-1.
41. **Mendes-Soares SH, Velicer GJ.** 2013. Decomposing predation: testing for parameters that correlate with predatory performance by a social bacterium. Microb Ecol **65:**415–423. http://dx.doi.org/10.1007/s00248-012-0135-6.
42. **Sullivan RF, Holtman MA, Zylstra GJ, White JF, Kobayashi DY.** 2003. Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA,

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

fatty acid composition and phenotypic characteristics. J Appl Microbiol **94:**1079–1086. http://dx.doi.org/10.1046/j.1365-2672.2003.01932.x.
43. **Jurkevitch E.** 2007. Predatory behaviors in bacteria—diversity and transitions. Microbe **2:**67–73.
44. **Stolp H, Starr MP.** 1963. *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. Antonie Van Leeuwenhoek **29:**217–248. http://dx.doi.org/10.1007/BF02046064.
45. **Li S, Jochum CC, Yu F, Zaleta-Rivera K, Du L, Harris SD, Yuen GY.** 2008. An antibiotic complex from *Lysobacter enzymogenes* strain C3: antimicrobial activity and role in plant disease control. Phytopathology **98:**695–701. http://dx.doi.org/10.1094/PHYTO-98-6-0695.
46. **Puopolo G, Giovannini O, Pertot I.** 2014. *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. Microbiol Res **169:**7–8.
47. **Xie Y, Wright S, Shen Y, Du L.** 2012. Bioactive natural products from *Lysobacter*. Nat Prod Rep **29:**1277–1287. http://dx.doi.org/10.1039/c2np20064c.
48. **Brakhage AA, Al-Abdallah Q, Tüncher A, Spröte P.** 2005. Evolution of β-lactam biosynthesis genes and recruitment of *trans*-acting factors. Phytochemistry **66:**1200–1210. http://dx.doi.org/10.1016/j.phytochem.2005.02.030.
49. **Hashizume H, Sawa Harada S, Igarashi M, Adachi H, Nishimura Nomoto A.** 2011. Tripropeptin C blocks the lipid cycle of cell wall biosynthesis by complex formation with undecaprenyl pyrophosphate. Antimicrob Agents Chemother **55:**3821–3828. http://dx.doi.org/10.1128/AAC.00443-11.
50. **Bonner DP, O'Sullivan J, Tanaka SK, Clark JM, Whitney RR.** 1988. Lysobactin, a novel antibacterial agent produced by *Lysobacter* sp. II. Biological properties. J Antibiot **41:**1745–1751.
51. **Kato A, Nakaya S, Ohashi Y, Hirata H.** 1997. WAP-8294A_2, a novel anti-MRSA antibiotic produced by *Lysobacter* sp. J Am Chem Soc **119:**6680–6681. http://dx.doi.org/10.1021/ja970895o.
52. **Hamamoto H, Urai M, Ishii K, Yasukawa J, Paudel A, Murai M, Kaji T, Kuranaga T, Hamase K, Katsu T, Su J, Adachi T, Uchida R, Tomoda H, Yamada M, Souma M, Kurihara H, Inoue M, Sekimizu K.** 2015. Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. Nat Chem Biol **11:**127–133. http://dx.doi.org/10.1038/nchembio.1710.
53. **Burnham JC, Collart SA, Melvin J.** 1984. Myxococcal predation of the cyanobacterium *Phormidium luridum* in aqueous environments. Arch Microbiol **137:**220–225. http://dx.doi.org/10.1007/BF00414547.

Downloaded from http://aem.asm.org/ on October 6, 2015 by THURINGER UNIVERSITATS

Supplemental Material – **A quantitative analysis of *Lysobacter* predation**

Ivana Seccareccia,[1] Christian Kost,[2] and Markus Nett[1]*

[1]Junior Research Group "Secondary Metabolism of Predatory Bacteria", Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute, 07745 Jena, Germany

[2]Research group "Experimental Ecology and Evolution", Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

*Corresponding author. Mailing address: Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute, Adolf-Reichwein-Str. 23, 07745 Jena, Germany. Phone: 49 3641 532 1297. Fax: 49 3641 532 0811. E-mail: markus.nett@hki-jena.de

Table of Contents

SI-2	Fig. S1	OD_{600}-CFU plots for *Lysobacter capsici*, *L. enzymogenes*, *L. oryzae*, and *Myxococcus fulvus*
SI-3	Fig. S2	OD_{600}-CFU plots for *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Chromobacterium pseudoviolaceum*, and *Escherichia coli*
SI-4	Fig. S3	OD_{600}-CFU plots for *Lactococcus lactis*, *Pseudomonas fluorescens*, *Ralstonia solanacearum*, and *Rhodococcus rhodochrous*
SI-5	Fig. S4	Work flow for the CFU-based predation assay
SI-6	Fig. S5	Plasmid stability analysis
SI-7	Fig. S6	Testing of *Myxococcus fulvus* in the CFU-based predation assay
SI-8	Table S1	Swarm expansion in the lawn predation assay
SI-9	Table S2	Evaluation of predation efficiency

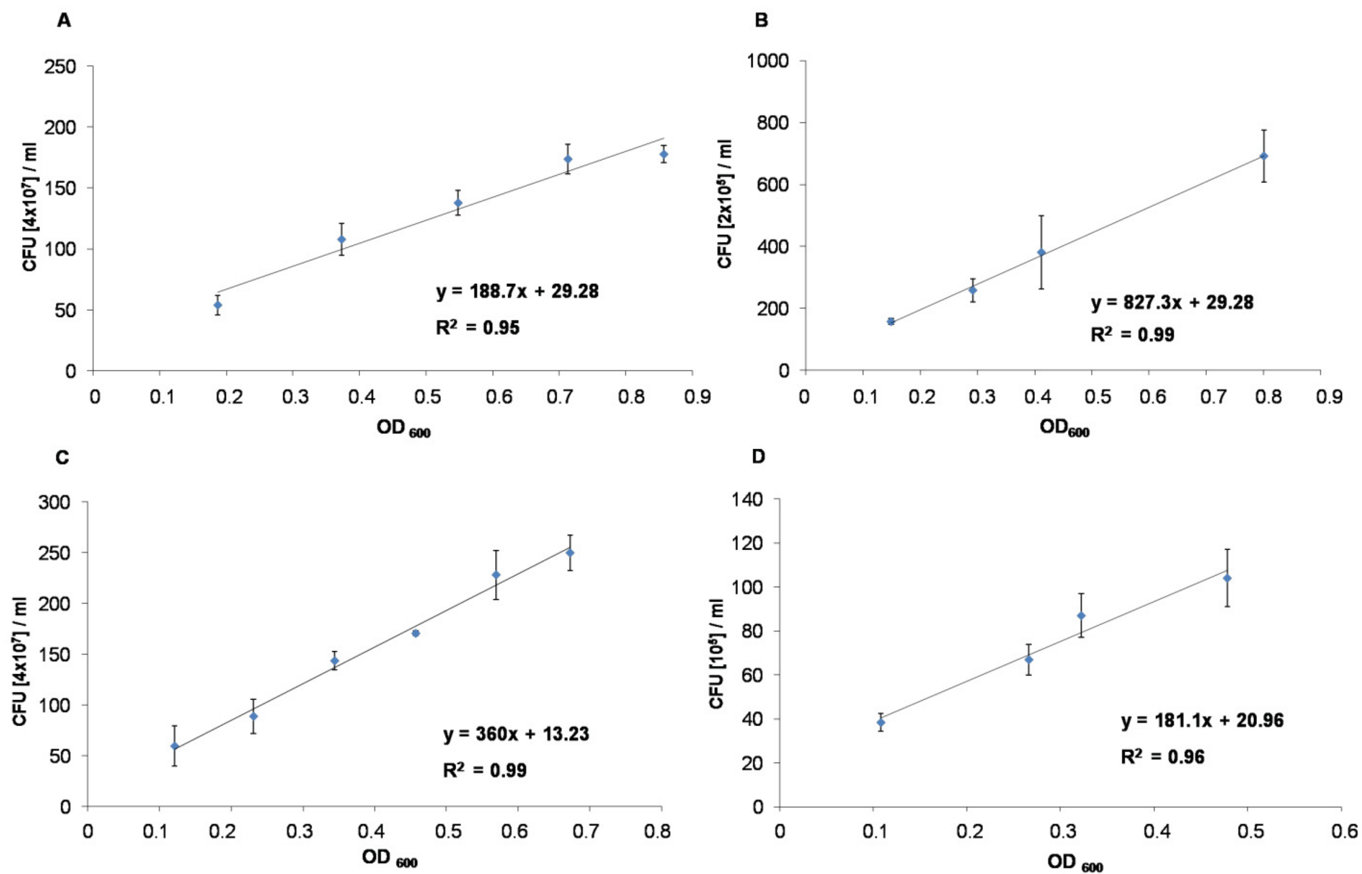


Figure S1. OD_{600}-CFU plot for *Lysobacter capsici* DSM 19286 (A), *L. enzymogenes* DSM 2043 (B), *L. oryzae* DSM 21044 (C), and *Myxococcus fulvus* ST035975 (D). Linear regression analysis was used for estimating the relationship between optical density at 600 nm (OD_{600}) and CFU/ml. Prediction was made within the range of values in the dataset used for model-fitting.

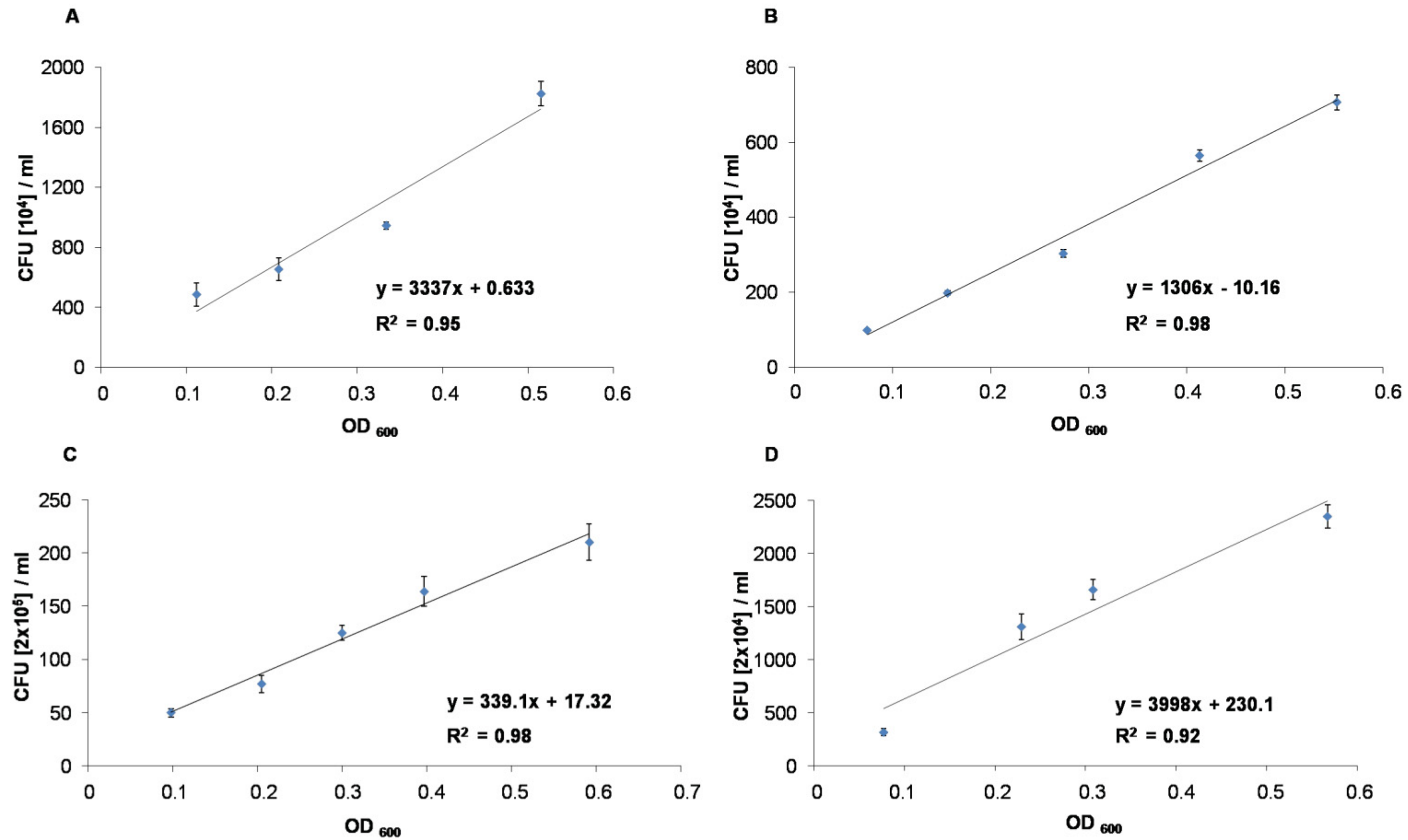


Figure S2. OD_{600}-CFU plot for *Agrobacterium tumefaciens* DSM 5172 (A), *Bacillus subtilis* DSM 347 (B), *Chromobacterium pseudoviolaceum* DSM 23279 (C), and *Escherichia coli* DSM 18039 (D). Linear regression analysis was used for estimating the relationship between optical density at 600 nm (OD_{600}) and CFU/ml. Prediction was made within the range of values in the dataset used for model-fitting.

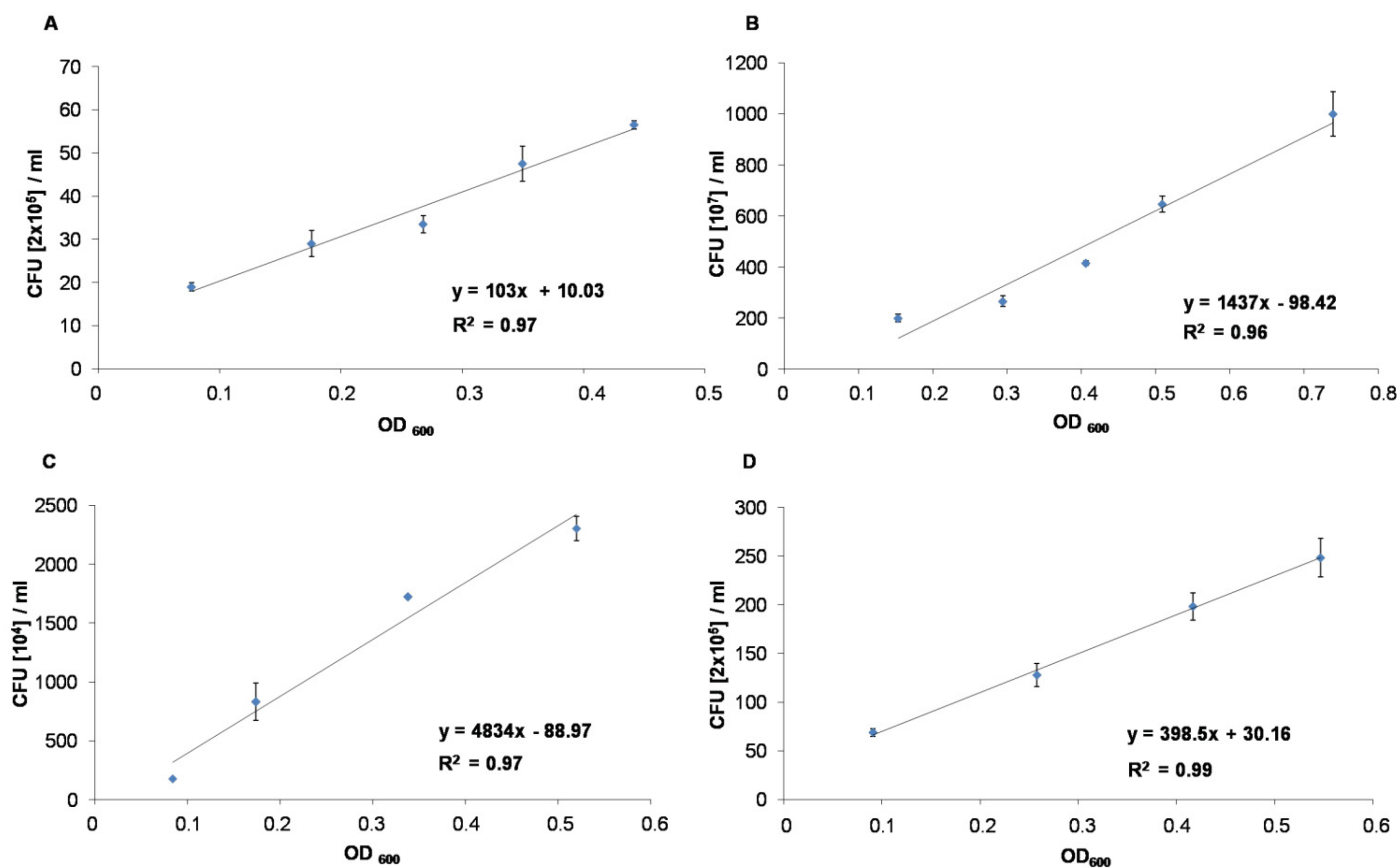


Figure S3. OD_{600}-CFU plot for *Lactococcus lactis* DSM 20069 (A), *Pseudomonas fluorescens* DSM 11532 (B), *Ralstonia solanacearum* GMI1000 (C), and *Rhodococcus rhodochrous* DSM 43334 (D). Linear regression analysis was used for estimating the relationship between optical density at 600 nm (OD_{600}) and CFU/ml. Prediction was made within the range of values in the dataset used for model-fitting.

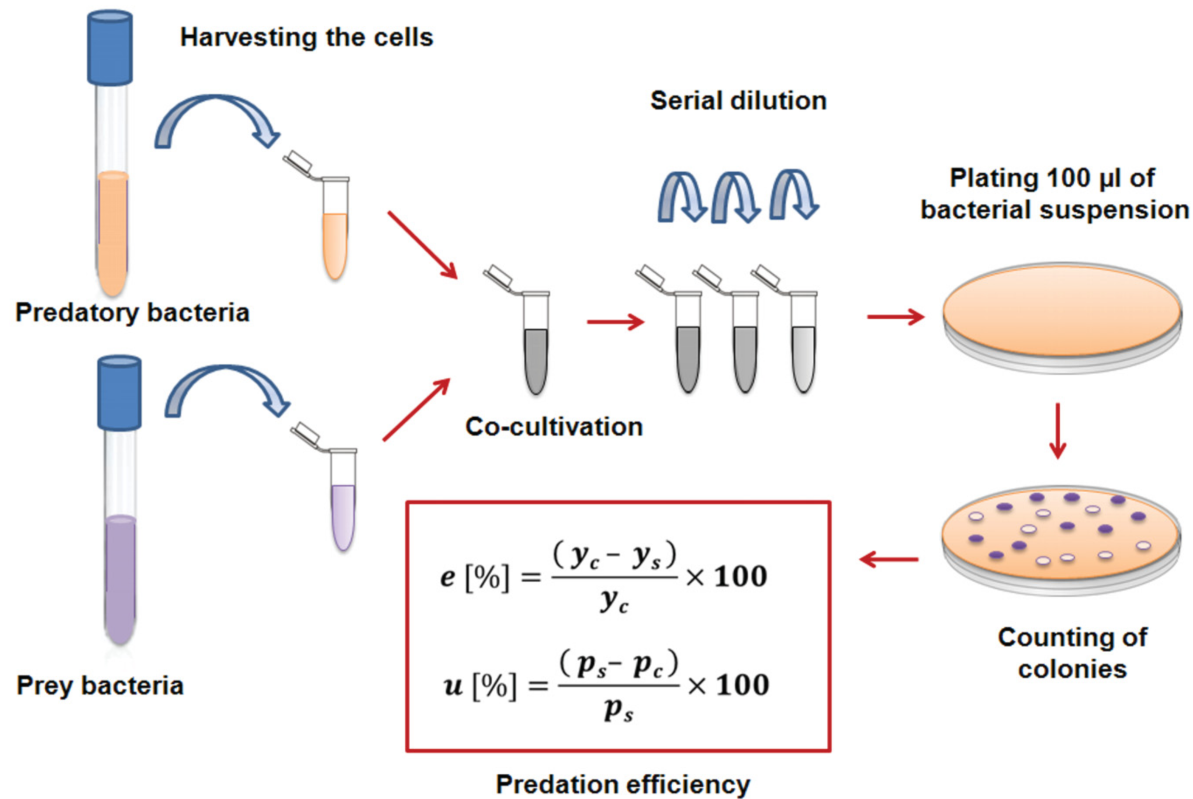


Figure S4. Work flow for the CFU-based predation assay and equations to calculate the predator's killing efficiency (e) and its utilization of prey (u). The variables were defined as follows: y_c = colony-forming units of the prey bacterium that had been grown in the absence of a predator; y_s = colony-forming units (CFUs) of the prey bacterium that had been cocultured with a predator; p_c = CFUs of the predatory bacterium that had been grown in the absence of prey; p_s = CFUs of the predatory bacterium that had been cocultured with a prey bacterium.

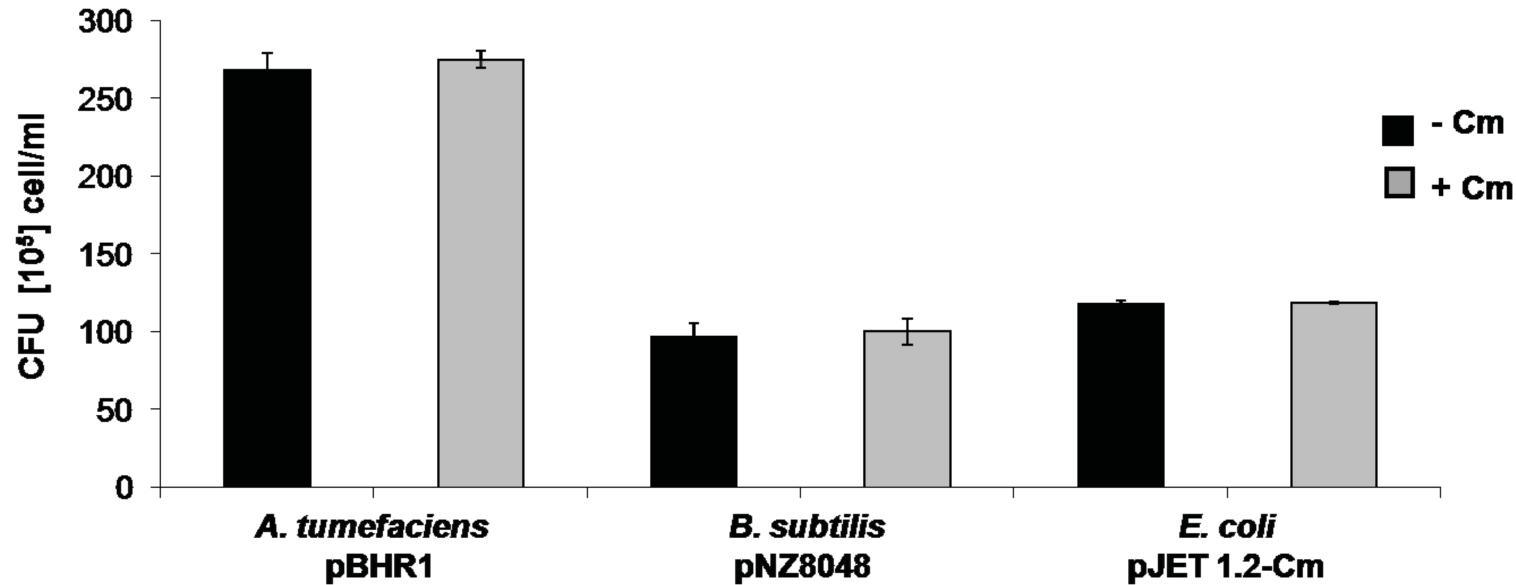


Figure S5. Plasmid stability analysis of *A. tumefaciens/*pBHR1, *B. subtilis/*pNZ8048, and *E. coli/*pJET1.2-cf. Bacteria were cultured in 5 ml LB medium supplemented with chloramphenicol (25 µg ml^{-1}). After 48 h, 2 ml of each bacterial culture were harvested by centrifugation (1,200 g, 4 °C, 5 min). The supernatant was removed and the cell pellet was washed three times with 2 ml of PBS buffer and, finally, resuspended in 1.6 ml of PBS buffer. From these suspensions, 370 µl aliquots (cell concentration adjusted to 1 x 10^6 cells ml^{-1}) were mixed with the same amount of either PBS buffer or PBS buffer supplemented with chloramphenicol (25 µg ml^{-1}). Incubation was then continued for 24 h at 30 °C, before serial dilutions of the cultures were prepared and spread on LB agar plates containing chloramphenicol (25 µg ml^{-1}). The CFU number was determined, as described for the CFU-based predation assay. None of the tested strains showed a significant plasmid loss, which is consistent with previous studies (cf. **Weber AE, San K-Y.** 1990. Population dynamics of a recombinant culture in a chemostat under prolonged cultivation. Biotechnol. Bioengineering. **36**:727-736).

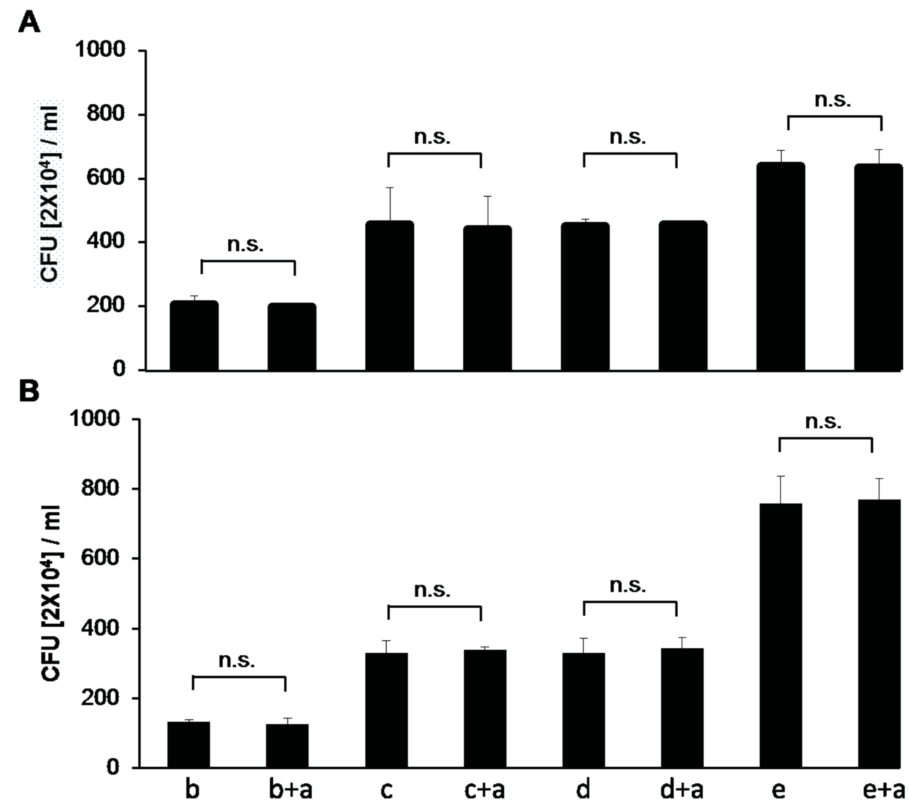


Figure S6. Testing of *Myxococcus fulvus* in the CFU-based predation assay. Mean (±95% confidence interval) CFU of (b) *B. subtilis*, (c) *C. pseudoviolaceum*, (d) *E. coli* and (e) *R. rhodochrous* grown in the absence or presence of (a) the predatory bacterium *M. fulvus*. Monocultures of prey served as controls to assess the reduction efficiency after (A) 24 h, and (B) and 48 h (n.s., not significant).

Table S1. Swarm expansion in the lawn predation assay. Mean (± 95% confidence interval, n=3) of the swarm diameter [in mm] on each prey bacterium after one and ten days (d) of incubation.

	Prey species									
	Bacillus subtilis		*C. pseudoviolaceum*		*Escherichia coli*		*Micrococcus luteus*		*R. rhodochrous*	
Predator species	**1 d**	**10 d**	**1 d**	**10 d**	**1 d**	**10 d**	**1 d**	**10 d**	**1 d**	**10 d**
Myxococcus fulvus	4.0 ± 1.0	14.6 ± 0.6	5.0 ± 1.2	6.0 ± 1.3	8.0 ± 1.0	42.6 ± 2.8	9.0 ± 1.0	24.6 ± 5.9	6.0 ± 1.0	8.0 ± 1.0
Lysobacter capsici	8.3 ± 0.6	9.6 ± 0.7	7.0 ± 1.0	7.4 ± 1.0	6.0 ± 1.0	7.6 ± 0.6	9.6 ± 1.3	10.3 ± 0.6	7.4 ± 1.0	7.4 ± 1.0
Lysobacter enzymogenes	7.0 ± 0.2	7.6 ± 0.7	4.6 ± 0.6	5.6 ± 0.6	9.6 ± 1.3	24.3 ± 1.3	8.0 ± 1.0	11.3 ± 0.6	7.4 ± 1.0	12.3 ± 0.6
Lysobacter oryzae	9.0 ± 0.2	9.6 ± 0.6	7.0 ± 1.0	7.0 ± 1.0	9.3 ± 0.6	9.6 ± 0.7	8.6 ± 1.7	9.6 ± 0.6	8.0 ± 1.0	8.0 ± 1.0

Table S2. Evaluation of predation efficiency in the CFU-based predation assay (*e* = killing efficiency; *u* = prey utilization; n.d. = not determined).

	Prey species							
	Agrobacterium tumefaciens		*Bacillus subtilis*		*C. pseudoviolaceum*		*Escherichia coli*	
Predator species	***e* [%]**	***u* [%]**	***e* [%]**	***u* [%]**	***e* [%]**	***u* [%]**	***e* [%]**	***u* [%]**
Lysobacter capsici	14.0 ± 1.0	n.d.	96.5 ± 0.4	19.0 ± 1.5	90.0 ± 1.5	23.7 ± 0.2	19.0 ± 1.5	3.8 ± 1.2
Lysobacter enzymogenes	8.5 ± 0.1	n.d.	62.6 ± 1.8	1.0 ± 0.5	9.4 ± 0.1	9.6 ± 0.4	14.6 ± 0.7	8.3 ± 1.5
Lysobacter oryzae	12.0 ± 0.2	n.d.	98.1 ± 1.0	15.3 ± 1.5	100.0 ± 0.0	26.6 ± 0.1	20.0 ± 1.8	13.4 ± 2.5

	Prey species							
	Lactococcus lactis		*Pseudomonas fluorescens*		*Ralstonia solanacearum*		*Rhodococcus rhodochrous*	
Predator species	***e* [%]**	***u* [%]**	***e* [%]**	***u* [%]**	***e* [%]**	***u* [%]**	***e* [%]**	***u* [%]**
Lysobacter capsici	68.4 ± 7.5	16.2 ± 1.5	1.0 ± 0.1	n.d.	2.69 ± 2.0	n.d.	97.6 ± 0.9	21.6 ± 0.4
Lysobacter enzymogenes	99.0 ± 1.5	13.7 ± 0.36	1.2 ± 0.1	n.d.	0.5 ± 1.0	n.d.	4.2 ± 0.4	10.6 ± 2.2
Lysobacter oryzae	99.1 ± 1.5	17.7 ± 0.7	1.4 ± 0.6	n.d.	11.6 ± 7.6	n.d.	96.8 ± 0.8	20.0 ± 0.1

4.2 Manuscript B: Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*

Seccareccia Ivana, Kovács Ákos T., Gallegos-Monterrosa Ramses and Nett Markus. Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*. Manuscript submitted to *Applied and Environmental Microbiol*ogy (manuscript ID: AEM04056-15).

1 **Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis***

2 Ivana Seccareccia,[a] Ákos T. Kovács,[b] Ramses Gallegos-Monterrosa,[b] and Markus Nett[a]#

3

4 Junior Research Group "Secondary Metabolism of Predatory Bacteria", Leibniz Institute for

5 Natural Product Research and Infection Biology, Hans-Knöll-Institute, Jena, Germany[a];

6 Terrestrial Biofilms Group, Institute of Microbiology, Friedrich Schiller University, Jena,

7 Germany[b]

8 Running Head: Predator-prey relation of *C. necator* and *B. subtilis*

9

10 #Address correspondence to Markus Nett, markus.nett@hki-jena.de

11 **Abstract**

12 *Cupriavidus necator* is a predatory soil bacterium that feeds on Gram-negative and Gram-
13 positive bacteria. In this study, we set out to determine the general conditions, which are
14 necessary to observe predatory behavior of *C. necator*. Using *Bacillus subtilis* as a prey
15 organism, we confirmed that the predatory performance of *C. necator* is correlated with the
16 available copper level, and that the killing is mediated, at least in part, by extracellular factors.
17 The predatory activity depends on the nutrition status of *C. necator*, but does not require a
18 quorum of predator cells. This suggests that *C. necator* is no group predator. Further analyses
19 revealed that sporulation enables *B. subtilis* to avoid predation by *C. necator*. However, the
20 resistance is not linked to the presence of an intact spore coat, but possibly mediated by
21 persistent cell state.

22 **Introduction**

23 Over the past decades it has become increasingly evident that the composition and dynamics of
24 microbial communities have a profound effect on Earth's ecosystems. Conversely, these
25 consortia are shaped by environmental cues and organismic interactions. Predator-prey
26 relationships are of particular interest in this context due to their evolutionary implications (1).
27 Surprisingly, our knowledge about predator-prey interactions in the bacterial world is still very
28 limited (2-4).

29 In this study, we set out to explore the predator-prey relationship of two common soil-dwelling
30 microbes, namely *Cupriavidus necator* and *Bacillus subtilis*. The former bacterium is
31 distinguished by an extraordinary metal resistance, and its growth responds favorably to high
32 concentrations of Cu^{2+}, which is tolerated up to 800 µM (5). *C. necator* was reported to prey
33 upon a wide range of Gram-negative and Gram-positive bacteria (6). The prey spectrum is not
34 restricted to soil bacteria, but also includes human commensals and pathogens, as well as other
35 predatory bacteria, such as *Agromyces ramosus* (5). The actinomycete *A. ramosus* is known to
36 kill its prey upon cell-to-cell contact (7). Previous studies revealed that a contact-mediated attack
37 on *C. necator* triggers a counter attack of the latter, which ultimately leads to lysis of the *A.*
38 *ramosus* mycelium (8). It has been proposed that the counter attack involves the secretion of a
39 copper-binding peptide, which is toxic to the actinomycete (8). The structure of the putative
40 peptide was not elucidated. Nevertheless, it has been speculated that *C. necator* also uses this
41 molecule for the killing of other prey bacteria (9).

42 *B. subtilis* is a known prey bacterium of *C. necator* (5). Furthermore, it has been established as a
43 model organism in the analysis of predator-prey interactions and is widely used to address basic

44 questions concerning the development of prey resistance (10-13). In theory, *B. subtilis* can resort
45 to a variety of possible defense strategies to evade predation (14, 15). Some resistance traits are
46 strain-specific and they provide protection only against selected predators. An example would be
47 the release of inhibitory or toxic secondary metabolites, such as the antibiotic bacillaene, which
48 is used to hold off the predatory myxobacterium *Myxococcus xanthus* (12). Other conceivable
49 defense mechanisms, among them motility and biofilm formation, are frequent features of *B.*
50 *subtilis* strains and can be expected to confer unspecific protection (16). Lastly, most strains of *B.*
51 *subtilis* are capable to sporulate under stressful conditions, which dramatically improves their
52 potential to resist predation by protozoa (10), nematodes (11), as well as myxobacteria (12).

53 Given the diversity of predation strategies that are used by bacteria (2, 17) and the observations
54 that had previously been made for *C. necator* (5, 8), we were particularly interested in clarifying
55 whether predatory activity of this species depends on specific triggers, among them nutrient
56 limitation and access to Cu^{2+}, and whether prey killing is exclusively mediated by extracellular
57 factors or requires cell contact. By analyzing prey preference we further sought to identify
58 defense mechanisms that confer resistance against *C. necator* predation. Taken together, our
59 analyses revealed that the predatory behavior of *C. necator* is distinct from other bacteria.
60 Predatory success does not depend on outnumbering the prey and must also not necessarily
61 involve prey contact. Although predation is positively correlated with the Cu^{2+} concentration, it
62 can also be observed at very low levels of this transient metal. Sporulation of *B. subtilis* grants
63 protection against *C. necator*, but an intact spore coat is surprisingly not required.

64

65 **Material and Methods**

66 **Bacterial strains and cultivation conditions.** *Cupriavidus necator* N-1 (DSM 545) was
67 cultivated in Lysogeny Broth (LB) or in H-3 mineral medium (0.1% aspartic acid, 0.23%
68 KH_2PO_4, 0.257% Na_2HPO_4, 0.1% NH_4Cl, 0.05% $MgSO_4 \times 7\ H_2O$, 0.05% $NaHCO_3$, 0.001%
69 $CaCl_2 \times 2\ H_2O$, and 0.5% SL-6 trace element solution). In specified experiments the growth
70 media were supplemented with $CuCl_2$ x 2 H_2O. The copper(II) chloride was added as a filter-
71 sterilized solution to give final concentrations ranging from 0.3 µM to 1.0 mM. The *Bacillus*
72 *subtilis* strains used in this study are listed in Table 1. All *B. subtilis* strains were routinely grown
73 in LB medium at 30 °C. For selection, chloramphenicol, tetracycline, or kanamycin were added
74 at a final concentration of 12.5 µg ml^{-1}, 10µg ml^{-1}, or 10 µg ml^{-1}, respectively.

75 **Strain constructions.** *B. subtilis* strains used for the predation assays were all endowed with
76 chloramphenicol resistance genes via transforming genomic DNA obtained from *B. subtilis*
77 strain TB48 (18) using natural competence (19). In these strains, the Phy-*gfp* (Cm) cassette is
78 recombined into the *amyE* locus of *B. subtilis* that was validated by the lack of α-amylase
79 activity and the presence of green fluorescence in the transformed strains. Further mutations (see
80 Table 1) were subsequently introduced into *B. subtilis* TB34 (NCIB 3610 natural competent
81 derivative DK1042 with Cm antibiotic marker) resulting marker exchanged mutants. In the case
82 of mutations harboring Cm markers (i.e., *cotA, cotC* and *cotE*), the mutations were transferred
83 directly into DK1042.

84 **Correlation of optical densities with viable cell count data.** For every bacterial strain, the
85 statistical relationship between colony-forming units (CFU) and optical density at 600 nm
86 (OD_{600}) was determined (20). For this, bacteria were grown in the appropriate growth medium

87 until they reached early stationary phase. At this time, cells were harvested by centrifugation
88 (2,400 × *g*, 5 min). The cell pellet was suspended in phosphate buffered saline (PBS buffer: 0.8%
89 NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.6). Serial dilutions of these
90 suspensions with defined OD_{600} values were streaked out on a suitable agar medium. Following
91 4 days of incubation at 30 °C, the number of CFU were determined and plotted against the
92 respective optical densities.

93 **CFU-based predation assay.** Glycerol stock cultures of *C. necator* and *B. subtilis* were used to
94 inoculate LB agar plates. Agar cultures were incubated at 30 °C until the appearance of the first
95 colonies. From every *C. necator* culture, six colonies were randomly selected and sub-cultured in
96 glass tubes containing either 5 ml LB or 15 ml H-3 medium with or without $CuCl_2$ x 2 H_2O.
97 Cultivation was conducted at 220 rpm and 30 °C for 24 h (LB medium) or 3 days (H-3 medium).
98 In parallel, colonies of *B. subtilis* were selected and individually cultured in 10 ml LB medium at
99 220 rpm and 30 °C for 24 h, if not stated otherwise. After cultivation, 2 ml of each bacterial
100 culture was harvested and centrifuged (1,200 × *g*, 4 °C, 5 min). The supernatant was removed,
101 and the cell pellet was washed three times with 2 ml of PBS buffer and then resuspended in 1.6
102 ml of PBS buffer. Aliquots (370 µl) of *C. necator* and *B. subtilis* suspensions were mixed in a 2-
103 ml tube. For this, the cell concentration of *C. necator* was adjusted to 2 x 10^8 cells ml^{-1} and the
104 cell concentration of *B. subtilis* was adjusted to 1 x 10^8 cells ml^{-1}. The predator control sample
105 contained 370 µl of *C. necator* suspension and the same volume of PBS buffer, and the prey
106 control sample contained 370 µl of *B. subtilis* suspension mixed with 370 µl PBS buffer. Control
107 experiments included only monocultures of predator or prey. Every experiment was replicated
108 six times. All cultures were incubated at 220 rpm and 30 °C for 24 h. After cultivation, serial
109 dilutions of cocultures and monocultures ranging from 10^{-3} to 10^{-5} were prepared by mixing with

110 PBS buffer and spread on LB agar plates. LB plates were either supplemented with
111 chloramphenicol (15 µg ml^{-1}) to determine killing efficiencies or with ampicillin (10 µg ml^{-1}) to
112 determine prey utilization.

113 **Evaluation of the predation efficiency.** To quantify predatory activity, both the killing
114 efficiency (*e*) and the utilization of prey (*u*) were determined for each experiment. The two
115 parameters were calculated using the following formulas: *e* = (CFU of control prey – CFU of
116 surviving prey)/CFU of control prey × 100 and *u* = (CFU of predator with prey – CFU of control
117 predator)/CFU of control predator × 100.

118 **Contact dependence of predatory behavior.** *C. necator* was grown in H-3 medium
119 supplemented with $CuCl_2 \times 2\ H_2O$ (50 µM) to an OD_{600} of 2.5. After centrifugation (2,400 × *g*, 5
120 min), the cell pellet was washed with PBS buffer and directly mixed with *B. subtilis*. In parallel,
121 the supernatant was filter-sterilized and mixed with *B. subtilis*. Control experiments included *B.*
122 *subtilis* suspensions treated with 370 µl of PBS buffer or H-3 medium supplemented with $CuCl_2$
123 $\times 2\ H_2O$ (50 µM).

124 ***B. subtilis* spore preparation.** *B. subtilis* was cultivated for 3-5 days on Schaeffer`s sporulation
125 medium (0.8% Difco Nutrient Broth supplemented with 1 mM $MgSO_4 \cdot 7H_2O$, 13.4 mM KCl, 1
126 mM $CaCl_2 \cdot 2H_2O$, 0.13 mM $MnSO_4 \cdot H_2O$) until more than 80 to 90% of the population
127 sporulated. Spores were purified following a previously described protocol (21). Briefly, cultures
128 were centrifuged (1,020 × *g*, 8 min, 4 °C) and washed two times with 10 mM PBS buffer (pH
129 7.4). Samples were incubated at -20 °C for 3 × 24 h and the washing step was repeated every
130 day. Afterwards, the spores were stored at -20 °C.

131 **Decoating of spores and lysozyme resistance assays.** Decoating of wild type and mutant spores
132 was performed as previously described (22). Briefly, a spore suspension of *B. subtilis* 3610 (1
133 ml; 10^{10}-10^{11} spores/ml) was mixed with the same volume of decoating solution (0.1 M NaCl;
134 0.1 M NaOH; 1% sodium dodecyl sulfate and 0.1 M dithiothreitol) and incubated for two hours
135 at 70 °C. Afterwards, the mixture was centrifuged (3,500 rpm, 5 min) and washed two times with
136 PBS buffer. Decoated spores were then used in the CFU-based predation assay. For lysozyme
137 resistance assays, 100 µl of lysozyme solution (1 mg ml^{-1}) was mixed with an equal volume of *B.*
138 *subtilis* spores and incubated for 20 minutes at 37 °C (22). Later, serial dilutions of the mixture
139 were made and plated on LB agar plates. After two days of incubation at 30 °C, CFU were
140 counted.

141 **Statistical Analysis.** Data was analyzed with a paired-sample *t* test and nonparametric statistical
142 tests, including Mann-Whitney U test and Wilcoxon median test. All statistical analyses were
143 done using SPSS software (version 22.0, IBM, USA).

144 **Results**

145 **General factors influencing predation.** To assess the impact of the nutrition status on the
146 predatory activity of *C. necator*, the bacterium was grown either in nutrient-rich (LB) or in
147 nutrient-poor (H-3 mineral salt) medium prior to the addition of the prey culture. Previous
148 studies on myxobacteria had unveiled that predatory performance can be species-dependent and
149 sometimes even strain-specific (3, 12, 23, 24). We thus tested *C. necator* against five *B. subtilis*
150 strains, including two domesticated strains (Marburg 168 and ATCC 6633), the ancestral strain
151 NCIB 3610, as well as two wild isolates (PS216 and RO-NN-1). Since no lysis of *B. subtilis*-
152 covered agar plates was observed under any condition tested, predator and prey were cultured
153 together in PBS buffer for 24 h. Afterwards the mixed populations were streaked out on growth-
154 selective agar media and the respective CFU number was determined following a previously
155 described protocol (4). The results of this predation assay indicated that *C. necator* was more
156 efficient in killing its prey after it had been grown in the low nutrient H-3 medium (Fig. 1A).
157 Since the observation was consistently made irrespective of the *B. subtilis* strain tested, we
158 concluded that nutrient deficiency is correlated with predatory performance. The type of prey
159 was also found to significantly affect the outcome of the predator-prey interaction. As expected,
160 the domesticated strains of *B. subtilis* were found to be more susceptible to *C. necator* predation
161 than the wild isolates. This initially suggested that the former might have lost resistance traits
162 due to lack of selection pressure in the laboratory. Contrasting previous observations with the
163 predatory myxobacterium *Myxococcus xanthus* (12), however, the ancestral strain NCIB 3610
164 exhibited almost no resistance against *C. necator*, even when the latter had been grown in the
165 rich LB medium. It was hence evident that strain-specific factors contribute to prey survival, and
166 that resistance cannot be generally associated with ancestral strains.

167 The consumption of *B. subtilis*, which was evaluated based on the increase of the predator
168 population in comparison to prey-free control experiments, was strongly affected by the nutrition
169 status of *C. necator*. After the predatory bacterium had been grown in the nutrient-rich LB
170 medium, significant feeding was only observed on the laboratory strain 168, whereas the other *B.*
171 *subtilis* strains were not consumed (Fig. 1B). This result was surprising considering the efficient
172 killing of strains ATCC 6633 and NCIB 3610 under the same conditions and demonstrated that
173 killing of bacteria is not necessarily an indication for predation. In cases where the assay was
174 carried out with starved *C. necator* cells, however, significant killing was always accompanied
175 by efficient prey utilization. Despite this consistency, we observed quantitative differences
176 concerning the growth stimulation of the predator. Again, it appeared that *C. necator* preferred
177 feeding on strain 168 over the other *B. subtilis* strains. Two conclusions were drawn from these
178 data. First, *C. necator* will only prey on other bacteria in the absence of alternative nutrient
179 sources that are more easily exploitable (even though exceptions such as strain 168 may occur).
180 Second, the predatory performance is definitely strain-specific.

