Mining into interspecific bacterial interactions

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Thesis

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“The role of the infinitely small in nature is infinitely great.”

-Louis Pasteur-

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Abstract

In terrestrial ecosystems bacteria live in complex multi-species networks. Within those networks bacteria are constantly interacting with each other and secondary metabolites like antibiotics have an important role in these interactions. Several studies revealed that the production of secondary metabolites by soil bacteria can be influenced by the presence of other microorganisms in their vicinity. Yet, not much is known on the frequency of interaction-mediated shifts of antibiotic production in microbes.

My thesis research focused on the importance of interaction-mediated shifts in secondary metabolite production in soil bacteria. To obtain more insight in the frequency of such events a high-through-put screening method was developed and applied. In total 146 bacteria were screened for the production of antimicrobials during one-to-one confrontations and during 2798 random interactions. Antimicrobial activity was recorded via an agar-overlay assay using two target organisms: *Escherichia coli* WA321 and *Staphylococcus aureus* 533R4. From all tested isolates, 33 % showed antimicrobial activity only in monoculture and 42 % of all isolates showed activity only during interactions. More bacterial isolates showed antimicrobial activity against *S. aureus* than against *E. coli*. The frequency of interaction-mediated induction of antimicrobial activity was 6 % (154 interactions out of 2798) indicating that only a specific set of species interactions induced antimicrobial activity. The screening revealed also interaction-mediated suppression of antimicrobial activity for 22 % of all tested combinations.

In another line of research, it was examined how interspecific bacterial interactions affect the composition and antimicrobial activity of volatile metabolites. The identities and antimicrobial activities of the volatiles were determined in monoculture as well as in mixed cultures of four bacterial strains belonging to different genera: *Chryseobacterium*, *Dyella*, *Janthinobacterium* and *Tsukamurella*. Antimicrobial activity of the produced volatiles was assessed against fungal, oomycetal and bacterial model organisms. The results revealed that interspecific bacterial interactions affected the composition of volatiles. Fungi and oomycetes showed high sensitivity towards bacterial volatiles whereas the effect of volatiles on bacteria varied. In total 35 volatile compounds were detected most of which were sulfur-containing compounds.

Two bacteria, namely a Gram-negative *Burkholderia* and a Gram-positive *Paenibacillus* strain, were selected to examine how interspecific bacterial interactions affect fitness, gene expression and the production of secondary metabolites. The bacteria were grown in monoculture and in mixed culture and subjected to detailed metabolome, volatolome and transcriptome analysis. One distinct volatile (2,5-bis(1-methylethyl)-pyrazine)-and one non-volatile compound (a pederin like compound) were detected in higher concentrations during interspecific interaction. The interacting bacteria as well as the identified volatile compound were tested in bioassays and showed strong inhibitory activity against a range of plant and human pathogens such as *Rhizoctonia solani*, *Fusarium culmorum*, *Candida albicans, S.aureus* and *E.coli.*

Furthermore, it was examined how nutrient conditions influence interactions between *Pseudomonas fluorescens* strain Pf0-1 and two other bacteria: *Pedobacter* sp. V48 and *Bacillus* sp. V102. Results of incubations in sand-microcosms revealed that under both nutrient conditions confrontation with the Gram-positive *Bacillus* led to significant lower cell numbers of *Pseudomonas*, whereas confrontation with the Gram-negative *Pedobacter* did not affected the growth of *Pseudomonas*. However, when *Pseudomonas* was confronted with the mixture of both strains, no significant effect on the growth of *Pseudomonas* was observed. Quantitative real-time PCR analysis revealed up-regulation of genes involved in the production of a broad-spectrum antibiotic by *Pseudomonas* when confronted with *Pedobacter* but not when confronted with *Bacillus*. Finally, possible costs of antibiotic production for *Pseudomonas fluorescens* Pf0-1 were measured by monitoring changes in growth rate with and without induction of antibiotic production. Our results did not reveal any significant costs for the production of the antibiotics produced by *Pseudomonas fluorescens* Pf0-1.

In summary, this thesis extends the knowledge about the effect of interspecific bacterial interactions on secondary metabolites production (soluble and volatiles), gene expression and fitness in bacteria. The exploitation of such bacterial interspecific interactions can be an important “tool” for the discovery of novel antimicrobial and agro-chemical compounds. Additionally, the obtained knowledge can help in selecting the right players for synthetic communities that fulfil important ecosystem services, like disease suppression, in agricultural crop systems.

Chapter 1

Introduction

Microbial soil ecology

Bacteria are the most ubiquitous and diverse living organisms on earth and surround us everywhere, with the highest diversity reported for soils (Curtis et al., 2002;Torsvik and Ovreas, 2002). Based on 16S rRNA bacterial gene sequences analysis 1 g of soil can contain up to 50.000 bacterial species and more than 10^8 bacterial cells (Torsvik and Ovreas, 2002;Huse et al., 2008;Uroz et al., 2010).

Soil is a very complex, heterogeneous and nutrient limited environment consisting of an inorganic-organic solid matrix, in which liquid and gaseous filled pores are present. Here micro- and macro-organisms are constantly interacting and competing for nutrients and micro-habitats. Microbial communities play a key role in many ecosystem processes e.g. decomposition, mineralization, carbon sequestration and plant growth promotion (Fitter et al., 2005;Hayat et al., 2010;Richardson and Simpson, 2011). Within the huge microbial diversity there is functional redundancy: many soil bacterial species have overlapping ecological niches as they are able to use similar substrates as an energy source for their growth (Yin, 2000;Demoling et al., 2007;Strickland, 2009). Since nutrient availability is one of the limiting factors for the growth of soil bacteria, interspecific competition for nutrients and space is ongoing and is consequently one of the most abundant forms of interaction occurring in soil (Demoling et al., 2007;Rousk and Baath, 2007). Besides biotic factors like competition also abiotic factors like soil moisture content, soil pH, matrix potential and other physio- chemical characteristics influence the survival of microorganisms. The dispersal of microorganisms in soil is strongly influenced by the moisture content, a low soil moisture content leads to lower connectivity between soil pores and thus to a lower number of accessible micro-habitats. The abiotic factors vary between soil habitats and can change locally very rapidly, thus creating a huge variety of micro-niches for growth and persistence of microbial species.

The rhizosphere

The plant root surfaces and the surrounding rhizosphere are a “hot-spot” of microbial life by providing significant sources of organic nutrients in nutrient poor surroundings. Easily degradable carbon compounds released by plant roots (root exudates) provide microorganisms with energy resources, thus microbial life intensifies in this narrow zone around the plant roots (Bais et al., 2006;De Boer et al., 2006;Dennis et al., 2010). In the rhizosphere many microbe-microbe as well as plant-microbe interactions are operating which

have a significant contribution to the performance of plants, for instance in acquisition of inorganic nutrients (Van Der Heijden et al., 2008;Bonkowski et al., 2009;Buée et al., 2009;Lambers et al., 2009). Additional to interactions with bacteria, plants also establish beneficial relationships with other microorganisms such as mycorrhizal fungi that colonize the inside of plant roots to exchange nutrients with their host (Bonfante and Anca, 2009). The lifestyle of bacteria can range from free living in soil and rhizosphere to endo- and epiphytic, including obligate endosymbionts and plant pathogens (Glick, 1995;Compant et al., 2005;Hardoim et al., 2008;Reinhold-Hurek and Hurek, 2011). Most of the interactions in the rhizosphere are beneficial for plants, however next to beneficial microorganisms the rhizosphere harbors also harmful microorganisms like pathogenic bacteria, fungi and plant-parasitic nematodes that can exert negative effects on plants and reducing crop yields worldwide (Oerke, 2006;Raaijmakers et al., 2009).