181 **Impact of copper(II).** It had previously been proposed that elevated concentrations of Cu^{2+}
182 foster the predatory activity of *C. necator* (8). To verify this assumption, we evaluated the effect
183 of different Cu^{2+} concentrations in H-3 mineral medium (0.01 µM; 12.5 µM; 25 µM; 50 µM; 100
184 µM; 200 µM and 400 µM) on the killing efficiency of *C. necator* in subsequent co-incubation
185 experiments with *B. subtilis* 168. To remove any bias due to Cu^{2+}-associated toxicity, the prey
186 survival was related to monocultures, in which the *B. subtilis* cell suspension was mixed with a
187 control solution that was obtained after processing predator-free H-3 medium with the respective
188 Cu^{2+} concentration according to the standard assay protocol. This analysis confirmed that the
189 predatory activity of *C. necator* increased after exposure to Cu^{2+} in a concentration-dependent

190 manner (Fig. 2). The maximum killing efficiency was observed at a Cu^{2+} concentration of 50
191 µM. Beyond this threshold, we did not observe significant changes in the number of surviving *B.*
192 *subtilis* cells. It was hence clear that Cu^{2+} has a strong impact on the predatory activity of *C.*
193 *necator*. Furthermore, it became obvious that the predation-enhancing effect of Cu^{2+} is limited
194 and that a defined fraction of the *B. subtilis* population will survive, possibly due to resistance.
195 Lastly, *C. necator* also preys on *B. subtilis* at low Cu^{2+} levels, although its killing efficiency is
196 reduced under these conditions.

197 **Group predation and proximity to prey.** Some predatory bacteria are assumed to hunt
198 collectively. They pool lytic enzymes and/or antibiotics to degrade the cell wall of their prey (2,
199 25-29). In order to test whether the predatory activity of *C. necator* depends on numerical
200 superiority over its prey, we varied the predator-prey ratio (PPR) in co-cultivation experiments
201 with *B. subtilis* 168. This analysis revealed that *C. necator* must not be present in large numbers
202 for effective predation (Fig. 3). Maximum killing efficiencies were already observed at a PPR of
203 5:1. Even when the experiment started with comparable concentrations of *C. necator* and *B.*
204 *subtilis*, the cell number of the latter severely declined during co-cultivation (e = 69.4%). We
205 hence reasoned that *C. necator* is capable of individual predation and does not rely on
206 cooperative feeding.

207 To clarify whether the killing of *B. subtilis* 168 requires physical contact or whether it is
208 mediated by secreted extracellular factors, such as enzymes and antibiotics, a defined number of
209 prey cells was individually exposed to the cell fraction and a cell-free supernatant of a *C. necator*
210 culture. In case of the harvested cells, the predation assay was carried out in PBS buffer, as
211 previously described, whereas the H-3 medium-derived supernatant was directly mixed with the
212 suspension of strain 168 in PBS buffer. Control experiments were also conducted in the presence

213 of Cu^{2+} (50 μM) to assess the effects of this transient metal on the growth of strain 168. The
214 number of *B. subtilis* CFUs that was obtained after an incubation for 24 h revealed that the
215 nutrient-poor H-3 medium still has a minor growth-promoting effect when compared to PBS
216 buffer, which lacks organic nutrients (Fig. 4). The addition of Cu^{2+} (50 μM) led to a negligible
217 decrease of the CFU number. To our surprise, both the cell fraction and the culture supernatant
218 of *C. necator* caused an almost complete eradication of the prey population. Because its
219 supernatant has strong antimicrobial effects, *C. necator* can kill *B. subtilis* 168 without making
220 physical contact. This indicates the release of a molecule or enzyme that is toxic to strain 168.

221 **Potential resistance factors of *Bacillus subtilis* against predation.** *B. subtilis* is a flagellated
222 bacterium and, thus, capable of active movement (30). Motility is required for surface spreading
223 of *B. subtilis* (31, 32), but also important during complex development, including biofilm
224 formation (33). Two integral membrane proteins, MotA and MotB, are essential components of
225 the flagellar motor (34, 35). To evaluate the impact of motility on predation resistance, we
226 analyzed the survival rate of a *B. subtilis* Δ*motA* mutant in the CFU-based predation assay.
227 However, there was no evidence for an increased susceptibility to bacterial predation when
228 compared to the wildtype strain (data not shown), indicating that active motion does not provide
229 protection against *C. necator*.

230 Another characteristic feature of *Bacillus* spp. is the formation of endospores. This
231 morphological adaptation can be triggered by nutritional limitation (36), but also by other
232 stressful conditions including predation (10-12). In order to assess the predation resistance of *B.*
233 *subtilis* spores, we grew the sporulation-competent strain NCIB 3610 for 24, 48, or 72 h, and
234 exposed the respective cultures to *C. necator*. Surviving *B. subtilis* were quantified after plating
235 on growth-selective agar media and heat treatment at 80 °C to eliminate the vegetative cells.

236 Consistent with our previous analysis (Fig. 1), *C. necator* exhibited very high killing efficiencies
237 against 24 h- and 48 h-old cultures of strain NCIB 3610. On the other hand, the 72 h-old culture
238 was hardly affected by the predator. In parallel experiments, the heat treatment was omitted, but
239 this did not significantly alter the number of *B. subtilis* CFU on the agar plates (Fig. 5).
240 Subsequent analyses confirmed that cultures harvested after 24 h or 48 h consisted mainly of
241 vegetative cells, whereas 72 h-old cultures were largely dominated by spores. This strongly
242 suggested that sporulation confers resistance against predation. Further evidence supporting this
243 assumption was obtained after testing isolated spores from strain NCIB 3610 in the CFU-based
244 predation assay. The co-cultivation with *C. necator* did not reduce the spore number in
245 comparison to control experiments lacking the predatory bacterium (data not shown). Finally, the
246 predation resistance of sporulation-deficient *spo0A* and *sigF* mutants was evaluated. The DNA-
247 binding protein Spo0A is a global transcriptional regulator which, once phosphorylated, activates
248 several genes that are required for early spore development in *B. subtilis* (37). SigF, which is
249 transcriptionally activated by Spo0A, is known as the first forespore specific sigma factor (38).
250 Both *B. subtilis* mutant strains were effectively killed by *C. necator* and the outcome of the co-
251 cultivation experiment was consistent for 24 h-, 48 h-, and 72 h-old cultures of every mutant
252 (Fig. 5).

253 **Spore components mediating resistance against predation.** Even though the precise
254 mechanism of spore persistence is not known yet, the spore coat is generally recognized as the
255 first line of defense against toxic molecules. Therefore, it might also have a role in protecting *B.*
256 *subtilis* from a secreted killing factor of *C. necator*. The spore coat consists of at least 70
257 different proteins, some of which have been associated with specific resistance functions (39). It
258 is hence plausible that a spore might even resist desiccation, extreme heat, UV radiation, or

259 predation when single spore proteins are absent. This raised the question whether a fully
260 maturated endospore is essential for *B. subtilis* to survive an attack by *C. necator* or whether the
261 predation resistance is due to defined spore components. In order to answer this question,
262 NCIB3610-derived mutant strains defective in the production of specific spore proteins were
263 chosen, their spores were isolated and subsequently tested in the CFU-based predation assay
264 (Fig. 6).

265 Initial experiments were carried out with spores lacking the outer coat proteins CotA, CotC, and
266 CotE, respectively. While CotA is needed for the biosynthesis of a melanin-type brown pigment
267 (39-41), CotE was found to be indispensable for the assembly of the outer coat (42). Spores from
268 *cotE* mutants still retain an intact inner coat, but they do not possess an outer coat. No specific
269 function has been assigned to the CotC protein yet (41, 43, 44). None of the mutant spores tested
270 showed an increased sensitivity to predation when compared to wildtype spores. We thus
271 inferred that the outer coat is not important for resisting bacterial predation. Next, we examined
272 the fate of *safA* and *spoVID* mutant spores during a 24-h co-incubation with *C. necator*. The *safA*
273 mutant spores lack an inner coat, whereas the outer coat is still present (39). Spores from *spoVID*
274 mutants exhibit a fully mature cortex, but they typically lack both inner and outer coat (45, 46).
275 Again, however, no change in predation resistance was apparent for any of these mutant spores
276 (Fig. 6), suggesting that the entire spore coat is expendable as a defense against *C. necator*. To
277 verify this, we chemically removed the coat of spores from the wildtype strain NCIB 3610. Such
278 decoated spores typically do not tolerate treatment with lysozyme or sodium hypochlorite (47).
279 Although we were able to confirm the lack of these properties of decoated spores, we did not
280 observe an increased sensitivity to predation by *C. necator* (Fig. 6).

281 **Discussion**

282 After some preliminary studies in the 1980s (5, 8), the predatory behavior of *C. necator* has not
283 received further attention, which is surprising considering the widespread occurrence of this
284 bacterium in nature and its industrial usage as a bioplastic producer (48). The aim of this study
285 was hence to determine molecular factors and environmental conditions that foster predatory
286 activity in *C. necator* as well as to identify possible mechanisms by which prey bacteria can
287 resist this micropredator. Initially, we observed that the nutrition status and also the prey type
288 significantly influenced the predatory performance. A starved *C. necator* population was more
289 likely to feed on *B. subtilis* than a culture that had previously been grown in a nutrient-rich
290 medium. Furthermore, there was significant variation in the killing and utilization of prey on the
291 subspecies level, which suggests some degree of specialization. Similar observations were made
292 when the predatory myxobacterium *Myxococcus xanthus* was feeding on *B. subtilis* strains (12).
293 In the corresponding study, the different survival rates of the prey bacteria could be traced to a
294 strain-specific production of defensive molecules (12). The same explanation might also hold
295 true for the two *B. subtilis* strains PS216 and RO-NN-1, which were found to be largely resistant
296 against predation by *C. necator*. Another possibility is that the resilience of PS216 and RO-NN-1
297 is due to an earlier onset of sporulation in the two strains.

298 Previous studies revealed that Cu^{2+} stimulates the growth of *C. necator* (5, 8). Furthermore, it
299 has been suggested that the bacterium secretes a peptide to scavenge Cu^{2+} from the environment
300 and that the resulting complex might also be used to deliver excess and toxic amounts of this
301 transient metal to prey cells (8). During our own analyses we noted that *C. necator* already
302 shows significant predatory activity in the presence of Cu^{2+} levels as low as 0.01 µM. Maximum
303 killing efficiencies, however, were only reached at a Cu^{2+} concentration of 50 µM. In natural

304 soil, the Cu^{2+} level is typically around 0.01 μM, whereas agricultural soil can harbor much higher
305 concentrations up to 20 μM (49). Although the killing efficiency of *C. necator* is suboptimal
306 under these conditions, ordinary soil still provides sufficient Cu^{2+} to support the predatory
307 behavior.

308 Bacterial predation can be distinguished by the mechanisms that are used to achieve a killing of
309 prey bacteria (17). In general, the hunting strategies of predatory bacteria presume a physical
310 contact with their prey, but exceptions are known as well (24). The supernatant of *C. necator*
311 was found to exhibit strong antibacterial effects. Secreted molecules are thus likely major
312 contributors to the killing of other bacteria. Still, it is possible that cell contact is needed for
313 efficient prey consumption. The numerical proportion between predator and prey cells can also
314 have a strong impact on the outcome of co-incubation experiments. In particular, bacteria
315 practicing group predation, such as *Lysobacter* spp., must outnumber their prey to achieve
316 appreciable killing efficiencies (4). In this study, we found that *C. necator* maintains predation at
317 comparatively low predator-to-prey ratios. Although the killing efficiency was shown to benefit
318 from a modest increase of the *C. necator* number, which might indicate a collaborative hunting
319 behavior, it is evident that large consortia are not needed for predation. We thus conclude that *C.*
320 *necator* does not pursue the so-called wolfpack strategy (17).

321 Lastly, the predatory performance also depends on the resistance of the prey. Bacteria, in general,
322 and *B. subtilis*, in particular, have evolved a variety of strategies in order to reduce predation
323 pressure (14). Our analyses confirmed that resistance mechanisms must not be common to all
324 members of a species, but instead can be rather strain-specific. Sporulation might represent a
325 conserved means of *B. subtilis* protection, which not only prevents predation by *C. necator*, but
326 also by myxobacteria (12), protozoa (10) and nematodes (11). While sporulation-deficient strains

of *B. subtilis* were readily killed by *C. necator*, an intact spore coat was surprisingly not found to be crucial for the spore resistance properties. Both chemically decoated wild-type spores and coat-defective spores from selected mutants (*e.g.*, *cotE*$^-$, *safA*$^-$, *spoVID*$^-$) were not destroyed by the predatory bacterium. This observation clearly contrasts previous findings of *B. subtilis* spore resistance against *Tetrahymena thermophila* and *Caenorhabditis elegans* (10, 11). The lack of essential coat morphogenetic proteins results in lysozyme sensitivity (43, 50, 51), which might explain an increased susceptibility of the spore to enzymatic digestion. Obviously, this mechanism is not relevant for predation resistance against *C. necator*, and we hence speculate that a non-growing state (*i.e.*, persister-like cell state) is sufficient for protection.

Taken together, the bacterium *C. necator* pursues a hitherto unique predation strategy, which is mainly distinguished by its relation to copper(II). Nutrient deficiency or the presence of preferred prey organisms may serve as triggers for predatory behavior. The subsequent attack does not require large numbers of *C. necator*. The killing of prey bacteria is likely mediated by secreted molecules, but further analyses are necessary to determine the chemical nature of these compounds. Some *B. subtilis* strains can reduce predation pressure by *C. necator*. This resistance has been traced to sporulation.

Acknowledgements

We thank Adam Driks, Adriano Henriques, Ines Mandic-Mulec, Ezio Ricca, Daniel Lopez, and Daniel Kearns for providing *B. subtilis* strains. I.S. thanks the graduate school Jena School for Microbial Communication, which is funded by the Deutsche Forschungsgemeinschaft (DFG), for a doctoral fellowship. R.G.-M. was supported by Consejo Nacional de Ciencia y Tecnología-German Academic Exchange Service (CONACyT-DAAD). The Kovács laboratory is supported

349 by grants KO4741/2-1 and KO4741/3-1 from the Deutsche Forschungsgemeinschaft (DFG) and
350 a Marie Skłodowska Curie career integration grant (PheHetBacBiofilm).

351 **References**

352 1. **Fussmann GF, Loreau M, Abrams PA.** 2007. Eco-evolutionary dynamics of communities and ecosystems.
353 Funct. Ecol. **21**:465-477.
354 2. **Jurkevitch E.** 2007. Predatory behaviors in bacteria – diversity and transitions. Microbe **2**:67-73.
355 3. **Morgan AD, MacLean RC, Hillesland KL, Velicer GJ.** 2010. Comparative analysis of *Myxococcus*
356 predation on soil bacteria. Appl. Environ. Microbiol. **76**:6920-6927.
357 4. **Seccareccia I, Kost C, Nett M.** 2015. Quantitative analysis of *Lysobacter* predation. Appl. Environ. Microbiol.
358 **81**:7098-7105.
359 5. **Makkar NS, Casida LE.** 1987. *Cupriavidus necator* gen. nov., sp. nov.: a nonobligate bacterial predator of
360 bacteria in soil. Int. J. Syst. Bacteriol. **37**:323-326.
361 6. **Sillman CE, Casida LE.** 1986. Isolation of non-obligate bacterial predators of bacteria from soil. Can. J.
362 Microbiol. **32**:760-762.
363 7. **Casida LE.** 1983. Interaction of *Agromyces ramosus* with other bacteria in soil. Appl. Environ. Microbiol.
364 **46**:881-888.
365 8. **Casida LE.** 1987. Relation to copper of N-1, a nonobligate bacterial predator. Appl Environ Microbiol.
366 **53**:1515-1518.
367 9. **Casida LE.** 1988. Nonobligate bacterial predation of bacteria in soil. Microb. Ecol. **15**:1-8.
368 10. **Klobutcher LA, Ragkousi K, Setlow P.** 2006. The *Bacillus subtilis* spore coat provides "eat resistance" during
369 phagocytic predation by the protozoan *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. U.S.A. **103**:165-170.
370 11. **Laaberki M-H, Dworkin J.** 2008. Role of spore coat proteins in the resistance of *Bacillus subtilis* spores to
371 *Caenorhabditis elegans* predation. J. Bacteriol. **190**:6197-6203.
372 12. **Müller S, Strack SN, Hoefler BC, Straight PD, Kearns DB, Kirby JR.** 2014. Bacillaene and sporulation
373 protect *Bacillus subtilis* from predation by *Myxococcus xanthus*. Appl. Environ. Microbiol. **80**:5603-5610.
374 13. **Müller S, Strack SN, Ryan SE, Kearns DB, Kirby JR.** 2015. Predation by *Myxococcus xanthus* induces
375 *Bacillus subtilis* to form spore-filled megastructures. Appl. Environ. Microbiol. **81**:203-210.

376 14. **Jousset A.** 2012. Ecological and evolutive implications of bacterial defences against predators. Environ.
377 Microbiol. **14**:1830-1843.
378 15. **Matz C, Kjelleberg S.** 2005. Off the hook – how bacteria survive protozoan grazing. Trends Microbiol.
379 **13**:302-307.
380 16. **Angelini TE, Roper M, Kolter R, Weitz DA, Brenner MP.** 2009. *Bacillus subtilis* spreads by surfing on
381 waves of surfactant. Proc. Natl. Acad. Sci. U.S.A. **106**:18109-18113.
382 17. **Martin MO.** 2002. Predatory prokaryotes: an emerging research opportunity. J. Mol. Microbiol. Biotechnol.
383 **4**:467-477.
384 18. **van Gestel J, Weissing FJ, Kuipers OP, Kovács ÁT.** 2014. Density of founder cells affects spatial pattern
385 formation and cooperation in *Bacillus subtilis* biofilms. ISME J. **8**:2069-2079.
386 19. **Anagnostopoulos C, Spizizen J.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol.
387 **81**:741-746.
388 20. **Madigan MT, Martinko JM.** 2006. Brock biology of microorganisms, 11th ed. Pearson Prentice Hall, Upper
389 Saddle River, NJ.
390 21. **Hornstra LM, de Vries YP, de Vos WM, Abee T, Wells-Bennik MH.** 2005. *gerR*, a novel *ger* operon
391 involved in L-alanine- and inosine-initiated germination of *Bacillus cereus* ATCC 14579. Appl. Envrion.
392 Microbiol. **71**:774-781.
393 22. **Ragkousi K, Setlow P.** 2004. Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis*
394 spores. J. Bacteriol. **186**:5567-5575.
395 23. **Hillesland KL, Lenski RE, Velicer GJ.** 2007. Ecological variables affecting predatory success in *Myxococcus*
396 *xanthus*. Microb. Ecol. **53**:571-578.
397 24. **Mendes-Soares H, Velicer GJ.** 2013. Decomposing predation: testing for parameters that correlate with
398 predatory performance by a social bacterium. Microb. Ecol. **65**:415-423.
399 25. **Kaiser D.** 2004. Signaling in myxobacteria. Annu. Rev. Microbiol. **58**:75-98.
400 26. **Rosenberg E, Keller KH, Dworkin M.** 1977. Cell density-dependent growth of *Myxococcus xanthus* on
401 casein. J. Bacteriol. **129**:770-777.
402 27. **Berleman JE, Kirby JR.** 2009. Deciphering the hunting strategy of a bacterial wolfpack. FEMS Microbiol.
403 Rev. **33**:942-957.

404 28. **Xiao Y, Wei X, Ebright R, Wall D.** 2011. Antibiotic production by myxobacteria plays a role in predation. J.
405 Bacteriol. **193**:4626-4633.
406 29. **Schieferdecker S, König S, Weigel C, Dahse H-M, Werz O, Nett M.** 2014. Structure and biosynthetic
407 assembly of gulmirecins, macrolide antibiotics from the predatory bacterium *Pyxidicoccus fallax*. Chem. Eur. J.
408 **20**:15933-15940.
409 30. **Blair DF, Berg HC.** 1988. Restoration of torque in defective flagellar motors. Science **242**:1678-1681.
410 31. **Kearns DB, Losick R.** 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. **49**:581-
411 590.
412 32. **Grau RR, de Oña P, Kunert M, Leñini C, Gallegos-Monterrosa R, Mhatre E, Vileta D, Donato V,**
413 **Hölscher T, Boland W, Kuipers OP, Kovács ÁT.** 2015. A duo of potassium-responsive histidine kinases
414 govern the multicellular destiny of *Bacillus subtilis*. mBio **6**:e00581.
415 33. **Hölscher T, Bartels B, Lin YC, Gallegos-Monterrosa R, Price-Whelan A, Kolter R, Dietrich LE, Kovács**
416 **ÁT.** 2015. Motility, chemotaxis and aerotaxis contribute to competitiveness during bacterial pellicle biofilm
417 development. J. Mol. Biol. **427**:3695-3708.
418 34. **Kojima S, Blair DF.** 2004. Solubilization and purification of the MotA/MotB complex of *Escherichia coli*.
419 Biochemistry **43**:26-34.
420 35. **Chan JM, Guttenplan SB, Kearns DB.** 2014. Defects in the flagellar motor increase synthesis of poly-γ-
421 glutamate in *Bacillus subtilis.* J. Bacteriol. **196**:740-753.
422 36. **Sonenshein AL.** 2000. Control of sporulation initiation in *Bacillus subtilis*. Curr. Opin. Microbiol. **3**:561-566.
423 37. **Molle V, Fujita M, Jensen ST, Eichenberger P, González-Pastor JE, Liu JS, Losick R.** 2003. The Spo0A
424 regulon of Bacillus subtilis. Mol. Microbiol. 50:1683-1701.
425 38. **Overkamp W, Kuipers OP.** 2015. Transcriptional profile of *Bacillus subtilis sigF*-mutant during vegetative
426 growth. PLoS ONE **10**:e0141553.
427 39. **McKenney PT, Driks A, Eichenberger P.** 2013. The *Bacillus subtilis* endospore: assembly and functions of
428 the multilayered coat. Nat. Rev. Microbiol. **11**:33-44.
429 40. **Hullo M-F, Moszer I, Danchin A, Martin-Verstraete I.** 2001. CotA of *Bacillus subtilis* is a copper-dependent
430 laccase. J. Bacteriol. **183**:5426-5430.

431 41. **Donovan W, Zheng L, Sandman K, Losick R.** 1987. Genes encoding spore coat polypeptides from *Bacillus*
432 *subtilis*. J. Mol. Biol. **196**:1-10.
433 42. **Zheng L, Donovan WP, Fitz-James PC, Losick R.** 1988. Gene encoding a morphogenic protein required in
434 the assembly of the outer coat of the *Bacillus subtilis* endospore. Genes Dev. **2**:1047-1054.
435 43. **Isticato R, Esposito G, Zilhão R, Nolasco S, Cangiano G, De Felice M, Henriques AO, Ricca E.** 2004.
436 Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol. **186**:1129-1135.
437 44. **Isticato R, Pelosi A, De Felice M, Ricca E.** 2010. CotE binds to CotC and CotU and mediates their interaction
438 during spore coat formation in *Bacillus subtilis*. J. Bacteriol. **192**:949-954.
439 45. **Wang KH, Isidro AL, Domingues L, Eskandarian HA, McKenney PT, Drew K, Grabowski P, Chua MH,**
440 **Barry SN, Guan M, Bonneau R, Henriques AO, Eichenberger P.** 2009. The coat morphogenetic protein
441 SpoVID is necessary for spore encasement in *Bacillus subtilis*. Mol. Microbiol. **74**:634-649.
442 46. **Beall B, Driks A, Losick R, Moran CP.** 1993. Cloning and characterization of a gene required for assembly of
443 the *Bacillus subtilis* spore coat. J. Bacteriol. **175**:1705-1716.
444 47. **Ragkousi K, Setlow P.** 2004. Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis*
445 spores. J. Bacteriol. **186**:5567-5575.
446 48. **Poehlein A, Kusian B, Friedrich B, Daniel R, Bowien B.** 2011. Complete genome sequence of the type strain
447 *Cupriavidus necator* N-1. J. Bacteriol. **193**:5017.
448 49. **Heijerick DG, Van Sprang PA, Van Hyfte AD.** 2006. Ambient copper concentrations in agricultural and
449 natural European soils: an overview. Environ. Toxicol. Chem. **25**:858-864.
450 50. **Ozin AJ, Henriques AO, Yi H, Moran CP.** 2000. Morphogenetic proteins SpoVID and SafA form a complex
451 during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. **182**:1828-1833.
452 51. **Ozin AJ, Samford CS, Henriques AO, Moran CP.** 2001. SpoVID guides SafA to the spore coat in *Bacillus*
453 *subtilis*. J. Bacteriol. **183**:3041-3049.
454 52. **Conn HJ.** 1930. The identity of *Bacillus subtilis*. J. Infect. Dis. **46**:341-350.
455 53. **Konkol MA, Blair KM, Kearns DB.** 2013. Plasmid-encoded ComI inhibits competence in the ancestral 3610
456 strain of *Bacillus subtilis*. J. Bacteriol. **195**:4085-4093.
457 54. **Roberts MS, Cohan FM.** 1995. Recombination and migration rates in natural populations of *Bacillus subtilis*
458 and *Bacillus mojavensis*. Evolution **49**:1081-1094.

459 55. **Stefanic P, Mandic-Mulec I.** 2009. Social interactions and distribution of *Bacillus subtilis* pherotypes at
460 microscale. J. Bacteriol. **191**:1756-1764.
461 56. **Xu K, Strauch MA.** 1996. Identification, sequence, and expression of the gene encoding gamma-
462 glutamyltranspeptidase in *Bacillus subtilis*. J. Bacteriol. **178**:4319-4322.
463 57. **Branda SS, González-Pastor JE, Ben-Yehuda S, Losick R, Kolter R.** 2001. Fruiting body formation by
464 *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **98**:11621-11626.
465 58. **López D, Vlamakis H, Losick R, Kolter R.** 2009. Paracrine signaling in a bacterium. Genes Dev. **23**:1631-
466 1638.
467 59. **Cangiano G, Mazzone A, Baccigalupi L, Isticato R, Eichenberger P, De Felice M, Ricca E.** 2010. Direct
468 and indirect control of late sporulation genes by GerR of *Bacillus subtilis*. J. Bacteriol. **192**:3406-3413.
469 60. **Costa T, Serrano M, Steil L, Völker U, Moran CP, Henriques AO.** 2007. The timing of *cotE* expression
470 affects *Bacillus subtilis* spore coat morphology but not lysozyme resistance. J. Bacteriol. **189**:2401-2410.
471 61. **Driks A, Roels S, Beall B, Moran CP, Losick R.** 1994. Subcellular localization of proteins involved in the
472 assembly of the spore coat of *Bacillus subtilis*. Genes Dev. **8**:234-244.
473 62. **Eichenberger P, Fawcett P, Losick R.** 2001. A three-protein inhibitor of polar septation during sporulation in
474 *Bacillus subtilis*. Mol. Microbiol. **42**:1147-1162.

475 TABLE 1. *Bacillus subtilis* strains used

Strain	**Description, genotype**	**Source or reference**
168	laboratory strain, *trpC2*	Bacillus Genetics Stock Center
NCIB 3610	wild isolate	52
DK1042	NCIB 3610 *comI*^{Q12I}	53
RO-NN-1	wild isolate	54
PS216	wild isolate	55
ATCC 6633	wild isolate, subtilin producer	ATCC collection
TB48	168 *trpC2 amyE*::Phy-*gfp* Cm	18
TB34	DK1042 *amyE*::Phy-*gfp* Cm	This study
TB268	RO-NN-1 *amyE*::Phy-*gfp* Cm	This study
TB269	PS216 *amyE*::Phy-*gfp* Cm	This study
TB270	ATCC6633 *amyE*::Phy-*gfp* Cm	This study
SWV215	168 *trpC2 spo0A*::Km	56
RL1265	PY79 *spoIIAC*::Km	57
RL50	168 *trpC2 cotA*::Cm	41
RL52	168 *trpC2 cotC*::Cm	41
DL1032	NCIB 3610 Δ*epsA-O*:tet, Δ*tasA*:Km	58
GC260	PY79 *gerR*::Km	59
AH2835	*cotE*::C006D	60
AD18	*spoIVA*::Neo	61
PE277	PY79 *safA*::Tet	62
PE697	PY 79 *spoVID*::Km	45
TB193	NCIB3610 Δ*motA amyE*::Phy-*gfp* Cm	33
TB421	DK1042 *spo0A*::Km *amyE*::Phy-*gfp* Cm	This study
TB422	DK1042 *spoIIAC*::Km *amyE*::Phy-*gfp* Cm	This study
TB423	DK1042 *tasA*::Km *amyE*::Phy-*gfp* Cm	This study
TB424	DK1042 *gerR*::Km *amyE*::Phy-*gfp* Cm	This study
TB425	DK1042 *cotA::*Cm	This study
TB426	DK1042 *cotC::*Cm	This study
TB567	DK1042 *cotE*::Cm	This study
TB568	DK1042 *safA*::Tet *amyE*::Phy-*gfp* Cm	This study
TB569	DK1042 *spoIVA*::Neo *amyE*::Phy-*gfp* Cm	This study
TB570	DK1042 *spoVID*::Km *amyE*::Phy-*gfp* Cm	This study

476

477 FIG. 1. Results from the CFU-based predation assay. (A) Mean killing efficiency ($\bar{e}$ ± 95%
478 confidence interval) of *C. necator* against different *B. subtilis* strains. Asterisks denote
479 significant differences between killing efficiencies (Mann-Whitney U-test: * = $P < 0.05$; d.f. =
480 2). (B) Mean prey utilization ($\bar{u}$ ± 95 % confidence interval) of *C. necator* when preying on
481 different *B. subtilis* strains. Asterisks denote significant differences in the prey consumption
482 (Wilcoxon test: * = $P < 0.05$; d.f. = 2).

483 FIG. 2. Copper(II) dependence of predatory behavior. Prey reduction of *B. subtilis* 168 after co-
484 cultivation with *C. necator* in the presence of different Cu^{2+} concentrations.

485 FIG. 3. Frequency dependence of predatory behavior. Different predator-prey ratios versus the
486 mean killing efficiency ($\bar{e}$ ± 95% confidence interval) of *C. necator* are shown against *B. subtilis*
487 168.

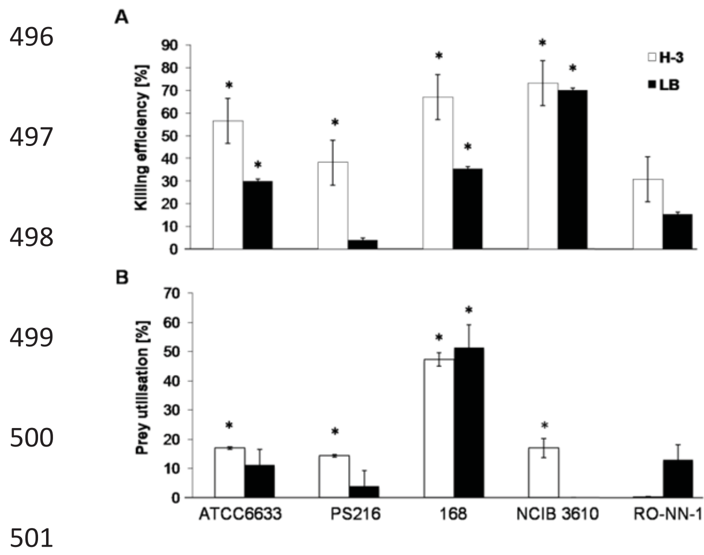
488 FIG. 4. Contact dependence of predatory behavior. Cells and supernatant from *C. necator*
489 cultures were tested in the CFU-based predation assay against *B. subtilis* 168. PBS buffer, H-3
490 medium supplemented with 50 µM of copper was mixed with *B. subtilis* and served as a control.

491 FIG. 5. Time dependence of predatory behavior. (A) Killing efficiency of *B. subtilis* NCIB 3610
492 and the nonsporulating (B) *spoA* and (C) *sigF* mutants (n.a., not applicable for statistic analysis).

493 FIG. 6. Survival of wild-type, mutant, or decoated spores of *B. subtilis* with or without exposure
494 to *C. necator*.

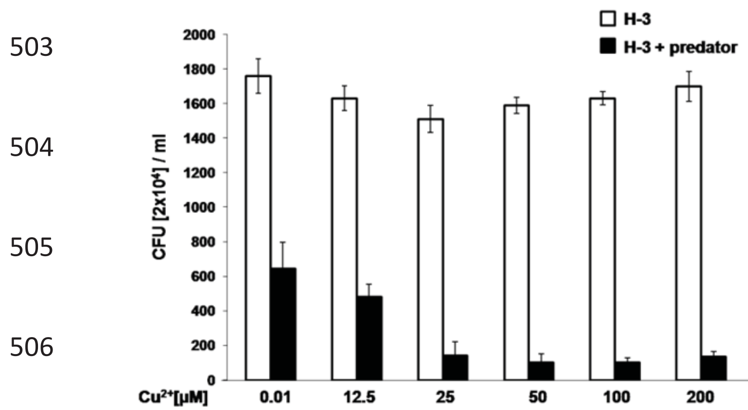
495 **FIG. 1.**

496
497
498
499
500
501



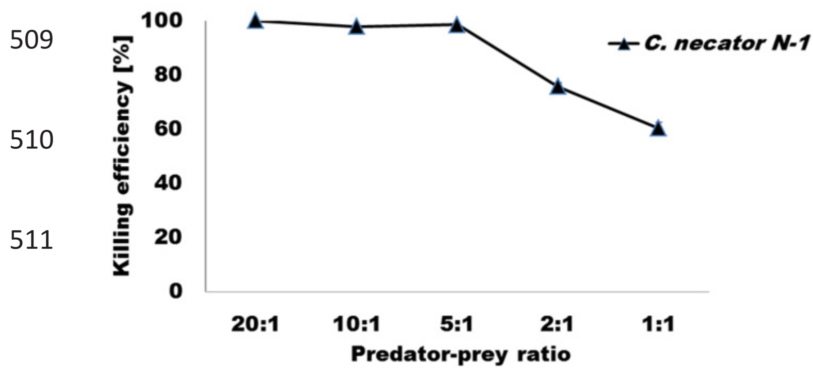
502 **FIG. 2.**

503
504
505
506
507



508 **FIG. 3.**

509
510
511

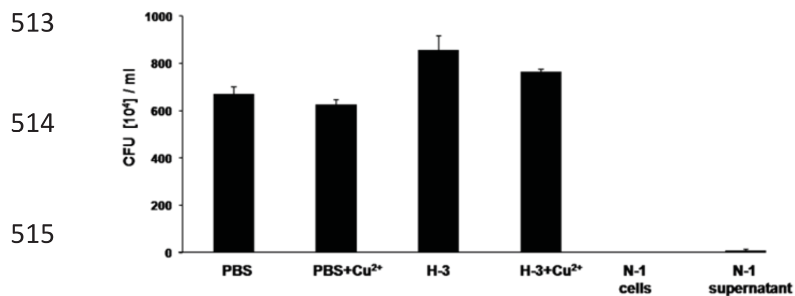


512 **FIG. 4.**

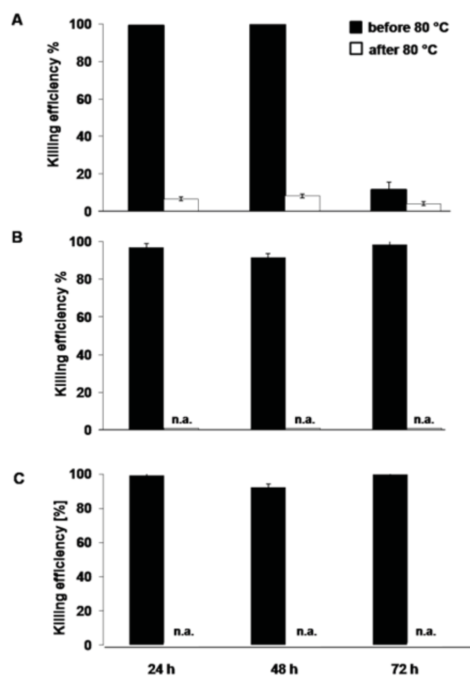
513

514

515

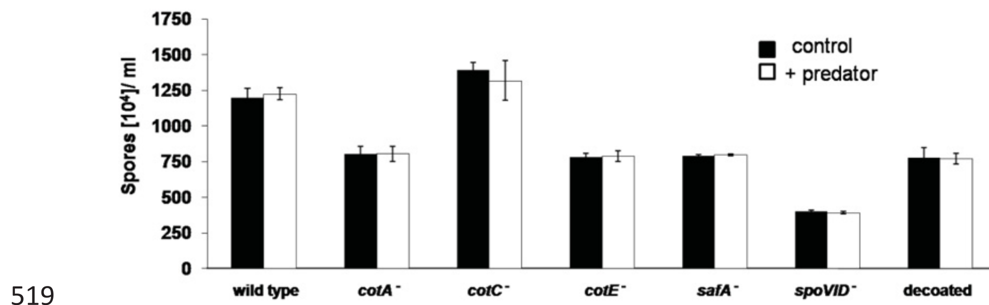


516 **FIG. 5.**



517

518 **FIG. 6.**



519

4.3 Manuscript C: Identification of new metallothioneins in the bacterium *Cupriavidus necator* N-1.

Seccareccia Ivana, Schmidt Andre, Hagen Matthias, Kothe Erika and Nett Markus. Identification of new metallothioneins in the bacterium *Cupriavidus necator* N-1. Manuscript in preparation.

Identification of new metallothioneins in the bacterium *Cupriavidus necator* N-1

Ivana Seccareccia[a], Andre Schmidt [b], Matthias Hagen[c], Erika Kothe [d] and Markus Nett[a#]

Junior Research Group "Secondary Metabolism of Predatory Bacteria", Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute, Jena, Germany[a];

Abteilung Geburtshilfe, Klinik für Frauenheilkunde und Geburtshilfe, Jena, Germany[b];

Big Data Analytics, Fakultät Medien, Bauhaus-Universität Weimar, Germany [c]

Institute of Microbiology, Microbial Communication, Friedrich Schiller University Jena, Germany[d]

#Address correspondence to Markus Nett, markus.nett@hki-jena.de

Abstract

Metallothioneins (MTs) are short peptides with high cysteine/histidine content that are able to bind metal ions. They play an essential role in detoxification and maintenance of optimal cell function. Based on their characteristic ability to scavenge heavy metals there is growing interest in these proteins for applications in bioremediation and drug development. However, there are many challenges with regard to the identification of new MTs owing to their short amino acid sequences that display little conservation. While bioinformatic tools can help to identify potential MT candidates, it is important to experimentally verify these predictions. In this study, the genome of *Cupriavidus necator* N-1 was bioinformatically screened for genes encoding ribosomally-derived peptides with high cysteine or histidine content. *C. necator* is a facultative microbial predator which exhibits high copper resistance. Not only was *C. necator* reported to secrete a peptidic molecule to sequester copper from the environment, but the bacterium was also proposed to use the resulting copper complex to kill other bacteria. Assuming that a metallothionein could represent the sought-after killing factor, we analyzed the genetic potential of *C. necator* for the production of these short peptides. After in *silico* analyses, all identified candidate peptides were heterologously produced and their capacity to bind different metals was evaluated.

Introduction

Metal homeostasis in bacteria is necessary for functioning of the cell (Hobman *et al.*, 2007; Moore & Helmann, 2005). When the intracellular concentration of essential macronutrients, like iron, magnesium, potassium, sodium or calcium, and micronutrients (e.g. zinc, nickel, copper, cobalt or manganese) is too low, specific acquisition mechanisms need to be activated. In case of a metal excess, resistance mechanisms are turned on to avoid toxic effects (Monsieurs *et al.*, 2011). High concentrations of essential metals may lead to the modification of enzymatic activities, the denaturation of proteins and structural damage of DNA or cell membranes (Dietz *et al.*, 1999; Hagemeyer, 2004; Vanassche & Clijsters, 1990). To minimize the damaging effects of high heavy metal concentrations, bacteria have developed detoxification reactions which include a range of efflux pumps, proteins that change the oxidation state of the metals, and intra- or extracellular metal binding mechanisms (Hossain *et al.*, 2012; Nies, 1999). Among them, immobilization is one of the major mechanisms for counteracting heavy metal toxicity in living organisms (Valls *et al.*, 2000). Bacteria, as well as higher organisms, use metallothioneins (MTs) for intracellular metal immobilization. MTs act as cytosolic binders and transporters of metal ions, especially Cu(I), Zn(II), and Cd(II) and Hg(II) (Sutherland & Stillman, 2014). They are small, cysteine and histidine-rich proteins. These proteins use their metal-thiolate clusters (Robinson *et al.*, 2001) to influence the cellular redox balance and they are known to play an essential role in metal homeostasis and in detoxification reactions (Hall, 2002). MTs are widespread across the eukaryotic kingdom, as well as in various bacteria (Valls *et al.*, 2000). Initially, MTs were thoroughly studied only in eukaryotes and for decades there were some misconceptions about their features, such as that they do not contain aromatic residues or secondary structure and that they exclusively use cysteine residues for metal binding (Blindauer, 2011). The discovery of bacterial MTs has led to a paradigm shift regarding these assumptions. It is now

generally accepted that MTs may also coordinate metal ions through imidazole nitrogens of histidine residues (Blindauer, 2011). The first bacterial MT that was demonstrated to bind metals via histidine residues was the zinc- and cadmium-binding SmtA. This peptide belongs to the BmtA family and is produced by the cyanobacterium *Synechococcus elongatus* PCC 7942 (Higham *et al.*, 1984). More recently, the second type of bacterial MT was identified in mycobacteria, featuring a copper-binding peptide (Gold *et al.*, 2008).

Due to the high capacity of MTs to immobilize heavy metals in the environment, there is a growing interest in these peptides for bioremediation purposes (Valls *et al.*, 2000). Some of these intriguing peptides may even have therapeutic potential. For instance, the MT azurin, a copper-binding protein isolated from *Pseudomonas aeruginosa* (Murphy *et al.*, 1993), shows a remarkable activity against breast cancer (Punj *et al.*, 2004). It is important to stress that MTs can be quite diverse. While they lack recognizable sequence similarity or even fold similarity, they are distinguished by an extraordinarily high abundance of cysteine and histidine residues as well as their metal ion binding ability. Because they must not display significant sequence homology with known MTs, there are some challenges in identifying new MTs (Blindauer, 2011). One example that illustrates this problem is the serendipitous discovery of MymT, which was found during screening for resistance mechanisms against a potential drug candidate and not as a consequence of the quest for new metal-binding proteins (Gold *et al.*, 2008). In summary, bacterial MTs are widespread, but largely overlooked and undetected.

In this study, a bioinformatic approach was used to identify new, putative bacterial MTs. The target organism was the predatory bacterium *Cupriavidus necator* N-1, which was already known to be resistant to high copper levels (Makkar & Casida, 1987). Moreover, for a long time it was speculated that *C. necator* produces a copper-chelating peptide, which can elevate the copper concentration to lethal levels for prey organisms (Casida, 1987). Only recently it

was experimentally confirmed that the presence of copper promotes the predatory behavior of this bacterium (Seccareccia *et al.*, 2016). We thus speculated that a copper-binding MT could be involved both in resistance and in the predatory strategy of *C. necator*. Therefore, MTs candidates that were identified based on their small protein size and a high content of cysteine and histidine residues (Schmidt *et al.*, 2010) were heterologously produced in *E. coli* and their ability to coordinate different metal ions was examined.

Materials and methods

Bioinformatic analysis. The sequenced genomes of four different *Cupriavidus* spp. were analyzed (Table 1). Gene products encoding small proteins of up to 200 amino acids were selected and subjected to an automated analysis that determined their cysteine and histidine contents. The selection criteria to qualify a protein as a putative metallothionein was an amino acid content of at least 15% cysteines and/or histidines. Qualified proteins were subjected to further *in silico* analyses and a manual search for known metal-chelating motifs in proteins was performed (Table S1). The analyses included the most commonly found short metal-binding motifs, namely CxxC, CxH and CxC (Usha *et al.*, 2009; Van Horn *et al.*, 2003; Zhou *et al.*, 2008). Additionally, the analyses included longer motifs such as CXCXXXXCXC (Cobine *et al.*, 2002) and CX21CXXXC, known as the TRASH domain (Ettema *et al.*, 2003).

Bacterial Strains and Growth Conditions. The *Cupriavidus necator* N-1 wild-type strain was cultivated at 30 °C in LB medium at 220 rpm. *Escherichia coli* strains were routinely grown in LB medium at 30 °C and 220 rpm. For protein expression experiments, terrific broth (TB, 1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4 and 0.4% glycerol) supplemented with 50 µg ml^{-1} kanamycin was used.

General DNA methods. Chromosomal DNA from *Cupriavidus necator* N-1 was isolated using an established protocol (Neumann *et al*., 1992). Plasmid isolation from *E. coli* strains was accomplished with a commercial plasmid isolation kit and DNA fragments from agarose electrophoresis gels were extracted with a QIAquick gel extraction kit. For the preparation of competent cells, *E. coli* DH5α and BL21(DE3) cultures were grown in LB medium at 30 °C until the OD_{600} reached a value of 0.4. The cultures were washed three times with ice-cold 10% glycerol and resuspended in 1 ml of 10% glycerol after final centrifugation. Competent cells (100 µl) were electrotransformed with 200 ng of purified DNA in 0.2-cm gapped ice-cold electroporation chambers using a Bio-Rad GenePulser II set to 200 Ω, 25 µF, and 2.5 kV.

Construction of expression plasmids. After *in silico* analyses of genomes, selected genes coding for putative MTs were heterologously expressed in *E. coli* BL21(DE3). The assembly of the respective expression vector is depicted in Fig. S1. In the first step, the nucleotide sequences of the selected genes were PCR amplified from *C. necator* genomic DNA with the specific primers listed in Table S2. The PCR reaction mixture included 2 mM $MgSO_4$, 0.2 mM each dNTP, 5% dimethyl sulfoxide, 50 pmol each primer, and 1.25 U *pfu* DNA polymerase. The following thermal cycling conditions were used: initial denaturation for 5 min at 95 °C; amplification steps during 30 cycles (95 °C for 1 min, 58 °C for 1 min, 72 °C for 5 min) and final extension for 10 min at 72 °C. The PCR products were first cloned into the vector pJET 1.2/blunt via blunt ligation. The resulting plasmids were then introduced in *E. coli* DH5α and subsequently sequence accuracy was verified by DNA sequencing. Afterwards, the gene inserts coding for targeted proteins were cloned into the NcoI-BamHI site of the expression plasmid pET28a(+). Lastly, the expression plasmids were introduced into the expression host *E. coli* BL21(DE3).

Heterologous expression and purification of proteins. The *E. coli* overexpression strains harboring the respective expression vectors were cultured in TB medium (containing 50 µg ml^{-1} kanamycin) at 37 °C to an OD_{600} of 0.6. At this point, 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the induced culture was further grown overnight at 16 °C. Finally, the cells were harvested by centrifugation at 9,600 x g for 5 min at 4 °C. The cell pellet was then resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol, 10% glycerol) and subjected to sonification (5 x 1 min., 4 cycles, 20% of power 200W). Afterwards, the suspension was centrifuged for 30 minutes at 4 °C and 9,600 x g. The supernatant was loaded onto a nickel-nitriloacetic acid column and, after a washing step with lysis buffer, the expressed native protein was eluted using increasing concentrations of imidazole in the buffer. Fractions that contained the target protein were identified via SDS-PAGE, pooled and desalted with PD-10 columns (GE Healthcare). The identity of the purified protein was confirmed by MALDI-TOF MS using a Bruker Ultraflex spectrometer (Bruker Daltonics).

Testing of metal binding. To investigate the metal binding properties of the recombinant peptides, immobilized metal ion affinity chromatography was performed. For this, 250 µL of Chelating Sepharose Fast Flow (GE Healthcare) were filled into a Pierce® Spin Column with screw cap and loaded with 300 µL of 4 different metal ion solutions: 200 mM iron(II) sulphate heptahydrate; 200 mM copper(II) sulphate; 200 mM nickel(II) sulphate hexahydrate and 200 mM zinc(II) sulphate heptahydrate. After 30 minutes of incubation the residual metal ion solution was removed by centrifugation at 500 x g for 1 min followed by washing with 300 µL washing buffer (20 mM sodium acetate, 200 mM sodium chloride, pH 4.0). Afterwards, the column was equilibrated twice with 300 µl of binding buffer (50 mM tris hydrochloride, 500 mM sodium chloride, pH 7.0). Then, the column was sealed and 100 µg of purified protein in 300 µL binding buffer were applied onto the sepharose and incubated

for 2 hours at 4 °C. After incubation, the column was washed with 5 x 300 µL binding buffer. Finally, the column was incubated twice at room temperature for 15 minutes with 300 µL binding buffer supplemented with 50 mM EDTA and then centrifuged at 500 g for 1 minute. Flow through, wash and elution fractions were collected and proteins were precipitated with acetone and subjected to SDS-PAGE.

Determination of dissociation constant. The affinity of metallothioneins towards different metal ions was determined by Microscale Thermophoresis (MST) using the Monolith NT.115 instrument (Nanotemper) as it was previously described for calcium binding protein (Nazari *et al.*, 2012). Selected proteins were labeled using the red fluorescent dye NT647 according to the manufacturer instructions. Different concentrations of $CuCl_2$, $NiCl_2$ and $MgCl_2$ (2 mM to 0.06 nM) were mixed with equivalent volumes of fluorescent labeled proteins (final concentration from 250 nM to 1.2 µM) supplemented with 0.1% of Tween-20. NT.115 Standard treated capillaries (Nanotemper) were used for all experiments. Data from two independent experiments and three capillary positions were averaged and evaluated using Kd model.

Results

Prediction of putative metallothioneins. Identifying new metallothioneins based on sequence similarity search is particularly challenging, because MTs possess comparatively short amino acid sequences, low complexity and they often lack significant homology to previously described MTs. Consequently, it is necessary to explore new approaches to identify promising MTs candidates. In the present study, genomes of four different *Cupriavidus* spp. (Table 1) were analyzed and *in silico* predictions of potential MTs were made based on high content of histidine and cysteine residues. We subsequently selected only small proteins of up to 200 amino acids in length that have cysteine and histidine contents of

higher than 15%. Of all proteins encoded by the *Cupriavidus* genomes, less than 1% fulfilled these criteria. Interestingly, while the total numbers of proteins differ greatly between the selected *Cupriavidus* strains (7,391 for *C. necator* N-1; 6,536 for *C. necator* H16; 6,169 for *C. metallidurans* CH34; 5686 for *C. taiwanensis* LMG 19424), the numbers of putative MTs in every strain are similar. A complete list of the putative MTs is given in the Supporting Information (Table S3). It is interesting to note that the most candidates were previously annotated as hypothetical proteins. Next, the 22 putative MTs were analyzed *in silico* for the presence of conserved metal binding motifs (Table S1). Three candidates did not feature known metal binding sequences and the remaining proteins, however, must not necessarily represent MTs. *C. metallidurans* CH34 has the highest number of identified putative metal binding proteins, seven, while five proteins were identified as MT candidates in the other three *Cupriavidus* species. The peptide with the highest content of cysteine and histidine had a size of 126 amino acids and was found in *C. taiwanensis* LMG 19424 (CAP62894). It contained an impressive histidine content of 21%, but completely lacked cysteines. Based on manual analysis, a few known metal-binding motifs were found such as HxxH, HHxH and HxH, which were reported to be potential zinc binding sites (Hooper, 1994). The identified protein with the second highest content of cysteine and histidine, 18.91%, belongs to *C. necator* N-1 (AEI78604). According to a protein BLAST search of the five putative MTs in *C. necator* N-1, four of them have homologs in other *Cupriavidus* spp. (Table S4). Since a relatively small number of putative MTs were obtained, all five candidate proteins from *Cupriavidus necator* N-1 (Table S3) were selected for overexpression studies and their potential to bind metals was investigated.

Heterologous production of the putative MTs from *C. necator* N-1. Genes for the five putative MTs were PCR amplified and individually cloned into pET28a. Subsequently, the genes were overexpressed in *E. coli* BL21(DE3) and the recombinant proteins were purified

by chromatography on the Ni-NTA resin exploiting their natural metal binding affinity (Fig. 1). Of the five tested candidates, only two proteins strongly bound to the Ni-NTA column. The identity of the two purified proteins was confirmed by MALDI-MS sequencing (Fig. S2).

Metal binding domains of recombinant peptides. The 74 aa-sized peptide with the accession number AEI78604 has the highest content of cysteine and histidine (18.91%) from all five putative MTs in *C. necator* N-1. It contains usual metal binding motifs like CxxC, CxH and CxC (Usha *et al.*, 2009; Zhou *et al.*, 2008), and it has at the C-terminus a 'natural His-tag' which consists of 7 histidines. Homologs of this protein are found in all 4 analyzed *Cupriavidus* proteomes (Fig. 2A), with 99% identity to that from *C. taiwanensis* H16 and 91% to that from *C. metallidurans* CH34. No conserved domains have been detected by BLAST analyses. The peptide AEI80891 has a size of 54 aa and contains 16.6% of cysteines and histidines. The cysteine and histidine content is lower than that of AEI78604 but the protein harbors conserved metal binding motifs, such as CxxCxH and HxxxHxxxHxxCxxCxxxxxxH. This protein has 96% sequence identity with WP_018008406 from *C. taiwanensis*, 94% with WP_041681830 from *Ralstonia pickettii* DTP0602 and 81% with WP_041680120 from *C. pinatubonensis* 1245 (Fig. 2B). A search for conserved domains revealed the presence of a zinc ribbon domain, which is characterized by two CxxC motifs separated by 17 amino acids (Zhou *et al.*, 2008). All presented homologs in Fig. 2B were annotated as FmdB family transcriptional regulators.

Testing of metal binding. The affinity of the purified proteins for binding divalent metal ions (Cu(II), Fe(II), Zn(II) and Ni(II)) was evaluated by means of immobilized metal ion affinity chromatography. Although both proteins were found to bind all tested metals, we observed some differences in their metal preferences. Protein AEI78604 exhibited the strongest affinity towards Ni(II) and Cu(II) based on the intensity of the corresponding elution band (Fig. 3A). For protein AEI80891, the strongest elution band was obtained after

chromatography over a Ni(II) column (Fig. 3B). Finally, the K_d values for the binding of Cu(II) and Ni(II) were determined by microscale thermohoresis (Table 2). The smaller the K_d values is, the more stronger the metal is bound and the higher the protein affinity towards tested metal is (Bisswanger, 2008). In this experiment as a reference the copper-binding protein azurin was used. Control experiments with Mg^{2+} showed that in the MST measurement indeed only Cu^{2+} and Ni^{2+} were binding to proteins. Azurin exhibited the highest affinity towards Cu(II) (1.0 ± 0.5 μM, n = 3), while no binding was detected when Ni(II) was used as ligand. On the other hand, both recombinant proteins showed binding affinity towards tested metals, although affinities of protein AEI80891 seem to be higher (Table 2). For protein AEI80891 Cu(II) K_d value was calculated to be 6.9 ± 1.8 μM (n = 3), while protein AEI78604 had ~ 6-fold less binding to Cu(II) (K_d = 43.9 ± 18.2 μM, n = 3). Binding affinities towards Ni(II) were weaker for both proteins AEI78604 (86.7 ± 45.1 μM) and again AEI80891 resulted in stronger binding (K_d = 24.0 ± 6.7 μM, n = 3).

Discussion

MTs play essential roles in metal homeostasis due to their capacity to neutralize high concentration of heavy metals. Therefore, the first step in their application is to identify novel MTs in different species. However, identifying new MTs based on homology to previously described ones is especially difficult since they are short peptides with low complexity. In general, it was recognized that the expected value (*e* value) in similarity searches is particularly low and promising MT candidates often remain undetected (Blindauer, 2011). Thus, in this study it was experimentally investigated whether an *in silico* approach for the discovery of putative MTs based on a high content of cysteine and histidine as described by Schmidt *et al*. (2010), leads to the identification of novel MTs. Four publicly available sequenced genomes belonging to species of the metal resistant *Cupriavidus* genus were analyzed in order to search for MT candidates. It is already known that peptides with high

amounts of cysteine or histidine are rare , which we confirmed after evaluation of four *Cupriavidus* genomes. From the total number of proteins encoded on each genome, less than 1% met the selection criteria, which is in line with the results reported in Schmidt *et al.* (2010). *Cupriavidus metallidurans* CH34 has the highest number of putative MTs and this is not surprising considering that this bacterium contains an exceptional number of genes involved in the resistance and processing of heavy metals (Mergeay *et al.*, 2003). Several metal resistance mechanisms have been elucidated in *C. metallidurans* CH34, whereas *C. necator* N-1 was not investigated in this respect, even though this predatory bacterium is also known for its high resistance against various metals (Makkar & Casida, 1987). *C. necator* N-1 had been proposed to use a copper-binding peptide for the killing of prey bacteria (Casida, 1987), which makes it hence an interesting candidate to explore MT biosynthesis and possible function of these peptides in predation. After *in silico* analyses of *C. necator* N-1 genome, five peptides were selected as putative MTs. In order to characterize the ability of these proteins to coordinate metal ions, selected genes were heterologously expressed in *E. coli* BL21(DE3). For pre-selection, the native proteins were initially isolated using standardized metal affinity chromatography with the Ni-NTA resin and from five candidates, two of them exhibit strong nickel binding properties (AEI78604 and AEI80891; Fig. 2). Both proteins exhibit very interesting features on the basis of their sequence (Table S3). Protein AEI78604 contains several metal binding motifs and the most unique feature is the natural His-tag consisting of 7 histidines at the C-terminus. Therefore, it was not surprising that the protein was able to bind to the Ni-NTA resin (Waugh, 2005). Homologous proteins were found in all four analyzed *Cupriavidus* genomes (Fig. 2A); however, the function is annotated as unknown. On the other hand, a possible zinc ribbon domain was detected during a BLAST search of protein AEI80891. The zinc ribbon fold has been conserved in a wide variety of differing protein structures and some of these proteins are recognized as important

transcription factors (Steven Hahn and Sadia Roberts, 2000). Further investigation confirmed that besides nickel, both isolated proteins can bind copper, iron and zinc (Fig 3). Applying MST measurement for both protein the binding affinity towards Cu^{2+} and Ni^{2+} was calculated. Isolated proteins showed significant binding towards tested metal ions and it seems that protein AEI80891 bind stronger than AEI80891. Overall, the approach to search putative MTs based on small size and high cysteine and histidine content showed to be very promising. From a total of four analyzed bacterial genomes, only 23 proteins qualified as putative MTs. Further evaluation revealed that 20 of these proteins feature known metal binding sequences, suggesting that the bioinformatic selection method yielded reasonable results. In summary, we were able to show that it is possible to predict metal-binding peptides via an *in silico* search based on high cysteine and histidine content in *C. necator* N-1.

Acknowledgment

We thank Mrs. Maria Poetsch for conducting MALDI-MS measurements and Prof. Dr. Peter Zipfel for providing the opportunity to conduct MST measurements. We are grateful to Prof. Dr. Alga Zuccaro and Robin Nostadt for sharing their protocol for immobilized metal affinity chromatography. I.S. thanks the graduate school Jena School for Microbial Communication, which is funded by the Deutsche Forschungsgemeinschaft (DFG), for a doctoral fellowship.

References

Bisswanger H (2008) Multiple Equilibria. In *Enzyme Kinetics*, pp 7-58. Wiley-VCH Verlag GmbH & Co. KGaA

Blindauer CA (2011) Bacterial metallothioneins: past, present, and questions for the future. *J Biol Inorg Chem* **16:** 1011-1024

Casida LE (1987) Relation to Copper of N-1, a Nonobligate Bacterial Predator. *Applied and environmental microbiology* **53:** 1515-1518

Cobine PA, George GN, Jones CE, Wickramasinghe WA, Solioz M, Dameron CT (2002) Copper transfer from the Cu(I) chaperone, CopZ, to the repressor, Zn(II)CopY: Metal coordination environments and protein interactions. *Biochemistry-Us* **41:** 5822-5829

Dietz KJ, Baier M, Krämer U (1999) *Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants*, Berlin: Springer.

Ettema TJG, Huynen MA, de Vos WM, van der Oost J (2003) TRASH: a novel metal-binding domain predicted to be involved in heavy-metal sensing, trafficking and resistance. *Trends Biochem Sci* **28:** 170-173

Gold B, Deng H, Bryk R, Vargas D, Eliezer D, Roberts J, Jiang X, Nathan C (2008) Identification of a copper-binding metallothionein in pathogenic mycobacteria. *Nat Chem Biol* **4:** 609-616

Hagemeyer J (2004) *Ecophysiology of plant growth under heavy metal stress*, Berlin: Springer.

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of experimental botany* **53:** 1-11

Higham DP, Sadler PJ, Scawen MD (1984) Cadmium-Resistant *Pseudomonas putida* Synthesizes Novel Cadmium Proteins. *Science* **225:** 1043-1046

Hobman JL, Yamamoto K, Oshima T (2007) *Transcriptomic responses of bacterial cells to sublethal metal ion stress*, Berlin: Springer.

Hooper NM (1994) Families of zinc metalloproteases. *FEBS letters* **354:** 1-6

Hossain MA, Piyatida P, da Silva JAT, Fujita M (2012) Molecular Mechanism of Heavy Metal Toxicity and Tolerance in Plants: Central Role of Glutathione in Detoxification of Reactive Oxygen Species and Methylglyoxal and in Heavy Metal Chelation. *Journal of Botany* **2012:** 37

Makkar NS, Casida LE (1987) *Cupriavidus necator* gen. nov., sp nov: a Nonobligate Bacterial Predator of Bacteria in Soil. *Int J Syst Bacteriol* **37:** 323-326

Mergeay M, Monchy S, Vallaeys T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D, Wattiez R (2003) *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS microbiology reviews* **27:** 385-410

Monsieurs P, Moors H, Van Houdt R, Janssen PJ, Janssen A, Coninx I, Mergeay M, Leys N (2011) Heavy metal resistance in *Cupriavidus metallidurans* CH34 is governed by an intricate transcriptional network. *Biometals* **24:** 1133-1151

Moore CM, Helmann JD (2005) Metal ion homeostasis in *Bacillus subtilis*. *Curr Opin Microbiol* **8:** 188-195

Murphy LM, Strange RW, Karlsson BG, Lundberg LG, Pascher T, Reinhammar B, Hasnain SS (1993) Structural Characterization of Azurin from *Pseudomonas aeruginosa* and Some of Its Methionine-121 Mutants. *Biochemistry-Us* **32:** 1965-1975

Nazari K, Kelay V, Mahmoudi A, Hashemianzadeh SM (2012) Binding of Divalent Metal Ions to Calcium-Free Peroxidase: Thermodynamic and Kinetic Studies. *Chem Biodivers* **9:** 1806-1822

Nies DH (1999) Microbial heavy-metal resistance. *Appl Microbiol Biot* **51:** 730-750

Punj V, Bhattacharyya S, Saint-Dic D, Vasu C, Cunningham EA, Graves J, Yamada T, Constantinou AI, Christov K, White B, Li G, Majumdar D, Chakrabarty AM, Das Gupta TK (2004) Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. *Oncogene* **23:** 2367-2378

Robinson NJ, Whitehall SK, Cavet JS (2001) Microbial metallothioneins. *Adv Microb Physiol* **44:** 183-213

Seccareccia I, **Kovács** ÁT, **Gallegos-Monterrosa R, Nett M** (2016) Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*. Manuscript in preparation

Schmidt A, Hagen M, Schutze E, Schmidt A, Kothe E (2010) In silico prediction of potential metallothioneins and metallohistins in actinobacteria. *J Basic Microb* **50:** 562-569

Sutherland DEK, Stillman MJ (2014) Challenging conventional wisdom: single domain metallothioneins. *Metallomics* **6:** 702-728

Usha B, Venkataraman G, Parida A (2009) Heavy metal and abiotic stress inducible metallothionein isoforms from *Prosopis juliflora* (SW) DC show differences in binding to heavy metals in vitro. *Mol Genet Genomics* **281:** 99-108

Valls M, Atrian S, de Lorenzo V, Fernández LA (2000) Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for immobilization of heavy metals in soil. *Nat Biotech* **18:** 661-665

Van Horn JD, Bulaj G, Goldenberg DP, Burrows CJ (2003) The Cys-Xaa-His metal-binding motif: {N} versus {S} coordination and nickel-mediated formation of cysteinyl sulfinic acid. *J Biol Inorg Chem* **8:** 601-610

Vanassche F, Clijsters H (1990) Effects of Metals on Enzyme-Activity in Plants. *Plant Cell Environ* **13:** 195-206

Waugh DS (2005) Making the most of affinity tags. *Trends Biotechnol* **23:** 316-320

Zhou L, Singleton C, Le Brun NE (2008) High Cu(I) and low proton affinities of the CXXC motif of *Bacillus subtilis* CopZ. *Biochem J* **413:** 459-465

Table 1. List of *Cupriavidus* genomes used in this study.

Organism	Replicon	Accession No.	Size (Mb)
Cupriavidus metallidurans CH34	chromosome	NC_007973.1	3.93
	megaplasmid	NC_007974.2	2.58
	plasmid pMOL30	NC_007971.2	0.23
	plasmid pMOL28	NC_007972.2	0.17
Cupriavidus necator H16	chromosome	NC_008313.1	4.05
	chromosome	NC_008314.1	2.91
	megaplasmid pHG1	NC_005241.1	0.45
Cupriavidus necator N-1	chromosome	NC_015726.1	3.80
	chromosome	NC_015723	2.60
	plasmid pBB1	NC_015727	1.50
	plasmid pBB2	NC_015724.1	0.42
Cupriavidus taiwanensis LMG 19424	chromosome	NC_010528.1	3.42
	chromosome	NC_010530.1	2.50
	plasmid pRALTA	NC_010529.1	0.56

Table 2. Dissociation constant of isolated recombinant proteins AEI78604 and AEI80891 for the binding of Cu(II), Ni(II), as determined by microscale thermophoresis. As negative control affinities towards Mg(II) ions were measured for both proteins and azurin was used as a reference. Calculations were made using obtained data from at least two measurements.

Metal	Proteins		
	AEI78604 K_d [µM]	AEI80891 K_d [µM]	Azurin K_d [µM]
Cu (II)	43.9 ± 18.2	6.9 ± 1.8	1.0 ± 0.5
Ni(II)	86.7 ±45.1	24.0 ± 6.7	No binding
Mg(II)	No binding	No binding	Not determined

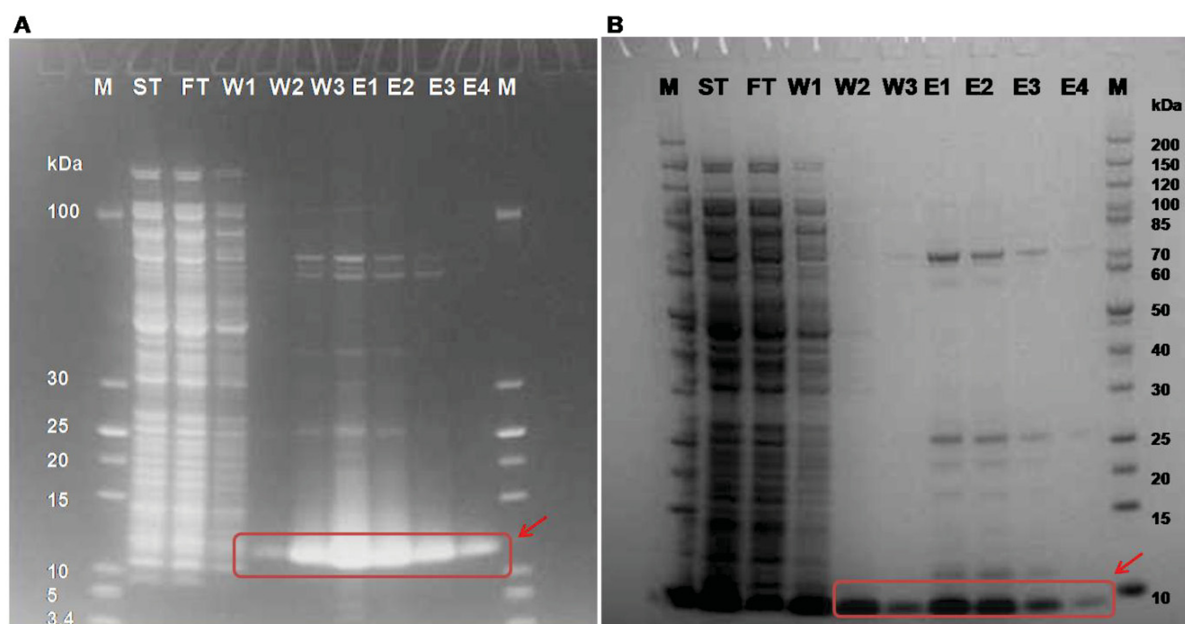


Figure 1. SDS-PAGE analysis of (A) AEI78604 and (B) AEI80891. Labeled columns on the gel image are: M-protein ladder 200-3.4 kDa; FT-flow through fraction; W1-W2 washing fractions; E1-E4 elution fractions.

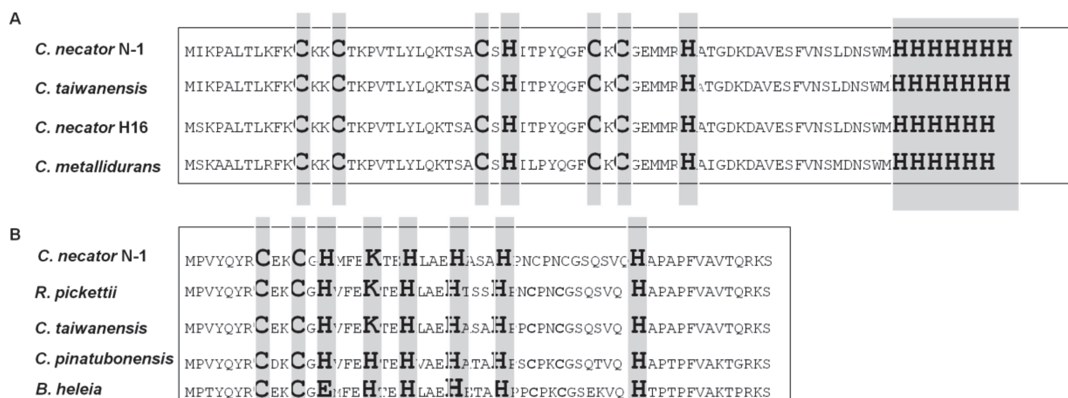


Figure 2. Alignment of (A) AEI78604 and (B) AEI80891.

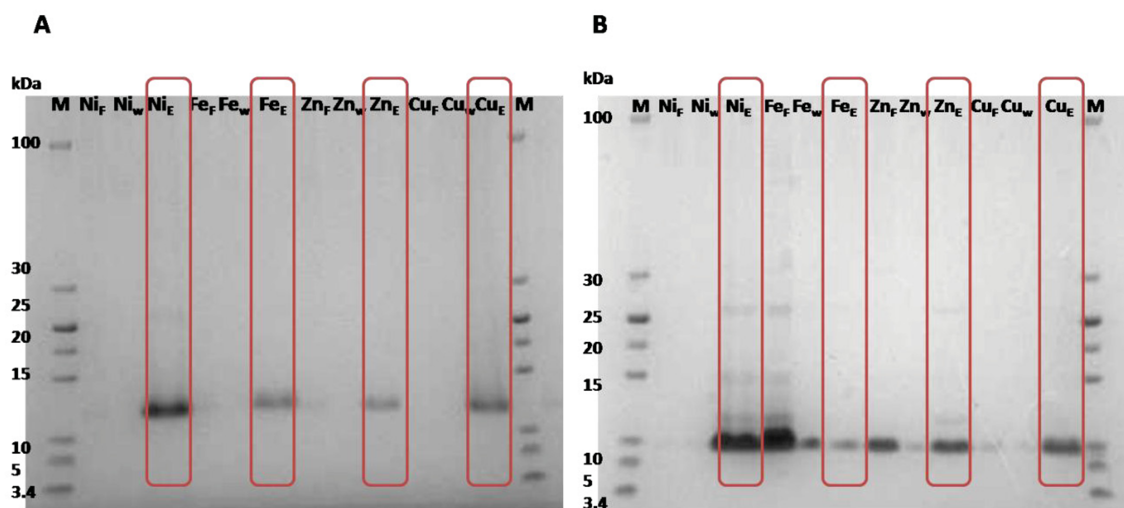


Figure 3. Metal binding affinity assay of (A) AEI78604 and (B) AEI80891. The peptides were subjected to SDS-PAGE after performing metal affinity chromatography using different metal resins. The flow through fraction is indicated by the letter F, W indicates the washing fraction and E indicates the elution fraction.

Identification of new putative bacterial metallothioneins of predatory bacteria *Cupriavidus necator* N-1

Ivana Seccareccia,[a] Andre Schmidt [b], Matthias Hagen[c], Erika Kothe[d] and Markus Nett[a#]

Junior Research Group "Secondary Metabolism of Predatory Bacteria", Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute, Jena, Germany[a];

Abteilung Geburtshilfe, Klinik für Frauenheilkunde und Geburtshilfe , Jena, Germany[b]

Big Data Analytics, Fakultät Medien, Bauhaus-Universität Weimar, Germany [c]

Institute of Microbiology, Microbial Communication, Friedrich Schiller University Jena, Germany[d]

#Address correspondence to Markus Nett, markus.nett@hki-jena.de

Table of Contents

SI-2	Table S1	Identified metal chelating motifs in proteins
SI-2	Table S2	Primer list
SI-3	Table S3	List of putative MTs that were identified during an *in silico* analysis of bacterial genomes
SI-4	Fig. S1	Work flow for construction of recombinant plasmids for heterologous expression of putative metallothioneins
SI-5	Table S4	Homologous proteins of putative MTs
SI-5	Fig. S2	MALDI-TOF/TOF data of purified putative metallothioneins

Table S1. Primer list used for the construction of metallothionein expression plasmids. Bold nucleotides represent restriction sites used for ligation.

Amplified gene	Name	Nucleotide sequence (5`--> 3`)
For_Cne1c32990	P1	5'-CAT**CCATGG**AAATGAGCAAACCCGCCCTGA-3'
Rev_Cne1c32990	P2	5'-CAT**GGATCC**TCAGTGGTGGTGGTGATGGT-3'
For_ 2c19350	P3	5'-CATG**CCATGG**AAATGCCCGTCTACCAATACCG-3'
Rev_2c19350	P4	5'-CATTG**GGATCC**TCAGCTCTTGCGCTGGGT-3'
For_2c08640	P5	5'-CATT**CCATGG**AAATGATGAGCGCCGTCAAGCT-3'
Rev_2c08640	P6	5'-CATG**GGATCC**TCAACGAACACACGAGCAAGAC-3'
For_2c00920	P7	5'-CAT**CCATGG**AAATGATCCGACCCACCGTGCA-3'
Rev_2c00920	P8	5'-CATTG**GGATCC**CTAAGCCGTCATCTTCAGGCAC-3'
For_BB1p13990	P9	5'-TG**CCATGG**AAATGAAGAAGCTTGCGACGGC-3'
Rev_ BB1p13990	P10	5'-CATTG**GGATCC**TCAATGGCAGTGGCGAATGC-3'

Table S2. Known metal chelating motifs in proteins.

Metal chelating motifs	Reference
CXCXXXXCXC	(Cobine *et al.*, 2002)
CXCC HXCXCC	(Monchy *et al.*, 2006)
CX21CXXXC-TRASH domain CSNSC	(Ettema *et al.*, 2003)
CXXEE	(Kotrba & Ruml, 2010)
CXXH	(Stephens & Bauerle, 1992)
CXXC	(Zhou *et al.*, 2008)
CXC CC	(Usha *et al.*, 2009)
CXH	(Horn D.V. *et al.*, 2003)
HXXC	(Wang *et al.*, 2003)

Table S1. List of putative MTs that were identified during an *in silico* analysis of bacterial genomes

Organism	GenBank accession #	Size (AA)	Sequence	CH%	Annotation
C. metallidurans CH 34	ABF07172	86	MALMITDDCINCDVCEPECPNEAISMGPEIYEIDPGKCTECVGHFDEPQCQQVCPVACIPKDPNHVETHEVLMQRYRLLTAAKHVA	15.1	Ferredoxin
	ABF10094	73	MSKAALTLRFKCKKCTKPVTLYLQKTSACSHILPYQGFCKCGEMMRHAIGDKDAVESFVNSMDNSWMHHHHHH	17.8	Conserved hypothetical protein
	ABF10238	58	MHSLCSGRSSSCMDVFWHGHSCKEGTREPKSNQACWVPRIAANHEQDCRQISELMELG	15.51	Hypothetical protein
	ADC45215	33	MIAFPHQGLCTRVAHLISIGSMQILYCEQFNCV	15.15	Hypothetical protein
	ADC45337	60	MLLEMSCLDYLVDPDSKPIRKILLLRRYNAGCPLSMNDHAQAHRVGHSWLSAHHVHCRSR	15	Hypothetical protein
	ABF12612	116	MIRPTVQENFSRYADCIAACNAAAAACLKCAAACLEEPDTRKMTRCIALDMDCAGIANLAASYMLRNSEFAPLVCEDCAEVCKWCKEECERYDHWHCQECAKACAACMEMCLKMTA	16.37	Conserved hypothetical protein
	ABF12828	100	MMGFLERLMGRHSGGHHGGGSEHGRRGGHHDGGGSYGYGNPLPPQSPAGVHCPNCGTVSAQGARFCQQCGSSLAPAPCSRCGTLLPRDAKFCGSCGNAAK	15	Conserved hypothetical protein
C. necator H16	CAJ92387	54	MDPDDTYFIIHCLDHADALPRRLASHDAHRSATLATLGLNLNVHLHISQNCHPT	16.6	Hypothetical protein
	CAJ92938	74	MHDVILESVVTCPHCGWAQRETMPMDACVFFYECRHCRVLLKPKSGDCCVFCSFGSVRCPQVQQGQCCERSRFE	18.9	Hypothetical protein
	CAJ94430	73	MSKPALTLKFKCKKCTKPVTLYLQKTSACSHITPYQGFCKCGEMMRHATGDKDAVESFVNSLDNSWMHHHHHH	17.88	Hypothetical protein
	CAJ94911	116	MIRPTVQENAARYADCIAACNAASAAALKCAAACLEEQDVRKMARCIALDMDCAGIAQLAASYMLRNSEFATLVCEDCAEVCKWCKEECEHHDNEHCQECARACAACMEQCLKMTA	16.4	Ferredoxin
	CAJ95696	89	MMSAVKLQSIITCPKCAHAKEETMPIDACQWCYECECCHAMLRPKAGDCCVFCSYGSERCPPMQQHSCSCVRRRRYSDARRTLDSVPPG	17.9	Hypothetical protein
C. necator N-1	AEI78604	74	MSKPALTLKFKCKKCTKPVTLYLQKTSACSHITPYQGFCKCGEMMRHATGDKDAVESFVNSLDNSWMHHHHHHH	18.91	Hypothetical protein
	AEI79084	116	MIRPTVQENAARYADCIAACNAAAAAALKCAAACLEEQDVRKMARCIALDMDCAGIAQLAASYMLRNSEFAPLVCEDCAEVCKWCKEECEHHDNEHCQDCARACAACMEQCLKMTA	16.37	Ferredoxin
	AEI79835	72	MMSAVKLQSIITCPKCAHAKEETMPIDACQWCYECERCHAVLRPKAGDCCVFCSYGTERCPPMQQQSCSCVR	19.4	Hypothetical protein
	AEI80891	54	MPVYQYRCEKCGHMFEKTEHLAEHASAHPNCPNCGSQSVQHAPAPFVAVTQRKS	16.6	Hypothetical protein
	AEI82791	44	MKKLATAIMALALFAAVGTANAHSGGTDRQGCHVDHSTGIRHCH	15.9	Hypothetical protein
C. taiwanensis LMG 19424	CAP62894	126	MRGTVHPFRLQQHRIMAHPVLFAALLAGLMSLLAARSAMAHVDVGVHIGPPVYAAPAPVYVAPPPPVVYAPRYHGWHGDRYWDGRRWYGRHEWRGRHHHHYRHHHHRHHHDRHHGHGHHGHRGHGH	21.42	Conserved hypothetical protein
	CAQ69704	30	MNASHCRETLQCGKVAVCHLIFYFYTTNLG	16.6	doubtful cds
	CAQ70746	74	MIKPALTLKFKCKKCTKPVTLYLQKTSACSHITPYQGFCKCGEMMRHATGDKDAVESFVNSLDNSWMHHHHHHH	18.9	Hypothetical protein
	CAP63299	116	MIRPTVQENAARYADCIAACNAAAAAALKCAAACLEEQDVRKMARCIALDMDCAGIAQLAASYMLRNSEFAPLVCEDCAEVCKWCKEECERHDAEHCQECARACAVCMEQCLKMTA	15.51	Hypothetical protein
	CAQ72461	104	MDRSILHRMSLCHCWKSAQAICRRHCKPSNRNGAARPASCVRHCQWTCRGFRRITWTKLHDQPVPCGLIEDVLARCAQKGHRLVQSRHLNVPVYTWTNVREWPN	15.38	Hypothetical protein

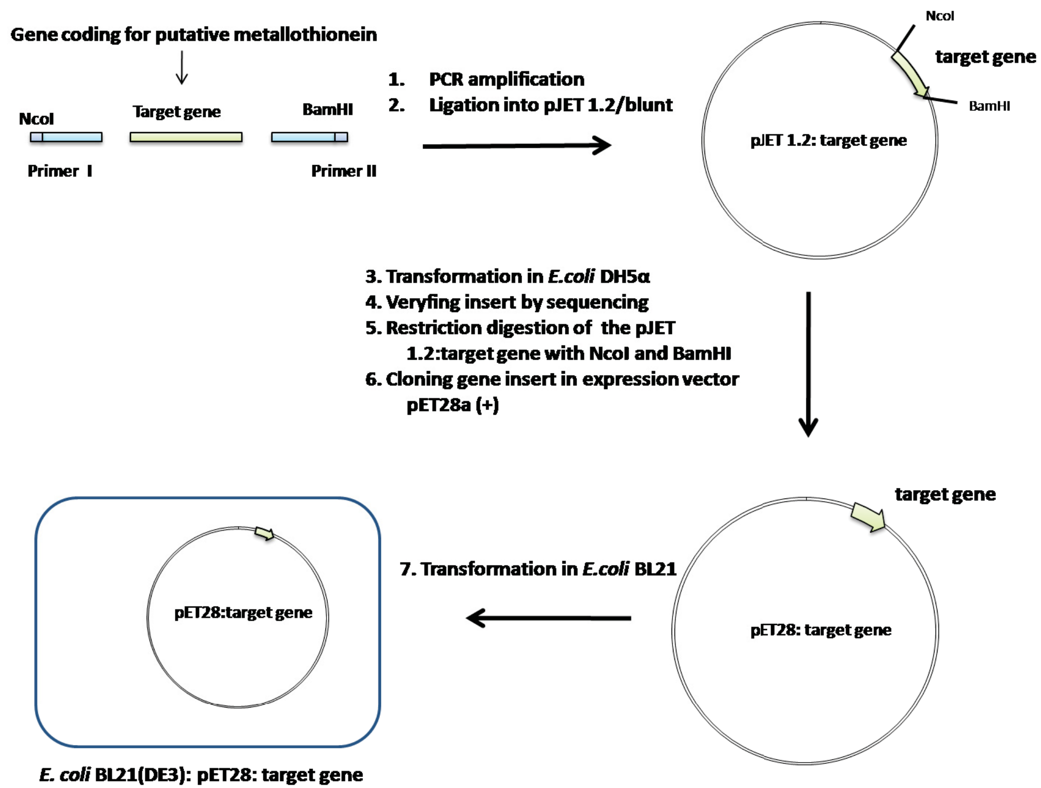
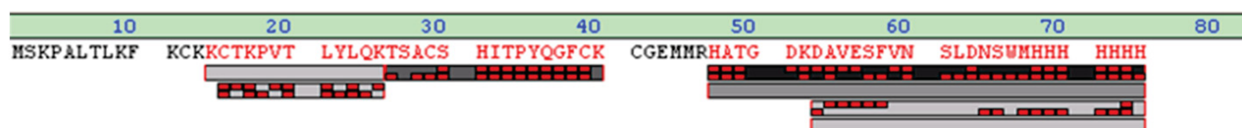


Figure S1. Work flow for construction of metallothionein expression plasmid

Table S4. Homologous proteins of putative MTs identified in *C. necator* N-1

Identified protein	Sequence identity [%]	GenBank Accession No	Organism
AEI78604	99	WP_029048367	*Cupriavidus taiwanensis* LMG19424
	99	AGW92127	*Ralstonia pickettii* DTP0602
	100	CAJ94430	*Cupriavidus necator H16*
	93	WP_011517705 WP_011517705	*Cupriavidus metalidurans CH 34*
	88 86	WP_043350086 WP_043420367.	*Cupriavidus basilensis* RK1
AEI79084	98	WP_018006016.1 WP_012354956	*Cupriavidus taiwanensis* LMG19424
	97	WP_022537330	*Ralstonia pickettii* DTP0602
	94	WP_042880569	*Cupriavidus necator* A5-1
	94	WP_011301627	*Cupriavidus pinatubonensis* 1245
AEI79835.1	94	CAJ95696	*Cupriavidus necator H16*
	75	EHP41788	*Cupriavidus basilensis* OR16
AEI80891	98	WP_042879673	*Cupriavidus necator H16*
	94	WP_041681830	*Ralstonia pickettii* DTP0602
	96	WP_018008406	*Cupriavidus taiwanensis* LMG19424
AEI82791	-	-	Unique

A



B

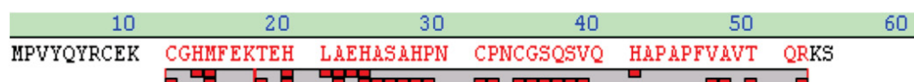


Figure S2: MALDI-TOF/TOF data of purified putative metallothioneins. (A) Protein AEI78604 confirmed with 74.3% MS/MS sequence coverage. (B) Protein AEI80891 confirmed with 77.8% MS/MS sequence coverage. Peptide fragments observed in MALDI-TOF/TOF are highlighted in red.

References

Cobine PA, George GN, Jones CE, Wickramasinghe WA, Solioz M, Dameron CT (2002) Copper transfer from the Cu(I) chaperone, CopZ, to the repressor, Zn(II)CopY: Metal coordination environments and protein interactions. *Biochemistry* **41:** 5822-5829

Ettema TJG, Huynen MA, de Vos WM, van der Oost J (2003) TRASH: a novel metal-binding domain predicted to be involved in heavy-metal sensing, trafficking and resistance. *Trends Biochem Sci* **28:** 170-173

Kotrba P, Ruml T (2010) Surface Display of Metal Fixation Motifs of Bacterial P1-Type ATPases Specifically Promotes Biosorption of Pb2+ by Saccharomyces cerevisiae. *Appl Environ Microbiol* **76:** 2615-2622

Monchy S, Benotmane MA, Wattiez R, van Aelst S, Auquier V, Borremans B, Mergeay M, Taghavi S, van der Lelie D, Vallaeys T (2006) Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in Cupriavidus metallidurans strain CH34. *Microbiology* **152:** 1765-1776

Stephens CM, Bauerle R (1992) Essential Cysteines in 3-Deoxy-D-Arabino-Heptulosonate-7-Phosphate Synthase from Escherichia-Coli - Analysis by Chemical Modification and Site-Directed Mutagenesis of the Phenylalanine-Sensitive Isozyme. *J Biol Chem* **267:** 5762-5767

Usha B, Venkataraman G, Parida A (2009) Heavy metal and abiotic stress inducible metallothionein isoforms from Prosopis juliflora (SW) DC show differences in binding to heavy metals in vitro. *Mol Genet Genomics* **281:** 99-108

Wang JY, Luthey-Schulten ZA, Suslick KS (2003) Is the olfactory receptor a metalloprotein? *Proc Natl Acad Sci USA* **100:**

5 Unpublished Results

5.1 Evaluation of predatory potential in *Cupriavidus* spp.

Cupriavidus necator was described as a non-obligate microbial predator capable of lysing different Gram-negative and Gram-positive bacteria [18]. In manuscript B it was demonstrated that copper(II) promotes the predation of *B. subtilis* by *C. necator* N-1. In addition, we set out to evaluate the predatory performance of *C. necator* N-1 against seven different prey bacteria and if the same correlation between the availability of copper(II) and killing efficiency exists. Until now, *C. necator* N-1 has been the only predatory strain described in the genus *Cupriavidus*. Therefore, a preliminary evaluation of the predatory potential of two additional *Cupriavidus* strains was also conducted. The first was the closely related *C. necator* H16, which is used for an industrial production of poly3-hydroxybutyrate (PHB) [110]. The second representative was *C. taiwanensis* LMG19424 which is a β-rhizobium capable of nodulating *Mimosa* species and fixing N2 within the nodules [111]. All *Cupriavidus* strains were tested in three different predation assays.

Bacterial strains and cultivation conditions. Three *Cupriavidus* spp. were selected as predators and different bacteria were tested as prey organisms. Strains used in this study were obtained from the German Strain Collection of Microorganisms and Cell Cultures (DSMZ) or the Jena Microbial Resource Collection (JMRC). *Ralstonia solanacearum* GMI1000 was kindly provided by C. Allen (Department of Plant Pathology, University of Wisconsin-Madison, USA). Strain numbers and cultivation conditions are provided in Table 2.

Table 2. Bacterial strains and used cultivation conditions

Predator species	Strain	Growth medium	Growth temperature (°C)
Cupriavidus necator	DSM 545 (N-1)	H-3 or LB	30
Cupriavidus necator	DSM 428 (H16)	H-3 or LB	30
Cupriavidus taiwanensis	DSM 17343 (LMG19424)	H-3 or LB	30
Prey species			
Agrobacterium tumefaciens	DSM 5172	LB	30
Bacillus subtilis	DSM 347	LB	30
Chromobacterium pseudoviolaceum	DSM 23279	LB	30
Escherichia coli	DSM 18039	LB	37

Lactococcus lactis	DSM 20069	SM17	30
Micrococcus luteus	DSM 14234	LB	30
Pseudomonas fluorescens	DSM 11532	LB	30
Ralstonia solanacearum	GMI1000	NB	30
Rhodococcus rhodochrous	DSM 43334	LB	30

H-3, Mineral minimal medium, LB, Luria Broth; NB, Nutrient Broth; SM17, M17 medium (Sigma) with 0.5% sucrose.

Prey spot plate assay. The predatory activity of *C. necator* N-1, *C. necator* H16 and *C. taiwanensis* was initially investigated in a prey spot plate assay against *E. coli*, *B. subtilis, M. luteus, R. rhodochrous* and *C. pseudoviolaceum*. *Myxococcus fulvus* was used as a control predator. None of the *Cupriavidus* strains were capable of lysing the prey bacteria under the experimental conditions tested (Fig. 5), paralleling observations previously made with *Lysobacter* spp. (see manuscript A).

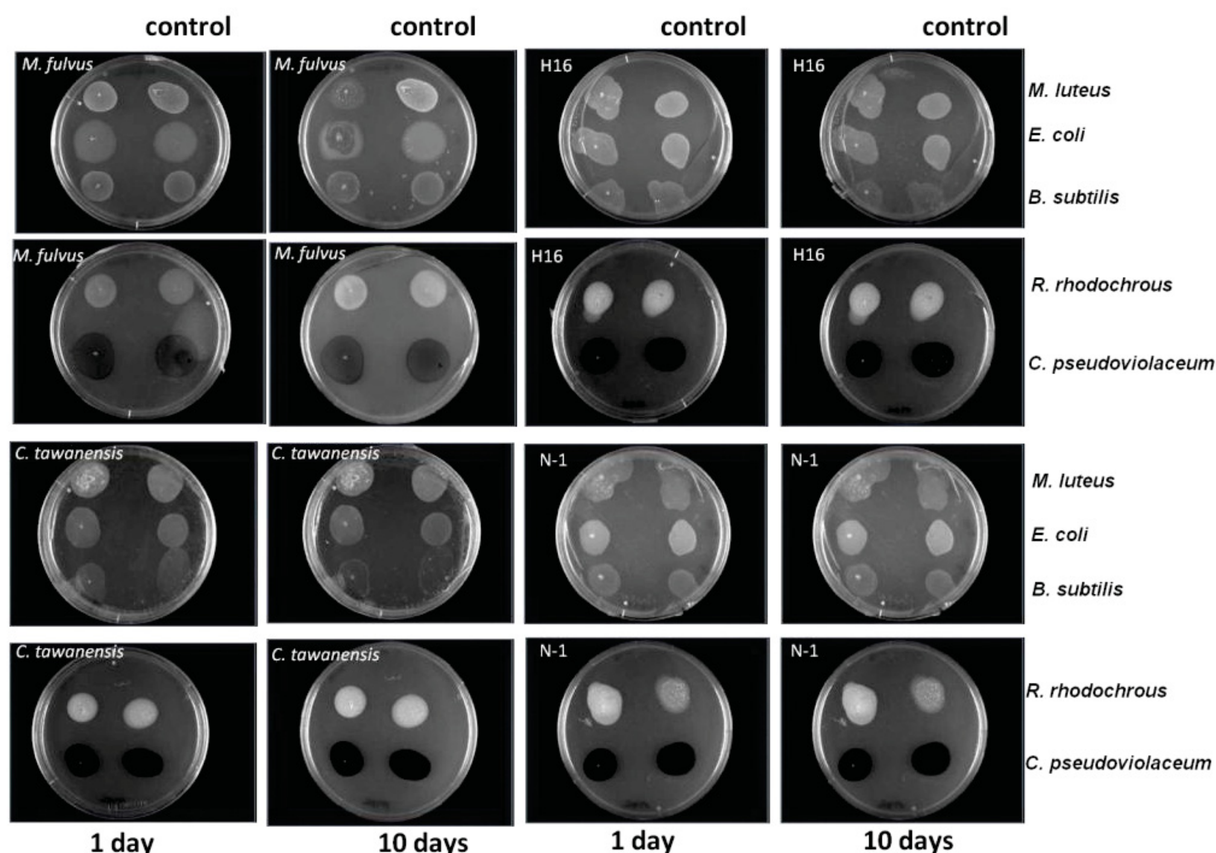


Figure 5. Prey spot plate assay of three different bacteria belonging to the genus *Cupriavidus*. *M. fulvus* was used as a control.

Lawn predation assay. The predatory activity of *C. necator* N-1, *C. necator* H16 and *C. taiwanensis* was also evaluated in a lawn predation assay against five different bacterial species (Fig. 6). *Myxococcus fulvus* was used as a positive control. Again, the *Cupriavidus*

spp. did not show predatory activity. Furthermore, swarming was also not observed for all three *Cupriavidus* spp. (Fig. 6).