Competitive strategies of soil microbes

To sustain under such demanding environmental conditions soil bacteria evolved different survival strategies. An important strategy to enhance the competitive abilities is the production of secondary metabolites with antimicrobial properties (e.g. antibiotics, siderophores, bacteriocins, volatiles and others) called interference competition**.** (Velicer, 2003;Hibbing et al., 2010). The competitive interactions of microorganisms for nutrients and space are one of the fundamental mechanisms determining the composition and diversity of soil microbial communities. Interference competition involves direct interactions between the competitors via production of antimicrobial metabolites that can be targeted against closely related strains e.g. bacteriocins or against a wide range of competitors e.g. broad-spectrum antibiotics (Riley and Wertz, 2002a;b;Riley et al., 2003).

The production of secondary metabolites with antimicrobial properties is one of the strongest bacterial weapons used in the competition for space and nutrients. Next to interference competition also exploitation competition occurs in natural soil microbial communities. This type of competition involves the fast utilization of available resources without direct interactions between the competitors (Crespi, 2001;Velicer, 2003;Fiegna and Velicer, 2005;Hibbing et al., 2010). Thus, the ability to cope with the presence of competing microorganisms is essential for the growth and survival of bacteria in soil ecosystems.

Impact of interspecific bacterial interactions on secondary metabolite production

Several studies have demonstrated that bacteria can be triggered to produce antimicrobial compounds during interspecific interactions with other bacteria (de Boer et al., 2007;Garbeva et al., 2011b;Seyedsayamdost et al., 2012;Bertrand et al., 2014). Apparently the majority of soil bacteria do not constantly produce secondary metabolites under standard laboratory conditions, as these conditions are very artificial compared to the complex situation bacteria experience in nature. So far, most of the antibiotics were identified from cultivable soil bacteria and in particular from bacteria of the genus *Streptomyces* spp. (Handelsman, 2005;Baltz, 2008). Indeed, next generation sequencing data of bacterial genomes revealed that many bacteria possess gene clusters encoding for so far unidentified secondary metabolites (Scherlach and Hertweck, 2009). Thus, it is possible that the actual screening procedures to explore antimicrobial compounds do overlook bacteria that possess cryptic genes encoding for novel antimicrobial compounds, due to the fact that they are only produced during interspecific interactions that are not presented in standard laboratory cultivation (Bertrand et al., 2014;Marmann et al., 2014). Triggering of antibiotic production via interactions would be one option to activate such cryptic genes.

The ecological roles of secondary metabolites with antimicrobial activity

In the soil antimicrobial compounds usually occur at concentrations below the inhibitory concentration. Therefore, there is a debate on the actual ecological role in nature which is to date not entirely known. Several studies showed that antimicrobial compounds have a variety of other roles, for example they can act as signalling molecules at sub-inhibitory concentrations and affect many cellular functions such as cellular development (Straight et al., 2006), biofilm formation, motility, virulence and nutrient use (Hoffman et al., 2005;Linares et al., 2006;Romero et al., 2011;Vaz Jauri et al., 2013). Additionally sub-inhibitory concentrations of antibiotics have shown to effect the global transcriptional pattern in bacteria by up-or- down-regulation of genes in bacteria (Goh et al., 2002;Yim et al., 2011). So far, there are only a limited number of studies demonstrating that antibiotic compounds are produced *in situ* by soil bacteria and at concentrations that reach their supposed functions as weapons in microbial warfare (Monier et al., 2011;Raaijmakers and Mazzola, 2012). However, sub-inhibitory concentrations of antibiotics might be still functional in interspecific competition for

microhabitats and act as repellent against competitors. Although antimicrobial compounds can inhibit or kill other strains competing for the same ecological niche, in rare cases it has been shown that antibiotics can act also as a source of nutrients promoting the growth of bacteria under nutrient deprived conditions (D'Costa et al., 2006;Dantas et al., 2008). However, contrary to the findings by Dantas a study done by Walsh and co-workers was not able to confirm the previous findings, thus it is still questionable if antibiotics can act as a source of nutrients (Walsh et al., 2013). Hence, there is so far no scientific consensus on the ecological role of antimicrobial compounds in nature (**Figure 1.1**). Bacterial secondary metabolites with antimicrobial properties can be useful tools in the so called microbial warfare e.g. to protect the eukaryotic host against diseases (Raaijmakers and Mazzola, 2012;Sengupta et al., 2013). Indeed, numerous studies have demonstrated that secondary metabolites like e.g. antibiotics, enzymes and others produced by plant-associated bacteria are important for the suppression of plant pathogens and useful for biocontrol against soil-borne plant diseases (Haas and Defago, 2005;Mendes et al., 2013;Berg et al., 2014).

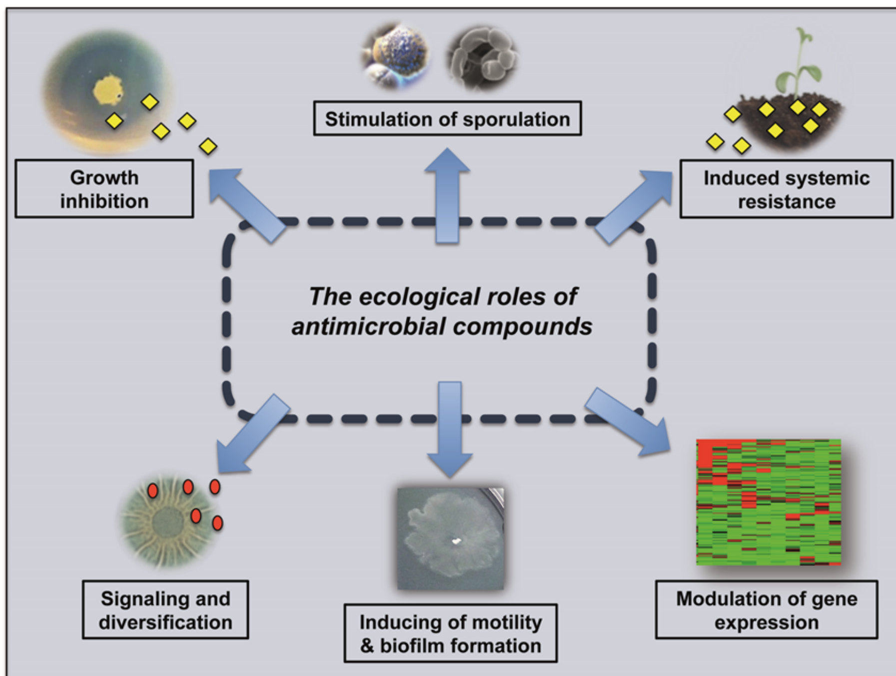

Figure 1.1: Schematic representation of the possible ecological roles of antimicrobial compounds in nature.

Volatile organic compounds (VOCs)

Next to soluble secondary metabolites bacteria produce also a vast repository of gaseous secondary metabolites, known as volatile organic compounds (VOCs). Volatile organic compounds are small molecules characterized by a low molecular weight (<300 Da), which evaporate and diffuse easily through air- and water-filled soil pores (Schulz and Dickschat, 2007;Penuelas et al., 2014). These physiochemical properties make VOCs ideal molecules for long-distance communication and antagonistic interactions between soil microorganisms. Volatile organic compounds emitted by soil microorganisms can have various functions such as info-chemicals, growth stimulants, growth inhibitors and inhibitors of quorum sensing (Kai et al., 2009;Chernin et al., 2011;Effmert et al., 2012;Kim et al., 2013). Moreover VOCs emitted by soil microorganism have shown to promote plant growth, induced systemic resistance (ISR) and induced systemic tolerance (IST) (Ryu et al., 2003;Ryu et al., 2004). The emitted volatile blend composition may vary according to the growth conditions, in particular to the growth media composition (Cleason, 2006;Groenhagen et al., 2013;Garbeva et al., 2014a), pH, moisture content, oxygen availability and cultivation temperature (Bjurman, 2007;Insam and Seewald, 2010;Romoli et al., 2014). The investigation of volatiles emitted by soil bacteria attracts great scientific interest since their ecological role in nature is not yet fully understood. However, volatile organic compounds emitted by bacteria may play an important role in the natural buffering against soil-borne plant- diseases (Garbeva et al., 2011a;van Agtmaal et al., 2015).

The need for novel antibiotics

Facing the worldwide problem of increasing antibiotic resistance in pathogenic bacteria (**Figure 1.2A**) novel antimicrobial compounds are urgently needed (Crisóstomo et al., 2001;Tenover et al., 2001;Al-Gheethi et al., 2013;Economou et al., 2013). Antibiotic resistance has become one of the major sanitary problems worldwide as many bacteria have developed antibiotic resistance against the most commonly used chemical classes of antibiotics. Unfortunately, only two families of new antibiotics (lipopeptides & oxazolidinones) have reached the clinical practice in the last four decades (**Figure 1.2B**) (Barbachyn and Ford, 2003;Kern, 2006;ECDC/EMEA, 2009) despite the fact the sanitary costs related to infections with antibiotic resistant microorganisms is continuously growing. The European Medicine Agency (EMA) estimates the costs that these infections provoke at approximately 1,5 billion euros only in the European Union (ECDC/EMEA, 2009). However,

there are still possibilities to change this current situation. One major strategy to combat this rising problem is the search for new antimicrobial compounds from natural sources like soil bacteria and other organisms by implementing innovative screening techniques. So far, most of the screenings for the discovery of novel bioactive compounds do often only target well-examined genera like e.g. *Streptomyces* spp. that produce antibiotics in monocultures in liquid and/or in solid media. These screening methods do not consider the ecological context of antibiotic production such as the importance of interspecific interactions. Thus, new strategies are needed to access the full genetic potential of terrestrial microbes as many bacterial species that might be considered to be of no interest for exploitation based upon individual screening methods may in fact possess cryptic genes encoding for novel antimicrobial compounds that are only produced during interspecific interactions or other natural conditions.

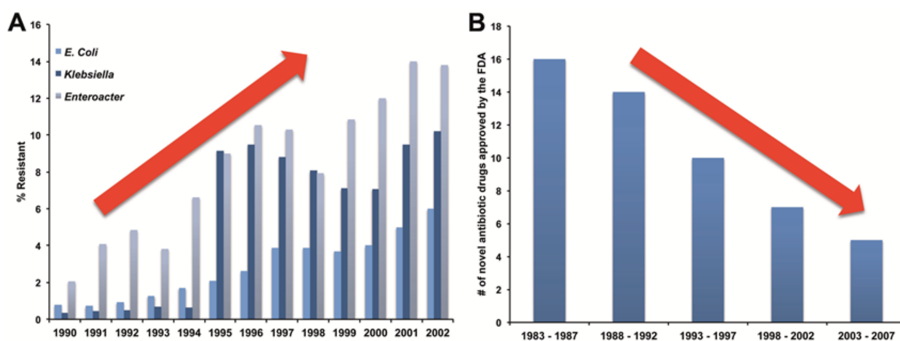

Figure 1.2: Increased drug resistance among diverse human pathogens versus the reduced development of novel antimicrobial drugs. (**A**) Growing proportion of human pathogens resistant against the antimicrobial ciprofloxacin. (**B**) The number of novel antimicrobial drugs approved by the FDA between 1983 – 2007. Data obtained and modified from (Livermore, 2004) (**A**) and (Spellberg et al., 2008)(**B**).

Objectives, research questions and thesis outline

The **major goal of this PhD research** was to explore the **effect** of **interspecific bacterial interactions** on the production of **secondary metabolites** (soluble and volatile) with inhibitory capacities for a range of soil bacteria. We started with the knowledge on a limited number of interactions between *Pseudomonas fluorescens* Pf0-1 and three other soil bacteria namely *Bacillus* sp V102, *Brevundimonas* sp. V52 and *Pedobacter* sp. V48. In previous research done by de Boer and Garbeva it was shown that interactions between *Pseudomonas fluorescens* Pf0-1, *Pedobacter* sp. V48 and *Bacillus* sp. V102 had profound effects on the secondary metabolite production, fitness and gene expression in *Pseudomonas fluorescens* Pf0-1. The interspecific interaction between the bacteria induced the production of an antimicrobial compound in *Pseudomonas fluorescens* Pf0-1 that was not produced in monocultures of *Pseudomonas fluorescens* Pf0-1 (Garbeva et al. 2010, 2011, Garbeva & de Boer 2009, de Boer et al., 2007). These studies revealed that apparently non-antagonistic bacteria can be triggered to produce antimicrobial compounds.

Part of this thesis dealt with the assessment of the frequency of competitor-induced antimicrobial compound production among soil bacteria, since the frequency of this event was not known so far. For this purpose I developed a high-through-put screening method to screen 146 soil bacterial isolates for antimicrobial activity in monocultures and in interactions.

Another aim of my thesis research was to investigate the effects of interspecific interactions on bacterial fitness, gene expression and on the global produced metabolome, including both soluble and volatile compounds.

Research questions related to the frequency of competition mediated triggering of antimicrobial activity in soil bacteria, the consequences of interspecific interactions on bacterial fitness, gene expression and the production of soluble and volatile secondary metabolites with inhibitory capacities are addressed in six chapters of this thesis.

The three main hypotheses for the thesis research are:

(1) Interspecific interactions have a major effect on antimicrobial compound production including both soluble and volatile antimicrobial compounds.
(2) Interspecific interactions have a significant effect on bacterial fitness, gene expression and consequently on soil bacterial community composition.
(3) The production of antimicrobial compounds is costly.

The research questions of this thesis are:

(1): What is the frequency of interspecific triggering of antimicrobial activity in soil bacteria?

The frequency of interaction mediated triggered antimicrobial activity among soil bacteria is addressed in the ***second*** **chapter**. In this chapter I highlight the importance of interspecific interactions for both induction and silencing of antimicrobial activity.

(2): How important are interspecific interactions for secondary metabolite production by soil bacteria?

The impact of bacterial interspecific interactions on secondary metabolite production in bacteria is addressed in **chapters** ***three*** and ***four***. These chapters report the results of research on the impact of interspecific interactions on the production of volatiles (chapter three and four) and soluble secondary metabolites (chapter four). Furthermore I evaluated the effect of interspecific interactions on gene expression and bacterial fitness (chapter four).

(3): What is the effect of interspecific bacterial interactions on the fitness of soil bacteria?

The effect of interspecific interactions on the fitness of soil bacteria is addressed in **chapter *five***. Here, I used a sand microcosm approach and applied the Malthusian growth model (population growth) to investigate how *Pseudomonas fluorescens* Pf0-1 responds to the presence of two phylogenetically different bacteria, under two different nutritional conditions.

(4): What are the possible costs for competitor induced antimicrobial compound production?