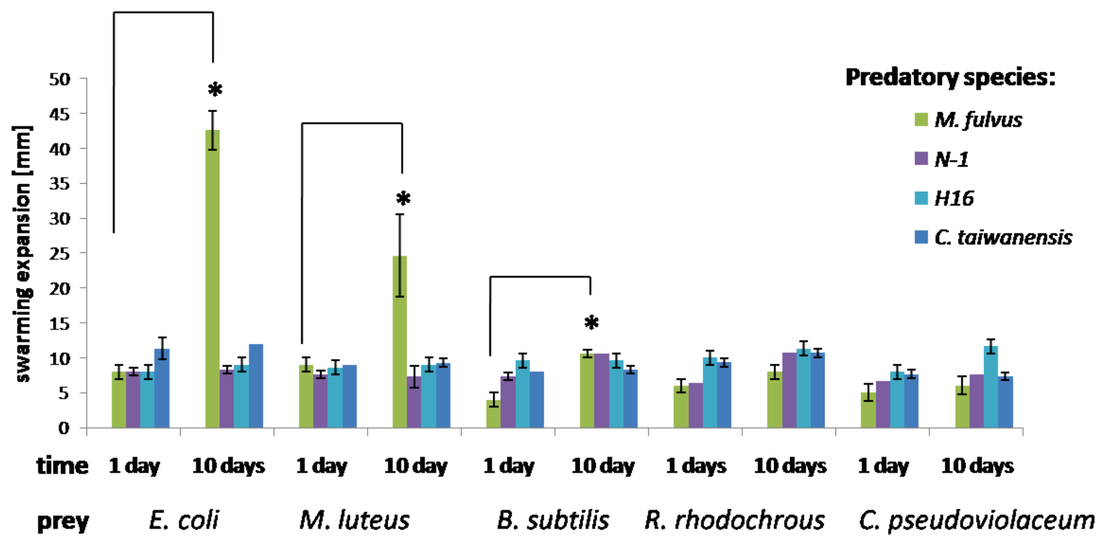


Figure 6. Results from the lawn predation assay of three different *Cupriavidus* spp. and *M. fulvus*. The mean swarm expansion is shown (±95% confidence interval [n=3]) (in millimeters) of four species of predatory bacteria. Paired t test: *, $P < 0.05$ between day 1 and day 10. All other comparisons were not significant ($P > 0.05$).

CFU-based predation assay. Finally, the predatory activities of the three *Cupriavidus* strains were tested in the CFU-based predation assay. Before testing in the assay, all selected predators were cultivated in nutrient-rich LB or, alternatively, in H-3 minimal medium in order to evaluate possible effects of the nutrition status on the predatory activity. When *C. necator* N-1 had been grown in LB medium and was subsequently tested against different prey organisms (Fig. 7A), prey reduction was only significant when *B. subtilis* and *P. fluorescens* served as a prey. However, when minimal H-3 mineral medium supplemented with copper (50 µM) was used for predator preculture, Mann-Whitney analysis revealed that prey reduction was more prominent for most tested prey bacteria (Fig. 7A). Moreover, *C. pseudoviolaceum* and *R. rhodochrous* were attacked only when N-1 was cultivated in H-3 medium but not in LB medium. Similar results were obtained for *M. luteus* and *E. coli*, and more pronounced predatory behavior was observed in H-3 medium. The phytopathogenic bacteria *A. tumefaciens* and *R. solanacearum* were not affected by *C. necator* N-1, while *B. subtilis* and *P. fluorescens* exhibited susceptibility in both tested media (Fig. 7A). Overall, the most susceptible bacteria were *B. subtilis* (95.5%) and *C. pseudoviolaceum* (97.9%), while the lowest significant prey reduction was for *E. coli* (14.7); *P. fluorescens* (27.8%) and for *R. rhodochrous* (33.6 %).

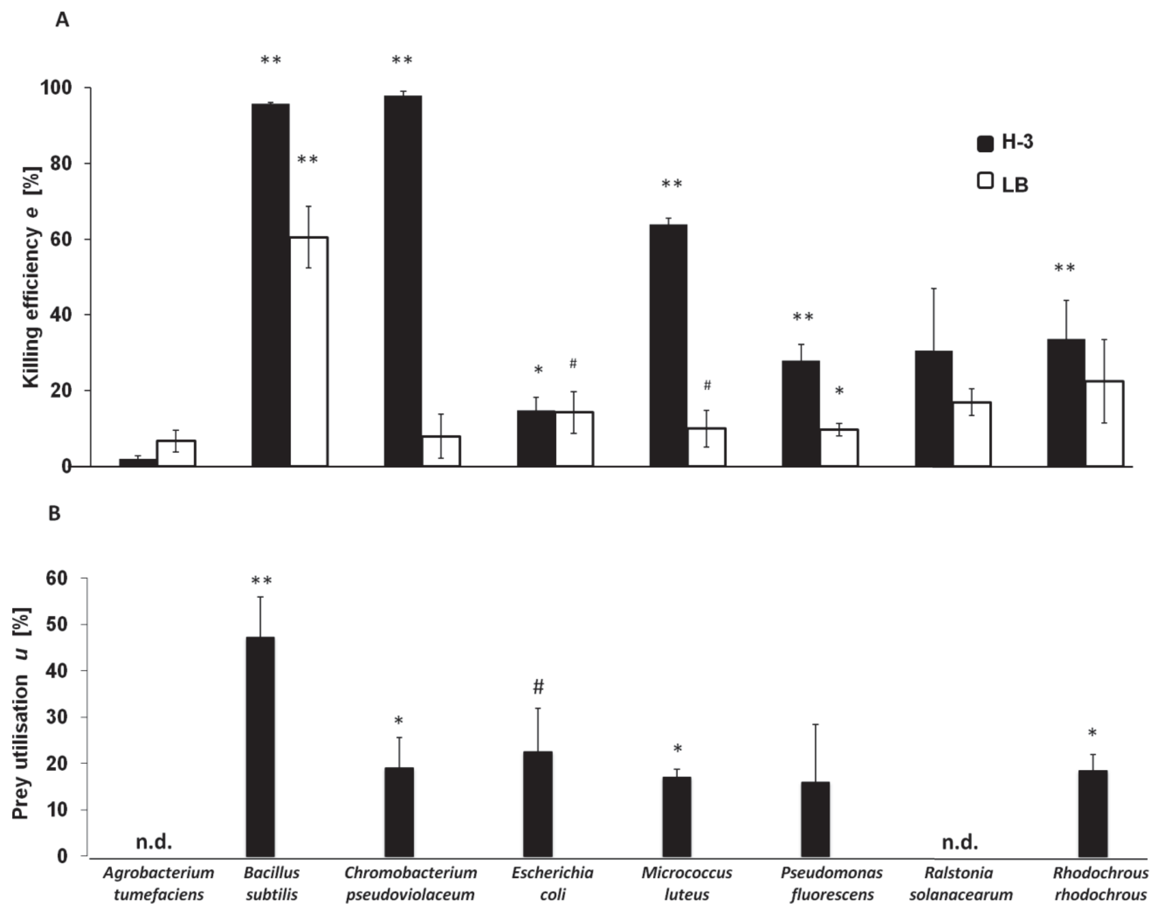


Figure 7. Mean killing efficiency ($\bar{e}$ ± 95% confidence interval) of *C. necator* N-1 against different species of prey bacteria (A). Asterisks denote significant differences between the number of prey CFU of the control group (i.e. monocultures) and samples containing both predator and prey (i.e. cocultures; Mann-Whitney U-test: ** = P < 0.01, * = P < 0.05; # = P < 0.07 d.f. =2). Mean prey utilization ($\bar{u}$ ± 95% confidence interval) of *C. necator* N-1 (B). Asterisks denote significant differences in the prey utilization when comparing control groups consisting exclusively of predators with samples containing both predators and prey (Wilcoxon test: * = P < 0.05; # = P < 0.07). n.d. = Prey species for which *u* was not determined.

Prey utilization was assessed for prey strains that were susceptible to N-1 predation. The populations of *C. necator* N-1 increased significantly in the presence of *B. subtilis*, *C. pseudoviolaceum, M. luteus* and *R. rhodochrous* (Fig. 7B). The highest prey utilization (*u*) was observed with *B. subtilis* (47.1 ± 8.7%), while the other prey bacteria were consumed at a comparable level: 19.0 ± 6.5% for *C. pseudoviolaceum*, 17.0 ± 1.8% for *M. luteus*, 18.4 ± 3.5% for *R. rhodochrous* and for *E.coli* 21.0 ± 7.1% (Wilcoxon test: P < 0.05, n = 6; Fig. 7B). However, when *P. fluorescens* was offered as prey, the population of N-1 did not significantly increase.

Subsequently, *C. necator* H16 and *C. taiwanensis* were tested against *B. subtilis*, *C. pseudoviolaceum*, *M. luteus* and *R. rhodochrous*, which had been identified as preferred prey bacteria of *C. necator* N-1. The latter was also included in this analysis and served as a control. Similar to *C. necator* N-1, prey reduction was more prominent when the predator had

been cultivated in H-3 minimal medium (Fig. 8). The only exception was *C. taiwanensis* which was more successful in eliminating *R. rhodochrous* when it had been pre-grown in LB medium. The highest killing efficiencies were found for *C. necator* N-1. Interestingly, *C. taiwanensis* was more efficient in killing the prey bacteria than *C. necator* H16. *B. subtilis* and *C. pseudoviolaceum* were consistently found to be highly susceptible to *Cupriavidus* spp. H16 failed to kill on *M. luteus* and *R. rhodochrous* under tested conditions (Fig. 8B). Overall, it seems that the genus *Cupriavidus* might harbor more than one predatory species and it is necessary to conduct more experiments in future to fully evaluate predatory potential of this genus.

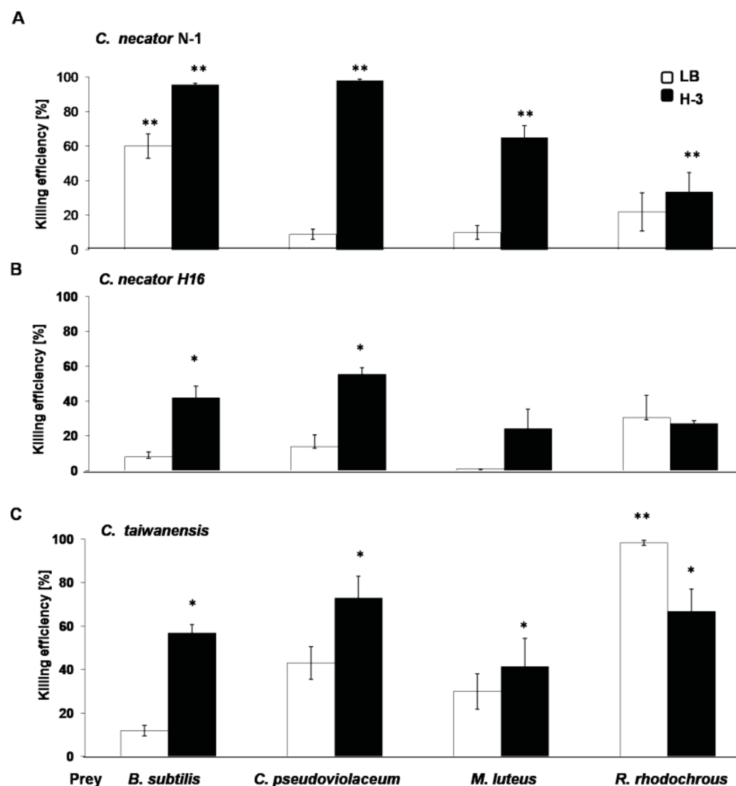


Figure 8. Mean killing efficiency ($\bar{e}$ ± 95% confidence interval) of *C. necator* N-1 (A), *C. necator* H16 (B) and *C. taiwanensis* (C) against different species of prey bacteria. Asterisks denote significant differences between the number of prey CFU of the control group (i.e. monocultures) and samples containing both predator and prey (i.e. cocultures; Mann-Whitney U-test: ** = $P < 0.01$, * = $P < 0.05$; # = $P < 0.07$ d.f. =2).

5.2 Antimicrobial activity of recombinant peptides from *C. necator* N-1

In previous reports, it was postulated that *C. necator* secretes a copper-binding peptide as a growth-promoting factor. This peptide was also proposed to play a key role in predation, but the underlying mechanism as well as the structure of the corresponding molecule has not been determined to date [108]. Therefore, in this study, the genome of *C. necator* strain N-1 was initially screened for genes that could be associated with the biosynthesis of a metal-binding

peptide. Additionally, the isolated metal-chelating proteins described in manuscript C were tested in the agar diffusion assay to evaluate if they possess antibacterial properties.

Agar diffusion assay. The recombinantly produced proteins (see manuscript C) were subjected to an agar diffusion assay to test their antimicrobial properties. Purified proteins (concentration 1 mg/ml) were spotted on the LB agar plate previously inoculated with *B. subtilis* 168. In addition, protein solutions were supplemented with 0.5 mM of copper(II) and spotted on the plate (Fig. 9). As a control, 50 µg/ml of kanamycin was applied and 0.5 mM of a copper(II) solution in PBS. The two proteins only inhibited the growth of *B. subtilis* in the presence of copper(II). However, when purified proteins where tested alone, the inhibition was not observed. Interestingly, the inhibition zone was more pronounced in the presence of copper(II), compared with the protein mixture. Obtain results may suggest that Cu(II) ions were binding to proteins and, therefore, reducing free Cu(II) ions which have inhibitory effect on the growth of *B. subtilis*.

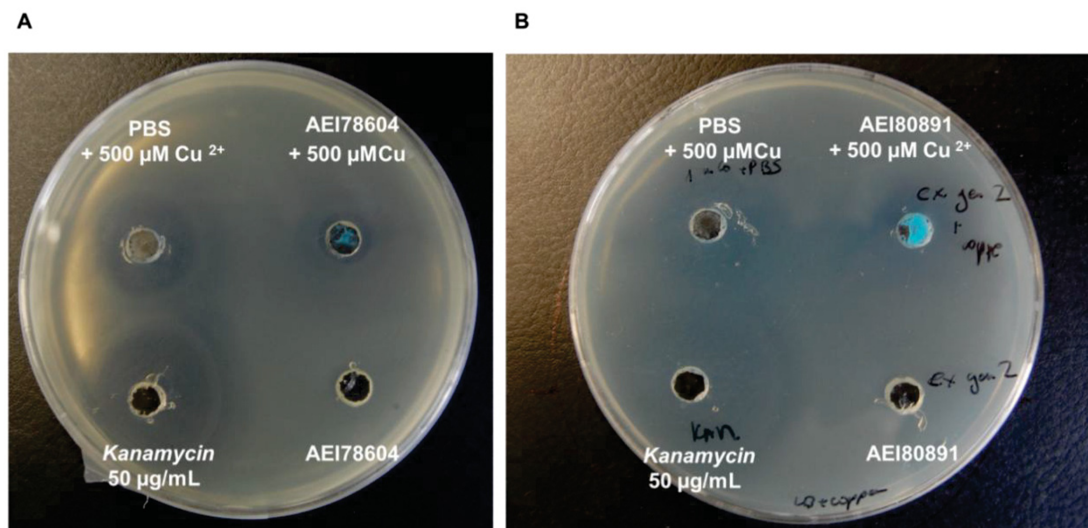


Figure 9. Agar diffusion assay of the overexpressed metal-chelating proteins AEI78604 (A) and AEI80891 (B). *B. subtilis* was used as a test organism.

6 Discussion

6.1 Analysis of bacterial predation in the laboratory

Although predatory behavior pervades the entire bacterial realm, research in this area has focused on a relatively small number of species. Moreover, bacterial hunters use different feeding strategies, making the establishment of a generally applicable predation assay challenging. As a consequence, the available predation assays were tailored to suit specific requirements. The solitary predators of the genus *Bdellovibrio* are most extensively investigated in this context. These bacteria were originally discovered while analyzing bacteriophages [21]. Typically, the double-layered plaque technique is used for both the isolation of *Bdellovibrio* spp. and for evaluating their predatory behavior [21, 112]. In the applied assay after liquid co-cultivation with the predator, numbers of plaques were counted on the plate [113]. This assay turned out to be highly useful for the analysis of obligate bacterial predator *Bdellovibrio* spp. Alternative predation assays are typically based on the clearing of prey suspensions or on microscopic analyses [113]. In the luminescence assay, fluorescent prey bacteria are used to correlate a decrease in luminescence with prey killing [36, 114]. Recently, the luminescence assay was integrated into a microfluidic platform [115], thereby supporting studies at the single cell level and enabling the analysis of chemotaxis between *Bdellovibrio* and prey organisms. The availability of complementary predation assays together with the development of new gene inactivation methods and proteomic studies has greatly facilitated research on *B. bacteriovorus* predation [116].

In contrast, only a few predation assays are available for non-obligate predatory bacteria. Casida and coworkers determined the predatory activities of *Cupriavidus necator* and *Ensifer adhaerens* by phage analysis using soil samples, from which the two proteobacteria had previously been isolated [16, 18]. The corresponding studies relied on the enumeration of predator-specific phages, whose numbers were postulated to correlate with the number of their hosts in soil [16]. However, the limitations of this approach for quantifying predation are obvious. Since non-obligate predatory bacteria, such as *C. necator* and *E. adhaerens*, can utilize non-living nutrient sources, an increase of their actual numbers in soil may not necessarily result from predation. Moreover, the reproducibility of this assay is low considering the variability of soil content in terms of microbial communities and mineral composition.

The majority of laboratory assays were established for myxobacteria, which are likely the most thoroughly investigated non-obligate predators [29]. Myxobacterial predation is

typically analyzed on agar plates (Fig. 10A&B). For this purpose, the myxobacterium is transferred to a spot or a lawn of prey bacteria. Afterwards, the emergence of lysis or the swarming of the predator is monitored [39, 117]. A variation of this methodology involves the recovery and enumeration of surviving prey cells after transferring the cell spots to LB agar plates, which were found to suppress myxobacterial growth [53]. There are several advantages of this type of assay. It is simple to conduct, relatively fast and it can also be used for detection and isolation of new predatory species from mixed soil samples, which is also known as baiting technique [109]. Indeed, agar-based assays turned out to be highly suited for observing myxobacterial predation. These bacteria are capable of gliding motility [87, 88] allowing them to move over solid surfaces [53, 54, 89]. This locomotion, together with the release of lytic enzymes, can result in visible clearing of prey that was previously spotted on agar plates.

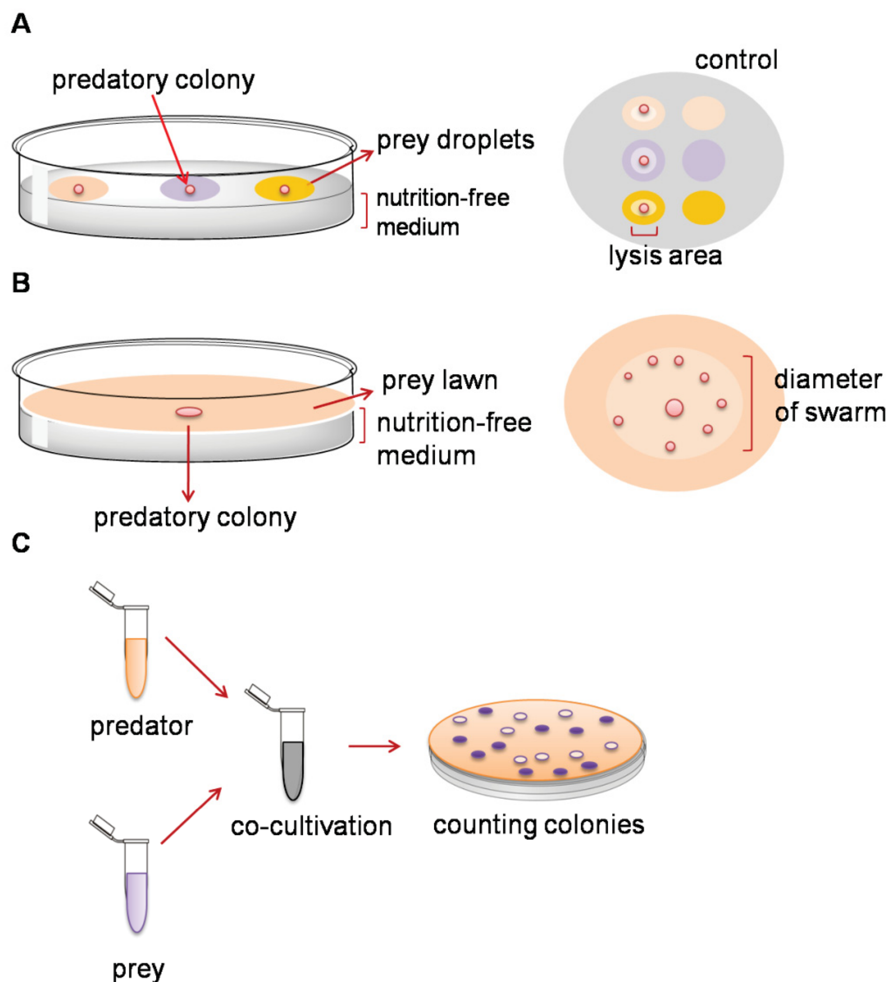


Figure 10. Laboratory assays for investigating bacterial predation: Prey spot plate assay (A), lawn predation assay (B) and CFU-based predation assay (C).

During this study it became obvious that the aforementioned assays are not applicable to all kinds of predatory bacteria (manuscripts A & B, Fig. 5 & 6). There could be several reasons

for this outcome. In general, only a single colony of the predator is spotted on the prey lawn and it is possible that under these conditions the production of toxins and lytic compounds is not optimal. Additionally, diffusion problems in agar might restrict transmission of predation factors to prey cells and in that way reduce killing efficiency. Furthermore, it is possible that the predatory bacterium must fulfill specific requirements in order to show activity in the prey spot or lawn predation assay. For example, motility could be one of the important factors that determine the outcome of these assays, since only the gliding bacteria can actively move on agar plates. Lastly, the number of predatory bacteria is critical to these assays. Numbers that are too low may not be sufficient to trigger predation (manuscript A). Therefore, significant effort was invested to establish and validate a bioassay for studying facultative predators other than myxobacteria. The colony-forming unit (CFU)-based predation assay can provide data on both the consumption of bacterial prey and the concomitant growth of predatory strains (Fig. 10C). For this, predator strains were incubated in a nutrient-free buffer together with prey bacteria featuring a distinctive pigmentation. After a defined period of incubation, co-cultures were streaked on agar plates and sizes of predator and prey populations were individually determined by counting colonies using their color as phenotypic markers. Besides pigmentation, antibiotic resistance could also be used to differentiate predator colonies and potential prey. It should also be noted that predator and prey populations are continuously mixed during one or two days of co-cultivation and this contributes to a homogeneous distribution of diffusible lytic factors. Using the CFU-based predation assay, it was possible to demonstrate that bacteria of the genera *Lysobacter* and *Cupriavidus* not only killed, but also consumed other bacteria (manuscripts A & B).

A prerequisite for the simultaneous determination of predator and prey populations from a mixed culture is the ability to distinguish both partners. If predator and prey cannot be discriminated on the basis of their natural pigmentation, the antibiotic resistance was introduced allowing individual counting of survived predator and prey colonies. Consequently, the CFU-based predation assay requires more effort and time for setting up the assay and evaluating predation efficiency, compared to predation assays that are based only on detection of lysis area. On the other hand, the CFU-based predation assay provides quantitative data on prey killing and consumption. It is hence possible to obtain further insights into the respective predation mode (manuscripts A & B).

6.2 Comparative analysis of predation behavior among bacteria

Bacterial hunters have developed specific strategies to capture and kill their prey. Our knowledge of predation mechanisms is largely based on few model organisms. In this study,

the neglected *Lysobacter* and *Cupriavidus* species were thus investigated. Bacteria of the genus *Lysobacter* are considered to be facultative predators that use a feeding strategy similar to myxobacteria. The assumption of group feeding was probably supported by the fact that many *Lysobacter* strains are potent antibiotic producers [92]. Actually, there is a strong discrepancy between the number of reviews on *Lysobacter* predation [19, 25, 28, 29, 118] and original research articles addressing this topic [99, 100].

The predatory behavior of *Cupriavidus necator* was initially observed in soil [22] and several hypotheses concerning its predation strategy were made. However, for decades, the research on *C. necator* predation was not continued. Therefore, the main goal of this research was to reveal conditions that influence the predatory behavior of *Lysobacter* and *Cupriavidus* in comparison to the well-characterized myxobacterium *Myxococcus fulvus*.

Table 3. Comparison of predation strategies in non-obligate predatory bacteria

		Myxobacteria	***Lysobacter***			***Cupriavidus***
		M. fulvus	*L. enzymogenes*	*L. capsici*	*L. oryzae*	*C. necator* N-1
Predation assays	Active in prey spot plate assay	+	+	-	-	-
	Active in lawn predation assay	+	+	-	-	-
	Active in CFU-based predation assay	-	-	-	-	-
Predator-prey ratio		Group and individual predator (<1:1) (1:1)	Group predator (10:1)	Group predator (100:1)	Group predator (100:1)	Individual predator (1:1)
Prey contact		Required	Required	Required	Required	Not required
Predation-triggering factors		Nutrient deficiency; Prey contact	Presence of prey	Presence of prey	Presence of prey	Nutrient deficiency, Presence of Cu^{2+},

6.2.1 Bacterial predation efficiency across different assays

In manuscript A, the predatory activity of three *Lysobacter* species was evaluated in two assays, namely the prey spot plate assay (Fig. 10A), which measures the progressive clearing of prey spotted on agar plates, and the lawn predation assay that determines the swarming diameter of the predator on a lawn of prey (Fig. 10B). Surprisingly, however, none of the three *Lysobacter* species showed a clear predatory behavior in these two assays. Only *Lysobacter enzymogenes* exhibited moderate predatory activity against *E. coli* and *R. rhodochrous*. Similar observation was made when predatory potential of *Cupriavidus* spp.

was evaluated using *C. necator* N-1 as predator (manuscript B, Fig. 5&6). Since *Cupriavidus* spp. are not capable of gliding locomotion, the absence of predatory activity in the agar-based assays was not surprising. On the other hand, *C. necator* is a flagellated bacterium [18] and the establishment of a liquid predation assay was hence the possible solutions to provide conditions that facilitate predator motility. However, in the natural habitat most likely *C. necator*, as many flagellated soil bacteria is actively moving though the soil only on the remained percolating water from the rainfall or irrigation [119] during which time it seeks for its prey.

Results obtained from the CFU-predation assay confirmed that *Lysobacter* spp. are capable of lysing both Gram-positive and Gram-negative bacteria (manuscript A). However, the phytopathogenic bacteria *A. tumefaciens* and *R. solanacearum* were not susceptible to *Lysobacter* predation under the experimental conditions tested. In this study, only bacteria were selected as prey organisms; thus, the results do not exclude the possibility that *Lysobacter* spp. can prey on phytopathogenic fungi. Indeed, it was previously demonstrated that various secondary metabolites isolated from this genus possess antimicrobial activity against different fungal pests [94, 95]. Concerning the predation efficiency towards susceptible bacterial species, certain differences within a genus were observed. For instance, *L. enzymogenes* killed fewer prey bacteria in the CFU-based predation assay than the other two tested *Lysobacter* species. Two possible conclusions can be drawn from this observation. Either *L. enzymogenes* is a less powerful bacterial hunter or it requires more time for prey killing and thus was not able to show its full predatory potential under the used assay conditions. It would be interesting to further optimize the CFU-based predation assay for predators that may require more time to grow and produce predation factors.

At first, *C. necator* N-1 was tested in the CFU assay under the same conditions established for *Lysobacter* spp. Significant killing was only observed for *B. subtilis* and *P. aeruginosa* (Fig. 7A). However, this predator was earlier described as a very potent predator with a much broader prey range and, therefore, further efforts were invested to optimize assay conditions. *C. necator* predation was initially observed in soil where nutrients were limited [22]. Hence, two different media were tested for a predator preculture: a nutrient rich LB medium and a nutrient poor H-3 minimal medium (manuscript B). Surprisingly, *C. necator* predation efficiency drastically changed when H-3 minimal medium was used for predator preculture (manuscript B, Fig. 7). The obtained result is in accordance with lifestyle of *C. necator* as a facultative predator that only attacks other bacteria in the absence of more easily accessible nutrient sources. Originally, the starvation step in the CFU assay was the co-cultivation in

nutrient free buffer where prey is offered as the only food source to predator. However, *C. necator* microscopic images (Fig. 4B) confirmed that this bacterium similarly to closely related bacterium *C. necator* H16 can form polyhydroxyalkanoates (PHA) granules which can be later used as reserves of carbon [110]. Therefore, N-1 possible requires longer time to reach starvation state and trigger predation behavior. Interestingly, the same phenomenon was observed when *C. necator* H16 and *C. taiwanensis* were subjected to CFU predation assay (Fig. 8). Under prolonged starvation conditions prey killing efficiency was more pronounced for both predators and *C. taiwanensis* exhibited a broader predation capability compared to *C. necator* H16. A specific feature of *C. necator* N-1 is its enhanced growth in the presence of copper [108]. Additionally, our results confirmed that the presence of copper(II) significantly promotes predation efficiency (manuscript B). This metal perhaps promotes predation by simply increasing the fitness of the predator and improving its growth. Another possible scenario is that copper acts as a signal that triggers the production of other toxic compounds, even copper chelating compounds, which subsequently kill the prey by increasing copper concentration to lethal levels for other organisms (see chapter 6.3).

The well-studied predatory bacterium *Myxococcus fulvus* was also tested in the CFU assay. Surprisingly, *M. fulvus* failed to reduce the prey population. Again, one possible explanation is that *M. fulvus* is a slow-growing bacterium which requires more time for co-cultivation with its prey in order to exhibit predatory behavior and further optimization of the assay conditions might be necessary.

6.2.2 Predator-prey ratio and cell contact

Outnumbering prey is an important feature of group predation [87, 88], and manipulating the initial ratio of predator and prey (PPR) confirmed that all tested *Lysobacter* spp. were restricted to group predation (manuscript A). Further analyses revealed that different optimal PPRs are required to achieve high killing efficiencies. In general, these predators needed to outnumber the prey by a factor of 100. Overall, it seems that *Lysobacter* predators must work together to successfully kill their prey, which could be mediated by the secretion of hydrolytic enzymes or toxic compounds. In contrast, the predatory behavior of *C. necator* was barely influenced when different predator-prey ratios were tested (manuscript B). Although the prey reduction modestly decreased in case of lower PPR, the killing efficiency stayed even relatively high (70%) when the numbers of predator and prey were equal. This result revealed that *C. necator* uses a predatory strategy that is distinct from *Lysobacter* spp.

In this study, minimal PPRs for myxobacterial predation were not determined. However, recent studies suggested that myxobacteria are single cell hunters rather than wolf pack

predators, and that close proximity to the prey cells might be necessary to penetrate and lyse prey colonies [29, 50]. In that case, another possible explanation why *M. fulvus* strain failed to exhibit predatory activity in the CFU-based predation assay is that myxobacterial cells do not just require close proximity to their prey, but instead they must establish physical contact with their prey for an extended period. This condition seems to prevent effective lysis in the CFU-based predation assay, as the shaking of the liquid cocultures likely prevented prolonged physical contact.

Further differences between these two predatory genera became evident. Some predators require close proximity to their prey in order to invade and lyse prey population. Sometimes they produce predation factors exclusively in the presence of the prey. *Lysobacter* is well-known for producing various secondary metabolites [92, 120] which could act as predation factors. However, cell-free supernatants from *Lysobacter* culture extracts did not show any activity on the growth of different prey bacteria (manuscript A). It seems that the main production of predation factors occur in the presence of the prey and that *Lysobacter* spp. may display a predatory behavior that is dependent on prey-contact. *C. necator* exhibited the opposite behavior. Cell-free culture supernatants retained antibacterial activity against tested prey (manuscript B). The predatory activity of *C. necator* does not depend on a physical contact with prey cells and possibly, it is solely mediated by extracellular predation factors like excreted enzymes, antibiotics or other toxic compounds. This result is in strong contrast to observations made with *Lysobacter* spp., suggesting that the tested *Lysobacter* species pursue a different predation strategy to *C. necator*.

6.3 Secondary metabolites of *C. necator*

The mechanisms by which *C. necator* seeks and kills other organisms have not been clarified to date. It has been proposed that a secreted peptidic copper-binding molecule is involved both in predation and in the interaction with other hunting bacteria, but a proof for this hypothesis is missing. Therefore, one of the goals of this research was to identify copper-binding molecules which could act as predation factors. Further efforts were invested in screening for genes associated with the biosynthesis of metallophores. Our *in silico* analyses aimed at genes whose products feature a high cysteine or histidine content, namely metallothioneins and metallohistins [121]. The selected proteins were heterologously produced and their ability to coordinate different metal ions was examined (manuscript C). Of five overexpressed proteins, two exhibited high affinity towards copper(II). A direct antimicrobial effect of the isolated proteins was not observed in an agar diffusion assay (Fig. 9). However, this result does not exclude that these proteins are involved in the predation

mechanism. Possibly, isolated proteins lost their function during extraction methods. Alternately, perhaps in order to display their biological function, these proteins require forming a complex to lipid or carbohydrate moieties in addition to protein which under the tested conditions was not possible. Future research is necessary to resolve if isolated proteins besides binding different metal ions play role in predation as well.

6.4 Prey-specific factors that influence predation

Some prey-dependent factors can directly affect the predator killing efficiency [3]. As a predator-prey model we chose the two soil bacteria *C. necator* and *B. subtilis*. The aim was to elucidate the conditions under which *B. subtilis* can avoid predation by *C. necator* (manuscript B). In this study, the predator showed the highest killing efficiencies on young *B. subtilis* cultures while three-day old prey cultures exhibited resistance. To identify the resistance factors, several *B. subtilis* mutants were tested. Initially, the contribution of motility and poly-γ-glutamic acid (PGA) production to predation resistance was evaluated. *B. subtilis* has flagella and it is capable of active movement [122]. The two integral membrane proteins MotA and MotB are essential components of the flagellar motor [123, 124]. Deletion of *motA* gene in *B. subtilis* results in overproduction of PGA around the cells [123]. To investigate a possible role of flagellar movement and PGA production in predation evasion, a *ΔmotA* mutant of *B. subtilis* was investigated in the CFU-based predation assay. However, we detected no differences in the survival rates of the wild-type *B. subtilis* strain and the *ΔmotA* mutant. To analyze if spore formation is the first line of *B. subtilis* defense against predation, two nonsporulating mutants, *Δspo0A* and *ΔsigF*, were co-incubated with *C. necator* N-1. All mutant cultures were completely eradicated by *C. necator*. Finally, to investigate whether *C. necator* has the ability to kill and consume *B. subtilis* spores, spores from strain NCIB 3610 were isolated and tested in the CFU-based predation assay. As expected, *C. necator* was not able to consume *B. subtilis* spores (manuscript B). Similar results were obtained when *Myxococcus xanthus* [60], *Streptomyces coelicolor* [15] as well as the protozoan *Tetrahymena thermophile* [72] were tested against *B. subtilis* spores. Additionally, we wanted to better understand whether certain components of the spore are crucial for the predation resistance against *C. necator*. Although the exact mechanism of spore persistence is unknown, the spore coat is recognized as the main protective shield against toxic molecules (Fig. 11). About 70 different proteins are involved in the formation of the spore coat [125].

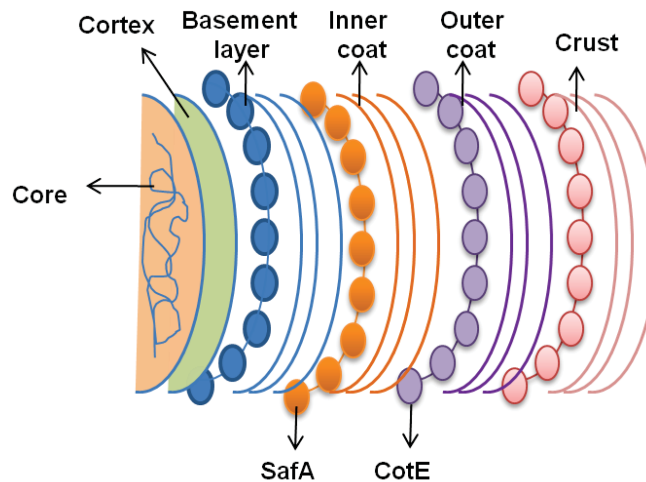


Figure 11. Spore structure according to [125]

Subsequently, seven mutants with altered sporulation properties were tested in order to identify which genes were sufficient for predation resistance (manuscript B). Surprisingly, all tested spores mutants exhibited similar resistance profiles to *C. necator* predation as the *B. subtilis* wild-type strain. Additionally, chemically decoated spores showed remarkable resistance to predation. One of the possible explanations is that *B. subtilis* use dormancy for escaping the *C. necator* predation. The spores are metabolically inactive and cells in this stage do not enter the replication cycle [126]. It is possible that *C. necator* secrets antimicrobial compounds that are only active against metabolically active cells, e.g. inhibitors of DNA replication or protein biosynthesis. Future research is necessary to test this hypothesis.

6.5 Distinguishing predation from competition

In the literature, predation among bacteria is often confused with competition. This unfortunate situation has caused some confusion concerning the assignment of bacteria as being predatory. Perhaps one of the major reasons for this is that facultative predatory bacteria do not require prey presence for their growth and new bacterial isolates are rarely tested for predatory behavior and as a consequence predatory potential across bacteria realm is overlooked. On the other hand, often bacterial isolates are analyzed for production of secondary metabolites with antimicrobial properties and their production in natural habitat is assigned to competitive rather than predatory interactions.

However, predation and competition can be easily distinguished. The latter always involves reciprocal negative effects between organisms that are often found to strive for the same resources in a confined habitat [127]. Long-term competition may lead to the extinction of a

competing organism. Alternatively, both competitors become more specialized [128]. Overall, competition presents a harmful relationship for both partners [128]. In stark contrast, predator-prey relationships are much more reminiscent of parasitic interactions. The predator benefits from the interaction, whereas the prey is negatively affected [129]. Competitors often try to avoid an encounter, while predators actively seek their prey [127].

In the prey spot assay (manuscript A), lysis areas are measured. The lysis of a bacterium could also be the result of a competitive relationship in which organisms kill each other to secure access to limited nutrient resources. On the other hand, the swarming of the predator on a lawn of prey is suggestive of predation, because the progressive clearing of agar prey spots from the inside out is described feeding strategy of gliding predators [87]. In the CFU-based predation assay (manuscripts A & B) both predator and prey populations were monitored. Whenever the prey population was significantly reduced, predator numbers were simultaneously increasing. This outcome clearly demonstrated a direct link between prey killing and increasing predator fitness, which is a fundamental trait of predation. The described CFU-based predation assay has potential to facilitate studies on further neglected predatory bacteria and assist in the evaluation of their predatory behavior.

7 Summary

It is known for decades that certain bacteria are capable to use other bacteria as nutrient sources. As a matter of fact, predatory behavior is not uncommon in prokaryotes. Predatory bacteria were found both in terrestrial and in aquatic habitats. To date, however, only a few of these micropredators have been thoroughly characterized with regard to their ecology and predation strategies. In this study, various aspects of the predatory behavior of the hitherto hardly investigated genera *Lysobacter* and *Cupriavidus* should be explored.

The first goal was to develop tools for investigating the predatory performance of *Lysobacter* and *Cupriavidus* strains under laboratory conditions. Since predatory bacteria pursue many different hunting strategies, the design of a generally applicable assay was challenging. Available predation assays are tailored to predator-specific features, such as growth rate, motility and prey range. The best characterized facultative predators are myxobacteria and several predation assays have been established for this bacterial group. Because myxobacterial predation assays were found to be unsuitable for testing of *Lysobacter* and *Cupriavidus* strains, an alternative predation assay was developed that provides quantitative data on the reduction of bacterial prey and the concomitant growth of the predator. For this purpose, as prey, soil bacteria that produce distinctive pigments or harbor specific antibiotic resistant gene were selected. Following a defined period in which the prey bacteria were incubated together with the predator, the individual populations could easily be distinguished based on the colors of their colonies or antibiotic resistance after plating. With CFU-based predation assay the predatory activity of both genera was verified. *Lysobacter* spp. and *Cupriavidus* spp. were shown to consume both Gram-negative and Gram-positive bacteria.

The second aim was to identify factors that trigger and promote predatory activity. Using the CFU-based predation assay, it was confirmed that the selected *Lysobacter* spp. were effectively feeding on both Gram-positive and Gram-negative prey under nutrient-deficient conditions. Furthermore, their predatory behavior was strongly promoted under starvation. Variation of the predator-prey ratio revealed that the tested strains had to outnumber prey bacteria for efficient killing. Overall, the obtained results suggested that the tested *Lysobacter* strains were restricted to the group predation. In addition, further analyses corroborate the assumption that *Lysobacter* spp. may display prey contact-dependent predatory behavior under tested conditions.

Likewise, exposure to low nutrient conditions for a prolonged period was found to significantly increase the predation efficiency of all tested *Cupriavidus* strains. Furthermore,

the addition of copper(II) to the predator pre-culture had a positive effect on the predatory activity of *C. necator*. Unlike *Lysobacter* spp., *C. necator* maintained high killing efficiencies at comparatively low predator-prey ratios. In addition, the *Cupriavidus* predatory activity does not depend on physical contact with prey cells and, possibly, it is solely mediated by extracellular predation factors like excreted enzymes, antibiotics or other toxic compounds. Taken together, the results are suggesting that *C. necator* pursues a different predation strategy than the tested *Lysobacter* spp. Additional research has been conducted aiming to address basic questions concerning the development of prey resistance. The wild-type *B. subtilis* was selected as prey since it was confirmed to be suitable a prey organism that shares the same natural habitat with *C. necator*. The established predation model of *C. necator* and *B. subtilis* revealed that this predator is not capable to consume the *B. subtilis* spores and after testing different *B. subtilis* spore mutants it is postulated that this bacterium in fact evades predation by entering dormancy.

The third objective of this research was to identify the copper-binding peptide which had previously been postulated to promote the predatory activity of *C. necator* N-1. For this purpose, the genome of *C. necator* N-1 was initially screened for genes that could be associated with the biosynthesis of a metallophore. In addition, genes for the production of ribosomally derived peptides with a high cysteine or histidine content, namely metallothioneins and metallohistins, were identified. After these *in silico* analyses, five identified candidate peptides were heterologous produced and their capacity to bind different metals was evaluated. Two of these peptides were shown to bind copper, nickel, iron and zinc. However, a connection between these peptides and the killing mechanism of *C. necator* N-1 could not be established. Future research will be required to elucidate the killing mechanism of this intriguing facultative predator.

8 Zusammenfassung

Seit Jahrzehnten ist bekannt, dass bestimmte Bakterien in der Lage sind, ihresgleichen als Nahrungsquelle zu nutzen. Tatsächlich ist räuberisches Verhalten bei Prokaryoten nicht ungewöhnlich. Räuberische Bakterien konnten sowohl in terrestrischen wie auch in aquatischen Habitaten nachgewiesen werden. Bislang sind jedoch nur wenige dieser Mikroprädatoren hinsichtlich ihrer Ökologie und ihres Jagdverhaltens näher charakterisiert worden. In dieser Arbeit sollten verschiedene Aspekte des räuberischen Verhaltens der bislang kaum beachteten Gattungen *Lysobacter* und *Cupriavidus* erforscht werden.

Das erste Ziel war die Entwicklung von Methoden, um das räuberische Verhalten von *Lysobacter*- und *Cupriavidus*-Stämmen unter Laborbedingungen untersuchen zu können. Da räuberische Bakterien unterschiedliche Jagdstrategien verfolgen, stellte die Entwicklung eines allgemein einsetzbaren Assays eine Herausforderung dar. Verfügbare Prädationsassays sind i.d.R. auf spezifische Räuber zugeschnitten. Sie sind auf die jeweilige Wachstumsrate, Motilität und das Beutespektrum optimiert. Die am besten charakterisierten fakultativen Räuber sind Myxobakterien, für die auch zahlreiche Prädationsassays etabliert worden sind. Da sich diese Assays für die Testung von *Lysobacter*- und *Cupriavidus*-Stämmen als ungeeignet erwiesen haben, wurde ein alternativer Assay entwickelt, mit dem quantitative Daten zur Tötung der bakteriellen Beute und zeitgleich zum damit einhergehenden Wachstum des Räubers erhoben werden können. Zu diesem Zweck wurden Bodenbakterien, die bestimmte Pigmente produzieren oder spezifische Antibiotika-resistente Gene haben, als Beute ausgewählt. Beute und Räuber wurden über einen definierten Zeitraum zusammen inkubiert. Nach Ausplattierung konnten die einzelnen Populationen leicht auf Grund der Koloniefarbe oder Antibiotikaresistenz unterschieden werden. Mit dem CFU-basierten Prädationsassay wurde die räuberische Aktivität beider Gattungen verifiziert. *Lysobacter* spp. und *Cupriavidus* spp. fraßen sowohl Gram-negative als auch Gram-positive Bakterien.

Das zweite Ziel war die Identifizierung von Faktoren, die die räuberische Aktivität induzieren und fördern.

Durch Verwendung des CFU-basierten Predationstests wurde bestätigt, dass die ausgewählten *Lysobacter* spp. unter Nährstoffmangelbedingungen sowohl Gram-positive als auch Gram-negative Beute effektiv als Nahrungsquelle verwenden. Darüber hinaus wurde die räuberische Aktivität unter nährstoffarmen Bedingungen stark stimuliert. Variation des Räuber-Beute-Verhältnisses ergab, dass eine zahlenmäßige Überlegenheit der *Lysobacter* spp. Voraussetzung für eine effiziente Tötung der Beutebacterien. Diese Ergebnisse legen nahe,

dass die untersuchten *Lysobacter* Stämme in der Gruppe auf Beutezug gehen. Weitere Untersuchungen erhärten die Annahme, dass ein beuteabhängiges Räuberverhalten vorliegt.

Die längerfristige Exposition gegenüber niedrigen Nährstoffbedingungen zeigte gleichfalls deutlich erhöhte Beuteeffizienz der getesteten *Cupriavidus* Stämme. Zusätzlich hatte die Zugabe von Cu^{+II} zur Vorkultur des Räubers einen positiven Effekt auf die räuberische Aktivität von *C. necator*. Im Gegensatz zu *Lysobacter* spp. behielt *C. necator* seine hohe Tötungseffizienz bei vergleichsweise niedrigem Räuber-Beute-Verhältnis. Die räuberische Aktivität von *C. necator* hängt zusätzlich nicht vom physikalischen Kontakt mit der Beute ab, sondern wird allein induziert durch extrazelluläre Faktoren wie z.B. sekretierte Enzyme, Antibiotika oder andere giftige Substanzen. Zusammengefasst legen diese Ergebnisse nahe, dass *C. necator* und die getesteten *Lysobacter* spp. verschiedene Prädationsstrategien verfolgen. Darüber hinaus wurde der Frage der Beuteresistenz nachgegangen. Als Beuteorganismus wurde Wildtyp *Bacillus subtilis* gewählt, da es als Beuteorganismus bereits bestätigt worden war und die gleiche natürliche Umgebung mit *C. necator* teilt. Die etablierten Räubermodelle von *C. necator* und *B. subtilis* zeigten, dass dieser Räuber nicht in der Lage ist *B. subtilis* Sporen für sich zu verwenden. Weitere Testungen mit sporendefizienten Mutanten von *B. subtilis* lassen vermuten, dass diese Bakterien diesen Ruhezustand der Sporenbildung nutzen um der Jagd zu entgehen.