Chapter *six* reports on the possible biological costs for the production of an antimicrobial compound triggered through interspecific bacterial interactions (competitor induced). Here it is shown that the facultative production of antibiotics do not mitigate any metabolic costs, but might be an advantageous survival strategy because it limits the risk of competitors evolving resistance, or the risk of competitors feeding on the antimicrobial compounds.

In **chapter *seven*** I summarize and discuss the outcome of all experimental chapters of this thesis and the importance of mining into interspecific bacterial interactions. Furthermore this chapter will give a critical outlook on the future of antimicrobial discovery.

Chapter 2

Impact of interspecific interactions on antimicrobial activity among soil bacteria

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Impact of interspecific interactions on antimicrobial activity among soil bacteria

Abstract

Certain bacterial species produce antimicrobial compounds only in the presence of a competing species. However little is known on the frequency of interaction-mediated induction of antibiotic compound production in natural communities of soil bacteria. Here we developed a high-throughput method to screen for the production of antimicrobial activity by monocultures and pair-wise combinations of 146 phylogenetically different bacteria isolated from similar soil habitats. Growth responses of two human pathogenic model organisms, *Escherichia coli* WA321 and *Staphylococcus aureus* 533R4, were used to monitor antimicrobial activity. From all isolates, 33 % showed antimicrobial activity only in monoculture and 42 % showed activity only when tested in interactions. More bacterial isolates were active against *S. aureus* than against *E. coli*. The frequency of interaction-mediated induction of antimicrobial activity was 6 % (154 interactions out of 2798) indicating that only a limited set of species combinations showed such activity.

The screening revealed also interaction-mediated suppression of antimicrobial activity for 22 % of all combinations tested. Whereas all patterns of antimicrobial activity (non-induced production, induced production and suppression) were seen for various bacterial classes, interaction-mediated induction of antimicrobial activity was more frequent for combinations of Flavobacteria and alpha- Proteobacteria.

The results of our study give a first indication on the frequency of interference competitive interactions in natural soil bacterial communities which may forms a basis for selection of bacterial groups that are promising for the discovery of novel, cryptic antibiotics.

Introduction

Production of antimicrobial compounds is an important strategy to increase competitiveness of soil bacteria. Soil is a heterogeneous, nutrient-poor and harsh environment harboring a huge diversity of bacteria (Gans et al., 2005;Uroz et al., 2010). There is also considerable functional redundancy as many soil bacterial species can use similar substrates as an energy source for growth and persistence (Yin, 2000;Strickland, 2009). Therefore, interspecific competition for nutrient resources is a major type of interaction in soil bacterial communities (Demoling et al., 2007;Rousk and Baath, 2007;Rousk et al., 2009). An important strategy in interspecific interactions, known as interference competition, is the production of growth inhibitory secondary metabolites (e.g. antibiotics, toxins, biosurfactants, volatiles and others) that can suppress or kill microbial opponents (Hibbing et al., 2010;Cornforth and Foster, 2013). Although the production of antimicrobial compounds could inhibit the growth of bacterial strains competing for resources, in some cases the produced antimicrobial compounds could also promote the growth of other bacteria (D'Costa et al., 2006;Dantas et al., 2008), act as signalling molecules (Linares et al., 2006;Romero et al., 2011) or modulate bacterial gene expression in sub inhibitory concentrations (Goh et al., 2002).

Whole genome sequencing has revealed that many soil microorganisms possess so-called cryptic gene clusters encoding for putative new secondary metabolites that are not produced during common in vitro conditions (Ikeda et al., 2003;Scherlach and Hertweck, 2009;Chiang et al., 2011;Saleh et al., 2012). In nature, however, antibiotics may be produced after perception of specific environmental signals (stress/nutrient signals) or signals from neighboring microorganisms (competitor sensing) (Firn, 2003;Cornforth and Foster, 2013;Zhu, 2014). Indeed, several studies have indicated that antibiotic production in soil bacteria can be induced when they are confronted with other bacterial species (Slattery et al., 2001;Lyon and Muir, 2003;Maurhofer et al., 2004;de Boer et al., 2007;Seyedsayamdost et al., 2012).

We hypothesize that competitor induced (facultative) rather than constitutive antibiotic production represents a key strategy in interference competition that is cost-effective and / or may reduce selection of antibiotic-resistant competitors (Garbeva et al., 2011c). Interaction-mediated induction of antibiotic production is also interesting from an applied perspective as it may lead to the discovery of novel antibiotics.

Impact of interspecific interactions on antimicrobial activity among soil bacteria

The aim of the current study was to obtain insight in the frequency of interaction-mediated induction of antibiotic production in natural soil bacterial communities. To this end, we screened a collection of bacterial isolates obtained from similar soil habitats. We developed and applied a high-throughput method to screen bacteria for the production of compounds that inhibit growth of Gram-positive and Gram-negative isolates that are closely related to human pathogens. By selecting these target organisms the study not only revealed information on the frequency of interaction-mediated antibiotic production, but also on specific soil bacterial genera or species that could be promising candidates for the discovery of novel antibiotics.

The obtained results revealed that interactions have a major impact on antimicrobial compound production albeit with effects in both directions i.e. induction and suppression of antimicrobial activity.

Materials and methods

Soil bacteria & culture conditions

We selected 146 bacterial isolates from organic-poor, sandy soils under vegetation patches of sand sedge (*Carex arenaria* L.) growing in natural field sites (de Ridder-Duine et al., 2005) (**Table S2.1**). The bacterial isolates were pre-cultured from -80 °C glycerol stocks on 1/10 TSBA (5.0 gL^{-1} NaCl, 1.0 gL^{-1} KH_2PO_4; 3 gL^{-1} Oxoid Tryptic Soy Broth; 20 gL^{-1} Merck Agar, pH 6.5) (Garbeva and de Boer, 2009) and incubated for 5 to 7 days at 20 °C prior to screening.

Control strains and target organisms

Reference strains that produce known antibiotics in monoculture were obtained from the DSMZ strain collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). These reference strains were: *Streptomyces kanamyceticus* (DSM 40500), producer of kanamycin, *Streptomyces rimosus* (DSM 40260), producer of oxytetracycline and *Streptomyces nodosus* (DSM 40109) producer of amphotericin A and B. These strains were pre-cultured from -80 °C glycerol stocks on GYM agar plates (4.0 gL^{-1} Glucose, 4.0 gL^{-1} BACTO™ Yeast extract, 10.0 gL^{-1} Malt extract, 2.0 gL^{-1} $CaCO_3$, 20 gL^{-1} Merck Agar, pH 7.2) and incubated for 7 days at 28 °C before inoculation into 96-well source plates (see below). In the agar-overlay assay, two bacterial strains were selected to act as model organisms for human pathogenic bacteria: *Escherichia coli* WA321 (DSM 4509) as Gram-negative target organism and *Staphylococcus aureus* 533R4 Serovar 3 (DSM 20231) as Gram-positive target organism. The target strains were pre-cultured from -80 °C glycerol stocks on Luria Bertani (LB) agar plates (10.0 gL^{-1} NaCl, 10 gL^{-1} Bacto™ Tryptone, 5 gL^{-1} Bacto™ Yeast extract, 20 gL^{-1} Merck Agar) Sambrook and Russell (2001) and incubated at 37 °C for 24 h before inoculation in the antimicrobial screening assay. Characteristics of the target and the control strains are listed in **Table S2.2**.

Preparation of OmniTray™ plates

For the high-throughput interaction assay polystyrene Nunc™ OmniTray™ - plates (size 128 × 86 mm; cap. 90 mL; Nunc™, Nalge Nunc International, Rochester, NY, USA Cat # 82-264728) were used. Each OmniTray™ plate was filled with 45 mL of 1/10 TSBA (2 %) agar. Plates were kept in the laminar flow cabinet until the agar was completely solidified.

Preparation of 96-well source-plates

96-well Microtiter plates (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 655180) were prepared to inoculate the selected bacterial isolates and the reference strains. Each well was filled with 150 µl liquid LB broth. Bacterial isolates were inoculated in 10 rows containing quadruplicates of each strain, the 11th row was kept empty and the 12th row was used as positive control by inoculating known antibiotic-producing *Streptomyces* strains in duplicate with one free well between each strain (**Figure 2.1**). Inoculation was done by picking cells from a single colony of each bacterial strain with a disposable inoculation loop (VWR international B.V., Amsterdam, The Netherlands Cat# 50806-404) and transferring to the designated well in the 96-well source plates. The plates were incubated for two days at 24 °C, after which the plates were prepared for long-term storage (-80 °C freezer) by adding 50 µl of 50 % (v/v) glycerol to achieve a final concentration of 12.5 % (v/v). In total, 15 Microtiter plates (source plates A - O) containing different compositions of monocultures of bacterial isolates were prepared for the high-throughput interaction assay.

High-throughput interaction assay

A Genetix QPix 2 colony-picking robot (Molecular Devices, UK Limited, Wokingham, United Kingdom) was used for the high-throughput interaction assay. The Genetix QPix 2 robot was mounted with a bacterial 96-pin picking head and programmed to replicate the source plates (96-well Microtiter plates) into the OmniTray™ plates (**Figure 2.1**). The source plates were replicated two times, one set of inoculated plates was removed from the robot and was used as control to estimate growth and antimicrobial activity of the monocultures. The remaining plates in the robot were used for the interaction assay by inoculating a second set of source-plates in various combinations. The second set of bacterial isolates was inoculated at the same position as the first set of bacteria, in this way the bacterial isolates had physical cell contact and could interact in one-to-one interactions (in quadruplicates). The inoculated OmniTray™ plates (monocultures and interaction plates) were incubated for 4 days at 24 °C. In total, 146 bacterial isolates were combined with each other in various arrangements and tested in 2798 unique interactions for the production of antimicrobial compounds.

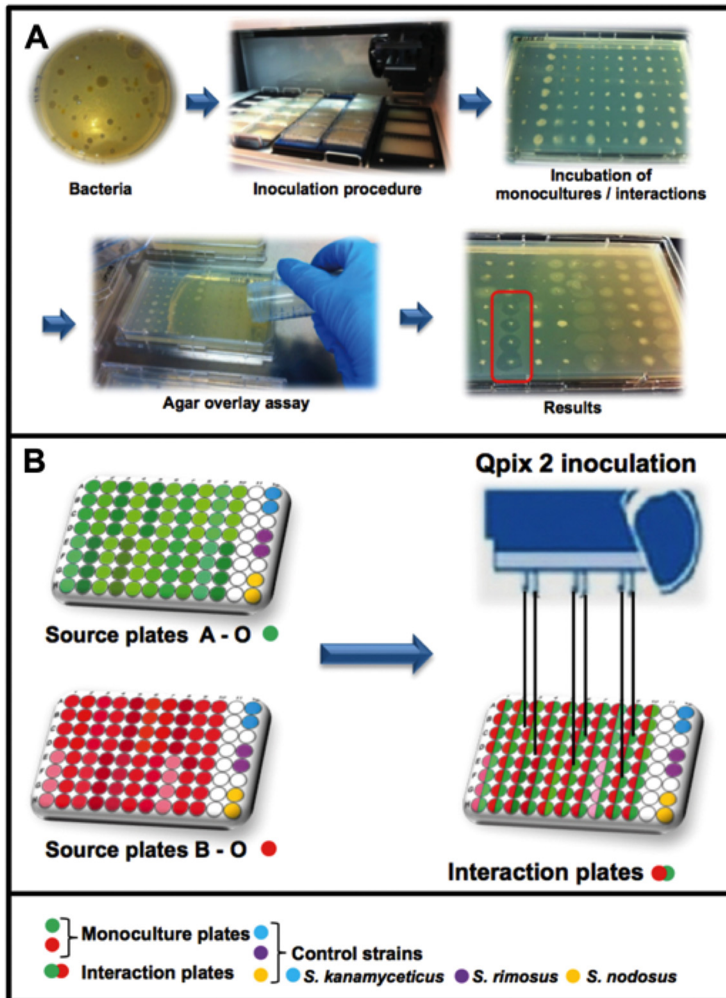

Figure 2.1: Workflow of the high-throughput interaction assay. (**A**) Overview of the antimicrobial screening: bacteria were inoculated with a Genetix Qpix 2 colony picking robot either in monoculture or in one-to-one interactions on OmniTray™ plates. For the detection of antimicrobial activity an agar overlay assay with two target organisms was performed on the fourth day of incubation. Antimicrobial activity was determined on the 5th day after overnight incubation at 37 °C by screening for visible zones of inhibition (ZOI) in the upper agar layer. (**B**) Overview of the 96-well plates design and the inoculation procedure using the Genetix QPix2 colony-picking robot.

Antimicrobial screening

For detection of antimicrobial activity, an agar overlay assay was performed on the 4th day of incubation (Nkanga and Hagedorn, 1978). The two target organisms *Escherichia coli* WA321 and *Staphylococcus aureus* 533R4 were grown overnight in liquid LB broth at 37 °C, 220 rpm. Fresh LB- agar (1.5 % Merck Agar) was prepared, cooled down to ~45 °C and the target organisms were added to a final OD_{600} of 0.002 corresponding to approximately 6 x 10^5 CFU/mL (*E.coli* WA321) or 4 x 10^5 CFU/mL (*S. aureus* 533R4) and mixed well. A volume of 15 mL liquid LB-agar containing the target organisms was poured over the OmniTray™ plates with the empty 11th row as the start position for pouring. After solidification of the overlay agar, the OmniTray™ plates were incubated overnight at 37 °C. The next day (5th day), plates were examined for visible zones of inhibition (ZOI). Monocultures or mixed-cultures of the soil bacterial isolates were scored as positive for antibiotic production if at least two out of four replicates produced zones of inhibition (**Figure 2.1A**). The majority of activity reported (>55 %) involved ≥3 out of 4 replicates. For confirmations of the high-throughput screening results, several of the antibiotic-triggering/suppressing interactions were tested outside the HTS setup (**Figure S2.3** and **S2.4**).

PCR and 16S rRNA gene sequencing

For identification of the bacterial isolates, PCRs were performed directly on colonies or with extracted genomic DNA. For genomic DNA extraction the QIAGEN QIAmp DNA Mini Kit (QIAGEN Benelux B.V., Venlo, The Netherlands cat# 51 304) was applied according to the manufacturer's manual. For the colony PCRs, a few colonies of each bacterial isolate were scraped from the plate with a disposable inoculation loop (VWR international B.V., Amsterdam, The Netherlands Cat# 50806-404) and re-suspended in 250 µl sterile MQ-water. The re-suspended bacterial cells were pulse vortexed and heated to 95 °C for 5 min. Tubes were centrifuged for 3 min at 12,000x g and 1 µl supernatant from each bacterial isolate was applied in a 50 µl PCR- master mix (Promega Corp. Madison, USA cat# M7505). For 16S rRNA gene amplification, one of the two primer combinations was used: (1) forward primer pA (5'- AGA GTT TGA TCC TGG CTC AG -3'), reverse primer 1492r (5'- GRT ACC TTG TTA CGA CTT -3'), amplifying ~1492 bp from the 16S rRNA gene or (2) forward primer 27f (5'- AGA GTTT GAT CMT GGC TCAG -3'), reverse primer 1492r amplifying ~1465 bp from the 16S rRNA gene (Edwards et al., 1989;Lane, 1991) (modified). All PCR reactions were performed on a MJ

Research Peltier thermal cycler 200 PCR machine (Harlow Scientific, Arlington, USA) with the following settings: initial cycle 95 °C for 5 min. and 30 cycles of 94 °C for 30 sec., 55 °C for 30 sec. and 72 °C for 1 min. After amplification, a volume of 5 µl of each PCR reaction was loaded on a 1.25 % (w/v) agarose gel and checked after electrophoresis for presence of PCR fragment. The PCR products were sent to MACROGEN (MACROGEN Europe, Amsterdam, The Netherlands) for sequencing.