Das dritte Ziel der Arbeit war die Identifizierung des kupferbindenden Peptids, dass zuvor als möglicher Jagdfaktor für *C. necator* N-1 postuliert worden ist. Dafür wurde zunächst das Genom von *C. necator* N-1 auf Gene untersucht, die an der Biosynthese von Metallophoren beteiligt sein könnten. Zusätzlich wurden Gene identifiziert, die für ribosomal gebildete Peptide mit einem hohen Cystein- oder Histidingehalt kodieren, namentlich Metallothioneine und Metallohistine. Nach diesen *in silico* Analysen wurden fünf Peptide ausgewählt, heterolog produziert und die Bindungskapazität der rekombinanten Peptide für verschiedene Metalle bestimmt. Zwei dieser Peptide können neben Kupfer auch Nickel, Eisen und Zink binden. Trotzdem konnte der Zusammenhang zwischen diesen Peptiden und dem Tötungsmechanismus von *C. necator* N-1 nicht aufgeklärt werden. Weitere Testungen sind nötig, um den Tötungsmechanismus von diesem faszinierenden fakultativen Räuber aufzuklären.

9 Manuscripts from side projects

9.1 Manuscript D: *Micromonospora schwarzwaldensis* sp. nov., a producer of telomycin, isolated from soil.

Gurovic Maria Soledad Vela, Müller Sebastian, Domin Nicole, **Seccareccia Ivana**, Nietzsche Sandor, Martin Karin, Nett Markus. *Micromonospora schwarzwaldensis* sp. nov., a producer of telomycin, isolated from soil. **2013**. *Int. J. Syst. Evol. Microbiol*. 63:3812-381

International Journal of Systematic and Evolutionary Microbiology (2013), **63**, 3812–3817 **DOI** 10.1099/ijs.0.051623-0

Micromonospora schwarzwaldensis sp. nov., a producer of telomycin, isolated from soil

Maria Soledad Vela Gurovic,[1]† Sebastian Müller,[2] Nicole Domin,[1] Ivana Seccareccia,[1] Sandor Nietzsche,[4] Karin Martin[3] and Markus Nett[1]

[1]Junior Research Group 'Secondary Metabolism of Predatory Bacteria', Leibniz Institute for Natural Product Research and Infection Biology e. V., Hans-Knöll-Institute, 07745 Jena, Germany

[2]Systems Biology/Bioinformatics, Leibniz Institute for Natural Product Research and Infection Biology e. V., Hans-Knöll-Institute, 07745 Jena, Germany

[3]Bio Pilot Plant, Leibniz Institute for Natural Product Research and Infection Biology e. V., Hans-Knöll-Institute, 07745 Jena, Germany

[4]Centre for Electron Microscopy, University Hospital Jena, 07745 Jena, Germany

Correspondence
Karin Martin
karin.martin@hki-jena.de
Markus Nett
markus.nett@hki-jena.de

A Gram-stain-positive, spore-forming actinomycete strain (HKI0641^T) was isolated from a soil sample collected in the Black Forest, Germany. During screening for antimicrobial natural products this bacterium was identified as a producer of the antibiotic telomycin. Morphological characteristics and chemotaxonomic data indicated that the strain belonged to the genus *Micromonospora*. The peptidoglycan of strain HKI0641^T contained *meso*-diaminopimelic acid, and the fatty acid profile consisted predominantly of anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{16:0}$. MK-10(H_4), MK-10(H_2) and MK-10 were identified as the major menaquinones. To determine the taxonomic positioning of strain HKI0641^T, we computed a binary tanglegram of two rooted phylogenetic trees that were based upon 16S rRNA and *gyrB* gene sequences. The comparative analysis of the two common classification methods strongly supported the phylogenetic affiliation with the genus *Micromonospora*, but it also revealed discrepancies in the assignment at the level of the genomic species. 16S rRNA gene sequence analysis identified *Micromonospora coxensis* DSM 45161^T (99.1 % sequence similarity) and *Micromonospora marina* DSM 45555^T (99.0 %) as the nearest taxonomic neighbours, whereas the *gyrB* sequence of strain HKI0641^T indicated a closer relationship to *Micromonospora aurantiaca* DSM 43813^T (95.1 %). By means of DNA–DNA hybridization experiments, it was possible to resolve this issue and to clearly differentiate strain HKI0641^T from other species of the genus *Micromonospora*. The type strains of the aforementioned species of the genus *Micromonospora* could be further distinguished from strain HKI0641^T by several phenotypic properties, such as colony colour, NaCl tolerance and the utilization of carbon sources. The isolate was therefore assigned to a novel species of the genus *Micromonospora*, for which the name *Micromonospora schwarzwaldensis* sp. nov. is proposed. The type strain is HKI0641^T (=DSM 45708^T=CIP 110415^T).

Micromonospora is the type genus of the family *Micromonosporaceae* Krasil'nikov 1938, emend. Zhi, Li and Stackebrandt 2009 within the suborder *Micromonosporineae* in the order *Actinomycetales* (Genilloud, 2012; Stackebrandt *et al.*, 1997; Zhi *et al.*, 2009). This family contains several genera which are morphologically distinct, but chemotaxonomically similar (Goodfellow *et al.*, 1990). At the time of writing, the 'List of Prokaryotic Names with Standing in Nomenclature' includes 50 species and seven subspecies in the genus *Micromonospora* (Euzéby, 2012). The majority of these species have been isolated from soil, freshwater or marine habitats (Carro *et al.*, 2012; Genilloud, 2012; Luedemann *et al.*, 1963). Like other actinomycetes, species of the genus *Micromonospora* are best known for synthesizing bioactive secondary metabolites, especially aminoglycoside, enediyne and oligosaccharide antibiotics (Bérdy, 2005). Their metabolic proficiency was confirmed in whole-genome sequencing projects, which showed that these organisms

†Present address: INQUISUR, Departamento de Química, Universidad Nacional del Sur, Av. Alem 1253, B8000CPB Bahía Blanca, Argentina

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA and *gyrB* gene sequences of strain HKI0641^T are KC517406 and KC517407, respectively.

Five supplementary tables and four supplementary figures are available with the online version of this paper.

051623 © 2013 IUMS *Printed in Great Britain*

dedicate a large portion of their genetic capacity to the biosynthesis of natural products (Nett *et al.*, 2009; Alonso-Vega *et al.*, 2012). While the biological function of most of these molecules still remains elusive, there is mounting evidence that some of them contribute to plant health, e.g. by alleviating metal-induced oxidative stress (Dimkpa *et al.*, 2009) or by suppressing the growth of phytopathogens (Raaijmakers & Mazzola, 2012). The importance of species of the genus *Micromonospora* for soil ecology, including plant growth and development, has recently been recognized (Hirsch & Valdés, 2010). During a survey of potential biocontrol agents from soil in the Black Forest (Schwarzwald), 48 strains were isolated. Extracts of strain HKI0641^T showed strong activities in the agar diffusion assay against various Gram-reaction-positive bacteria as well as fungi, suggesting the production of antimicrobial natural products. Here, we report a comprehensive phenotypic and phylogenetic characterization of strain HKI0641^T.

The soil samples were collected in the Black Forest near Forbach, Germany, in 2000. Strain HKI0641^T was isolated from the flooding zone of the Schwarzenbach dam. The corresponding sample (pH 5.5) contained significant amounts of loam, but also some plant debris. To promote the isolation of spore-forming actinomycetes, all samples were initially dried and heated for 1 h at 80 °C. Afterwards, 1 g of each sample was suspended in 10 ml 0.85 % NaCl (w/v) and mixed on a shaker for 30 min. After sedimentation of the soil particles the supernatants were diluted to 10^{-4}, 10^{-5} and 10^{-6} with 0.85 % NaCl (w/v). Aliquots of these suspensions were spread over plates containing humic acid-vitamin agar (Hayakawa & Nonomura, 1987) supplemented with nalidixic acid (20 μg ml^{-1}) and cycloheximide (30 μg ml^{-1}). The plates were incubated at 28 °C for three weeks. All isolates were purified and maintained on yeast extract–malt extract (ISP-2) agar (Shirling & Gottlieb, 1966). Pure cultures were preserved at −80 °C as a mixture of hyphae and few spores in liquid ISP-2 medium and glycerol medium [8.8 % glycerol, 0.18 % $(NH_4)_2SO_4$, 0.09 % Na-citrate, 1.26 % K_2HPO_4, 0.36 % H_2PO_4 and 0.01 % $MgSO_4$]. Stock cultures in liquid ISP-2 medium supplemented with 5 % DMSO were additionally maintained in the vapour phase of liquid nitrogen. To identify potential producers of bioactive metabolites, the supernatants of isolated strains, grown in liquid ISP-2 medium for 7 days, were subjected to an agar diffusion assay as previously described (Nett & Hertweck, 2011). Based upon the antimicrobial effects in this primary screening, strain HKI0641^T was selected for further taxonomic analysis.

Genomic DNA as template for PCR was extracted using the DNeasy Blood and Tissue kit (Qiagen). PCR amplification of the 16S rRNA gene was performed using primers Fw-16S (5′-GTCTCTGGGCCGATACTGACGC-3′) and Rev-16S (5′-CGGCTACCTTGTTACGAC TTCGTC-3′). The sequencing of the *gyrB* gene was performed as described by Garcia *et al.* (2010). 16S rRNA gene and *gyrB* sequences of strain HKI0641^T served as probes to search for similar sequences using the BLASTN module of EPoS (Griebel *et al.*, 2008). Representative sequences were manually selected (Table S1 available in IJSEM Online) and aligned with the CLUSTAL W module of EPoS using default parameters. This approach resulted in a multiple alignment of 1413 and 1032 sites after removing all gap columns. The alignments were subsequently used to compute sequence similarities using the R-package APE (Paradis *et al.*, 2004) as well as the phylogenetic trees employing the neighbour-joining (NJ) module of EPoS based on the Kimura model. The NJ calculation was performed utilizing *Actinoplanes regularis* DSM 43151^T as the outgroup and 500 bootstrap replicates to assess the stability of the grouping.

The almost complete 16S rRNA gene sequence of strain HKI0641^T was a continuous stretch of 1460 bp between positions 25 and 1518 of the *Escherichia coli* numbering (Brosius *et al.*, 1978). The complete signature nucleotide patterns associated with the order *Actinomycetales* and the family *Micromonosporaceae* were identified (Table S2; Zhi *et al.*, 2009). The sequence-based similarity calculations indicated that the closest relatives of strain HKI0641^T were *Micromonospora coxensis* DSM 45161^T (99.1 %) and *Micromonospora marina* DSM 45555^T (99.0 %). Due to the high levels of relatedness of strains of species of the genus *Micromonospora* based on their 16S rRNA gene sequences (Carro *et al.*, 2010; Koch *et al.*, 1996), we set out to verify the 16S rRNA gene-derived phylogenetic classification by applying a *gyrB*-based method (Kasai *et al.*, 2000). According to this analysis, however, strain HKI0641^T should be affiliated with *Micromonospora. aurantiaca* DSM 43813^T rather than with the aforementioned species. We illustrated the observed discrepancy by comparing both inferred trees in a tanglegram (Böcker *et al.*, 2009). This graphical juxtaposition showed several lines crossing, thereby indicating significant methodological bias (Fig. 1). To resolve the phylogenetic grouping of strain HKI0641^T, spectroscopic DNA–DNA hybridization experiments were performed in duplicate according to the methods of De Ley *et al.* (1970) and Huss *et al.* (1983). The required DNA was obtained following cell disruption and purification of the resulting crude lysate via column chromatography on hydroxyapatite (Cashion *et al.*, 1977). The highest DNA–DNA reassociation value was obtained between strain HKI0641^T and *M. aurantiaca* DSM 43813^T (mean value, 44.3 %), whereas the corresponding values with *M. marina* DSM 45555^T (34.9 %) and *M. coxensis* DSM 45161^T (11.5 %) were significantly lower (Table S3). This result corroborated the superiority of *gyrB* gene sequence analysis for inferring intrageneric relationships in the genus *Micromonospora.* Since the phylogenetic definition of a species generally excludes strains with <70 % DNA–DNA relatedness, it was evident that the isolate HKI0641^T represented a distinct species (Wayne *et al.*, 1987).

Subsequently, the phenotypic features of the novel strain were analysed. Gram staining and cell morphology were examined under a phase-contrast microscope using 24 h-old cultures grown on ISP-2 agar at 28 °C. For scanning

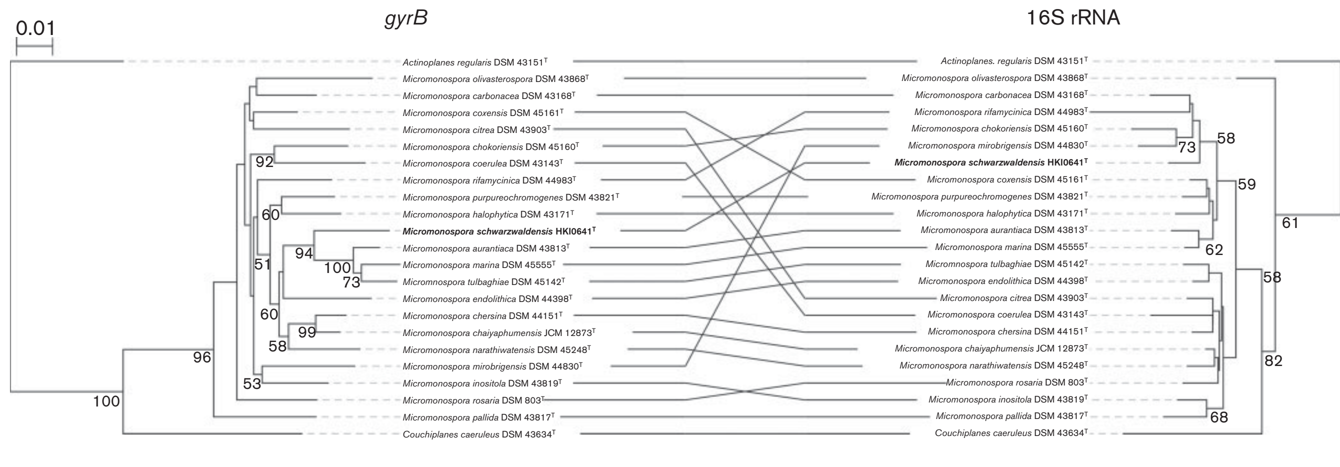


Fig. 1. Tanglegram comparing neighbour-joining phylogenetic trees based upon *gyrB* (left) and 16S rRNA (right) gene sequences from strain HKI0641^T and members of the family *Micromonosporaceae*. *Actinoplanes regularis* DSM 43151^T was used as an outgroup. The numbers on the branches indicate the bootstrap values (%) from 500 resamplings; only values >50 % are indicated. Bar, 0.01 substitutions per nucleotide position.

electron microscopy, a 28 day-old agar culture was suspended in a phosphate-buffered salt solution. The cells were fixed with 0.5 % glutaraldehyde, washed and dehydrated in ascending ethanol concentrations. Afterwards the samples were critical-point dried using liquid CO_2 and sputter coated with platinum using a SCD005 sputter coater (BAL-TEC) to avoid surface charging. Finally the specimens were investigated with a field emission scanning electron microscope (LEO-1530 Gemini; Carl Zeiss NTS). The fatty acid profile was determined according to the method described by Groth *et al.* (1996). For quinone and polar lipid analysis, cells were grown in ISP-2 medium at 28 °C. Quinone analysis was performed according to the procedure described by Collins *et al.* (1977). Polar lipids were determined according to the methods of Minnikin *et al.* (1979) and Collins & Jones (1980). Isomers of diaminopimelic acid in whole cells hydrolysates were analysed by TLC on cellulose (Schleifer & Kandler, 1972). The occurrence of mycolic acids was determined by TLC as described by Minnikin *et al.* (1975). Whole-cell sugars were examined according to the method of Schumann (2011). The utilization of carbon sources was investigated using the API 50 CH B system (bioMérieux). Temperature-dependent growth was analysed on ISP-2 agar at the following incubation temperatures: 4, 10, 20, 25, 28, 37 and 45 °C. Tolerance to NaCl and pH were determined on ISP-2 agar at 28 °C by the addition of 2, 4, 6, 8, 10 or 15 % (w/v) NaCl and using a pH range from 4 to 10. Susceptibility to antibiotics was tested on ISP-2 agar at 28 °C.

The morphological and chemical properties of strain HKI0641^T are consistent with its classification as a member of the genus *Micromonospora* (Genilloud, 2012). The isolate developed substrate hyphae on ISP-2 agar, oatmeal agar (ISP-3) and on inorganic salts–starch agar (ISP-4) (Shirling & Gottlieb, 1966). In comparison with the former three media, the growth on glycerol–asparagine agar (ISP-5) was delayed (Table S4). Abundant black spores were observed on ISP-2 agar (Fig. 2, Fig. S1), but no soluble pigments were observed in any of the media tested. Aerial mycelium was always absent. *M. aurantiaca* DSM 43813^T, *Micromonospora purpureochromogenes* DSM 43821^T and *Micromonospora tulbaghiae* DSM 45142^T exhibited the same growth profile as strain HKI0641^T, albeit they differed in sporulation and in the colour of their colonies. The growth temperature of strain HKI0641^T ranged from 20 to 37 °C, with optimal growth occurring at 28 °C. Except for *M. aurantiaca* DSM 43813^T, which did not grow below 25 °C, all other tested strains of species of the genus

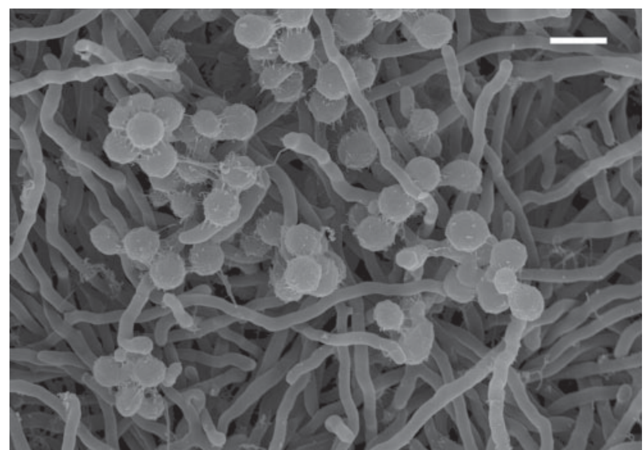


Fig. 2. Scanning electron micrograph of strain HKI0641^T cultivated at 28 °C on ISP-2 agar for 28 days. Bar, 1 μm.

Micromonospora thrived in the same temperature range. Most strains tolerated pH 6–10 and up to 2 % NaCl. Only *M. tulbaghiae* DSM 45142^T was restricted to pH 7–9. Strain HKI0641^T also grew at elevated NaCl concentrations up to 4 %. The fatty acid profile of strain HKI0641^T was dominated by branched-chain fatty acids in accordance with those of other species of the genus *Micromonospora*. However, some qualitative and quantitative differences were found. While anteiso-$C_{15:0}$ was a major constituent of strain HKI0641^T (19.9 %) and *M. aurantiaca* DSM 43813^T (16.4 %), the same fatty acid was much less notable in *M. marina* DSM 45555^T (4.9 %) and *M. purpureochromogenes* DSM 43814^T (3.1 %). Instead the latter two type strains were distinguished by increased levels of iso-$C_{17:0}$. All four strains shared significant amounts of iso-C_{15} and iso-C_{16} (Table S5). The cell wall of strain HKI0641^T contained *meso*-diaminopimelic acid while mycolic acids were not detected, which is in congruence with the taxonomic position in the genus *Micromonospora*. Whole-cell sugars included arabinose, galactose, glucose, mannose, ribose and xylose. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylinositol mannoside (PIM) were the major polar lipids. Furthermore, trace amounts of phosphatidylglycerol as well as one unknown phospholipid, two glycolipids and three other lipids were found (Fig. S2). The predominant menaquinones were MK-10(H_4) (54 %), MK-10(H_2) (20 %) and MK-10 (13 %), besides small amounts of MK-9(H_4) (4 %), MK-10(H_8) (3 %) and MK-9(H_2) (2 %). The phenotypic properties that differentiate HKI0641^T from the type strains of phylogenetically related species are given in Table 1. In particular, the ability to resort to the glycosides amygdalin or arbutin as sole carbon source appear to be highly distinctive metabolic traits. Furthermore, the type strains *M. aurantiaca* DSM 43813^T and *M. marina* DSM 45555^T, which are phylogenetically most closely related to strain HKI0641^T, can be easily distinguished based upon the colour of their colonies on ISP-4 agar (Table S4).

Resistance genes are widely encountered among antibiotic-producing bacteria to confer self-protection (Cundliffe & Demain, 2010) and, in some cases, the resistance profile of a bacterial strain reflects its potential for the biosynthesis of certain antibiotics (Hotta & Okami, 1996). A set of different antibacterial compounds was hence profiled against the isolated strain HKI0641^T and also against closely related species of the genus *Micromonospora*. Except for novobiocin, all tested antibiotics that are known to be derived from actinomycete bacteria, such as kanamycin, streptomycin, tetracycline, chloramphenicol or vancomycin, were active against the strains of species of the genus *Micromonospora* (Table 1). It appeared thus unlikely that the observed antimicrobial effects of strain HKI0641^T could be ascribed to any of these compounds. To identify the metabolites that account for its biological activity, repeat fermentations were carried out on a 200 l scale in production medium (2 % D-sucrose, 0.2 % casitone, 0.5 % cane molasses, 0.01 % $FeSO_4 . 7H_2O$, 0.02 % $MgSO_4 . 7H_2O$, 0.05 % NaI and 0.5 % $CaCO_3$) at 30 °C. At the end of cultivation, the culture supernatant was separated from the cells by centrifugation at 11 710 ***g*** and extracted with ethyl acetate. The extract was fractionated by open column chromatography on silica gel 60 using a dichloromethane-methanol gradient and, subsequently, on Polygoprep 60-50 C18 (Macherey–Nagel) using a methanol–water gradient. Fractions that showed activity in the agar diffusion assay were pooled and subjected to semipreparative reverse phase-HPLC. After an initial separation on a Nucleodur C18 HTec column (5 µm, VP 250/10, Macherey–Nagel; eluent: 80 % methanol) the final purification of the active component was achieved on a Nucleodur C18 PAH column (3 µm, VP 250/8, Macherey–Nagel; eluent: 80 % acetonitrile). This approach yielded 7.6 mg of the known antibiotic telomycin (Figs S3 and S4), which was identified by comparison of its spectroscopic data with those published in the literature (Kumar & Urry, 1973). Further testing revealed that the observed bioactivity of strain HKI0641^T is largely due to the production of telomycin. The activity profile of the isolated peptide antibiotic was consistent with previous reports (Sheehan *et al.*, 1968). To the best of our knowledge, this is the first report of telomycin production in a species of the genus *Micromonospora*.

Consolidating morphological, biochemical and genetic data, it is evident that strain HKI0641^T exhibits all characteristic features of the genus *Micromonospora*. The strain can be distinguished from the most closely related species of the genus *Micromonospora* by both physiological and genetic traits. The deviations in the 16S rRNA and the *gyrB* gene sequences from those of species of the genus *Micromonospora* with validly published names as well as DNA–DNA hybridization data suggest that HKI0641^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora schwarzwaldensis* sp. nov. is proposed.

Description of *Micromonospora schwarzwaldensis* sp. nov.

Micromonospora schwarzwaldensis (schwarz.wald.en′sis. N.L. fem. adj. *schwarzwaldensis* of or belonging to Schwarzwald, the region where the type strain was isolated).

Gram-stain-positive and strictly aerobic, mesophilic actinomycete. Colonies on ISP-2 agar are orange. Well-developed and branched substrate hyphae bear black, smooth-surfaced spores with a diameter of 600 nm. Aerial hyphae are not produced. Growth is good on ISP-2, ISP-3 and ISP-4 agar and moderate on ISP-5 agar. The growth temperature range is 20–37 °C. Optimal growth occurs at 28 °C. Grows at pH 6–9 and in the presence of $<$4 % NaCl. Utilizes cellobiose, lactose, maltose and salicin as sole carbon sources for energy, but not fucose, melicitose or raffinose. The diagnostic diamino acid of the cell-wall peptidoglycan is *meso*-diaminopimelic acid. Whole-cell sugars include arabinose, galactose, glucose, mannose, ribose and xylose. The predominant menaquinone is

Table 1. Phenotypic features of strain HKI0641^T and closely related species of the genus *Micromonospora*

Strains: 1, *Micromonospora schwarzwaldensis* sp. nov. HKI0641^T; 2, *M. aurantiaca* DSM 43813^T; 3, *M. coxensis* DSM 45161^T; 4, *M. marina* DSM 45555^T; 5, *M. purpureochromogenes* DSM 43821^T; 6, *M. carbonacea* DSM43168^T; 7, *M. tulbaghiae* DSM 45142^T. All strains were positive for utilization of cellobiose, aesculin, galactose, glucose, glycerol, glycogen, lactose, maltose, sucrose and starch and negative for utilization of *N*-acetylglucosamine, D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, inositol, D-lyxose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol and L-xylose. All strains showed resistance to penicillin G (10 units), ampicillin + sulbactam (10 + 10 μg), methicillin (5 μg), lincomycin (2 μg) and novobiocin (5 μg), but were sensitive to kanamycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), chlortetracycline (30 μg), oxytetracycline (30 μg), rifampicin (5 μg), vancomycin (30 μg), azlocillin (75 μg), ciprofloxacin (5 μg), imipenem (10 μg), polymyxin B (300 units), amoxicillin + clavulanic acid (20 + 10 μg), norfloxacin (10 μg). +, positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4	5	6	7
Utilization of:							
Amygdalin	+	−	−	−	w	−	w
L-Arabinose	+	+	+	w	−	−	w
Arbutin	+	−	−	−	w	−	−
Fructose	+	+	+	+	w	−	−
Mannitol	−	+	−	−	w	−	−
Mannose	w	−	w	+	w	−	w
Melibiose	−	+	w	w	+	−	w
Raffinose	−	−	+	−	+	w	w
D-Ribose	w	−	+	w	w	−	−
Salicin	+	+	+	w	w	+	−
Trehalose	w	w	w	−	w	+	−
D-Xylose	+	−	+	+	+	w	w
Temperature for growth (°C)	20–37	25–37	20–37	20–37	20–37	20–37	20–37
pH for growth	6–9	6–10	6–10	6–10	6–10	6–10	7–9
Tolerance to 2 % NaCl	+	+	+	+	+	+	+
Tolerance to 4 % NaCl	+	+	−	−	+	+	+
Resistance to sulphonamide (200 μg)	+	−	+	−	−	+	−

*Results were taken after incubation at 28 °C for 7 days.
†Spores observed after 7 days of cultivation.

MK-10(H_4). The phospholipid profile comprises DPG, PE, PI and PIM. Major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{15:0}$ and $C_{16:0}$.

The type strain is HKI0641^T (=DSM 45708^T=CIP 110415^T), isolated from soil near the Schwarzenbach dam, Germany. The type strain produces the antibiotic telomycin.

Acknowledgements

We are grateful to Carmen Schult, Karin Perlet and Bettina Bardl for excellent technical assistance and Jean Euzéby for his advice on the specific epithet. Research in the group 'Secondary Metabolism of Predatory Bacteria' is generously supported by the Deutsche Forschungsgemeinschaft (DFG)-funded graduate school Jena School for Microbial Communication (JSMC). Dr Vela Gurovic gratefully acknowledges the German Academic Exchange Service (DAAD) for a postdoctoral research fellowship (grant# A1071666).

References

Alonso-Vega, P., Normand, P., Bacigalupe, R., Pujic, P., Lajus, A., Vallenet, D., Carro, L., Coll, P. & Trujillo, M. E. (2012). Genome sequence of *Micromonospora lupini* Lupac 08, isolated from root nodules of *Lupinus angustifolius*. *J Bacteriol* **194**, 4135.

Bérdy, J. (2005). Bioactive microbial metabolites. *J Antibiot* (*Tokyo*) **58**, 1–26.

Böcker, S., Hüffner, F., Truss, A. & Wahlström, M. (2009). A faster fixed-parameter approach to drawing binary tanglegrams. *Lect Notes Comput Sci* **5917**, 38–49.

Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.

Carro, L., Spröer, C., Alonso, P. & Trujillo, M. E. (2012). Diversity of *Micromonospora* strains isolated from nitrogen fixing nodules and rhizosphere of *Pisum sativum* analyzed by multilocus sequence analysis. *Syst Appl Microbiol* **35**, 73–80.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Collins, M. D. & Jones, D. (1980). Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2,4-diaminobutyric acid. *J Appl Bacteriol* **48**, 459–470.

Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221–230.

Cundliffe, E. & Demain, A. L. (2010). Avoidance of suicide in antibiotic-producing microbes. *J Ind Microbiol Biotechnol* **37**, 643–672.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Dimkpa, C. O., Merten, D., Svatoš, A., Büchel, G. & Kothe, E. (2009). Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. *Soil Biol Biochem* **41**, 154–162.

Euzéby, J. P. (2012). List of prokaryotic names with standing in nomenclature (LPSN). Société de Bactériologie Systématique et Vétérinaire (SBSV). http://www.bacterio.cict.fr

Garcia, L. C., Martínez-Molina, E. & Trujillo, M. E. (2010). *Micromonospora pisi* sp. nov., isolated from root nodules of *Pisum sativum*. *Int J Syst Evol Microbiol* **60**, 331–337.

Genilloud, O. (2012). Genus I. Micromonospora Ørskov 1923, 156[AL]. In: *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 5, pp. 1039–1057. Edited by M. Goodfellow, P. Kämpfer, H.-J. Busse, M. E. Trujillo, K. Suzuki, W. Ludwig & W. B. Whitman. New York: Springer.

Goodfellow, M., Stanton, L. J., Simpson, K. E. & Minnikin, D. E. (1990). Numerical and chemical classification of *Actinoplanes* and some related actinomycetes. *J Gen Microbiol* **136**, 19–36.

Griebel, T., Brinkmeyer, M. & Böcker, S. (2008). EPoS: a modular software framework for phylogenetic analysis. *Bioinformatics* **24**, 2399–2400.

Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.

Hayakawa, M. & Nonomura, H. (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J Ferment Technol* **65**, 501–509.

Hirsch, A. M. & Valdés, M. (2010). *Micromonospora*: an important microbe for biomedicine and potentially for biocontrol and biofuels. *Soil Biol Biochem* **42**, 536–542.

Hotta, K. & Okami, Y. (1996). Diversity in aminoglycoside antibiotic resistance of actinomycetes and its exploitation in the search for novel antibiotics. *J Ind Microbiol* **17**, 352–358.

Huss, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Kasai, H., Tamura, T. & Harayama, S. (2000). Intrageneric relationships among *Micromonospora* species deduced from *gyrB*-based phylogeny and DNA relatedness. *Int J Syst Evol Microbiol* **50**, 127–134.

Koch, C., Kroppenstedt, R. M., Rainey, F. A. & Stackebrandt, E. (1996). 16S Ribosomal DNA analysis of the genera *Micromonospora*, *Actinoplanes*, *Catellatospora*, *Catenuloplanes*, *Couchioplanes*, *Dactylosporangium*, and *Pilimelia* and emendation of the family *Micromonosporaceae*. *Int J Syst Bacteriol* **46**, 765–768.

Kumar, N. G. & Urry, D. W. (1973). Proton magnetic resonance assignments of the polypeptide antibiotic telomycin. *Biochemistry* **12**, 3811–3817.

Luedemann, G. M. & Brodsky, B. C. (1963). Taxonomy of gentamicin-producing *Micromonospora*. *Antimicrob Agents Chemother* (*Bethesda*) **161**, 116–124.

Minnikin, D. E., Alshamaony, L. & Goodfellow, M. (1975). Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. *J Gen Microbiol* **88**, 200–204.

Minnikin, D. E., Collins, M. D. & Goodfellow, M. (1979). Fatty acid and polar lipid composition in the classification of *Cellulomonas*, *Oerskovia* and related taxa. *J Appl Bacteriol* **47**, 87–95.

Nett, M. & Hertweck, C. (2011). Farinamycin, a quinazoline from *Streptomyces griseus*. *J Nat Prod* **74**, 2265–2268.

Nett, M., Ikeda, H. & Moore, B. S. (2009). Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat Prod Rep* **26**, 1362–1384.

Paradis, E., Claude, J. & Strimmer, K. (2004). APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289–290.

Raaijmakers, J. M. & Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu Rev Phytopathol* **50**, 403–424.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.

Schumann, P. (2011). Peptidoglycan Structure. *Methods Microbiol* **38**, 101–129.

Sheehan, J. C., Mania, D., Nakamura, S., Stock, J. A. & Maeda, K. (1968). The structure of telomycin. *J Am Chem Soc* **90**, 462–470.

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.

Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Zhi, X.-Y., Li, W.-J. & Stackebrandt, E. (2009). An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class *Actinobacteria*, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int J Syst Evol Microbiol* **59**, 589–608.

Supplementary Material

Micromonospora schwarzwaldensis sp. nov., a producer of telomycin, isolated from soil in the Black Forest

Maria Soledad Vela Gurovic, Sebastian Müller, Nicole Domin, Ivana Seccareccia, Sandor Nietzsche, Karin Martin, Markus Nett

* E-mail: karin.martin@hki-jena.de, markus.nett@hki-jena.de

Table of contents

Table S1. Accession numbers of 16S rRNA and *gyrB* gene sequences **S2**

Table S2. 16S rRNA signature nucleotides in strain HKI0641^T **S3**

Table S3. Levels of DNA relatedness **S4**

Table S4. Cultural characteristics **S5**

Table S5. Cellular fatty acids composition **S6**

Figure S1. Scanning electron micrographs of strain HKI0641^T **S7**

Figure S2. Two-dimensional TLC of polar lipid extracts from strain HKI0641^T **S8**

Figure S3. ^{1}H NMR spectrum of telomycin in dimethylsulfoxide-d_6 **S9**

Figure S4. Mass spectrum of telomycin **S10**

References **S11**

Table S1. Accession numbers of 16S rRNA and *gyrB* genes of *Micromonospora* species and other actinomycete bacteria used for phylogenetic analyses.

Species	Strain number	16S rRNA gene sequence accession number	*gyrB* gene sequence accession number
M. schwarzwaldensis	HKI0641^T	KC517406	KC517407
M. aurantiaca	DSM 43813^T	NR_026279	AB015621
M. carbonacea	DSM 43168^T	NR_037043	AB014147
M. chaiyaphumensis	DSM45246^T	NR_041265	HQ231755
M. chersina	DSM 44151^T	X92628.1	AB014148
M. chokoriensis	DSM 45160^T	AB241454	JF272469
M. citrea	DSM 43903^T	NR_044886	AB014150
M. coerulea	DSM 43143^T	X92598	AB014151
M. coxensis	DSM 45161^T	AB241455	FN662498
M. endolithica	DSM 44398^T	AJ560635	FN662502
M. halophytica	DSM 43171^T	X92601	AB014157
M. inositola	DSM 43819^T	NR_026280	AB014158
M. marina	DSM 45555^T	AB196712	JF272468
M. mirobrigensis	DSM 44830^T	AJ626950	FN662505
M. narathiwatensis	DSM 45248^T	AB193559	HM631872
M. olivasterospora	DSM 43868^T	X92613	AB014159
M. pallida	DSM 43817^T	X92608	AB014153
M. purpureochromogenes	DSM 43821^T	X92611	AB014161
M. rifamycinica	DSM 44983^T	AY561829	JN051663
M. rosaria	DSM 803^T	X92631	AB014163
M. tulbaghiae	DSM 45142^T	EU196562	EU434806
Actinoplanes regularis	DSM 43151^T	NR_026288	AB014133
Couchioplanes caeruleus	DSM 43634^T	NR_037054	AB014137

Table S2. 16S rRNA signature nucleotides in strain HKI0641^{T}.

Taxonomic rank: 1, *Actinomycetales*; 2, *Micromonosporaceae*; 3, *Micromonospora schwarzwaldensis*

Conserved position(s)	1[a]	2[a]	3	Nucleotide position(s) in KC517406
127 : 234		A–U	A–U	74 : 181
209		G	G	156
534		G	G	481
688 : 699	G–C		G–C	635 : 646
701	C		C	648
823 : 877	G–C		G–C	770 : 826
831 : 855		U–G	U–G	778 : 804
832 : 854		G–Y	G–T	779 : 803
833 : 853		U–G	U–G	780 : 802
840 : 846		Y–G	C–G	787 : 795
845		G	G	794
955 : 1225		A–U	A–U	904 : 1172
986 : 1219		U–A	U–A	935 : 1166
987 : 1218		G–C	G–C	936 : 1165
1060 : 1197	U–A		U–A	1006 : 1144

[a] The data was taken from Zhi *et al.* [3].

Table S3. Levels of relatedness between *Micromonospora schwarzwaldensis* HKI0641^T and the type strains of other *Micromonospora* species.

Strain	Sequence similarity or relatedness (%) with *M. schwarzwaldensis* HKI0641^T		
	16S rRNA	*gyr*B	DNA-DNA hybridization
M. coxensis DSM 45161^T	99.1	93.0	6.5 (16.5)[1]
M. marina DSM 45555^T	99.0	94.5	31.0 (38.8)[1]
M. carbonacea DSM 43168^T	99.0	93.3	
M. chokoriensis DSM 45160^T	99.0	91.4	
M. halophytica DSM 43171^T	98.9	93.5	
M. purpureochromogenes DSM 43821^T	98.8	92.6	
M. narathiwatensis DSM 45248^T	98.7	94.3	
M. mirobrigensis DSM 44830^T	98.7	91.2	
M. tulbaghiae DSM 45142^T	98.7	94.2	
M. aurantiaca DSM 43813^T	98.6	95.1	39.4 (49.1)[1]
M. chaiyaphumensis DSM 45246^T	98.6	93.6	
M. chersina DSM 44151^T	98.5	93.7	
M. olivasterospora DSM 43868^T	98.5	90.7	
M. endolithica DSM 44398^T	98.5	93.5	
M. citrea DSM 43903^T	98.4	92.0	
M. rosaria DSM 803^T	98.4	92.7	
M. inositola DSM 43819^T	98.3	92.0	
M. pallida DSM 43817^T	98.3	89.6	
M. rifamycinica DSM 44983^T	98.1	91.5	
M. coerulea DSM 43143^T	98.0	91.0	
Couchioplanes caeruleus DSM 43634^T	97.2	84.9	
Actinoplanes regularis DSM 43151^T	97.1	84.5	

[1]The numbers set in parentheses refer to a second, independent hybridization experiment.

Table S4. Cultural characteristics of strain HKI0641^T and the type strains of closely related *Micromonospora* species.

Media	***M. schwarzwaldensis* sp. nov. HKI0641^T**		***M. aurantiaca* DSM 43813^T**		***M. coxensis* DSM 45161^T**		***M. marina* DSM 45555^T**	
	Growth	**Colour of colony**	**Growth**	**Colour of colony**	**Growth**	**Colour of colony**	**Growth**	**Colour of colony**
ISP-2	Abundant	Orange	Moderate	Orange	Abundant	Orange	Abundant	Orange
ISP-3	Abundant	Orange	Moderate	Orange	Moderate	Orange	Poor	Orange
ISP-4	Abundant	Orange	Abundant	Grey	Poor	Orange	No	
ISP-5	Moderate	Orange	Abundant	Orange	Poor	Orange	No	

Media	***M. purpureochromogenes* DSM 43821^T**		***M. carbonacea* DSM 43168^T**		***M. tulbaghiae* DSM 45142^T**	
	Growth	**Colour of colony**	**Growth**	**Colour of colony**	**Growth**	**Colour of colony**
ISP-2	Moderate	Red-orange	Moderate	Orange	Moderate	Orange
ISP-3	Moderate	Orange	Poor	Orange	Abundant	Orange-brown
ISP-4	Abundant	Brown	Abundant	Orange	Abundant	Orange
ISP-5	Abundant	Brown	Abundant	Orange-white	Abundant	Orange

Table S5. Cellular fatty acid compositions (%) of strain HKI0641^T and the type strains of phylogenetically related *Micromonospora* species.

Fatty acid[a]	1	2	3[b]	4[b]	5	6	7
14:0	1.95	0.61	-	-	-	0.62	-
14:0 iso	4.20	4.71	1.36	0.70	1.14	0.98	6.17
15:0	3.09	3.03	-	0.80	1.58	-	1.65
15:0 iso	11.58	18.44	12.26	25.10	19.29	11.63	21.82
15:0 anteiso	19.91	16.36	1.57	4.90	3.12	11.09	14.04
15:1 B	-	0.76	-	-	-	-	-
15:1 iso G	-	-	-	0.30	1.14	-	-
16:0	10.36	4.30	-	0.90	0.98	5.57	3.03
16:0 iso	14.61	15.81	-	16.40	26.50	19.21	33.97
16:0 9-methyl	-	1.37	-	-	9.14	4.10	-
16:1 *ω9cis*	3.67	1.66	-	0.90	-	2.95	-
16:1 2-OH	-	-	4.13	-	-	-	-
16:1 iso G	-	-	7.94	-	3.41	-	-
16:1 iso H	-	0.58	-	0.60	-	-	1.54
17:0	8.55	8.21	1.75	4.90	7.80	1.36	5.99
17:0 iso	1.25	3.67	3.71	7.90	7.54	3.24	2.36
17:0 anteiso	5.13	5.88	8.67	6.90	4.69	8.90	5.93
17:0 10-methyl	-	-	21.58	1.20	1.17	2.71	-
17:1 *ω6cis*	-	-	1.67	-	-	-	-
17:1 *ω8cis*	-	-	4.02	12.70	-	-	-
17:1 *ω9cis*	6.24	6.08	-	6.40	6.47	6.01	1.74
17:1 iso (*ω9cis*)	-	-	12.59	-	-	-	-
17:1 anteiso C	-	-	1.83	0.50	1.02	0.76	-
18:0	5.90	4.81	3.55	1.40	2.12	2.68	1.76
18:0 iso	-	-	2.53	0.30	0.65	-	-
18:0 10-methyl (TBSA)	-	-	2.13	0.40	-	2.73	-
18:1 *ω9cis*	3.57	3.16	3.08	4.50	1.58	14.47	-
18:1 iso			1.97				
19:0	-	0.58	-	0.40	0.67	-	-
Summed feature 6[c]	-	-	-	0.40	-	-	-
Summed feature 7[c]	-	-	-	-	-	0.99	-

Strains : **1**, strain HKI0641^T ; **2**, *M. aurantiaca* DSM 43813^T ; **3**, *M. coxensis* DSM 45161^T ; **4**, *M. marina* DSM 45555^T ; **5**, *M. purpureochromogenes* DSM 43814^T ; **6**, *M. carbonacea* subsp. *carbonaceae* DSM 43168^T ; **7**, *M. tulbaghiae* DSM 45142^T.

[a] The double-bond position indicated by a capital letter is unknown. [b] Data was taken from Ara and Kudo [1] and from Tanasupawat *et al.* [2], respectively. [c] Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 6 includes 19:1 *ω11cis* and/or 19:1 *ω9cis*. Summed feature 7 includes 18:1 *ω11cis,* 18:1 *ω9trans* and/or 18:1 *ω6trans*.

Figure S1. Scanning electron micrographs of strain HKI0641^{T} cultivated at 28 °C on ISP-2 agar. **A**: 14-day old culture. Spores were observed on a 28-day old culture (**B** and **C**). Bars: A, 1 µm; B, 10 µm; C, 2 µm.

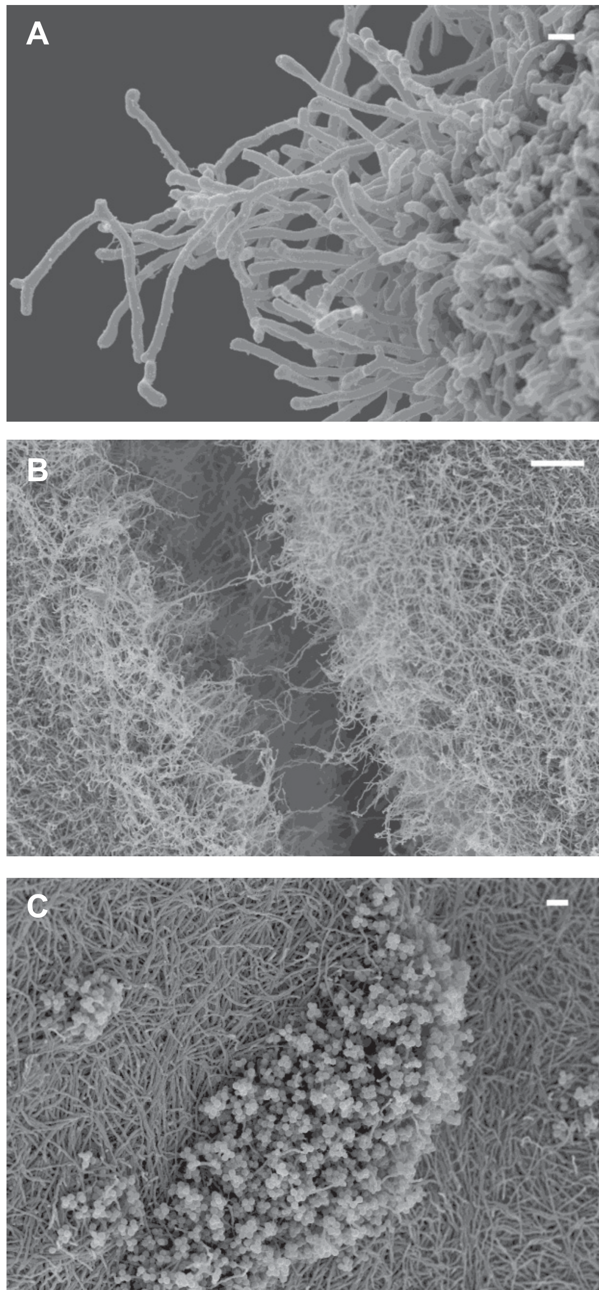
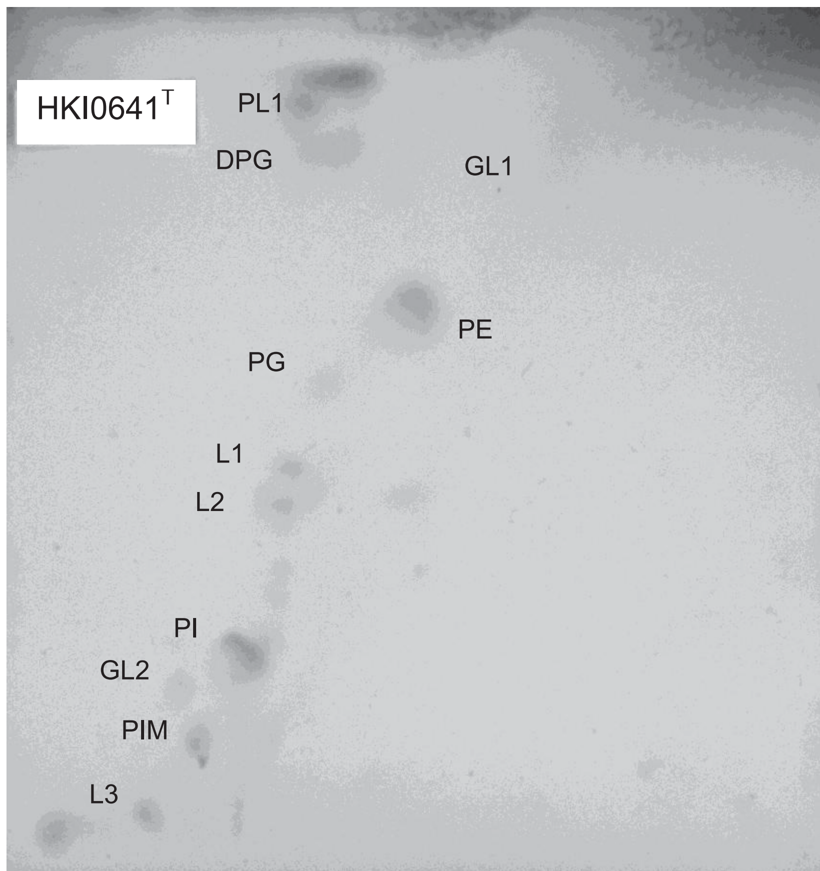


Figure S2. Two-dimensional TLC of polar lipid extracts from strains HKI 641^T, stained with molybdatophosphoric acid.



Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL1, unknown phospholipid; L1, L2, L3, unknown lipids; GL1, GL2, glycolipids.

Figure S3. ^{1}H NMR spectrum of telomycin in dimethylsulfoxide-d_6 recorded with water suppression using presaturation.

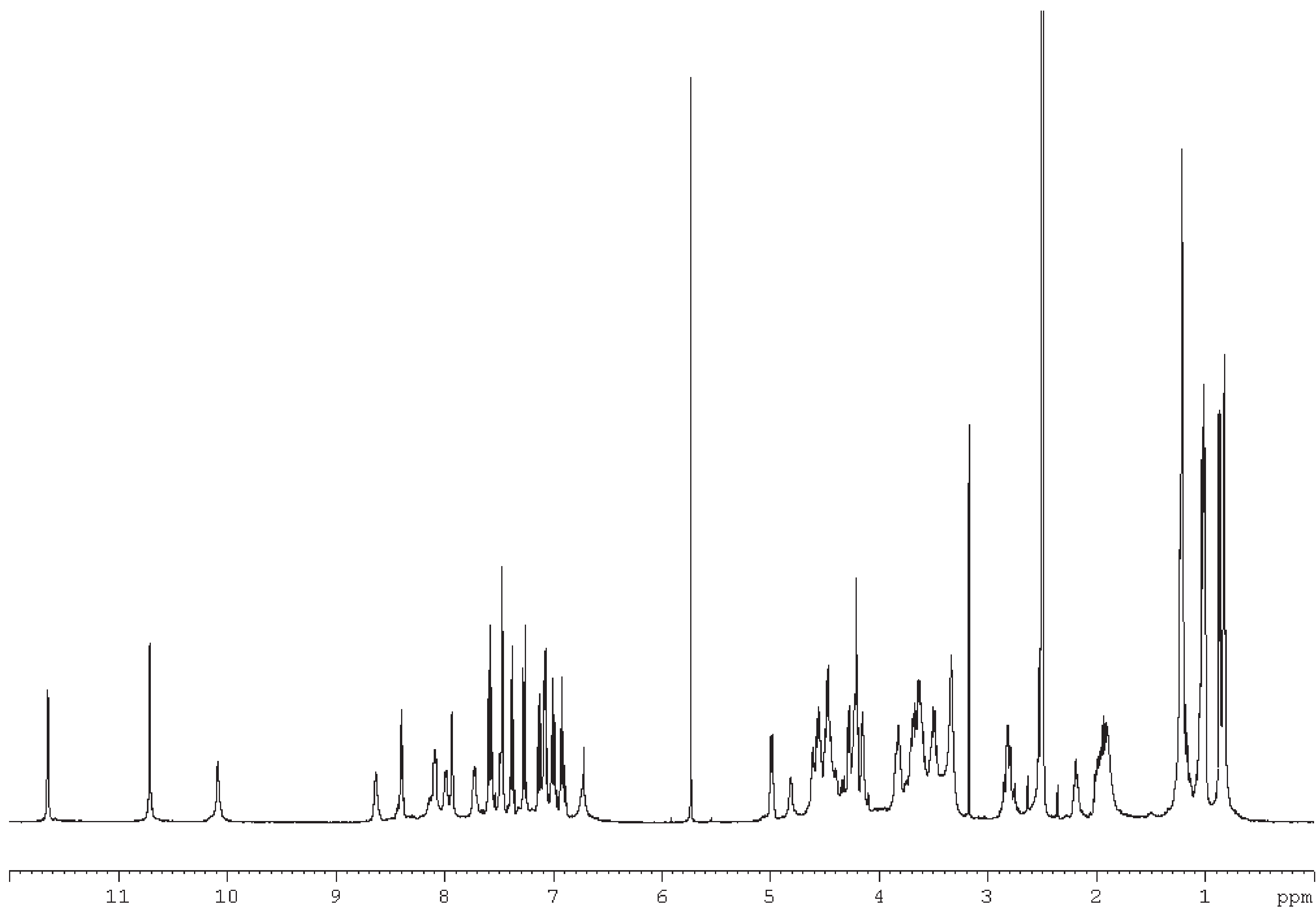
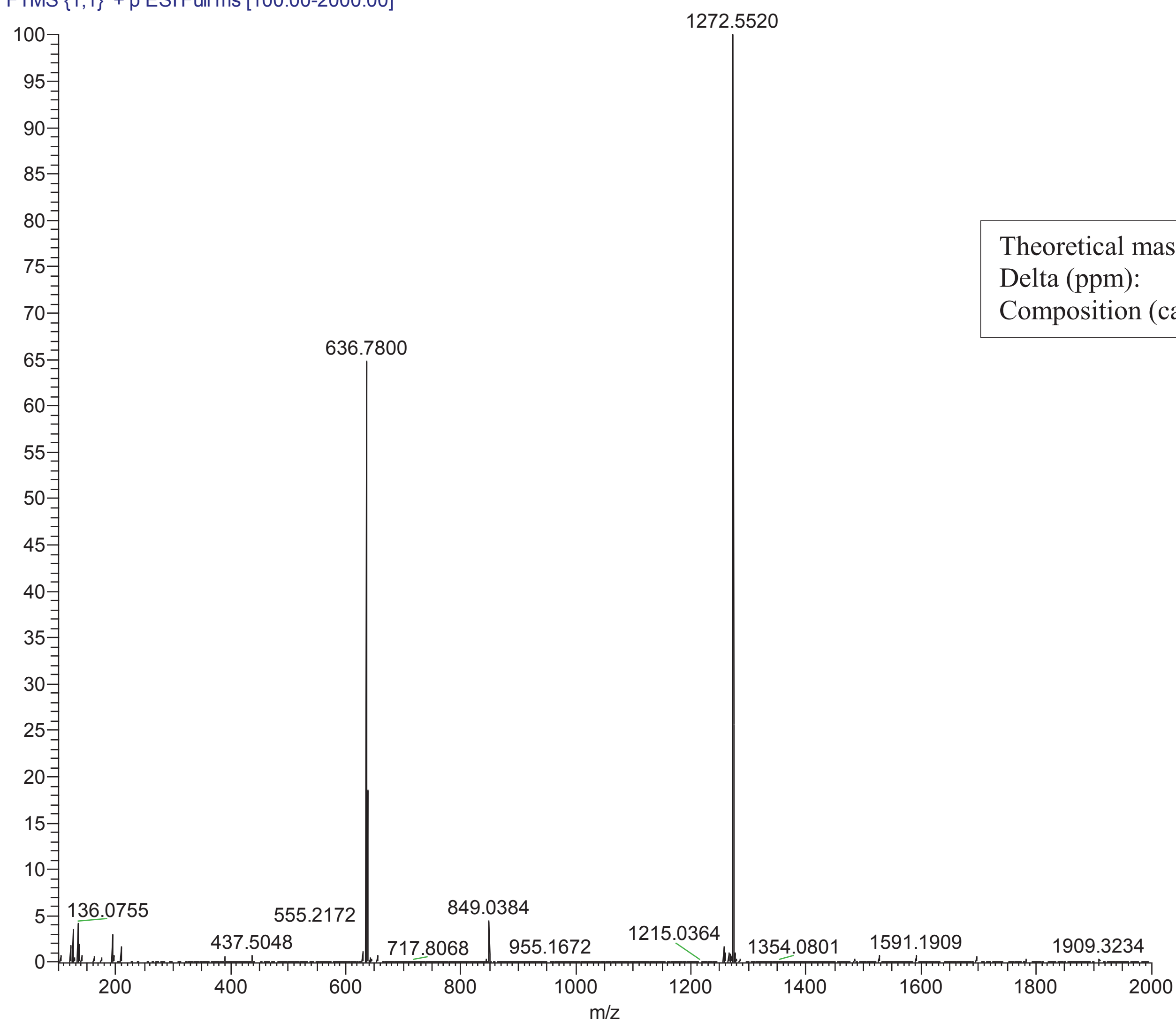


Figure S4. HR-ESIMS spectrum of telomycin.



Theoretical mass (*m/z*):	1272.5531 $[M+H]^+$
Delta (ppm):	0.90
Composition (calculated):	$C_{59}H_{78}O_{19}N_{13}$

References

[1] **Ara, I. & Kudo, T. (2007).** Two new species of the genus *Micromonospora*: *Micromonospora chokoriensis* sp. nov. and *Micromonospora coxensis* sp. nov., isolated from sandy soil. *J Gen Appl Microbiol* **53**, 29-37.

[2] **Tanasupawat, S., Jongrungruangchok, S. & Kudo, T. (2010).** *Micromonospora marina* sp. nov., isolated from sea sand. *Int J Syst Evol Microbiol* **60**, 648-652.

[3] **Zhi, X.-Y., Li, W.-J. & Stackebrandt, E. (2009).** An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class *Actinobacteria*, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa. *Int J Syst Evol Microbiol* **59**, 589-608.

9.2 Manuscript E: Variochelins, lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining.

Kurth Colette, Schieferdecker Sebastian, Athanasopoulou Kalliopi, **Seccareccia Ivana**, and Nett Markus. Variochelins, novel lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining. Manuscript submitted to *Journal of Natural Product*

Variochelins, lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining

*Colette Kurth, Sebastian Schieferdecker, Kalliopi Athanasopoulou, Ivana Seccareccia, and Markus Nett**

Junior Research Group "Secondary metabolism of Predatory Bacteria", Leibniz Institute for Natural Product Research and Infection Biology e.V., Hans-Knöll-Institute, Beutenbergstr. 11a, 07745 Jena, Germany

ABSTRACT

Photoreactive siderophores have a major impact on the growth of planktonic organisms. To date, these molecules have mainly been reported from marine bacteria, although evidence is now accumulating that some terrestrial bacteria also harbor the biosynthetic potential for their production. In this paper, we describe the genomics-driven discovery and characterization of variochelins, lipopeptide siderophores from the bacterium *Variovorax boronicumulans*, which thrives in soil and freshwater habitats. Variochelins are different from most other lipopeptide siderophores in that their biosynthesis involves a polyketide synthase. We demonstrate that the ferric iron complex of variochelin A possesses photoreactive properties and present the MS-derived structure of a degradation product that emerges upon light exposure.

INTRODUCTION

Iron is an essential nutrient for virtually all forms of life. It plays a crucial role in many biological key processes, such as photosynthesis, respiration, N_2 fixation, methanogenesis, oxygen transport, gene regulation, and DNA biosynthesis.[1] Despite its abundance in the Earth's crust, iron's biological availability is severely limited in aerobic environments, which is amongst others due to the formation of insoluble oxides or hydroxides.[2] In order to secure a sufficient iron uptake, many bacteria and fungi thus secrete low molecular weight compounds, so-called siderophores, which have an extremely high affinity towards ferric iron.[3,4] After binding of the metal, the resulting complex is actively transported back into the cell, where the metal is released by a reductive or hydrolytic mechanism.[5]

Siderophores not only support the growth of the producing organism, but also play a significant role in the structuring of microbial communities.[6-9] Moreover, some lipopeptide siderophores were shown to serve as chemical mediators for bacteria-algal interactions in the oceans.[10] These molecules are often distinguished by iron-binding α-hydroxycarboxylate ligand groups.[11] The latter absorb photons in the presence of UV light, thereby triggering a ligand-to-metal charge transfer reaction.[10,12] As a result, ferrous iron is released into the environment, which is then available for direct uptake by surrounding microalgae.[13] Since the latter provide organic nutrients in exchange, the bacterial siderophore producers still benefit from their expenditure for siderophore biosynthesis.[14] This mutualism has important ecological implications and was even proposed to contribute to the occurrence of algal blooms.[15]

The production of photoreactive siderophores was reported from taxonomically distinct genera of marine bacteria, such as *Halomonas*, *Marinobacter*, *Ochrobactrum*, *Synechococcus*, and *Vibrio*.[16-19] The widespread occurrence of these natural products suggests that the carbon-for-iron exchange is a common feature of bacteria-algal interactions.

Recently, three classes of photoreactive lipopeptide siderophores were isolated from non-marine strains.[20-22] While the cupriachelins are produced by a freshwater bacterium and could thus inhere a biological function similar to their marine counterparts,[20] taiwachelin and serobactins are made by rhizosphere bacteria.[21,22] Not only do these findings raise questions concerning the ecological significance of photoreactivity and amphiphilicity in a soil environment, but also whether additional non-recognized producers of such siderophores exist.

Here, we report our recent results on the discovery of novel lipopeptide siderophores from terrestrial bacteria. Using a genome mining strategy,[23-26] we analyzed various strains for the presence of genes that are involved in the biosynthesis of such compounds. This approach resulted in the identification of the genus *Variovorax* as a potential source of structurally new siderophores. Subsequent screening efforts, which were guided by the chrome azurol S (CAS) assay,[27] led to the isolation of variochelins A and B from the bacterium *Variovorax boronicumulans*. The structures of the two natural products were elucidated by NMR and MS measurements as well as Marfey's analysis. We present and discuss the gene cluster involved in variochelin biosynthesis and evaluate the photoreactive properties of variochelin A.

RESULTS AND DISCUSSION

Genome Mining and Siderophore Screening. A common structural motif of photoreactive acyl peptide siderophores is the presence of one or more *β*-hydroxyaspartate residues. Biosynthetically, these moieties derive from aspartate, which is incorporated into the respective siderophore by a non-ribosomal peptide synthetase (NRPS) and, subsequently, subjected to an enzymatic oxidation reaction to introduce the *β*-hydroxyl moiety.[28,29] The oxidation is carried out by an α-ketoglutarate dependent dioxygenase, which is highly similar to the well-studied taurine dioxygenase (TauD).[20,30] In order to identify siderophore gene clusters with these features, BLASTP homology searches were conducted using an aspartate-activating NRPS adenylation domain as well as a TauD-like protein from cupriachelin biosynthesis as probes. Cross-searches against the fatty acyl-AMP ligase (FAAL) domain from taiwachelin biosynthesis or, alternatively, the starter condensation domain from cupriachelin biosynthesis were used to narrow down the initial results to siderophore loci that possess genetic hallmarks of fatty acid incorporation.[31] The hits that were retrieved from this combined analysis were validated by bioinformatic software to confirm the predictions concerning the molecular architecture of the encoded natural products.[32] In this way, we identified a total of 16 non-marine strains that are likely to produce acyl peptide siderophores with a *β*-hydroxyaspartate motif (Table S1). After excluding those strains whose biosynthetic potential had already been confirmed in previous investigations,[20-22] 13 strains remained, covering six different genera. Among the newly identified producers, the genus *Variovorax* appeared to be of particular interest. Unlike the known acyl peptide siderophore gene clusters,[29] the loci in the three *Variovorax paradoxus* strains include distinctive polyketide synthase (PKS) genes (Figure 1). A thorough inspection of the NRPS and PKS domain architecture and substrate specificities[32-35] unveiled the close relatedness of the biosynthetic enzymes in *V. paradoxus* B4 and *V. paradoxus* S110, suggesting that they catalyze the

production of structurally identical molecules (Table S2). In contrast, the gene cluster of *V. paradoxus* EPS clearly differs from the other two loci in size and gene organization. A total of ten NRPS and PKS modules in the EPS assembly line outnumbers the six modules from B4 and S110 and indicates the biosynthesis of a significantly larger siderophore (Table S3).

To test the secretion of iron-chelating metabolites, we subjected five *Variovorax* strains available in our laboratory to a modified CAS assay, in which the siderophore detection is spatially separated from the growth area of the respective bacterium.[36] All five strains produced an orange halo in the CAS zone of the agar plate (Figure S1), thereby indicating the release of iron-chelating agents.[27] A comparison of the different halo sizes in three independently conducted experiments revealed that *V. boronicumulans* BAM-48 consistently gave the strongest assay response when compared to the other strains. Since the bacterium also grew vigorously under established siderophore production conditions (data not shown),[20,21] we decided to carry out all following chemical investigations with this organism.

Isolation and Structure Determination of Variochelins. In order to induce siderophore biosynthesis in *V. boronicumulans* BAM-48, the bacterium was cultivated in H-3 minimal medium under iron starvation conditions. Secreted metabolites were recovered from the fermentation broth by adsorption onto XAD-2 resin. After removal of the supernatant, the resin was eluted with methanol to release the bound molecules. The resulting extract was concentrated and subjected to HPLC. Two peaks in the metabolic profile corresponded to iron-chelating compounds, as evidenced by a positive response of the respective fractions in the liquid CAS assay (Figure S2).[27] The associated compounds were hence isolated and subjected to ESI-MS. Distinctive pseudomolecular ions appeared at *m/z* 1074 $[M + H^+]$ for compound **1** and *m/z* 1102 $[M + H]^+$ for compound **2**, respectively. A preliminary inspection of their ^1H NMR spectra suggested both metabolites to be acyl peptides.

High resolution ESI-MS of variochelin A (**1**) yielded *m/z* 1074.6040 for the $[M + H]^+$ ion, indicating a molecular formula of $C_{47}H_{83}N_{11}O_{17}$. The constituents in the peptidic headgroup of **1** and their connectivity were initially deduced by tandem mass spectrometry.[37] A sequential loss of 191, 172, 97, 87, and 131 mass units during MALDI-TOF/TOF fragmentation was attributed to an amino acid sequence of N^{δ}-acetyl-N^{δ}-hydroxyornithine, N^{δ}-acetyl-N^{δ}-hydroxyornithine, proline, serine, and β-hydroxyaspartic acid from the carboxylate terminus (Figure 2, Figure S3). The configurations of the amino acid residues were determined by Marfey's method upon acidic hydrolysis of **1**.[38] This analysis established the proline residue to be in L configuration, whereas the serine was found to be D-configured (Figures S4, S5). Marfey's method also revealed the presence of *threo*-β-hydroxyaspartic acid. The elution order of the diastereomeric pairs of L-FDAA-derivatized *threo*-β-hydroxyaspartic acid had previously been shown to be D→L under reversed-phase conditions.[39] The variochelin A hydrolysate contained only one single peak of the correct mass upon conversion with L-FDAA, which eluted at the same retention time as the second peak of L-FDAA-derivatized D,L-*threo*-β-hydroxyaspartic acid (Figure S6). Therefore, we concluded that, out of the four possible stereoisomers of β-hydroxyaspartic acid, the L-*threo* form is present in **1**. Surprisingly, the derivatization of the released ornithine units with L-FDAA failed. To determine the configuration of these amino acid residues, we treated the hydrolysate of **1** with (1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyl carbonochloridate. Cochromatography of the resulting ornithine carbamate against synthetic standards eventually established the L configuration for both N^{δ}-acetyl-N^{δ}-hydroxyornithine moieties in **1** (Figure S7).

The amino acid sequence that had been inferred from the interpretation of the tandem mass spectra was subsequently confirmed by NMR data (Table 1, Figures S8-S12). Still, however, a significant portion of the molecular structure of **1** was not resolved. The

concluding NMR-based structure elucidation started with the quaternary carbon C35, which had not been assigned yet and was distinguished by its chemical shift at 156.5 ppm. This value was lower than those observed for the amide carbonyl groups and could also not be traced to an aromatic moiety. Instead a comparison with literature data strongly suggested that C35 is part of a guanidino group.[40-42] HMBC interactions then enabled the identification of a 4-amino-7-guanidino-3-hydroxy-2-methylheptanoate fragment. The relative stereochemistry of the three chiral centers in this moiety was deduced as (28*S**,29*S**,30*S**) by selective NOESY experiments. Upon irradiation at the resonance frequency of H34, an NOE was observed with H30, but not with H29. Likewise, irradiation on H28 revealed an NOE with H29, but not with H30. Eventually, the unusual γ-amino acid was connected to the β-hydroxyaspartate residue of **1** due to long range correlations from H24 and NH4 to C27. The remaining non-assigned signals in the ^{1}H and ^{13}C spectra were distinctive of an unbranched acyl chain. The latter was attributed to a dodecanoyl residue in consideration of the molecular formula of **1**. HMBC interactions from H30 and NH5 to C36 linked the acyl moiety with the rest of the molecule and, thereby, established the complete structure of **1**.

The sum formula of variochelin B (**2**) was calculated as $C_{49}H_{87}N_{11}O_{17}$ from its high-resolution mass. Tandem mass spectrometry revealed the same y fragments as those observed during the fragmentation of **1**. However, the corresponding b fragments were increased by 28 mass units each. This data suggested that both variochelins differ in their fatty acid tail with **2** featuring a tetradecanoic acid residue.

Variochelin Gene Cluster and Biosynthetic Model. The guanidino-containing γ-amino acid that is present in both variochelins can be biosynthetically rationalized as the product of a decarboxylative Claisen condensation of arginine and methylmalonate, which is then subject to a β-keto reduction. This scenario suggests the involvement of a PKS and could indicate that the variochelin gene cluster is organizationally closely related to the siderophore

loci that were previously discovered in the three *V. paradoxus* strains. To find out whether this assumption is correct, we sequenced and annotated the entire genome of *Variovorax boronicumulans* BAM-48. Gene clusters with a putative role in secondary metabolism were identified using antiSMASH 3.0.1.[32] Out of the seven loci detected, only one met the defined criteria for the biosynthesis of an acyl peptide with a *β*-hydroxy aspartate motif. The cluster boundaries that had been predicted by antiSMASH 3.0.1 were manually refined on the basis of functional annotations, gene distances and GC content shifts.[25] According to this analysis, the variochelin (*var*) locus (Figure 3A) consists of 18 genes, covers 43.2 kb of contiguous DNA and displays a significant similarity to the *V. paradoxus* siderophore clusters. However, substrate specificity predictions suggest different metabolic products (Tables S2, S3).[33-35] The seven NRPS and PKS modules encoded by *varF*, *varG*, *varH*, *varI* and *varJ* are assumed to assemble the molecular backbones of **1** and **2** (Figure 3B). The biosynthesis would hence start with the activation of dodecanonoic (or tetradecanoic) acid by the FAAL domain of VarF. It then follows the co-linear logic of assembly-line enzymology, where the activated substrates are incorporated into a linear oligomer by successive condensation steps.[25,43] The PKS VarG contains the typical *β*-ketoacylsynthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains, as well as a ketoreductase (KR) domain. Each NRPS module harbors the complete set of condensation (C), adenylation (A) and peptidyl carrier protein (PCP) domains. The TauD domain of VarG would be responsible for the hydroxylation of the incorporated aspartic acid residue. Additionally, VarI harbors an epimerization (E) domain, which would be required for the stereochemical inversion of L-serine. The domain architecture is hence consistent with the experimentally deduced configurations. Although Marfey's analysis provided no information on the stereochemistry of the 4-amino-7-guanidino-3-hydroxy-2-methylheptanoate moiety, the missing E domain in VarF strongly suggests an L configuration for the primary arginine building block. We thus propose an

(28*S*,29*S*,30*S*) absolute configuration for **1** and **2**. Once the chain elongation is completed, the terminal thioesterase (TE) domain in VarJ releases the newly synthesized lipopeptide.

The *var* cluster also features several accessory proteins that are essential for the proper functioning of the NRPS and PKS enzymes. Small MbtH-like proteins, such as VarC, are assumed to influence amino acid activation by NRPS,[44] whereas the role of type II thioesterases, such as VarD, lies in the removal of aberrant intermediates that may block the NRPS/PKS assembly line. Furthermore, type II thioesterases are possibly involved in substrate selection and in product release.[45] The phosphopantetheinyl transferase VarE is essential to convert the carrier protein domains of the NRPS and PKS from the inactive *apo* into the active, substrate-binding *holo* forms.[46] VarN and VarO were annotated as L-ornithine 5-monooxygenase and N^{δ}-hydroxyornithine acetyltransferase, respectively. Similar to the homologous IucD and IucB in aerobactin biosynthesis,[47] the two enzymes are assumed to act in a concerted manner to generate the hydroxamate ligand groups in variochelins. The remaining genes that are located in the *var* cluster are likely involved in siderophore transport. Uptake of ferric iron-variochelin complexes should occur via the TonB-dependent receptor VarK and possibly also via the peptide transporter VarR. Intracellular iron release from the siderophore would then be mediated by the ferric iron reductase VarP.[48] VarB, VarL and VarQ are homologs of FecR, a protein responsible for the regulation of Fe^{3+}-dicitrate uptake in *Escherichia coli*.[49] Together with the encoded sigma-factors VarA and VarM, we expect these proteins to regulate gene expression within the cluster depending on iron availability.

Complexing Properties and Photoreactivity of 1. The variochelins possess three bidentate ligand groups for the coordination of metal ions, including an *α*-hydroxycarboxylate (*i.e.*, the *β*-hydroxyaspartate residue) and two hydroxamate functions. To test the complexing properties of **1**, the compound was treated with an equimolar quantity of

a metal salt and directly subjected to HR-MS. This analysis revealed that **1** is capable to form monomeric 1:1 complexes with Fe^{3+} and Ga^{3+} (Figure S12). No masses corresponding to Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+} complexes could be detected and we also did not observe any complex formation of **1** in the presence of boron salts. The observed discrimination between divalent and trivalent metal ions and the iron-responsive production suggest a siderophore function for **1**.

Depending on their coordination state, bidentate ligand groups are sensitive to light exposure. While hydroxamates are in general photochemically stable, catechols are prone to photooxidation in the absence of metal coordination, but stable once bound to ferric iron.[50] In contrast, α-hydroxy acid moieties are stable in their metal-free form, but undergo light-induced oxidation after complexation to ferric iron.[10,11] In order to test the photostability of **1**, we exposed an aqueous solution of its ferric iron complex to direct sunlight and analyzed product formation via LC-ESI-MS. The most prominent photoproduct (**3**) that was observed exhibited *m/z* 414.3440 $[M + H]^+$, corresponding to a molecular formula of $C_{21}H_{43}N_5O_3$. The postulated structure of **3** is consistent with a preceding cleavage reaction in the β-hydroxyaspartate residue of **1** (Figure 4). A mass of the remaining peptide headgroup was not detected, suggesting that it further decomposes into smaller, as yet undefined fragments. In a parallel experiment, we confirmed the light-induced ligand-to-metal charge-transfer reaction, which leads to a reduction of the coordinated ferric iron. Samples of Fe^{3+}-variochelin A that were exposed to sunlight gradually turned red in the presence of the Fe^{2+}-trapping agent bathophenantrolinedisulphonate (BPDS). Absorption at 535 nm increased from 0.004 ± 0.001 to 0.0438 ± 0.001 within 4 h of light exposure. The negative control that was shielded from light remained colorless in the same time period. Here, the absorption at 535 nm increased from 0.0027 ± 0.001 to 0.0032 ± 0.001.

In summary, we reported the discovery of a new class of acyl peptides from the bacterium *V. boronicumulans* BAM-48. Although the variochelins show typical hallmarks of marine siderophores, the producing strain originates from soil,[51] where it was shown to contribute to plant growth.[52] After taiwachelin[21] and the serobactins[22], the variochelins already represent the third class of photoreactive lipopeptide siderophores that are produced by plant-associated bacteria. Amphiphilic siderophores that might be added to this group include corrugatin and ornicorrugatin from *Pseudomonas* spp.,[53] ornibactins from *Burkholderia* spp.,[54] as well as rhizobactin 1021 from *Sinorhizobium meliloti*,[55] even though the photoreactivity of these compounds still awaits experimental confirmation. The occurrence of such siderophores in bacteria thriving in vicinity to plants raises two possible conclusions. Either amphiphilicity and/or photoreactivity are somewhat beneficial in the rhizosphere or the chemical properties inherent to these siderophores represent an evolutionary relict. It is noteworthy in this context, that bacteria of the genus *Variovorax* are also commonly found in freshwater habitats,[56] where an ecological advantage of amphiphilicity and photoreactivity would be plausible.[3]

During the preparation of this manuscript, a NRPS-PKS assembly line with a domain architecture almost identical to that of *V. boronicumulans* BAM-48 was reported from the bacterium *V. paradoxus* P4B.[57] A product from this assembly line named variobactin A possesses the same elemental composition as variochelin A. However, the structure of variobactin A was proposed as a cyclic depsipeptide[57] with an amino acid sequence that deviates from that of variochelin A and apparently does not follow the co-linearity rule.

EXPERIMENTAL SECTION

General Experimental Procedures. LC-MS experiments were conducted on an Accela UHPLC system equipped with a C18 column (Betasil C18, 150 × 2.1 mm, 3 µm; Thermo Scientific) coupled to a Finnigan Surveyor PDA plus detector (Thermo Scientific). For metabolic profiling, a gradient of acetonitrile (ACN) in water + 0.1% formic acid and a flow rate of 0.2 mL/min was used. The ACN concentration was increased from 5% to 98% within 16 min, was kept for 3 min at 98%, and was subsequently decreased to 5% within 14 min. High-resolution mass determination was carried out on an Exactive Mass Spectrometer (Thermo Scientific). One- and two-dimensional MALDI-TOF/MS data using post-source decay were acquired on a Bruker Ultraflex Spectrometer (Bruker Daltonics). NMR spectra were recorded at 300 K on Bruker Avance III 500 or 600 MHz spectrometers with DMSO-d_6 as solvent and internal standard. The solvent signals were referenced to δ_H 2.50 ppm and δ_C 39.5 ppm.

Siderophore Screening. CAS agar plates were prepared as previously reported.[27,36] Half of the CAS agar layer was cut out, and the gap was filled with iron-free H-3 mineral medium (1.0 g/L aspartic acid, 2.3 g/L KH_2PO, 2.57 g/L Na_2HPO_4, 1.0 g/L NH_4Cl, 0.5 g/L $MgSO_4 \times 7\ H_2O$, 0.5 g/L $NaHCO_3$, 0.01 g/L $CaCl_2 \times 2\ H_2O$ and 5 mL/L SL-6 trace element solution).[58] *V. boronicumulans* BAM-48, *V. paradoxus* 351, B13-0-1 D, *V. paradoxus* B4, *V. paradoxus* S110 and *V. soli* GH9-3 were streaked out on the H-3 half of the plates. The secretion of iron-complexing metabolites was detected by a color change from blue to orange in the CAS half of the plates after incubation at 30 °C.

Isolation of Variochelins. *V. boronicumulans* BAM-48 was grown in a 10 L scale in iron-free H-3 mineral medium.[58] The strain was shaken (130 rpm) at 30 °C for 5 days. The culture supernatant was then separated from the cells by centrifugation (8000 rpm) and extracted with 150 g/L XAD-2 (Supelco). The resin was thoroughly washed with distilled water before the adsorbed metabolites were eluted with MeOH. The eluate was dried under

vacuum, resuspended in 1 mL MeOH and initially fractionated by flash column chromatography over Polygoprep 60-50 C_{18} (Macherey-Nagel) using an increasing concentration of MeOH in water. CAS-positive fractions were further purified by high performance liquid chromatography on a Shimadzu UFLC liquid chromatography system equipped with a Nucleodur C18 HTec column (VP 250 × 10 mm, 5 µm; Macherey-Nagel) using a MeOH/H_2O gradient from 50% to 100% over 20 min and keeping 100% MeOH for 10 min, with 0.1% trifluoroacetic acid.

Amino Acid Analysis by Marfey's Method. Amino acid configurations were determined following acid hydrolysis and derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, L-FDAA, Sigma Aldrich)[38] by coelution experiments with L-FDAA-derivatized amino acids. For this purpose, 1 mg purified **1** was dissolved in 500 µL concentrated HI and heated at 110 °C for 8 h. The solution was lyophilized, and the dried hydrolysate was resuspended in 50 µL of water and 20 µL of aqueous $NaHCO_3$ (1 M). Derivatization was carried out with 100 µL of L-FDAA (1% w/v in acetone) at 40 °C for 1 h. Afterwards the reaction was quenched with 20 µL of HCl (1 M). The products were lyophilized and prepared for LC-HR-MS analysis by dissolving in MeOH. Standards for cochromatography were prepared by reacting 50 µL of an aqueous amino acid solution (50 mM) with 20 µL of $NaHCO_3$ (1 M) and 100 µL of L-FDAA (1% w/v in acetone) at 40 °C for 1 h. The dried reaction mixture was dissolved in MeOH and subsequently analyzed by LC-HR-MS.

Configurational analysis of the released ornithine residues. Triphosgene (0.065 mmol) and pyridine (0.45 mmol) were added to a solution of (-)-menthol (0.13 mmol) in 3 mL DCM in an ice bath. The solution was stirred for 30 min and subsequently allowed to warm to room temperature. The stirring was continued for 30 min. The resulting (1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyl carbonochloridate was used directly for derivatization. For

this purpose, D and L ornithine standards (0.2 mmol) or hydrolysed **1** were dissolved in a 2:1 DMSO/H_2O mixture (3 mL) in the presence of $NaHCO_3$ (0.2 mmol) and added to the (1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyl carbonochloridate solution. After stirring for 1 h at room temperature, the samples were lyophilized, dissolved in MeOH and analyzed by HR-LC-MS.

Genome Sequencing, Assembly and Annotation. Genomic DNA of *V. boronicumulans* BAM-48 was isolated via phenol chloroform extraction. The purity, quality and size of the bulk gDNA preparation were assessed according to DOE-JGI guidelines.[43] Sequencing was performed at GATC Biotech AG (Konstanz, Germany) by means of single molecule real time (SMRT) sequencing.[59] The reads extracted from the resulting dataset were assembled using the hierarchical genome assembly process (HGAP).[60] Variochelin biosynthesis genes were first identified using antiSMASH 3.0.1.[32] Refinement of the cluster analysis was conducted as previously described.[25,43] The annotated nucleotide sequence for the variochelin gene cluster has been deposited in GenBank under accession number xxx (*currently processed under submission # 18635224; the accession number will be provided once this manuscript is accepted*).

Photoreactivity Tests. Photoreactivity tests of variochelins were performed as previously described.[20] Reduction of the complexed ferric iron to ferrous iron was investigated via the bathophenantrolinedisulfonic acid (BPDS) assay. Each reaction contained 100 µM variochelin A, 10 µM $FeCl_3$ and 40 µM BPDS (Sigma Aldrich) in PBS buffer (pH 7.5). The reactions were either exposed to sunlight or kept in the dark for 4 h. The formation of $Fe(BPDS)_3^{2+}$ was monitored before and after exposure to sunlight/darkness by measuring the absorption at 535 nm using a Genesys 10 UV spectrophotometer (Thermo). The experiments were run in duplicate. In order to identify photolysis products, a 2 mM solution of ferric **1** in PBS buffer (pH 7.5) was exposed to sunlight for 6 h. An identical solution that

was shielded from sunlight served as a negative control. After photoexposure, both samples were dried in vacuo. The samples were then taken up in 100 μL MeOH and analyzed by HR-ESI-MS.

FIGURES.

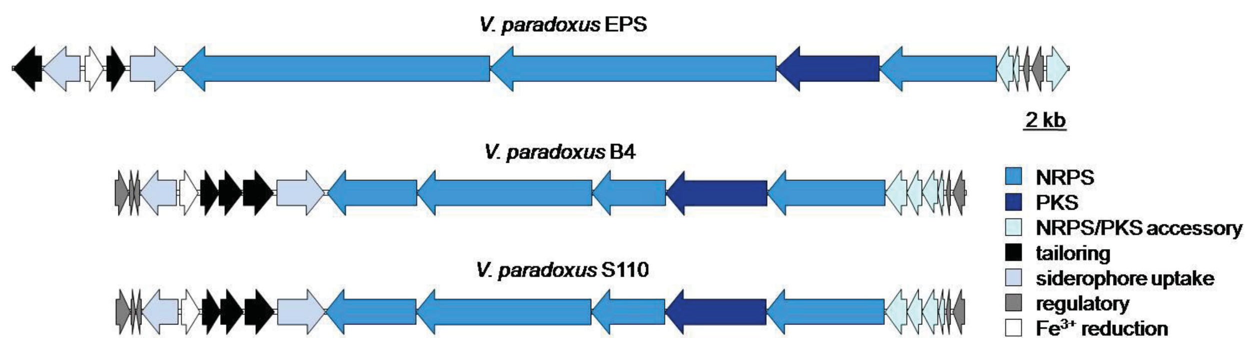


Figure 1. Putative siderophore biosynthesis gene clusters in bacteria of the genus *Variovorax*.

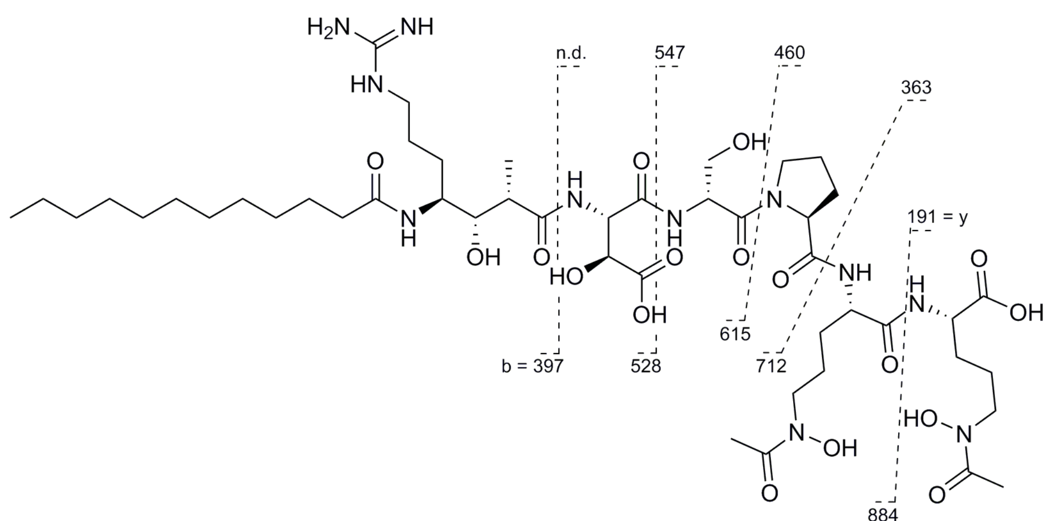


Figure 2. Structure of variochelin A (**1**). The dashed lines through the structure show the “y” and “b” fragments obtained in a tandem MS experiment. The depicted numbers indicate the corresponding *m/z* values.

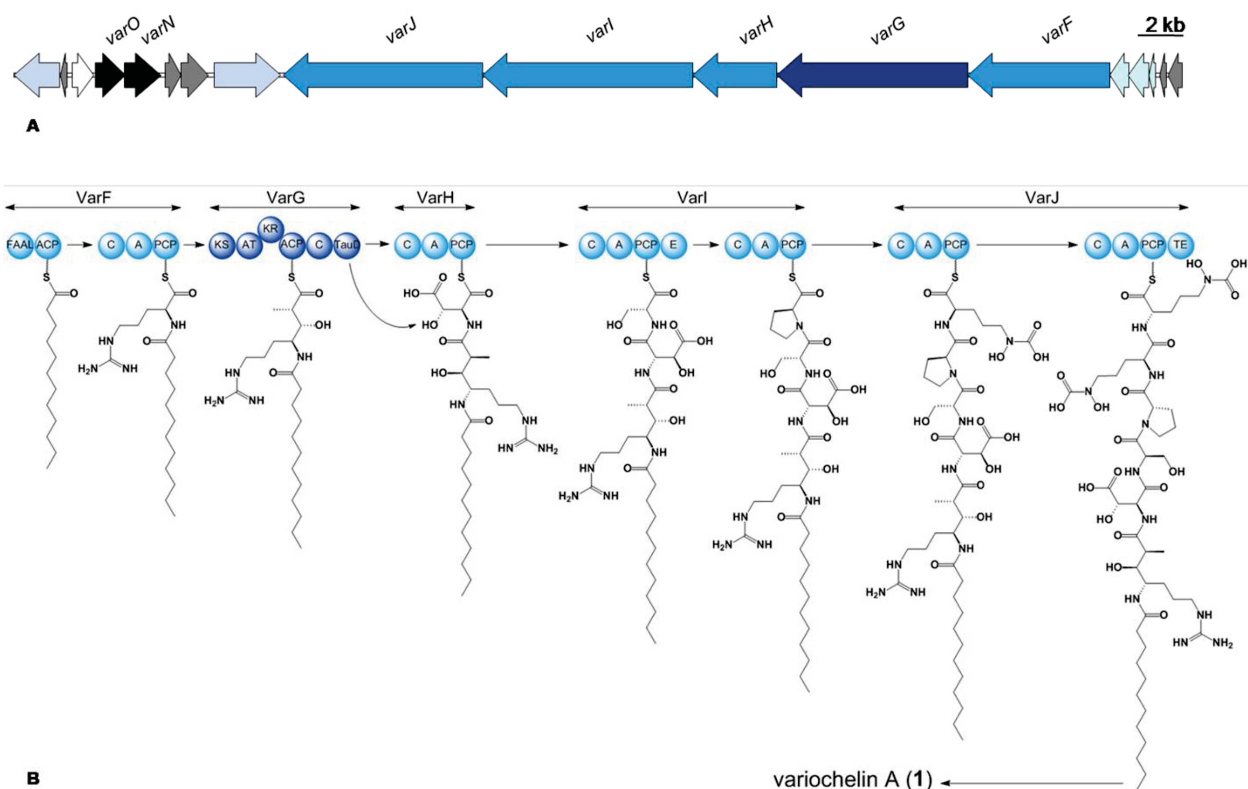


Figure 3. Organization of the variochelin biosynthesis cluster (for annotations see Table S4) (A). Molecular assembly line deduced from *varF-varJ* and proposed biosynthesis of **1** (B). Domain annotation: FAAL, fatty acyl-AMP ligase; ACP, acyl carrier protein; C, condensation; A, adenylation; PCP, peptidyl carrier protein; KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; TauD, hydroxylase; E, epimerization; TE, thioesterase.

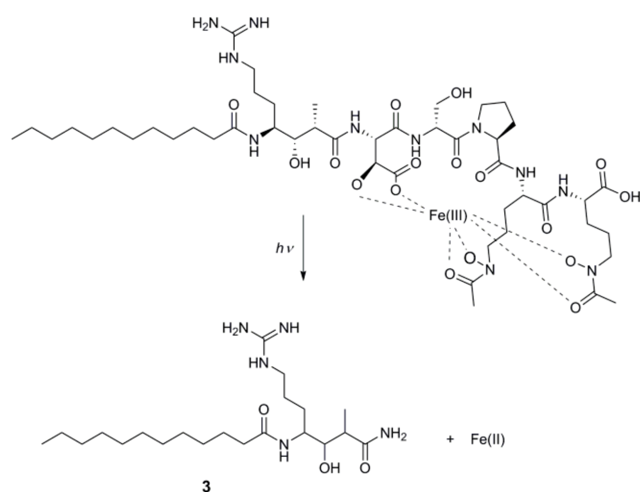


Figure 4. Proposed reaction scheme for the photolysis of ferric **1**. The depicted cleavage product **3** was detected by HR-ESI-MS and its structure was deduced by tandem MS. The complexed ferric iron is likely to be reduced via ligand-to-metal charge transfer.

Table 1. NMR Spectroscopic Data for Variochelin A in DMSO-d_6

pos.	δ_C, type	δ_H (J in Hz)	HMBC[a]
	N^{δ}-acetyl-N^{δ}-hydroxy-ornithine		
C1	173.4, C		
C2	51.6, CH	4.17, dt (7.8, 4.4)	1, 3
C3	29.0, CH_2	a: 1.70, m	2, 4, 5
		b: 1.56, m	2, 4, 5
C4	22.9, CH_2	1.53, m	2, 3, 5
C5	46.5, CH_2	3.48, m	3, 4
C6	170.3, C		
C7	20.3, CH_3	1.95, s	6
N1		8.08, d (7.8)	8
	N^{δ}-acetyl-N^{δ}-hydroxy-ornithine		
C8	171.4, C		
C9	51.6, CH	4.35, m	8, 11
C10	29.8, CH_2	a: 1.61, m	9, 11, 12
		b: 1.50, m	9, 11, 12
C11	22.8, CH_2	1.53, m	9, 10, 12
C12	46.8, CH_2	3.48, m	10, 11
C13	170.3, C		
C14	20.3, CH_3	1.96, s	13
N2		7.96, d (8.7)	15
	proline		
C15	171.3, C		
C16	59.7, CH	4.38, dd (8.4, 3.7)	15, 17, 18
C17	29.8, CH_2	a: 2.05, m	15, 16
		b: 1.83, m	16
C18	24.1, CH_2	1.49, m	17, 19
C19	47.0, CH_2	3.48, m	17, 18, 20
	serine		
C20	168.5, C		
C21	52.8, CH	4.65, q (7.4)	20, 22, 23
C22	61.8, CH_2	3.48, d (7.4)	20, 21
N3		7.72, d (7.4)	21, 23

pos.	δ_C, type	δ_H (J in Hz)	HMBC[a]
	β-hydroxyaspartic acid		
C23	168.4, C		
C24	55.0, CH	4.74, dd (9.1, 2.7)	23, 25, 26, 27
C25	70.1, CH	4.51, d (2.7)	23, 24, 26
C26	173.0, C		
N4		7.75, d (9.1)	24, 25, 27
	4-amino-7-guanidino-3-hydroxy-2-methylheptanoic acid		
C27	175.5, C		
C28	41.8, CH	2.49, m	27, 29, 34
C29	73.3, CH	3.56, m	27, 28, 30, 31, 34
C30	49.5, CH	3.66, m	29, 31, 32, 36
C31	27.5, CH_2	1.22, m	29, 30, 32
C32	25.1, CH_2	a: 1.44, m	30, 31, 33
		b: 1.36, m	30, 31, 33
C33	40.8, CH_2	a: 3.10, m	31, 32, 35
		b: 3.02, m	31, 32, 35
C34	11.3, CH_3	1.00, d (6.9)	27, 28, 29
C35	156.5, C		
N5		7.56, d (9.2)	29, 30, 36
N6		7.40, t (5.8)	33
	dodecanoic acid		
C36	172.1, C		
C37	35.5, CH_2	2.07, t (7.4)	36, 38, 39
C38	25.4, CH_2	1.47, m	36, 37, 39, 40
C39	28.7, CH_2	1.23, m	n.r.
C40	28.8, CH_2	1.23, m	n.r.
C41	29.0, CH_2	1.23, m	n.r.
C42	29.1, CH_2	1.23, m	n.r.
C43	29.0, CH_2	1.23, m	n.r.
C44	29.0, CH_2	1.23, m	n.r.
C45	31.3, CH_2	1.23, m	n.r.
C46	22.1, CH_2	1.26, m	45, 47
C47	14.0, CH_3	0.85, t (7.0)	45, 46

[a] HMBC correlations, optimized for 7.7 Hz, are from proton(s) stated to the indicated carbon; n.r., not resolved.