Phylogenetic analysis and sequence analysis

Obtained sequence chromatograms of the 16S rRNA gene were examined for quality and trimmed to approximately the same size (~650 bp) using 4 PEAKS V1.7.2 for MAC OS X (www.nucleobytes.com) © 2006 Mek&Tosj.com and Clustal W. The aligned 16S rRNA gene sequences were compared against those available in the NCBI database by BLASTN (blast.ncbi.nlm.nih.gov) (Altschul et al., 1997). The sequences obtained during this study are deposited in NCBI GenBank under accession numbers KJ685218 - KJ685361. For two isolates, the 16S rRNA sequences were available from previous work: *P. fluorescens* (strain AD21): DQ778036, *Pedobacter* sp. (strain V48): DQ778037 (de Boer et al., 2007).

Network visualization of interactions

The bacterial interaction pairs that triggered or suppressed antimicrobial activity against the target organisms were visualized with Cytoscape 3.0.2 (www.cytoscape.org) for MAC OS X (Shannon et al., 2003). Interaction visualizations were performed with the following parameters: each phylogenetic class was visualized as a single node with different symbols for each phylogenetic class, the interactions between the phylogenetic classes (nodes) were visualized by links (edges) connecting each interacting phylogenetic class. Node colours were scaled to the number of interactions between the different phylogenetic classes (see Figure legends). For visualization, self-loops (interactions within the same phylogenetic class) and edges (interactions between phylogenetic classes) were bundled to single links between the respective phylogenetic classes (the darker the line the higher the number of interactions between the phylogenetic classes).

Statistical analysis

Statistical analyses on frequencies for induction and/or suppression of antimicrobial compound production between the different Gram-groups were performed with http://math.hws.edu/javamath/ryan/ChiSquare.html using online chi-square tests. Results of the chi-square test are shown in **Table S2.5** and **S2.6**.

Results

Phylogeny of the tested bacterial isolates

16S rRNA gene sequence analysis revealed that the 146 bacterial isolates tested in this study belonged to 4 phyla covering 7 classes and 9 genera: Proteobacteria (14 alpha- Proteobacteria, 65 beta -Proteobacteria, 29 gamma-Proteobacteria), Bacteroidetes (19 Flavobacteria, 1 Sphingobacteria), Actinobacteria (11 Actinobacteria) and Firmicutes (7 Bacilli) (**Table 2.1** and **S2.1**).

Table 2.1: Frequencies of antimicrobial activity for the phyla included in this study.

Phylum / phylogenetic class	Total abundance	AM active vs *E. coli* in monoculture	AM active vs *E. coli* in interaction	AM active vs *S. aureus* in monoculture	AM active vs *S. aureus* in interaction
Actinobacteria					
Actinobacteria	11	3	3	4	5
Bacteroidetes					
Flavobacteria	19	1	3	3	11
Sphingobacteria	1		1		
Firmicutes					
Bacilli	7	2	2	3	2
Proteobacteria					
a-proteobacteria	14		1	3	9
β-proteobacteria	65	17	8	26	25
γ-proteobacteria	29	2	2	12	7
(n) isolates	**146**	**25**	**20**	**51**	**59**

High-throughput screening for antimicrobial activity

We developed a high-throughput assay to screen for production of antimicrobial compounds by interacting bacteria (**Figure 2.1**). In total 146 isolates were screened in monocultures and in 2798 random one-to-one interactions. For 17 isolates (11 %), no activity against *E. coli* and *S. aureus* was detected not in monocultures nor in mixed cultures (**Table S2.7** and **Figure 2.2A**). For 20 isolates (14 %) antibacterial activity was observed in both monoculture and mixed cultures. For 48 isolates (33 %), this was restricted to monocultures only and for 61 isolates (42 %) antibacterial activity was only apparent during interactions (**Figure 2.2A** and **Figure 2.3**).

The number of isolates (110) involved in activity against the Gram-positive target strain *S. aureus* 533R4 was more than twice the number of isolates (45) with activity against the Gram-negative target strain *E. coli* WA321 (**Table 2.1**, **Table S2.1**). Despite the high number of bacterial isolates involved in antimicrobial activity in interactions, the frequency of interaction-mediated induction of antimicrobial activity was low ~6 % (154 interactions out of 2798). This implies that interaction-mediated induction was only occurring in a limited number of combinations (**Table 2.2** and **Table 2.3**).

Most interactions (72 %) did not have an effect on antimicrobial activity (induction or suppression) and about 22 % of the interactions suppressed antimicrobial activity in isolates that revealed activity in monoculture (**Figure 2.2B**).

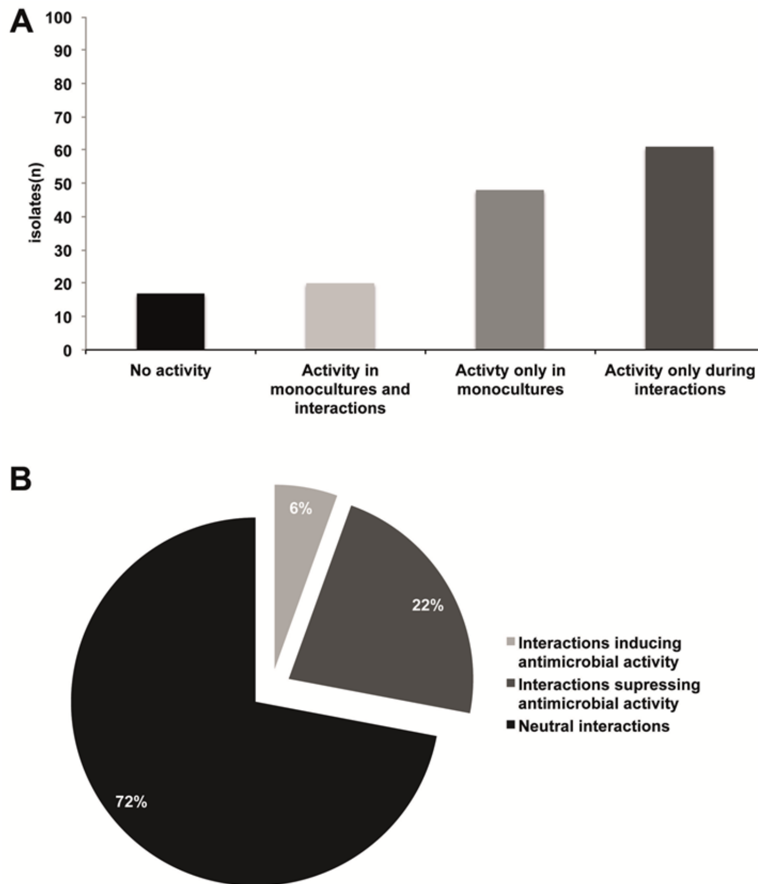

Figure 2.2: (**A**) Number of bacterial isolates exhibiting different patterns of antimicrobial activity against *E.coli* WA321 and / or *S.aureus* 533R4; in total 146 bacterial isolates were studied (**B**) Frequencies of interactions (1) inducing antimicrobial activity, (2) suppressing antimicrobial activity and (3) neutral interactions (no induction/suppression). Number of tested combinations (n=2798).