ASSOCIATED CONTENT

Complete annotation of the *Variovorax* siderophore gene clusters, including GenBank accession numbers; Testing of *Variovorax* strains in the CAS assay; HPLC purification of **1** and **2**; MS data and NMR spectra of **1**; Amino acid analysis; Photoreactivity test of ferric **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49 3641 5321297 Fax: +49 3641 5322297. E-mail: markus.nett@hki-jena.de

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This project was supported by the Collaborative Research Center ChemBioSys (CRC 1127 ChemBioSys) and funded by the Deutsche Forschungsgemeinschaft (DFG). We thank A. Perner and H. Heinecke (Hans-Knöll-Institute Jena, Department of Biomolecular Chemistry) for recording high-resolution ESI-MS and NMR spectra. We also thank M. Poetsch (Hans-Knöll-Institute Jena, Department of Molecular and Applied Microbiology) for MALDI-TOF/TOF measurements.

REFERENCES

(1) Andrews, S. C.; Robinson A. K.; Rodríguez-Quiñones, F. *FEMS Microbiol Rev.* **2003**, *27*, 215-237.

(2) Shaked, Y.; Erel, Y.; Sukenik, A. *Environ. Sci. Technol.* **2002**, *36*, 460-467.

(3) Sandy, M.; Butler, A. *Chem. Rev.* **2009**, *109*, 4580-4595.

(4) Hider, R. C.; Kong, X. *Nat. Prod. Rep.* **2010**, *27*, 637-657.

(5) Hopkinson, B. M.; Morel, F. M. M. *Biometals* **2009**, *22*, 659-669.

(6) Matthijs, S.; Tehrani, K. A.; Laus, G.; Jackson, R. W.; Cooper, R. M.; Cornelis, P. *Environ. Microbiol.* **2007**, *9*, 425-434

(7) D'Onofrio, A.; Crawford, J. M.; Stewart, E. J.; Witt, K.; Gavrish, E.; Epstein, S.; Clardy, J.; Lewis, K. *Chem. Biol.* **2010**, *17*, 254-264.

(8) Traxler, M. F.; Seyedsayamdost, M. R.; Clardy, J.; Kolter, R. *Mol. Microbiol.* **2012**, *86*, 628-644.

(9) Böttcher, T.; Clardy, J. *Angew. Chem. Int. Ed.* **2014**, *53*, 3510-3513.

(10) Barbeau, K.; Rue, E. L.; Bruland, K. W.; Butler, A. *Nature* **2001**, *413*, 409-413.

(11) Butler, A.; Theisen, R. M. *Coord. Chem. Rev.* **2010**, *254*, 288-296.

(12) Küpper, F. C.; Carrano, C. J., Kuhn, J.-U.; Butler, A. *Inorg. Chem.* **2006**, *45*, 6028-6033.

(13) Yarimizu, K.; Polido, G.; Gärdes, A.; Carter, M. L.; Hilbern, M.; Carrano, C. J. *Metallomics* **2014**, *6*, 1156-1163.

(14) Amin, S. A.; Green, D. H.; Hart, M. C.; Küpper, F. C.; Sunda, W. G.; Carrano, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 17071-17076.

(15) Amin, S. A.; Parker, M. S.; Armbrust, E. V. *Microbiol. Mol. Biol. Rev.* **2012**, *76*, 667-684.

(16) Gauglitz, J. M.; Zhou, H.; Butler, A. *J. Inorg. Biochem.* **2012**, *107*, 90-95.

(17) Martinez, J. S.; Zhang, G. P.; Holt, P. D.; Jung, H.-T.; Carrano, C. J.; Haygood, M. G.; Butler, A. *Science* **2000**, *287*, 1245-1247.

(18) Ito, Y.; Butler, A. *Limnol. Oceanogr.* **2005**, *50*, 1918-1923.

(19) Homann, V. V.; Sandy, M.; Tincu, J. A.; Templeton, A. S.; Tebo, B. M.; Butler, A. *J. Nat. Prod.* **2009**, *72*, 884-888.

(20) Kreutzer, M. F.; Kage, H.; Nett, M. *J. Am. Chem. Soc.* **2012**, *134*, 5415-5422.

(21) Kreutzer, M. F.; Nett, M. *Org. Biomol. Chem.* **2012**, *10*, 9338-9343.

(22) Rosconi, F.; Davyt, D.; Martínez, V.; Martínez, M.; Abin-Carriquiry, J. A.; Zane, H.; Butler, A.; de Souza E. M.; Fabiano, E. *Environ. Microbiol.* **2013**, *15*, 916-927.

(23) Zerikly, M.; Challis, G. L. *ChemBioChem* **2009**, *10*, 625-633.

(24) Gross, H. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 267-277.

(25) Nett, M. In *Progress in Chemistry of Organic Natural Products*; Kinghorn, D. A.; Falk, H.; Kobayashi, J., Eds.; Springer: Switzerland, 2014; Vol. 99, pp 199-246.

(26) Kleigrewe, K.; Almaliti, J.; Tian, I. Y.; Kinnel, R. B.; Korobeynikov, A.; Monroe, E. A.; Duggan, B. M.; Di Marzo, V.; Sherman, D. H.; Dorrestein, P. C.; Gerwick, L.; Gerwick, W. H. *J. Nat. Prod.* **2015**, *78*, 1671-1682.

(27) Schwyn, B.; Neilands, J. B. *Anal. Biochem.* **1987**, *160*, 47-56.

(28) Kem, M. P.; Naka, H.; Iinishi, A.; Haygood, M. G.; Butler, A. *Biochemistry* **2015**, *54*, 744-752.

(29) Kem, M. P.; Butler, A. *Biometals* **2015**, *28*, 445-459.

(30) Singh, G. M.; Fortin, P. D.; Koglin, A.; Walsh, C. T. *Biochemistry* **2008**, *47*, 11310-11320.

(31) Zane, H. K.; Naka, H.; Rosconi, F.; Sandy, M.; Haygood, M. G.; Butler, A. *J. Am. Chem. Soc.* **2014**, *136*, 5615-5618.

(32) Weber, T.; Blin, K.; Duddela, S.; Krug, D.; Kim, H. U.; Bruccoleri, R.; Lee, S. Y.; Fischbach, M. A.; Müller, R.; Wohlleben, W.; Breitling, R.; Takano, E.; Medema, M. H. *Nucleic Acids Res.* **2015**, 43, W237-W243.

(33) Bachmann B. O.; Ravel, J. *Method. Enzymol.* **2009**, *458*, 181-217.

(34) Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. *Chem. Biol.* **1999**, *6*, 493-505.

(35) Challis, G. L.; Ravel, J.; Townsend, C. A. *Chem. Biol.* **2000**, *7*, 211-224.

(36) Milagres, A. M.; Machuca, A.; Napoleao, D. *J. Microbiol. Methods* **1999**, *37*, 1-6.

(37) Papayannopoulos, I. A. *Mass Spectrom. Rev.* **1995**, *14*, 49-73.

(38) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591-596.

(39) Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 3346-3352.

(40) Fuchs, S. W.; Sachs, C. C.; Kegler, C.; Nollmann, F. I.; Karas, M.; Bode, H. B. *Anal. Chem.* **2012**, *84*, 6948-6955.

(41) Bosello, M.; Zeyadi, M.; Kraas, F. I.; Linne, U.; Xie, X.; Marahiel, M. A. *J. Nat. Prod.* **2013**, *76*, 2282-2290.

(42) Johnston, C. W.; Wyatt, M. A.; Li, X.; Ibrahim, A.; Shuster, J.; Southam, G.; Magarvey, N. A. *Nat. Chem. Biol.* **2013**, *9*, 241-243.

(43) Schieferdecker, S.; König, S.; Weigel, C.; Dahse, H.-M.; Werz, O.; Nett, M. *Chem. Eur. J.* **2014**, *20*, 15933-15940.

(44) Felnagle, E. A.; Barkei, J. J.; Park, H.; Podevels, A. M.; McMahon, M. D.; Drott, D. W.; Thomas, M. G. *Biochemistry* **2010**, *49*, 8815-8817.

(45) Kotowska M.; Pawlik, K. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 7735-7746.

(46) Lambalot, R. H.; Gehring, A. M.; Flugel, R. S.; Zuber P.; LaCelle, M.; Marahiel, M. A.; Reid, R.; Khosla, C.; Walsh. C. T. *Chem. Biol.* **1996**, *3*, 923-936.

(47) Challis, G. L. *ChemBioChem* **2005**, *6*, 601-611.

(48) Matzanke, B. F.; Anemüller, S.; Schünemann, V.; Trautwein, A. X.; Hantke, K. *Biochemistry* **2004**, *43*, 1386-1392.

(49) Braun, V.; Killmann, H. *Trends Biochem. Sci.* **1999**, *24*, 104-109.

(50) Barbeau, K.; Rue, E. L.; Trick, C. G.; Bruland, K. W.; Butler, A. *Limnol. Oceanogr.* **2003**, *48*, 1069-1078.

(51) Miwa, H.; Ahmed, I.; Yoon, J.; Yokota, A.; Fujiwara, T. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 286-289.

(52) Liu, Z. H.; Cao, Y. M.; Zhou, Q. W.; Guo, K.; Ge, F.; Hou, J. Y.; Hu S. Y.; Yuan, S.; Dai, Y. J. *Biodegradation* **2013**, *24*, 855-864.

(53) Matthijs, S.; Budzikiewicz, H.; Schäfer, M.; Wathelet, B.; Cornelis, P. *Z. Naturforsch. C* **2008**, *63*, 8-12.

(54) Stephan, H.; Freund, S.; Beck, W.; Jung, G.; Meyer, J. M.; Winkelmann, G. *BioMetals* **1993**, *6*, 93-100.

(55) Smith, M. J.; Shoolery, J. N.; Schwyn, B.; Holden, I.; Neilands, J. B. *J. Am. Chem. Soc.* **1985**, *107*, 1739-1743.

(56) Satola, B.; Wübbeler, J. H.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 541-560.

(57) Johnston, C. W.; Skinnider, M. A.; Wyatt, M. A.; Li, X.; Ranieri, M. R. M.; Yang, L.; Zechel, D. L.; Ma, B.; Magarvey, N. A. *Nat. Commun.* **2015**, *6*, 8421.

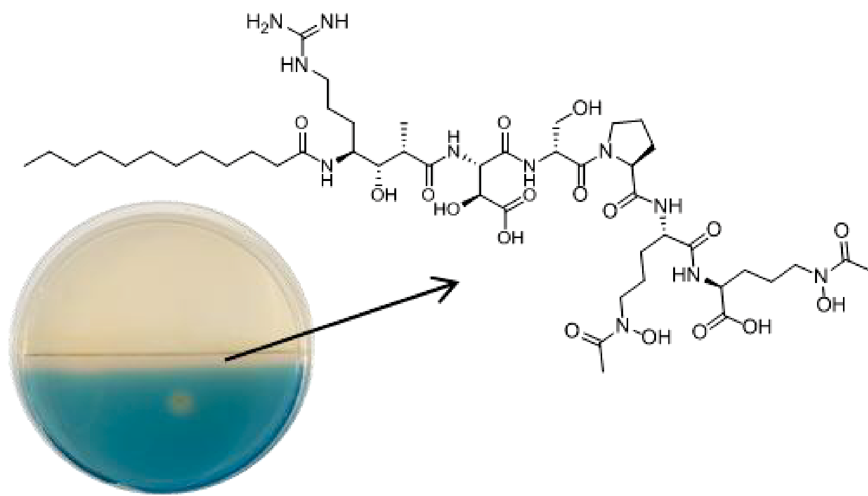
(58) Pfennig, N. *Arch. Microbiol.* **1974**, *100*, 197-206.

(59) Eid, J.; Fehr, A.; Gray, J.; Luong, K.; Lyle, J.; Otto, G.; Peluso, P.; Rank, D.; Baybayan, P.; Bettman, B.; Bibillo, A.; Bjornson, K.; Chauduri, B.; Christians, F.; Cicero, R.; Clark, S.; Dalal, R.; deWinter, A.; Dixon, J.; Foquet, M.; Gaertner, A.; Hardenbol, P.; Heiner, C.; Hester, K.; Holden, D.; Kearns, G.; Kong, X.; Kuse, R.; Lacroix, Y.; Lin, S.; Lundquist, P.; Ma, C.; Marks, P.; Maxham, M.; Murphy, D.; Park,

I.; Pham, T.; Phillips, M.; Roy, J.; Sebra, R.; Shen, G.; Sorenson, J.; Tomaney, A.; Travers, K.; Trulson, M.; Vieceli, J.; Wegener, J.; Wu, D.; Yang, A.; Zaccarin, D.; Zhao, P.; Zhong, F.; Korlach, J.; Turner, S. *Science* **2009**, *323*, 133-138.

(60) Chin, C. S.; Alexander, D. H.; Marks, P.; Klammer, A. A.; Drake, J.; Heiner, C.;Clum, A.; Copeland, A.; Huddleston, J.; Eichler, E. E.; Turner, S. W.; Korlach, J. *Nat. Methods* **2013**, *10*, 563-569.

TOC graphic



Supporting Information
Variochelins, lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining

*Colette Kurth, Sebastian Schieferdecker, Kalliopi Athanasopoulou, Ivana Seccareccia, and Markus Nett**

Table of Contents

SI2	Figure S1	Response of *Variovorax* strains in the CAS assay
SI3	Figure S2	HPLC separation of variochelin A (**1**) and B (**2**)
SI4	Figure S3	MALDI-TOF/TOF spectrum of variochelin A (**1**)
SI5	Figure S4	Marfey's analysis of the proline residue in **1**.
SI5	Figure S5	Marfey's analysis of the serine residue in **1**.
SI6	Figure S6	Marfey's analysis of the β-hydroxyaspartic acid residue in **1**.
SI6	Figure S7	Configurational analysis of the ornithine residues in **1**.
SI7	Figure S8	^{1}H NMR spectrum of **1** in DMSO-d_6
SI8	Figure S9	^{1}H-decoupled ^{13}C NMR spectrum of **1** in DMSO-d_6
SI9	Figure S10	^{1}H, ^{1}H COSY spectrum of **1** in DMSO-d_6
SI10	Figure S11	^{1}H, ^{13}C HSQC spectrum of **1** in DMSO-d_6
SI11	Figure S12	^{1}H, ^{13}C HMBC spectrum of **1** in DMSO-d_6
SI12	Figure S13	HR-MS spectra of variochelin A, Fe^{3+}-variochelin A and Ga^{3+}-variochelin A
SI13	Table S1	Soil and freshwater bacteria that are assumed to produce photoreactive acyl peptide siderophore
SI14	Table S2	Annotation of siderophore gene clusters from *Variovorax paradoxus* B4 and *V. paradoxus* S110
SI15	Table S3	Annotation of the siderophore gene cluster from *Variovorax paradoxus* EPS
SI16	Table S4	Annotation of the variochelin gene cluster from *Variovorax boronicumulans* BAM-48

Figure S1. Response of *Varivorax paradoxus* B4 (a), *V. paradoxus* S110 (b), *V. paradoxus* 351, B13-0-1 D (c), *V. boronicumulans* BAM-48 (d), and *V. soli* GH9-3 (e) in the CAS assay. Plate (f) shows a negative control.

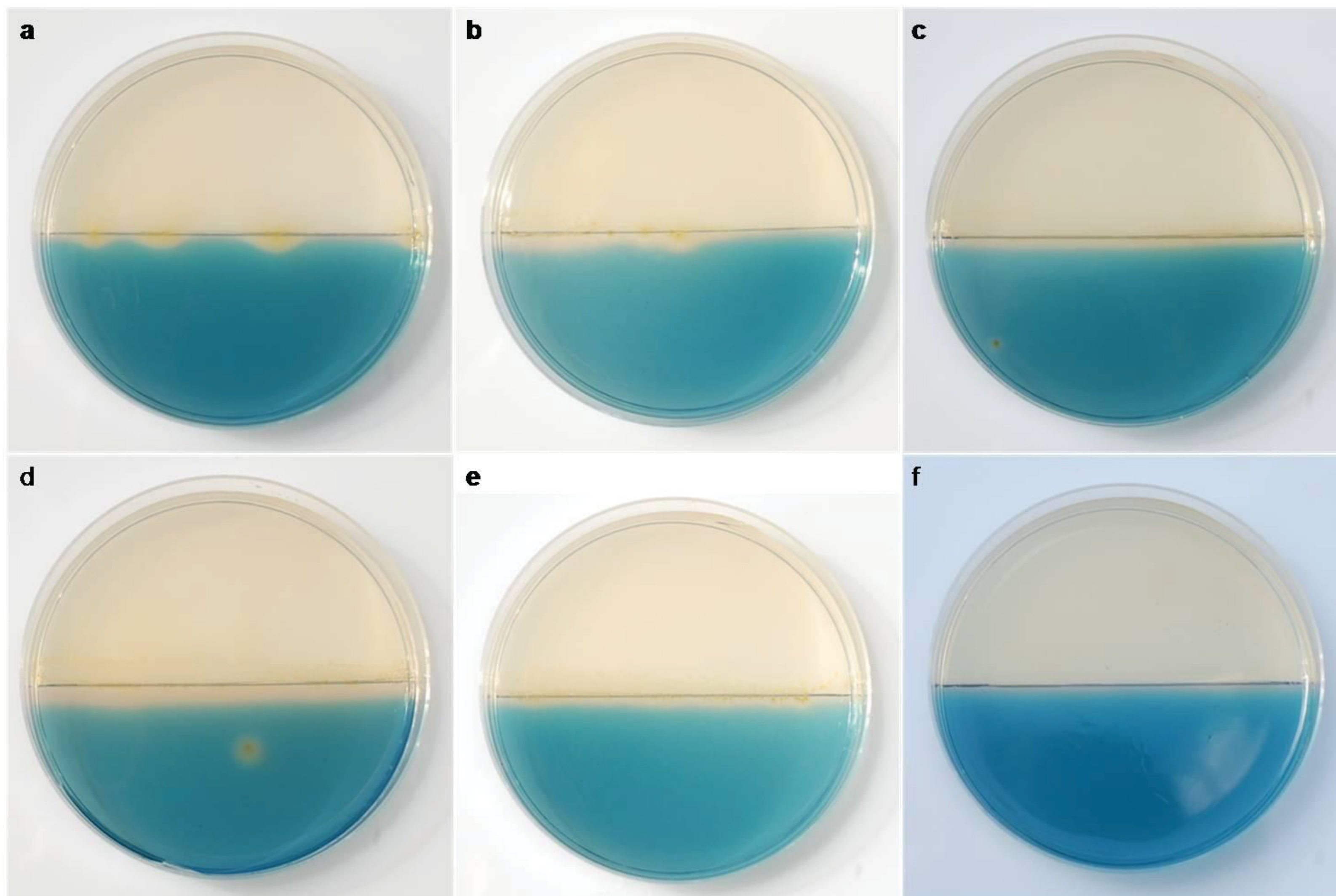


Figure S2. HPLC separation of variochelin A (**1**) and B (**2**) monitored at 190 nm (A). Both compounds were positive in the liquid CAS assay (B).

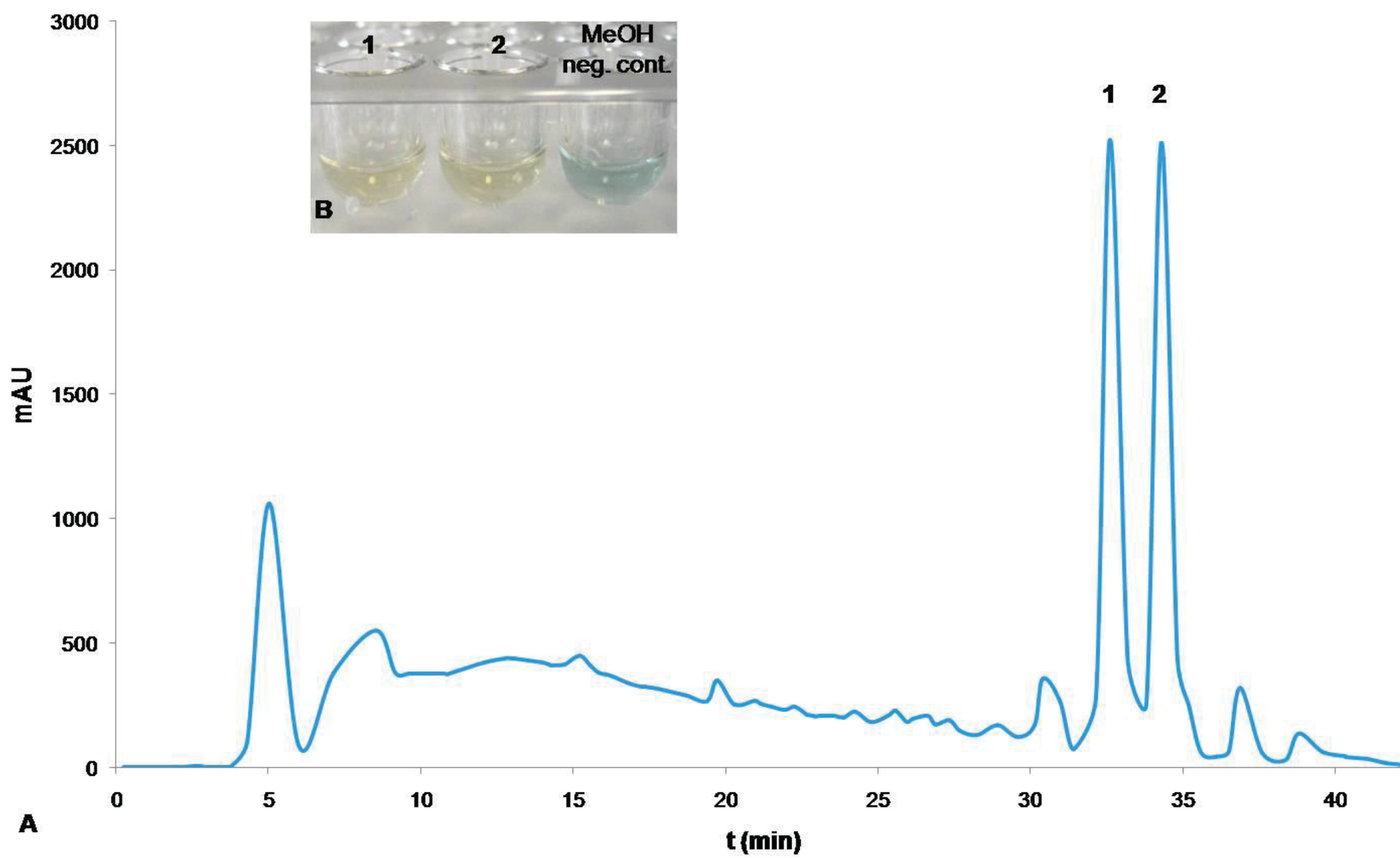


Figure S3. MALDI-TOF/TOF spectrum of variochelin A (**1**).

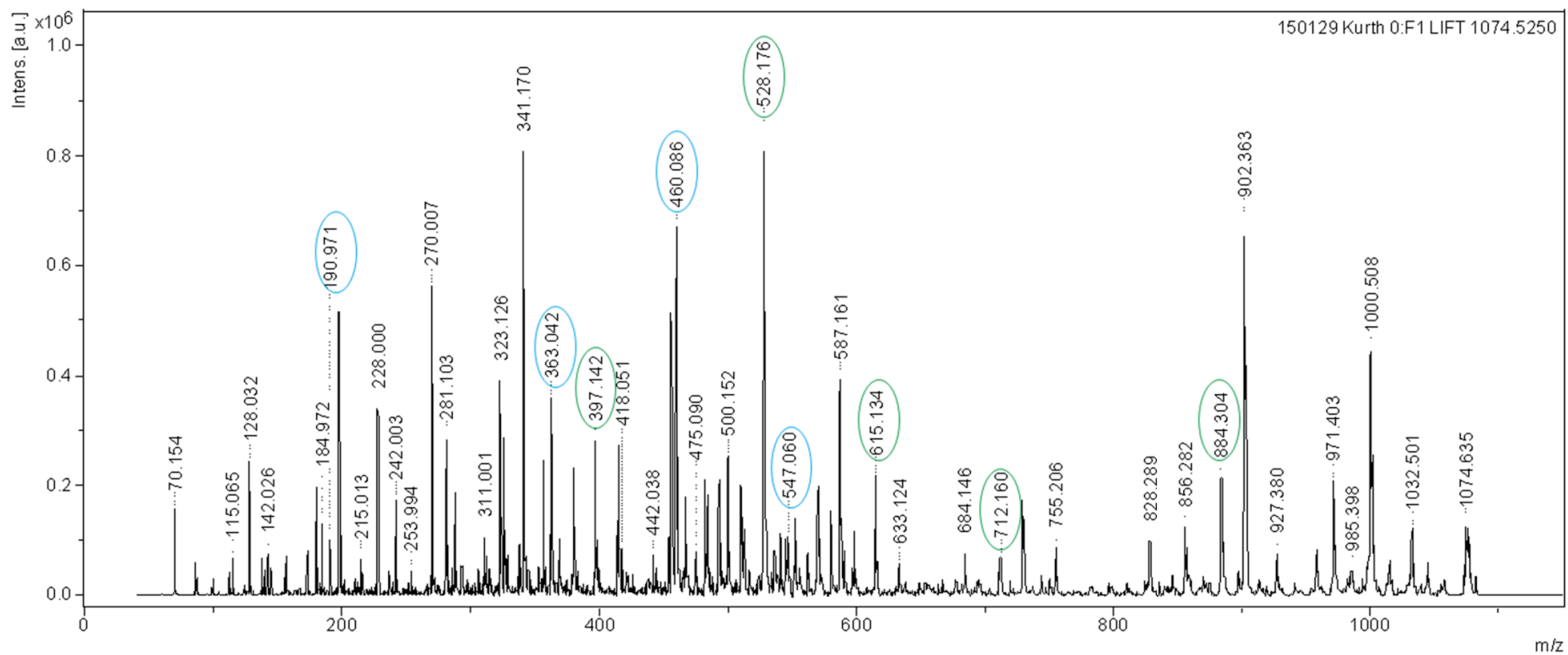


Figure S4. Marfey's analysis of the proline residue in **1**. Extracted ion chromatograms of Marfey products after HI cleavage of **1** (A), from commercial L-proline (B) and D-proline (C).

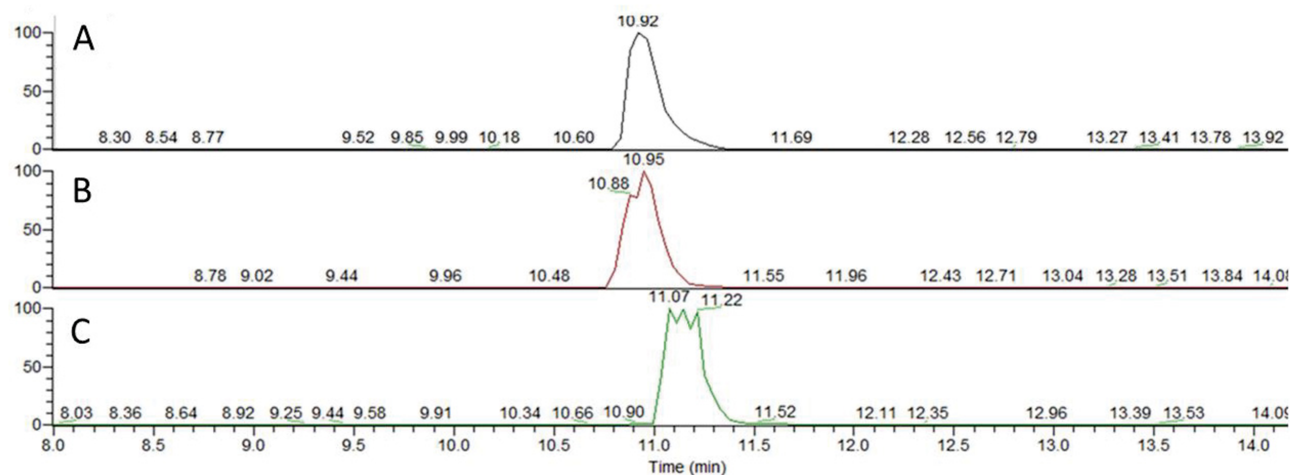


Figure S5. Marfey's analysis of the serine residue in **1**. Extracted ion chromatograms of Marfey products after HI cleavage of **1** (A), from commercial L-serine (B) and D-serine (C).

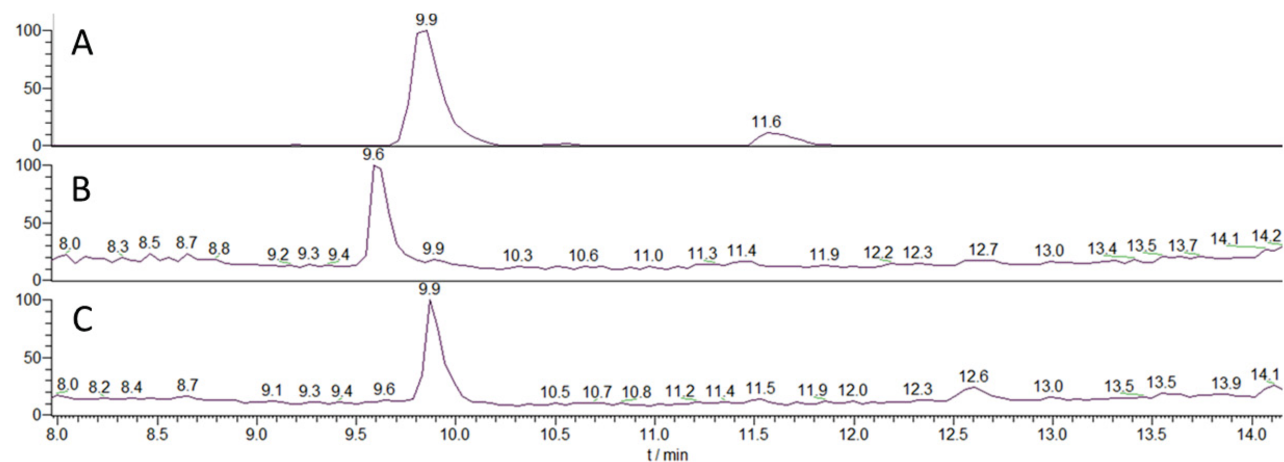


Figure S6. Marfey's analysis of the β-hydroxyaspartic acid residue in **1**. UV profiles of Marfey products after HI cleavage of **1** (A) and from commercial D/L-*threo*-β-hydroxyaspartic acid (B).

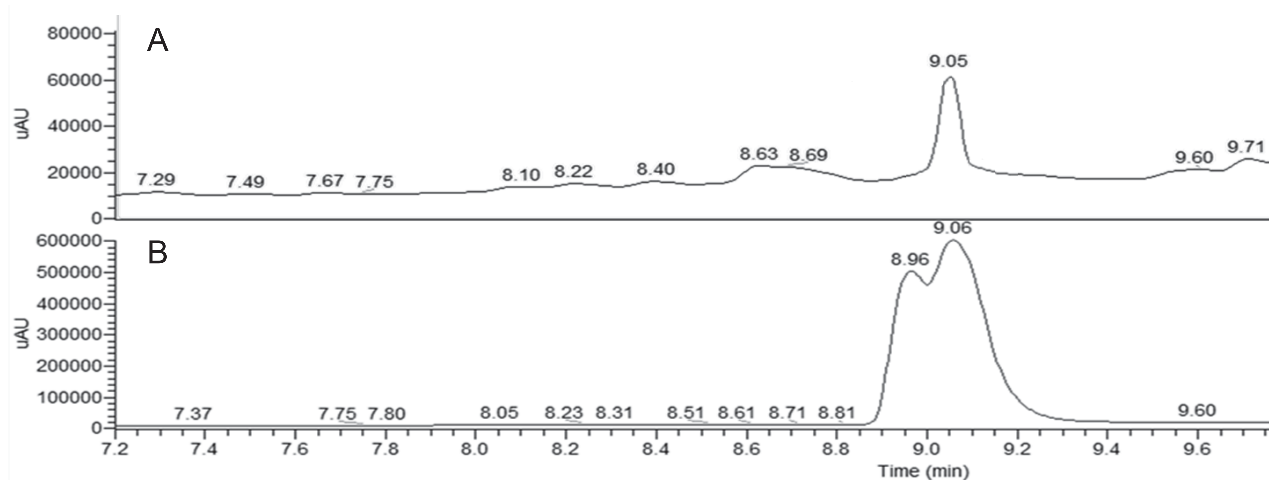


Figure S7. Configurational analysis of the ornithine residues in **1**. Extracted ion chromatograms of bis-carbamate products after HI cleavage of **1** (A), from commercial D/L-ornithine (B), and from L-ornithine (C).

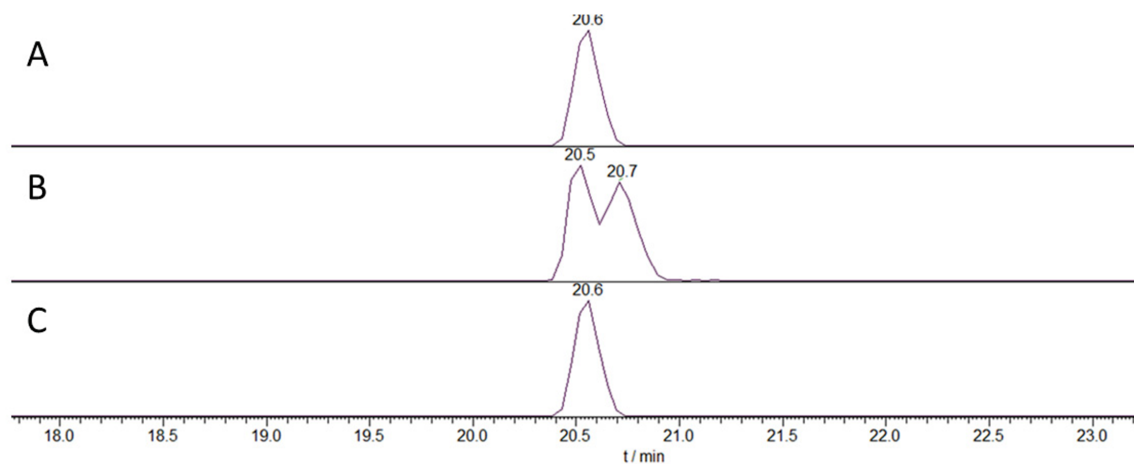


Figure S8. ^{1}H NMR (600 MHz) spectrum of **1** in DMSO-d_6

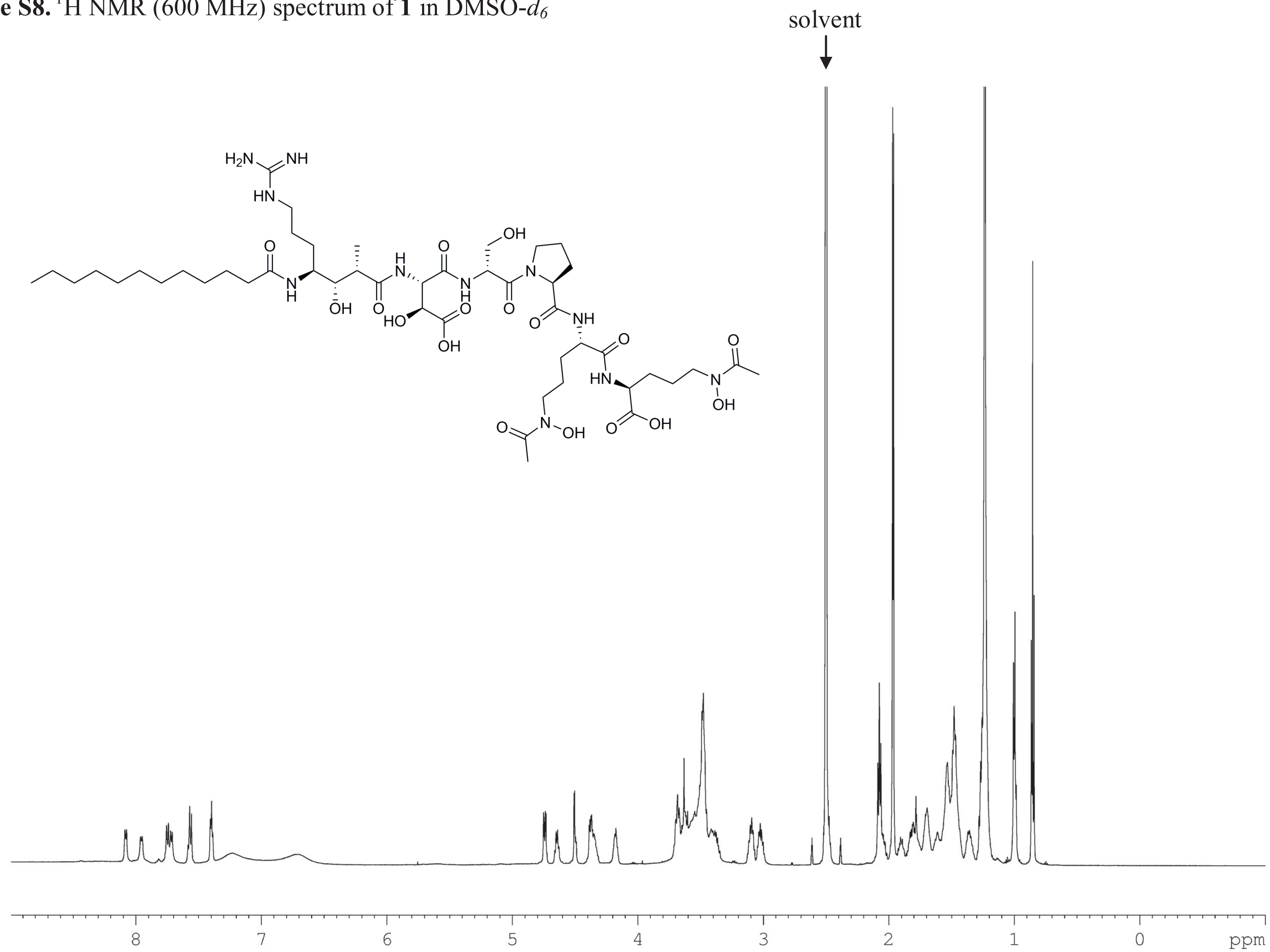


Figure S9. ^{1}H-decoupled ^{13}C NMR (125 MHz) spectrum of **1** in DMSO-d_6

Figure S10. ^{1}H, ^{1}H COSY spectrum (500 MHz) of **1** in DMSO-d_6

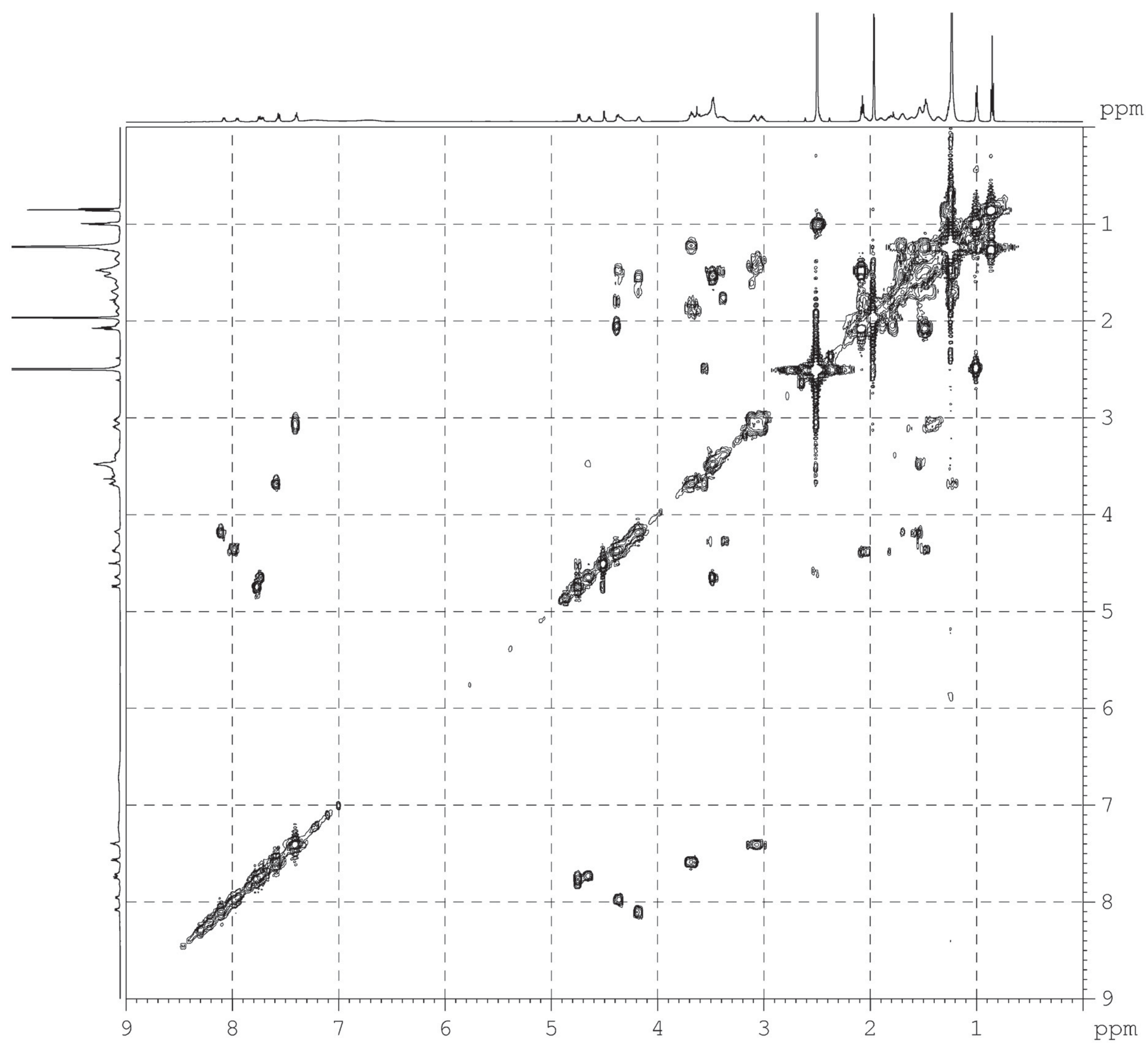


Figure S11. ^{1}H, ^{13}C HSQC (500 MHz) spectrum of **1** in DMSO-d_6

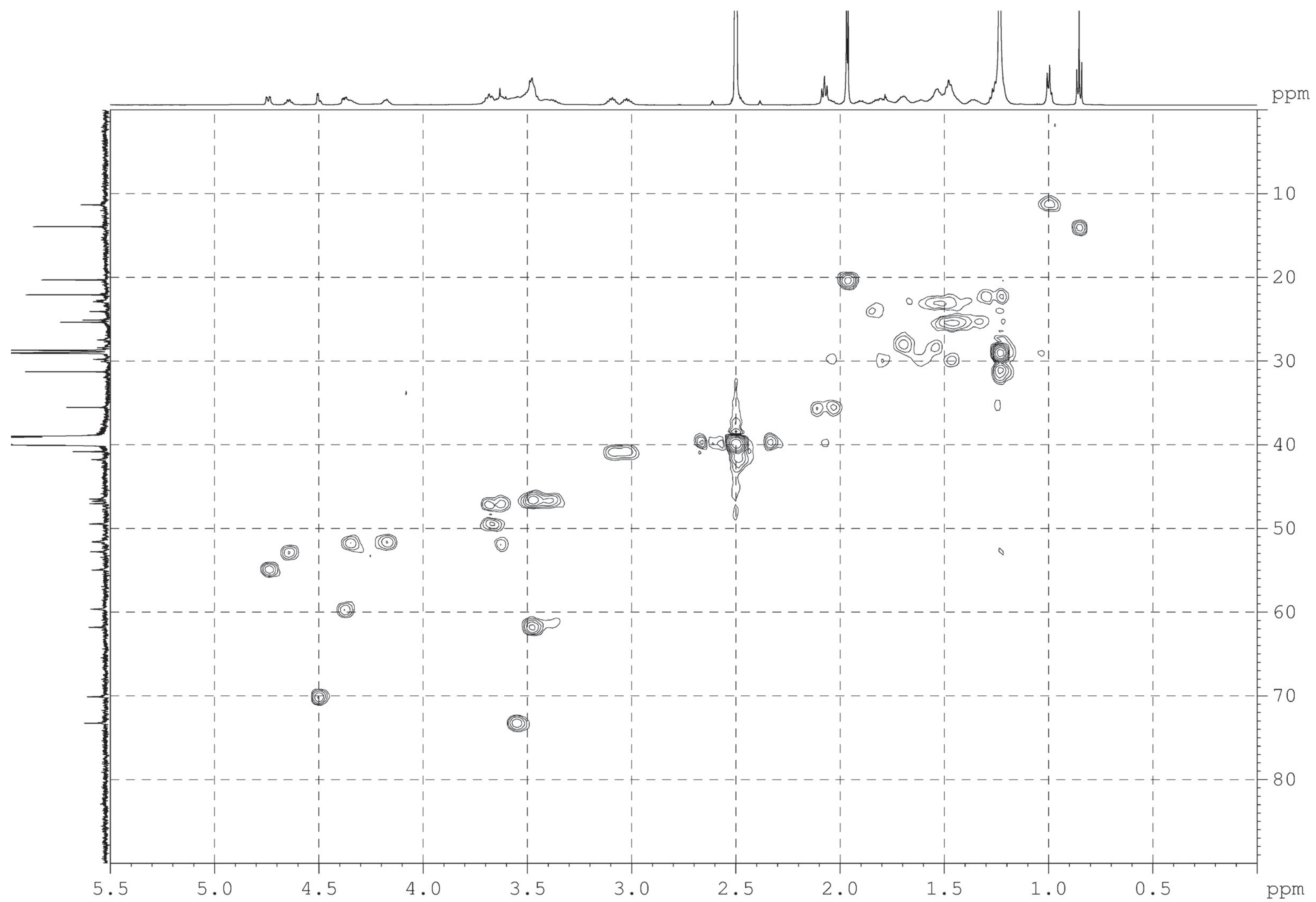


Figure S12. ^{1}H, ^{13}C HMBC (600 MHz) spectrum of **1** in DMSO-d_6

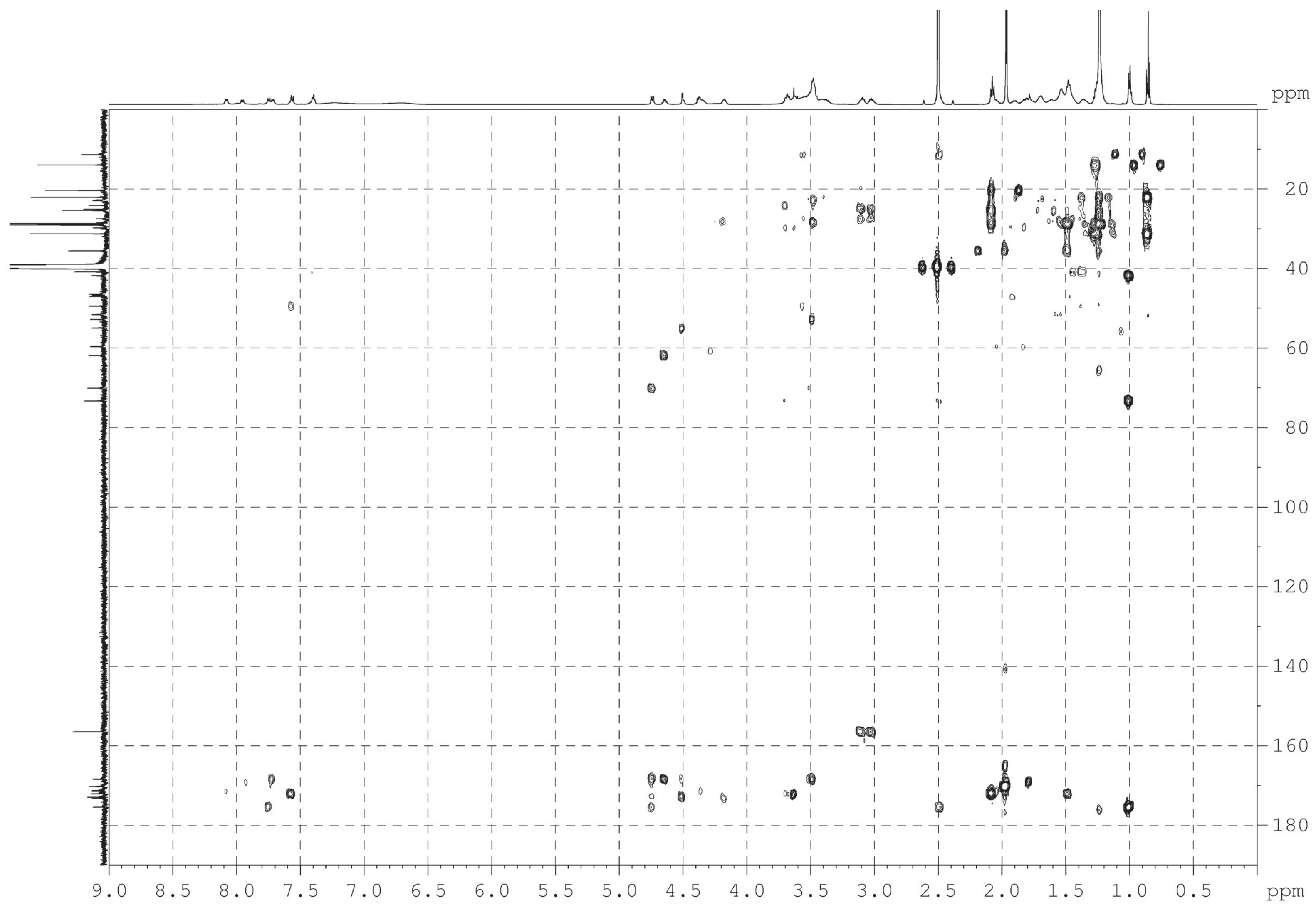


Figure S13. HR-ESI-MS spectra of variochelin A as a free ligand (top), and in complex with Fe^{3+} (middle) and Ga^{3+} (bottom).