Antimicrobial activity during interactions

Interaction-mediated activity against *E. coli* WA321

Growth of *E.coli* WA321 was inhibited by 14 pair-wise combinations involving 20 isolates that did not show antimicrobial activity in monoculture (**Table 2.2**). Some isolates were present in different combinations. For example, *Janthinobacterium* sp. AD80 and *Streptomyces* sp. AD108 were present in 4 combinations with induced activity (**Table 2.2**). Combinations inhibiting growth of *E. coli* WA321 consisted of Gram-negative/Gram-positive isolates (7 interactions) or Gram-negative/Gram-negative (6 interactions). Only in one case, a combination of two Gram-positive isolates (*Micrococcus* & *Microbacterium*) showed activity against *E.coli*.

Table 2.2: Bacterial pairs with induced antimicrobial activity against *E.coli* WA 321.

Phylogenetic class	Genus A	Phylogenetic class	Genus B
alpha-proteobacteria	*Phyllobacterium* sp. AD152	gamma-proteobacteria	*Pseudomonas* sp. AD114
beta-proteobacteria	*Burkholderia* sp. AD24	beta-proteobacteria	*Collimonas* sp. AD68
beta-proteobacteria	*Burkholderia* sp. AD32	beta-proteobacteria	*Janthinobacterium* sp. AD80
beta-proteobacteria	*Janthinobacterium* sp. AD72	Flavobacteria	*Flavobacterium* sp. AD151
beta-proteobacteria	*Janthinobacterium* sp. AD80	gamma-proteobacteria	*Dyella* sp. AD56
beta-proteobacteria	*Janthinobacterium* sp. AD80	beta-proteobacteria	*Variovorax* sp. AD133
Actinobacteria	*Streptomyces* sp. AD108	beta-proteobacteria	*Burkholderia* sp. AD37
Actinobacteria	*Streptomyces* sp. AD108	Flavobacteria	*Flavobacterium* sp. AD47
Actinobacteria	*Streptomyces* sp. AD108	Flavobacteria	*Flavobacterium* sp. AD84
Actinobacteria	*Streptomyces* sp. AD108	Sphingobacteria	*Pedobacter* sp. V48
Actinobacteria	*Microbacterium* sp. AD141	beta-proteobacteria	*Janthinobacterium* sp. AD80
Bacilli	*Bacillus* sp. AD78	beta-proteobacteria	*Burkholderia* sp. AD11
Bacilli	*Paenibacillus* sp. AD83	beta-proteobacteria	*Burkholderia* sp. AD24
Actinobacteria	*Micrococcus* sp. AD31	Actinobacteria	*Microbacterium* sp. AD141

Interaction-mediated activity against *S. aureus* 533R4

Growth of *S. aureus* 533R4 was inhibited by 63 pair-wise combinations involving 59 isolates. Several isolates were present in multiple combinations that inhibited growth of *S. aureus* (**Table 2.3**). *Burkholderia* sp. AD37, *Collimonas* sp. AD65, *Collimonas* sp. AD98, *Janthinobacterium* sp. AD72, *Micrococcus* sp. AD31, *Pseudomonas* sp. AD104, *Streptomyces* spp. AD92 and AD108, *Variovorax* sp. AD143 were all involved in more than five combinations that inhibited the growth of *S. aureus*. Most of the combinations consisted of Gram-negative/Gram-negative isolates (35 interactions) or Gram-negative/Gram-positive isolates (25 interactions). Activity against *S. aureus* was only observed 3 times for Gram-positive/Gram-positive combinations (**Figure S2.8**).

Table 2.3: Bacterial pairs with induced antimicrobial activity against *S. aureus* 533R4.