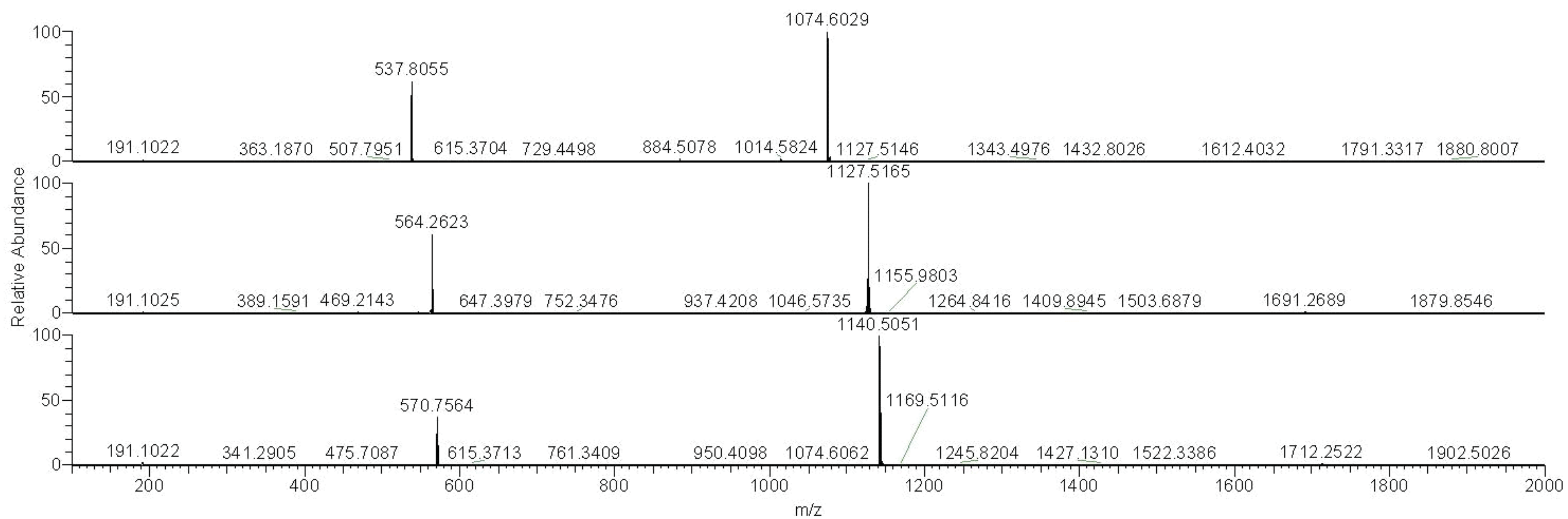


Table S1. Soil and freshwater bacteria that are assumed to produce photoreactive acyl peptide siderophore.

	CucG A domain homolog (Identity [%])	CucF TauD domain homolog (Identity [%])	CucF starter C domain homolog (Identity [%])	TaiD FAAL domain homolog (Identity [%])	Predicted siderophore
Achromobacter spanius CGMCC9173	WP_050444824 (51)	WP_050444825 (71)	-	WP_050444825 (71)	new
Burkholderia sordidicola S170	WP_051887899 (42)	WP_051887896 (68)	-	WP_051887899 (56)	new
Cupriavidus basilensis OR16	EHP40329 (38)	EHP40328 (73)	-	EHP40327 (76)	taiwachelin
Cupriavidus gilardii CR3	WP_053823544 (44)	ALD92493 (45)	-	WP_053823547 (52)	new
Cupriavidus necator H16	WP_011617407 (100)	WP_011617408 (100)	WP_011617408 (100)	-	cupriachelin
Cupriavidus sp. amp6	WP_051320452 (44)	WP_051320452 (94)	WP_051320452 (83)	-	cupriachelin
Cupriavidus sp. SK-4	EYS85590 (97)	EYS85589 (99)	EYS85589 (97)	-	cupriachelin
Cupriavidus sp. WS	WP_020206421 (46)	WP_020206420 (75)	-	WP_020206420 (72)	taiwachelin
Cupriavidus taiwanensis LMG 19424	WP_012356046 (47)	WP_012356045 (73)	-	WP_012356045 (100)	taiwachelin
Herbaspirillum seropedicae Z67	AKN68207 (43)	AKN68207 (71)	-	AKN68207 (36)	serobactin
Janthinobacterium agaricidamnosum NBRC 102515	CDG82376 (43)	CDG82376 (72)	-	CDG82375 (57)	new
Ralstonia pickettii DTP0602	AGW94292 (93)	AGW94293 (98)	AGW94293 (93)	-	cupriachelin
Ralstonia sp. GA3-3	EON20600 (99)	EON20601 (100)	EON20601 (99)	-	cupriachelin
Variovorax paradoxus B4	WP_021008405 (40)	WP_021008410 (42)	-	WP_021008409 (55)	new
Variovorax paradoxus EPS	WP_013542707 (37)	ADU35203 (55)	-	WP_013542707 (55)	new
Variovorax paradoxus S110	WP_015866520 (41)	WP_015866525 (42)	-	WP_015866524 (56)	new

Table S2. Annotation of siderophore gene clusters from *Variovorax paradoxus* B4 and *V. paradoxus* S110.

Gene	Protein accession no. (GenBank)	Size (aa)	Proposed function (domain architecture)	Predicted substrate specificity[1]
VAPA_1c38580 / Vapar_3733	WP_021008396 / WP_015866511	206 / 207	DNA-directed RNA polymerase sigma-70 factor	
VAPA_1c38590 /Vapar_3734	WP_021008397 / WP_015866512	72 / 72	anti-FecI sigma factor FecR	
VAPA_1c38600 / Vapar_3735	WP_021008398 / WP_015866513	78 / 78	hypothetical protein	
VAPA_1c38610 / Vapar_3736	WP_021008399 / WP_015866514	563 / 563	peptide transporter	
VAPA_1c38620 / Vapar_3737	WP_021008400 / WP_015866515	281 / 281	ferric iron reductase	
VAPA_1c38630 / Vapar_3738	WP_021008401 / WP_015866516	281 / 281	formyl transferase	
VAPA_1c38640 / Vapar_3739	WP_021008402 / WP_015866517	344 / 344	acetyltransferase	
VAPA_1c38650 / Vapar_3740	WP_021008403 / WP_015866518	450 / 439	monooxygenase	
VAPA_1c38660 / Vapar_3741	WP_021008404 / WP_015866519	722 / 723	TonB-dependent receptor	
VAPA_1c38670 / Vapar_3742	WP_021008405 / WP_015866520	1357 / 1358	non-ribosomal peptide synthetase (C-A-PCP-TE)	aspartic acid
VAPA_1c38680 / Vapar_3743	WP_021008406 / WP_015866521	2625 / 2626	non-ribosomal peptide synthetase (C-A-PCP-E-C-A-PCP)	N^{δ}-hydroxyornithine + threonine
VAPA_1c38690 / Vapar_3744	WP_021008407 / WP_015866522	1113 / 1110	non-ribosomal peptide synthetase (C-A-PCP)	serine
VAPA_1c38700 / Vapar_3745	WP_021008408 / WP_015866523	1520 / 1520	polyketide synthase (KS-AT-KR-ACP)	malonyl-CoA
VAPA_1c38710 / Vapar_3746	WP_021008409 / WP_015866524	1771 / 1776	non-ribosomal peptide synthetase (FAAL-ACP-C-A-PCP)	fatty acid + threonine
VAPA_1c38720 / Vapar_3747	WP_021008410 / WP_015866525	330 / 330	TauD-like hydroxylase	
VAPA_1c38730 / Vapar_3748	WP_021008411 / WP_015866526	229 / 229	4'-phosphopantetheinyl transferase	
VAPA_1c38740 / Vapar_3749	WP_021008412 / WP_015866527	245 / 246	type II thioesterase	
VAPA_1c38750 / Vapar_3750	WP_021008413 / WP_015866528	85 / 85	MbtH domain-containing protein	
VAPA_1c38760 / Vapar_3751	WP_021008414 / WP_015866529	67 / 67	anti-FecI sigma factor FecR	
VAPA_1c38770 / Vapar_3752	WP_021008415 / WP_015866530	181 / 181	DNA-directed RNA polymerase sigma-70 factor	

[1]according to references [32-35] in the main manuscript

Table S3. Annotation of the siderophore gene cluster from *Variovorax paradoxus* EPS.

Gene	Protein accession no. (GenBank)	Size (aa)	Proposed function (domain architecture)	Predicted substrate specificity[1]
Varpa_4319	WP_013542699	433	L-ornithine 5-monooxygenase	
Varpa_4320	WP_013542700	559	peptide transporter	
Varpa_4321	WP_013542701	288	ferric iron reductase	
Varpa_4322	WP_013542702	280	hypothetical protein	
Varpa_4323	WP_013542703	721	TonB-dependent siderophore receptor	
Varpa_4324	WP_013542704	4633	non-ribosomal peptide synthetase (C-A-PCP-C-A-PCP-C-A-PCP-C-A-PCP-TE)	aspartic acid + threonine + threonine +serine
Varpa_4325	WP_013542705	4313	non-ribosomal peptide synthetase (C-A-PCP-C-A-PCP-C-A-PCP-C-A-PCP)	threonine + threonine + threonine + glycine
Varpa_4326	WP_013542706	1542	polyketide synthase (KS-AT-KR-ACP)	malonyl-CoA
Varpa_4327	WP_013542707	1766	non-ribosomal peptide synthetase (FAAL-ACP-C-A-PCP)	fatty acid + threonine
Varpa_4328	WP_013542708	249	type II thioesterase	
Varpa_4329	WP_013542709	84	MbtH domain-containing protein	
Varpa_4330	WP_013542710	82	anti-FecI sigma factor FecR	
Varpa_4331	WP_013542711	179	DNA-directed RNA polymerase sigma-70 factor	
Varpa_4332	WP_013542712	321	4'-phosphopantetheinyl transferase	

[1]according to references [32-35] in the main manuscript

Tables S4. Annotation of the variochelin gene cluster from *Variovorax boronicumulans* BAM-48.

Gene	Size of protein (aa)	Proposed function (domain architecture)	Predicted substrate specificity[1]
varR	560	peptide transporter	
varQ	78	anti-FecI sigma factor FecR	
varP	262	ferric iron reductase	
varO	369	acetyl transferase	
varN	440	L-ornithine 5-monooxygenase	
varM	193	RNA polymerase subunit sigma-24	
varL	343	anti-FecI sigma factor FecR	
varK	816	TonB-dependent receptor	
varJ	2459	nonribosomal peptide synthetase (C-A-PCP-C-A-PCP-TE)	N^{δ}-hydroxyornithine + N^{δ}-hydroxyornithine
varI	2586	nonribosomal peptide synthetase (C-A-PCP-E-C-A-PCP)	serine + proline
varH	1035	nonribosomal peptide synthetase (C-A-PCP)	aspartic acid
varG	2351	polyketide synthase (KS-AT-KR-ACP-C-TauD)	malonyl-CoA
varF	1756	nonribosomal peptide synthetase (FAAL-ACP-C-A-PCP)	fatty acid + arginine
varE	234	4'-phosphopantetheinyl transferase	
varD	249	type II thioesterase	
varC	81	MbtH domain-containing protein	
varB	82	anti-FecI sigma factor FecR	
varA	178	DNA-directed RNA polymerase sigma-70 factor	

[1]according to references [32-35] in the main manuscript

10 List of abbreviations

CFU	Colony form unit
dNTP	Deoxynucleotide
EPS	Exopolysaccharides
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
Kb	Kilobase
LB	Luria Broth
MALDI	Matrix-assisted laser desorption/ionisation
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MST	Microscale thermophoresis
MTs	Metallothioneins
NB	Nutrient Broth
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoates
PHB	Poly3-hydroxybutyrate
PKS	Polyketide synthase
PGA	Poly-γ-glutamic acid
rpm	Rounds per minute
SDS	Sodium dodecylsulfate
SNP	Single nucleotide polymorphism
TOF	Time of flight
^{13}C	Isotope of carbon with a nucleus containing 6 protons and 7 neutrons

11 References

1. Faust, K. and J. Raes, *Microbial interactions: from networks to models.* Nat Rev Micro, 2012. **10**(8): p. 538-550.
2. Abrams, P.A., *The Evolution of Predator-Prey Interactions: Theory and Evidence.* Annual Review of Ecology and Systematics, 2000. **31**(1): p. 79-105.
3. Jousset, A., *Ecological and evolutive implications of bacterial defences against predators.* Environ Microbiol, 2012. **14**(8): p. 1830-43.
4. Matz, C. and K. Jurgens, *High motility reduces grazing mortality of planktonic bacteria.* Appl Environ Microbiol, 2005. **71**(2): p. 921-9.
5. Creed, R.P., *Direct and Indirect Effects of Crayfish Grazing in a Stream Community.* Ecology, 1994. **75**(7): p. 2091-2103.
6. Zhang, R., M.G. Weinbauer, and P.Y. Qian, *Viruses and flagellates sustain apparent richness and reduce biomass accumulation of bacterioplankton in coastal marine waters.* Environ Microbiol, 2007. **9**(12): p. 3008-18.
7. Rothhaupt, K.O., *Stimulation of Phosphorus-Limited Phytoplankton by Bacterivorous Flagellates in Laboratory Experiments.* Limnology and Oceanography, 1992. **37**(4): p. 750-759.
8. Caron, D.A., J.C. Goldman, and M.R. Dennett, *Experimental Demonstration of the Roles of Bacteria and Bacterivorous Protozoa in Plankton Nutrient Cycles.* Hydrobiologia, 1988. **159**(1): p. 27-40.
9. Rose, J.M., N.M. Vora, and D.A. Caron, *Effect of temperature and prey type on nutrient regeneration by an antarctic bacterivorous protist.* Microb Ecol, 2008. **56**(1): p. 101-11.
10. Pernthaler, J., *Predation on prokaryotes in the water column and its ecological implications.* Nat Rev Microbiol, 2005. **3**(7): p. 537-46.
11. Sallal, A.K., *Lysis of cyanobacteria with Flexibacter spp isolated from domestic sewage.* Microbios, 1994. **77**(310): p. 57-67.
12. Daft, M.J., J.C. Burnham, and Y. Yamamoto, *Lysis of Phormidium-Luridum by Myxococcus-Fulvus in Continuous-Flow Cultures.* Journal of Applied Bacteriology, 1985. **59**(1): p. 73-80.
13. Daft, M.J. and W.D.P. Stewart, *Bacterial Pathogens of Freshwater Blue-Green Algae.* New Phytologist, 1971. **70**(5): p. 819-&.
14. Sigee, D.C., *et al.*, *Biological control of cyanobacteria: principles and possibilities.* Hydrobiologia, 1999. **395**: p. 161-172.
15. Kumbhar, C., *et al.*, *Widespread predatory abilities in the genus Streptomyces.* Arch Microbiol, 2014. **196**(4): p. 235-48.
16. Germida, J.J. and L.E. Casida, *Ensifer-Adhaerens Predatory Activity against Other Bacteria in Soil, as Monitored by Indirect Phage Analysis.* Applied and Environmental Microbiology, 1983. **45**(4): p. 1380-1388.
17. Lambina, V.A., *et al.*, *Micavibrio-Admirandus Gen Et Sp-Nov.* Microbiology, 1982. **51**(1): p. 103-106.
18. Makkar, N.S. and L.E. Casida, *Cupriavidus-Necator Gen-Nov, Sp-Nov - a Nonobligate Bacterial Predator of Bacteria in Soil.* International Journal of Systematic Bacteriology, 1987. **37**(4): p. 323-326.
19. Reichenbach, H., *The genus Lysobacter*. The prokaryotes: an evolving electronic resource for the microbiological community, ed. F.S. Dworkin M, Rosenberg E, Schleifer KH, Stackebrandt E. 2006, BerlinHeidelberg New York: Springer.

20. Singh, B.N., *Myxobacteria in Soils and Composts - Their Distribution, Number and Lytic Action on Bacteria.* Journal of General Microbiology, 1947. **1**(1): p. 1-&.
21. Stolp, H. and M.P. Starr, *Bdellovibrio Bacteriovorus Gen. Et Sp. N., a Predatory, Ectoparasitic, and Bacteriolytic Microorganism.* Antonie Van Leeuwenhoek Journal of Microbiology and Serology, 1963. **29**(3): p. 217-&.
22. Byrd, J.J., L.R. Zeph, and L.E. Casida, *Bacterial Control of Agromyces-Ramosus in Soil.* Canadian Journal of Microbiology, 1985. **31**(12): p. 1157-1163.
23. Sangkhobol, V. and V.B.D. Skerman, *Saprospira Species - Natural Predators.* Current Microbiology, 1981. **5**(3): p. 169-174.
24. Quinn, G.R. and V.B.D. Skerman, *Herpetosiphon - Natures Scavenger.* Current Microbiology, 1980. **4**(1): p. 57-62.
25. Jurkevitch E and D. Y., *Phylogenetic diversity and evolution of predatory prokaryotes*. Predatory prokaryotes - biology, ecology, and evolution, ed. J. E. 2007, Heidelberg: Springer.
26. Davidov, Y. and E. Jurkevitch, *Diversity and evolution of Bdellovibrio-and-like organisms (BALOs), reclassification of Bacteriovorax starrii as Peredibacter starrii gen. nov., comb. nov., and description of the Bacteriovorax-Peredibacter clade as Bacteriovoracaceae fam. nov.* International Journal of Systematic and Evolutionary Microbiology, 2004. **54**: p. 1439-1452.
27. Esteve, I. and N. Gaju, *Bacterial symbioses. Predation and mutually beneficial associations.* Int Microbiol, 1999. **2**(2): p. 81-6.
28. Martin, M.O., *Predatory prokaryotes: An emerging research opportunity.* Journal of Molecular Microbiology and Biotechnology, 2002. **4**(5): p. 467-477.
29. Berleman, J.E. and J.R. Kirby, *Deciphering the hunting strategy of a bacterial wolfpack.* FEMS Microbiol Rev, 2009. **33**(5): p. 942-57.
30. Beebe, J.M., *The Morphology and Cytology of Myxococcus xanthus, N. Sp.* J Bacteriol, 1941. **42**(2): p. 193-223.
31. Jeschke, J.M., M. Kopp, and R. Tollrian, *Predator functional responses: Discriminating between handling and digesting prey.* Ecological Monographs, 2002. **72**(1): p. 95-112.
32. Jurkevitch, E., *The Genus Bdellovibrio*, in *The Prokaryotes*, M. Dworkin, *et al.*, Editors. 2006, Springer New York. p. 12-30.
33. Gray, K.M. and E.G. Ruby, *Prey-Derived Signals Regulating Duration of the Developmental Growth-Phase of Bdellovibrio-Bacteriovorus.* Journal of Bacteriology, 1990. **172**(7): p. 4002-4007.
34. Lamarre, A.G., S.C. Straley, and S.F. Conti, *Chemotaxis toward Amino-Acids by Bdellovibrio-Bacteriovorus.* Journal of Bacteriology, 1977. **131**(1): p. 201-207.
35. Straley, S.C. and S.F. Conti, *Chemotaxis by Bdellovibrio bacteriovorus toward prey.* J Bacteriol, 1977. **132**(2): p. 628-40.
36. Lambert, C., M.C. Smith, and R.E. Sockett, *A novel assay to monitor predator-prey interactions for Bdellovibrio bacteriovorus 109 J reveals a role for methyl-accepting chemotaxis proteins in predation.* Environ Microbiol, 2003. **5**(2): p. 127-32.
37. Zusman, D.R., *et al.*, *Chemosensory pathways, motility and development in Myxococcus xanthus.* Nature Reviews Microbiology, 2007. **5**(11): p. 862-872.
38. Baker, M.D., P.M. Wolanin, and J.B. Stock, *Systems biology of bacterial chemotaxis.* Current Opinion in Microbiology, 2006. **9**(2): p. 187-192.
39. Pham, V.D., *et al.*, *Mutations affecting predation ability of the soil bacterium Myxococcus xanthus.* Microbiology-Sgm, 2005. **151**: p. 1865-1874.
40. Kearns, D.B. and L.J. Shimkets, *Chemotaxis in a gliding bacterium.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(20): p. 11957-11962.

41. Shi, W.Y., T. Kohler, and D.R. Zusman, *Chemotaxis Plays a Role in the Social-Behavior of Myxococcus-Xanthus.* Molecular Microbiology, 1993. **9**(3): p. 601-611.
42. Matz, C., *et al.*, *Role of bacterial phenotypic traits in selective feeding of the heterotrophic nanoflagellate Spumella sp.* Aquatic Microbial Ecology, 2002. **27**(2): p. 137-148.
43. Wootton, E.C. and E.C. Roberts, *Biochemical recognition of prey by planktonic protozoa.* Journal of Eukaryotic Microbiology, 2007. **54**(2): p. 56s-56s.
44. Wildschutte, H., *et al.*, *Protozoan predation, diversifying selection, and the evolution of antigenic diversity in Salmonella.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(29): p. 10644-10649.
45. Freyth, K., *et al.*, *Reproductive Fitness and Dietary Choice Behavior of the Genetic Model Organism Caenorhabditis elegans under Semi-Natural Conditions.* Molecules and Cells, 2010. **30**(4): p. 347-353.
46. Jousset, A., *et al.*, *Predator-Prey Chemical Warfare Determines the Expression of Biocontrol Genes by Rhizosphere-Associated Pseudomonas fluorescens.* Applied and Environmental Microbiology, 2010. **76**(15): p. 5263-5268.
47. Rotem, O., *et al.*, *Cell-cycle progress in obligate predatory bacteria is dependent upon sequential sensing of prey recognition and prey quality cues.* Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(44): p. E6028-E6037.
48. Rosenberg, E. and M. Varon, *Antibiotics and lytic enzymes*. Myxobacteria: development and cell interactions, ed. E. Rosenberg. 1984, New York: Springer.
49. Margolith, P., *Bacteriolytic Principles of Myxococcus Fulvus.* Nature, 1962. **196**(4861): p. 1335-&.
50. McBride, M.J. and D.R. Zusman, *Behavioral analysis of single cells of Myxococcus xanthus in response to prey cells of Escherichia coli.* Fems Microbiology Letters, 1996. **137**(2-3): p. 227-231.
51. Gerth, K., *et al.*, *Antibiotics from Gliding Bacteria .8. The Myxovirescins, a Family of Antibiotics from Myxococcus-Virescens (Myxobacterales).* Journal of Antibiotics, 1982. **35**(11): p. 1454-1459.
52. Rosenberg, E. and M. Dworkin, *Autocides and a paracide, antibiotic TA, produced byMyxococcus xanthus.* Journal of Industrial Microbiology, 1996. **17**(5-6): p. 424-431.
53. Xiao, Y., *et al.*, *Antibiotic Production by Myxobacteria Plays a Role in Predation.* Journal of Bacteriology, 2011. **193**(18): p. 4626-4633.
54. Schieferdecker, S., *et al.*, *New myxothiazols from the predatory bacterium Myxococcus fulvus.* Journal of Antibiotics, 2014. **67**(7): p. 519-525.
55. Jansen, R., et al., *Antibiotics from Gliding Bacteria .24. Corallopyronin-a, Corallopyronin-B, and Corallopyronin-C - 3 Novel Antibiotics from Corallococcus-Coralloides, Cc C127 (Myxobacterales).* Liebigs Annalen Der Chemie, 1985(4): p. 822-836.
56. Cain, C.C., *et al.*, *Synergistic antimicrobial activity of metabolites produced by a nonobligate bacterial predator.* Antimicrobial Agents and Chemotherapy, 2003. **47**(7): p. 2113-2117.
57. Dworkin, M., *Mechanisms of Action of Antibiotics.* American Journal of Medical Technology, 1972. **38**(4): p. 138-&.
58. Goldman, B.S., *et al.*, *Evolution of sensory complexity recorded in a myxobacterial genome.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(41): p. 15200-15205.
59. Evans, A.G.L., *et al.*, *Predatory activity of Myxococcus xanthus outer-membrane vesicles and properties of their hydrolase cargo.* Microbiology-Sgm, 2012. **158**: p. 2742-2752.

60. Muller, S., *et al.*, *Bacillaene and Sporulation Protect Bacillus subtilis from Predation by Myxococcus xanthus.* Applied and Environmental Microbiology, 2014. **80**(18): p. 5603-5610.
61. Butcher, R.A., *et al.*, *The identification of bacillaene, the product of the PksX megacomplex in Bacillus subtilis.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(5): p. 1506-1509.
62. Barger, S.R., *et al.*, *Imaging secondary metabolism of Streptomyces sp Mg1 during cellular lysis and colony degradation of competing Bacillus subtilis.* Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 2012. **102**(3): p. 435-445.
63. Deines, P., C. Matz, and K. Jurgens, *Toxicity of violacein-producing bacteria fed to bacterivorous freshwater plankton.* Limnology and Oceanography, 2009. **54**(4): p. 1343-1352.
64. Matz, C., *et al.*, *Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates.* Applied and Environmental Microbiology, 2004. **70**(3): p. 1593-1599.
65. Kothe, M., *et al.*, *Killing of Caenorhabditis elegans by Burkholderia cepacia is controlled by the cep quorum-sensing system.* Cellular Microbiology, 2003. **5**(5): p. 343-351.
66. Lainhart, W., G. Stolfa, and G.B. Koudelka, *Shiga Toxin as a Bacterial Defense against a Eukaryotic Predator, Tetrahymena thermophila.* Journal of Bacteriology, 2009. **191**(16): p. 5116-5122.
67. Hilliard, M.A., C.I. Bargmann, and P. Bazzicalupo, *C-elegans responds to chemical repellents by integrating sensory inputs from the head and the tail.* Current Biology, 2002. **12**(9): p. 730-734.
68. Pradel, E., *et al.*, *Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(7): p. 2295-2300.
69. Gomez, W., *et al.*, *Evidence for gluconic acid production by Enterobacter intermedium as an efficient strategy to avoid protozoan grazing.* Soil Biology & Biochemistry, 2010. **42**(5): p. 822-830.
70. Hahn, M.W., H. Lunsdorf, and L. Janke, *Exopolymer production and microcolony formation by planktonic freshwater bacteria: defence against protistan grazing.* Aquatic Microbial Ecology, 2004. **35**(3): p. 297-308.
71. Hahn, M.W., E.R.B. Moore, and M.G. Hofle, *Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla.* Applied and Environmental Microbiology, 1999. **65**(1): p. 25-35.
72. Klobutcher, L.A., K. Ragkousi, and P. Setlow, *The Bacillus subtilis spore coat provides "eat resistance" during phagocytic predation by the protozoan Tetrahymena thermophila.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(1): p. 165-170.
73. Laaberki, M.H. and J. Dworkin, *Role of spore coat proteins in the resistance of Bacillus subtilis spores to Caenorhabditis elegans predation.* Journal of Bacteriology, 2008. **190**(18): p. 6197-6203.
74. Queck, S.Y., *et al.*, *The role of quorum sensing mediated developmental traits in the resistance of Serratia marcescens biofilms against protozoan grazing.* Environmental Microbiology, 2006. **8**(6): p. 1017-1025.
75. Muller, S., *et al.*, *Predation by Myxococcus xanthus Induces Bacillus subtilis To Form Spore-Filled Megastructures.* Applied and Environmental Microbiology, 2015. **81**(1): p. 203-210.

76. Callahan, H.S., H. Maughan, and U.K. Steiner, *Phenotypic Plasticity, Costs of Phenotypes, and Costs of Plasticity Toward an Integrative View.* Year in Evolutionary Biology 2008, 2008. **1133**: p. 44-66.
77. Leibold, M.A., *A graphical model of keystone predators in food webs: Trophic regulation of abundance, incidence, and diversity patterns in communities.* American Naturalist, 1996. **147**(5): p. 784-812.
78. Mazzola, M., *et al.*, *Protozoan-Induced Regulation of Cyclic Lipopeptide Biosynthesis Is an Effective Predation Defense Mechanism for Pseudomonas fluorescens.* Applied and Environmental Microbiology, 2009. **75**(21): p. 6804-6811.
79. Jousset, A. and M. Bonkowski, *The model predator Acanthamoeba castellanii induces the production of 2,4, DAPG by the biocontrol strain Pseudomonas fluorescens Q2-87.* Soil Biology & Biochemistry, 2010. **42**(9): p. 1647-1649.
80. Keller, L. and M.G. Surette, *Communication in bacteria: an ecological and evolutionary perspective.* Nature Reviews Microbiology, 2006. **4**(4): p. 249-258.
81. Matz, C., *et al.*, *Microcolonies, quorum sensing and cytotoxicity determine the survival of Pseudomonas aeruginosa biofilms exposed to protozoan grazing.* Environmental Microbiology, 2004. **6**(3): p. 218-226.
82. Harcke, E., Hutterma.A, and H. Kuhlwein, *Studies on Lytic Activities of Chondrococcus-Coralloides (Myxobacterales) .2. Identification of Bacteriolytic Enzyme as a Muramidase.* Archiv Fur Mikrobiologie, 1972. **85**(1): p. 6-&.
83. Hart, B.A. and S.A. Zahler, *Lytic Enzyme Produced by Myxococcus Xanthus.* Journal of Bacteriology, 1966. **92**(6): p. 1632-&.
84. Ensign, J.C. and R.S. Wolfe, *Lysis of Bacterial Cell Walls by an Enzyme Isolated from a Myxobacter.* Journal of Bacteriology, 1965. **90**(2): p. 395-&.
85. Guerrero, R., *et al.*, *Predatory prokaryotes: predation and primary consumption evolved in bacteria.* Proc Natl Acad Sci U S A, 1986. **83**(7): p. 2138-42.
86. Kadouri, D.E., *et al.*, *Predatory Bacteria: A Potential Ally against Multidrug-Resistant Gram-Negative Pathogens.* Plos One, 2013. **8**(5).
87. Hillesland, K.L., R.E. Lenski, and G.J. Velicer, *Ecological variables affecting predatory success in Myxococcus xanthus.* Microbial Ecology, 2007. **53**(4): p. 571-578.
88. Kaiser, D., *Coupling cell movement to multicellular development in myxobacteria.* Nature Reviews Microbiology, 2003. **1**(1): p. 45-54.
89. Reichenbach, H. and G. Hofle, *Biologically active secondary metabolites from myxobacteria.* Biotechnol Adv, 1993. **11**(2): p. 219-77.
90. Berleman, J.E., *et al.*, *The lethal cargo of Myxococcus xanthus outer membrane vesicles.* Frontiers in Microbiology, 2014. **5**.
91. CHRISTENSEN, P. and F.D. COOK, *Lysobacter, a New Genus of Nonfruiting, Gliding Bacteria with a High Base Ratio.* International Journal of Systematic and Evolutionary Microbiology, 1978. **28**(3): p. 367-393.
92. Nett, M. and G.M. Konig, *The chemistry of gliding bacteria.* Natural Product Reports, 2007. **24**(6): p. 1245-1261.
93. Hayward, A.C., *et al.*, *Stenotrophomonas and Lysobacter: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology.* Journal of Applied Microbiology, 2010. **108**(3): p. 756-770.
94. Li, S., *et al.*, *An antibiotic complex from Lysobacter enzymogenes strain C3: Antimicrobial activity and role in plant disease control.* Phytopathology, 2008. **98**(6): p. 695-701.
95. Puopolo, G., O. Giovannini, and I. Pertot, *Lysobacter capsici AZ78 can be combined with copper to effectively control Plasmopara viticola on grapevine.* Microbiological Research, 2014. **169**(7-8): p. 633-642.

96. Yuen, G.Y., *et al.*, *Bean rust biological control using bacterial agents.* Crop Protection, 2001. **20**(5): p. 395-402.
97. Zhang, Z. and G.Y. Yuen, *Biological Control of Bipolaris sorokiniana on Tall Fescue by Stenotrophomonas maltophilia Strain C3.* Phytopathology, 1999. **89**(9): p. 817-22.
98. Ji, G.H., *et al.*, *Biological control of rice bacterial blight by Lysobacter antibioticus strain 13-1.* Biological Control, 2008. **45**(3): p. 288-296.
99. Shilo, M., *Lysis of Blue-Green Algae by Myxobacter.* Journal of Bacteriology, 1970. **104**(1): p. 453-&.
100. Safferman, R.S. and M.E. Morris, *Growth Characteristics of Blue-Green Algal Virus Lpp-1.* Journal of Bacteriology, 1964. **88**(3): p. 771-&.
101. Lueders, T., *et al.*, *Identification of bacterial micropredators distinctively active in a soil microbial food web.* Appl Environ Microbiol, 2006. **72**(8): p. 5342-8.
102. Islam, M.T., *Mode of antagonism of a biocontrol bacterium Lysobacter sp SB-K88 toward a damping-off pathogen Aphanomyces cochlioides.* World Journal of Microbiology & Biotechnology, 2010. **26**(4): p. 629-637.
103. Park, J.H., *et al.*, *Lysobacter capsici sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus Lysobacter.* Int J Syst Evol Microbiol, 2008. **58**(Pt 2): p. 387-92.
104. Puopolo, G., A. Raio, and A. Zoina, *Identification and Charactherization of Lysobacter Capsici Strain Pg4: A New Plant Health-Promoting Rhizobacterium.* Journal of Plant Pathology, 2010. **92**(1): p. 157-164.
105. Begunova, E.A., *et al.*, *The effect of the extracellular bacteriolytic enzymes of Lysobacter sp on gram-negative bacteria.* Microbiology, 2004. **73**(3): p. 267-270.
106. Berleman, J.E., *et al.*, *Rippling is a predatory behavior in Myxococcus xanthus.* Journal of Bacteriology, 2006. **188**(16): p. 5888-5895.
107. Vandamme, P. and T. Coenye, *Taxonomy of the genus Cupriavidus: a tale of lost and found.* International Journal of Systematic and Evolutionary Microbiology, 2004. **54**: p. 2285-2289.
108. Casida, L.E., *Relation to Copper of N-1, a Nonobligate Bacterial Predator.* Applied and Environmental Microbiology, 1987. **53**(7): p. 1515-1518.
109. Sillman, C.E. and L.E. Casida, *Isolation of Nonobligate Bacterial Predators of Bacteria from Soil.* Canadian Journal of Microbiology, 1986. **32**(9): p. 760-762.
110. Tian, J., *et al.*, *Analysis of transient polyhydroxybutyrate production in Wautersia eutropha H16 by quantitative Western analysis and transmission electron microscopy.* J Bacteriol, 2005. **187**(11): p. 3825-32.
111. Gyaneshwar, P., *et al.*, *Legume-nodulating betaproteobacteria: diversity, host range, and future prospects.* Mol Plant Microbe Interact, 2011. **24**(11): p. 1276-88.
112. Varon, M. and M. Shilo, *Interaction of Bdellovibrio Bacteriovorus and Host Bacteria .I. Kinetic Studies of Attachment and Invasion of Escherichia Coli B by Bdellovibrio Bacteriovorus.* Journal of Bacteriology, 1968. **95**(3): p. 744-&.
113. Lambert, C. and R.E. Sockett, *Laboratory maintenance of Bdellovibrio.* Curr Protoc Microbiol, 2008. **Chapter 7**: p. Unit 7B 2.
114. Im, H., *et al.*, *Shedding light on microbial predator-prey population dynamics using a quantitative bioluminescence assay.* Microb Ecol, 2014. **67**(1): p. 167-76.
115. Park, S., *et al.*, *A microfluidic concentrator array for quantitative predation assays of predatory microbes.* Lab on a Chip, 2011. **11**(17): p. 2916-2923.
116. Sockett, R.E., *Predatory Lifestyle of Bdellovibrio bacteriovorus.* Annual Review of Microbiology, 2009. **63**: p. 523-539.
117. Morgan, A.D., *et al.*, *Comparative Analysis of Myxococcus Predation on Soil Bacteria.* Applied and Environmental Microbiology, 2010. **76**(20): p. 6920-6927.

118. Davidov, Y. and E. Jurkevitch, *Comments of Poole and Penny's essay "Evaluating hypotheses for the origin of eukaryotes", BioEssays 29 : 74-84.* Bioessays, 2007. **29**(6): p. 615-616.
119. Whalen, J.K. and L. Sampedro, *Soil ecology and management*. 2010, Cambridge: CABI.
120. Xie, Y.X., *et al*., *Bioactive natural products from Lysobacter.* Natural Product Reports, 2012. **29**(11): p. 1277-1287.
121. Schmidt, A., *et al*., *In silico prediction of potential metallothioneins and metallohistins in actinobacteria.* Journal of Basic Microbiology, 2010. **50**(6): p. 562-569.
122. Blair, D.F. and H.C. Berg, *Restoration of Torque in Defective Flagellar Motors.* Science, 1988. **242**(4886): p. 1678-1681.
123. Chan, J.M., S.B. Guttenplan, and D.B. Kearns, *Defects in the Flagellar Motor Increase Synthesis of Poly-gamma-Glutamate in Bacillus subtilis.* Journal of Bacteriology, 2014. **196**(4): p. 740-753.
124. Kojima, S. and D.F. Blair, *Solubilization and purification of the MotA/MotB complex of Escherichia coli.* Biochemistry, 2004. **43**(1): p. 26-34.
125. McKenney, P.T., A. Driks, and P. Eichenberger, *The Bacillus subtilis endospore: assembly and functions of the multilayered coat.* Nature Reviews Microbiology, 2013. **11**(1): p. 33-44.
126. Ghosh, S., *et al*., *Superdormant spores of Bacillus species have elevated wet-heat resistance and temperature requirements for heat activation.* J Bacteriol, 2009. **191**(18): p. 5584-91.
127. Schluter, D., *Ecological causes of adaptive radiation.* American Naturalist, 1996. **148**: p. S40-S64.
128. Hibbing, M.E., *et al*., *Bacterial competition: surviving and thriving in the microbial jungle.* Nat Rev Microbiol, 2010. **8**(1): p. 15-25.
129. Habte, M. and M. Alexander, *Further evidence for the regulation of bacterial populations in soil by protozoa.* Arch Microbiol, 1977. **113**(3): p. 181-3.

12 Acknowledgement

My first and foremost gratitude goes to my supervisor Dr. habil. Markus Nett for giving me the opportunity to conduct my PhD project in his group and introducing me to the fascinating world of predatory bacteria. He was a supervisor who was always available for his students (even when he was very busy), open for new ideas, showing great interest in research and being very understandable and polite even when experiments were not going in the right direction. Now, at the verge of finishing my studies, I look back and say thanks to Markus for his guidance, constant encouragement, support and for helping me successfully come to the end of my PhD journey.

I am very grateful to my co-supervisor, Prof. Dr. Dirk Hofmeister for his constructive suggestions and support during progress meetings. Dr. Christian Kost for his indispensable help in establishing predation assay and helping me to get published my first author paper. I am truly grateful to Dr. Ákos T. Kovács and Ramses Gallegos-Monterrosa for a very fruitful collaboration in designing predator-prey model and for their support and ideas. My special thanks go to Dr. Andre Schmidt for introducing me to interesting field of metal resistant bacteria and supporting me in incorporating this new knowledge in my research project. But the most of I am grateful for is his never-ending enthusiasm and fascination with science, even after finishing PhD. I would like to thank Mrs. Andrea Perner for MS measurements, Mrs. Heike Heinecke for NMR measurements, Mrs. Maria Poetsch for conducting MALDI-MS measurements and Prof. Dr. Peter Zipfel for providing opportunity to conduct MST measurements. Dr. Sandor Nietzsche for introducing me to electron microscopy and, naturally, for making nice photos of my copper loving bacteria.

Thanks to all who, knowingly or unknowingly, helped me to get to this stage of nearly 200 page document especially giving look on the written version. First of all, to my supervisor Markus for critical proofreading of this manuscript and his invaluable comments. Secondly, to my dear friends Riya for proofreading the text, and Evelyn for providing aspects as a native English speaker and my special thanks go to my colleague Angela who helped me with German translation and constructive suggestions. I would also like to thank the whole SRB working group for being pleasant company and supporting me over the last years.

I sincerely acknowledge the Jena School for Microbial Communication for selecting me for their PhD fellowship, for helping me in various ways during my studies and making my stay

in Jena fulfilled with the most cherishable memories. Also I would like to express my sincere gratitude to the Leibniz Institute for Natural Product Research and Infection Biology for providing excellent platform with state of the art laboratory facilities for my research work. Additionally, I would like to express my gratitude to Serbian Business Foundation for financial support.

And at the last but not the least, I would like to thank to my dearest friends and my family. In the first place, I would like to express my sincere gratitude to my grandparents who supported my curiosity from early childhood and bought me so many encyclopedia and books about life sciences before I could even read. To my mother who celebrated my successes and for being my emotional support in hard times. Next, I would like to thank to my biology teacher Milena Jeličić who made me fell in love in biology during her lectures and with age of 11 I knew already, when I grow up I would become a biologist. To my 'intellectual role model' Vesna Kukić, for teaching me so many rhetorical, social and other life skills and above of all, for being one of the most important persons in my life. In the end, I would like express my deepest gratitude to my husband Nikola for his patience, the greatest love and support.

13 Eigenständigkeitserklärung

Die zurzeit gültige Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt. Die vorliegende Arbeit wurde von mir selbst und nur unter Verwendung der angegebenen Hilfsmittel erstellt und all benutzen Quellen angegeben. All Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind benannt. Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen. Die vorliegende Arbeit wurde bisher weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch bei einer anderen Hochschule als Dissertation eingereicht.

Jena, den 11.10.2015 Ivana Seccareccia

14 Curriculum Vitae

Personal data:

Name: Ivana Seccareccia

Date and place of birth:

8th of April 1986 in Rijeka, Croatia

Education:

Since October 2011: **PhD student** at the Leibniz-Institute for Natural Product Research and Infection Biology (HKI) in the Junior Research Group *Secondary Metabolism of Predatory Bacteria* in Jena

2009-2010 M.Sc. in microbiology at the University of Novi Sad, Faculty of Science, Department of Biology and Ecology in Serbia

2005-2009 Dipl. in biology at the University of Novi Sad, Faculty of Science, Department of Biology and Ecology in Serbia

Stay and study abroad: 2008-2009 Attended 4th academic exchange year at the University of Alabama in Huntsville, USA

Publications during PhD studies:

Seccareccia Ivana, Kost Christian, Nett Markus. Quantitative analysis of *Lysobacter* predation. *Appl. Environ. Microbiol*. **2015**. 81:7098 –7105.

Gurovic Maria Soledad Vela, Müller Sebastian, Domin Nicole, **Seccareccia Ivana**, Nietzsche Sandor, Martin Karin, Nett Markus. *Micromonospora schwarzwaldensis* sp. nov., a producer of telomycin, isolated from soil. **2013**. *Int. J. Syst. Evol. Microbiol*. 63:3812-3817.

Seccareccia Ivana, T. Kovács Ákos, Gallegos-Monterrosa Ramses and Nett Markus. Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*. Manuscript submitted

Kurth Colette, Schieferdecker Sebastian, Athanasopoulou Kalliopi, **Seccareccia Ivana**, and Nett Markus. Variochelins, novel lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining. Manuscript submitted to *Journal of Natural Products*

Oral presentations

Ivana Seccareccia, Ramses Gallegos Monterrosa, Ákos T. Kovács and Markus Nett**.** The unseen battlefield- predatory behavior of *Cupriavidus necator* N-1, Biomicroworld 2015, Barcelona

Ivana Seccareccia, Markus Nett. To kill or to be killed - predatory mechanism of *Cupriavidus necator*, MiCom 2015, Jena

Ivana Seccareccia and Markus Nett. Metallophores as predation factors in *Cupriavidus necator*, Annual Conference of the Association for General and Applied Microbiology(VAAM) 2013, Bremen
Ivana Seccareccia, Markus Nett. Copper-dependent predatory behavior of *Cupriavidus necator*, JSMC Symposium 2014, Jena

Poster presentations

Ivana Seccareccia, Christian Kost and Markus Nett. Development of an assay for studying predator-prey interactions. MiCom 2014, Jena

Ivana Seccareccia and Markus Nett. Decoding the Predatory Behavior of *Cupriavidus necator,* VAAM Workshop "Biology of Bacteria Producing Natural Products", 2013 Frankfurt

Ivana Seccareccia and Markus Nett. Small molecules as predation factors in *Cupriavidus necator*, MiCom 2012, Jena

Awards received:

2015 Received the lecture award on the *4th International Conference on Microbial Communication*
2014 Received the best scientific talk award on Symposium *Jena School for Microbial Communication (JSMC)*
2012 Received the best scientific poster award on the *3rd International Conference on Microbial Communication*
2010 3rd place in biology, Republic competition of Universities in Serbia

Workshops and seminars:

- **2015** seminar: Teaching in English
- **2015** 2-day workshop: Project Proposal Writing
- **2014** 2-day workshop: Project Management for Doctoral Candidates
- **2013** seminar: Applying Statistics – Understanding Your data with SPSS
- **2013** 2-day workshop: Personalized Leadership: How to lead intelligent people
- **2013** 2-day workshop: Introduction to the GxPs, with special focus on GMP and GLP
- **2012** 2-day workshop: Scientific Imaging and Processing
- **2011** seminar: Structure elucidation of small molecules using NMR- and MS-based methods