Phylogenetic class	Genus A	Phylogenetic class	Genus B
alpha-proteobacteria	*Phyllobacterium* sp. AD34	beta-proteobacteria	*Collimonas* sp. AD89
alpha-proteobacteria	*Phyllobacterium* sp. AD153	beta-proteobacteria	*Collimonas* sp. AD65
beta-proteobacteria	*Collimonas* sp. AD69	Flavobacteria	*Flavobacterium* sp. AD43
beta-proteobacteria	*Janthinobacterium* sp. AD72	gamma-proteobacteria	*Dyella* sp. AD46
beta-proteobacteria	*Janthinobacterium* sp. AD72	beta-proteobacteria	*Collimonas* sp. AD97
beta-proteobacteria	*Janthinobacterium* sp. AD72	alpha-proteobacteria	*Agrobacterium* sp. AD140
beta-proteobacteria	*Collimonas* sp. AD61	beta-proteobacteria	*Collimonas* sp. AD98
beta-proteobacteria	*Collimonas* sp. AD67	beta-proteobacteria	*Collimonas* sp. AD68
beta-proteobacteria	*Janthinobacterium* sp. AD75	beta-proteobacteria	*Burkholderia* sp. AD37
beta-proteobacteria	*Collimonas* sp. AD69	Flavobacteria	*Flavobacterium* sp. AD146
beta-proteobacteria	*Collimonas* sp. AD71	alpha-proteobacteria	*Rhizobium* sp. AD148
beta-proteobacteria	*Collimonas* sp. AD88	beta-proteobacteria	*Burkholderia* sp. AD37
beta-proteobacteria	*Collimonas* sp. AD102	Flavobacteria	*Flavobacterium* sp. AD45
beta-proteobacteria	*Collimonas* sp. AD98	Flavobacteria	*Flavobacterium* sp. AD142
beta-proteobacteria	*Burkholderia* sp. AD37	gamma-proteobacteria	*Pseudomonas* sp. AD104
beta-proteobacteria	*Collimonas* sp. AD99	beta-proteobacteria	*Burkholderia* sp. AD138
beta-proteobacteria	*Collimonas* sp. AD89	alpha-proteobacteria	*Mesorhizobium* sp. AD38
beta-proteobacteria	*Variovorax* sp. AD143	beta-proteobacteria	*Collimonas* sp. AD65
beta-proteobacteria	*Variovorax* sp. AD143	alpha-proteobacteria	*Mesorhizobium* sp. AD112
beta-proteobacteria	*Variovorax* sp. AD143	alpha-proteobacteria	*Phyllobacterium* sp. AD153
beta-proteobacteria	*Collimonas* sp. AD98	alpha-proteobacteria	*Phyllobacterium* sp. AD159
beta-proteobacteria	*Collimonas* sp. AD98	gamma-proteobacteria	*Pseudomonas* sp. AD105
beta-proteobacteria	*Collimonas* sp. AD137	gamma-proteobacteria	*Pseudomonas* sp. AD157
beta-proteobacteria	*Collimonas* sp. AD97	beta-proteobacteria	*Collimonas* sp. AD62
beta-proteobacteria	*Roseateles* sp. AD145	beta-proteobacteria	*Collimonas* sp. AD67
gamma-proteobacteria	*Pseudomonas* sp. AD124	beta-proteobacteria	*Collimonas* sp. AD65
gamma-proteobacteria	*Pseudomonas* sp. AD114	beta-proteobacteria	*Burkholderia* sp. AD18
gamma-proteobacteria	*Pseudomonas* sp. AD105	alpha-proteobacteria	*Bosea* sp. AD132
gamma-proteobacteria	*Pseudomonas* sp. AD104	alpha-proteobacteria	*Phyllobacterium* sp. AD136
gamma-proteobacteria	*Pseudomonas* sp. AD104	Flavobacteria	*Chryseobacterium* sp. AD48
Flavobacteria	*Flavobacterium* sp. AD91	beta-proteobacteria	*Variovorax* sp. AD143
Flavobacteria	*Flavobacterium* sp. AD91	alpha-proteobacteria	*Phyllobacterium* sp. AD153
Flavobacteria	*Flavobacterium* sp. AD42	Flavobacteria	*Flavobacterium* sp. AD146
Flavobacteria	*Flavobacterium* sp. AD155	beta-proteobacteria	*Collimonas* sp. AD98
Flavobacteria	*Flavobacterium* sp. AD44	beta-proteobacteria	*Collimonas* sp. AD62
Actinobacteria	*Micrococcus* sp. AD31	beta-proteobacteria	*Collimonas* sp. AD65
Actinobacteria	*Micrococcus* sp. AD31	beta-proteobacteria	*Collimonas* sp. AD69
Actinobacteria	*Micrococcus* sp. AD31	beta-proteobacteria	*Collimonas* sp. AD70
Actinobacteria	*Micrococcus* sp. AD31	Flavobacteria	*Flavobacterium* sp. AD85
Actinobacteria	*Micrococcus* sp. AD31	beta-proteobacteria	*Collimonas* sp. AD88
Actinobacteria	*Micrococcus* sp. AD31	alpha-proteobacteria	*Phyllobacterium* sp. AD136
Actinobacteria	*Micrococcus* sp. AD31	gamma-proteobacteria	*Stenotrophomonas* sp. AD147
Actinobacteria	*Micrococcus* sp. AD31	Flavobacteria	*Flavobacterium* sp. AD156
Actinobacteria	*Streptomyces* sp. AD92	beta-proteobacteria	*Collimonas* sp. AD65
Actinobacteria	*Streptomyces* sp. AD92	beta-proteobacteria	*Variovorax* sp. AD143
Actinobacteria	*Streptomyces* sp. AD92	beta-proteobacteria	*Burkholderia* sp. AD18
Actinobacteria	*Streptomyces* sp. AD92	alpha-proteobacteria	*Phyllobacterium* sp. AD153
Actinobacteria	*Tsukamurella* sp. AD106	beta-proteobacteria	*Collimonas* sp. AD89
Actinobacteria	*Tsukamurella* sp. AD106	Flavobacteria	*Chryseobacterium* sp. AD48
Actinobacteria	*Streptomyces* sp. AD108	beta-proteobacteria	*Burkholderia* sp. AD37
Actinobacteria	*Streptomyces* sp. AD108	Flavobacteria	*Chryseobacterium* sp. AD48
Actinobacteria	*Streptomyces* sp. AD108	beta-proteobacteria	*Janthinobacterium* sp. AD73
Actinobacteria	*Streptomyces* sp. AD108	beta-proteobacteria	*Janthinobacterium* sp. AD75
Actinobacteria	*Streptomyces* sp. AD108	beta-proteobacteria	*Collimonas* sp. AD88
Actinobacteria	*Streptomyces* sp. AD108	beta-proteobacteria	*Collimonas* sp. AD101
Actinobacteria	*Streptomyces* sp. AD108	gamma-proteobacteria	*Pseudomonas* sp. AD104
Actinobacteria	*Microbacterium* sp. AD141	beta-proteobacteria	*Burkholderia* sp. AD37
Bacilli	*Paenibacillus* sp. AD83	beta-proteobacteria	*Collimonas* sp. AD62
Bacilli	*Paenibacillus* sp. AD83	beta-proteobacteria	*Burkholderia* sp. AD24
Bacilli	*Paenibacillus* sp. AD116	gamma-proteobacteria	*Pseudomonas* sp. AD104
Actinobacteria	*Micrococcus* sp. AD31	Actinobacteria	*Tsukamurella* sp. AD106
Actinobacteria	*Tsukamurella* sp. AD106	Actinobacteria	*Microbacterium* sp. AD141
Actinobacteria	*Streptomyces* sp. AD108	Actinobacteria	*Microbacterium* sp. AD141

Interaction-mediated activity against both target organisms

Nine isolates were present in pair-wise combinations that exhibited antimicrobial activity against both target organisms (**Table S2.1**). Two combinations were inhibitory for both target organisms. These were the combinations of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD83 (**Figure 2.3**) and of *Streptomyces* sp. AD108 and *Burkholderia* sp. AD37.

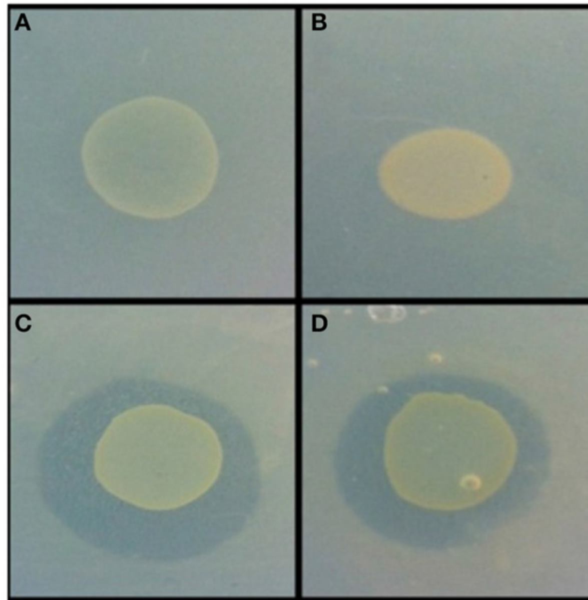

Figure 2.3: Example of antimicrobial activity revealed via the agar overlay assay: *Burkholderia* sp. AD24 monoculture (**A**), *Paenibacillus* sp. AD83 monoculture (**B**), Interaction *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD83 antimicrobial activity against *S. aureus* 533R4 (**C**) and antimicrobial activity against *E. coli* WA321 (**D**).

Interactions inducing antimicrobial activity against *E.coli* or *S.aureus*

The number of pair-wise combinations with induced antimicrobial activity against *S. aureus* 533R4 was higher than against *E. coli* WA321. Most combinations with induced activity against *E. coli* WA321 involved beta-Proteobacteria, Actinobacteria, Flavobacteria and Bacilli (**Figure 2.4A**). Combinations with induced activity against *S. aureus* 533R4 involved all classes of Proteobacteria, Actinobacteria, Flavobacteria, and Bacilli (**Figure 2.4B**). Two phylogenetic classes, Flavobacteria and alpha– Proteobacteria, were 3 times more represented in pair-wise combinations with antimicrobial activity than in monocultures (**Table 2.1**).

Interactions suppressing antimicrobial activity against *E.coli* or *S.aureus*

22 % of the isolates with antimicrobial activity in monoculture lost this activity during interactions. This apparent suppression of antimicrobial activity was found among all bacterial classes included in this study (**Figure 2.5A, 2.5B**). Suppression of antimicrobial activity was more frequently found for *S. aureus* than for *E. coli* (**Figure 2.3B**). The lists of bacterial pairs that suppressed antimicrobial activity against *S.aureus* and/or *E.coli* are shown in **Table S2.9** and **S2.10**.

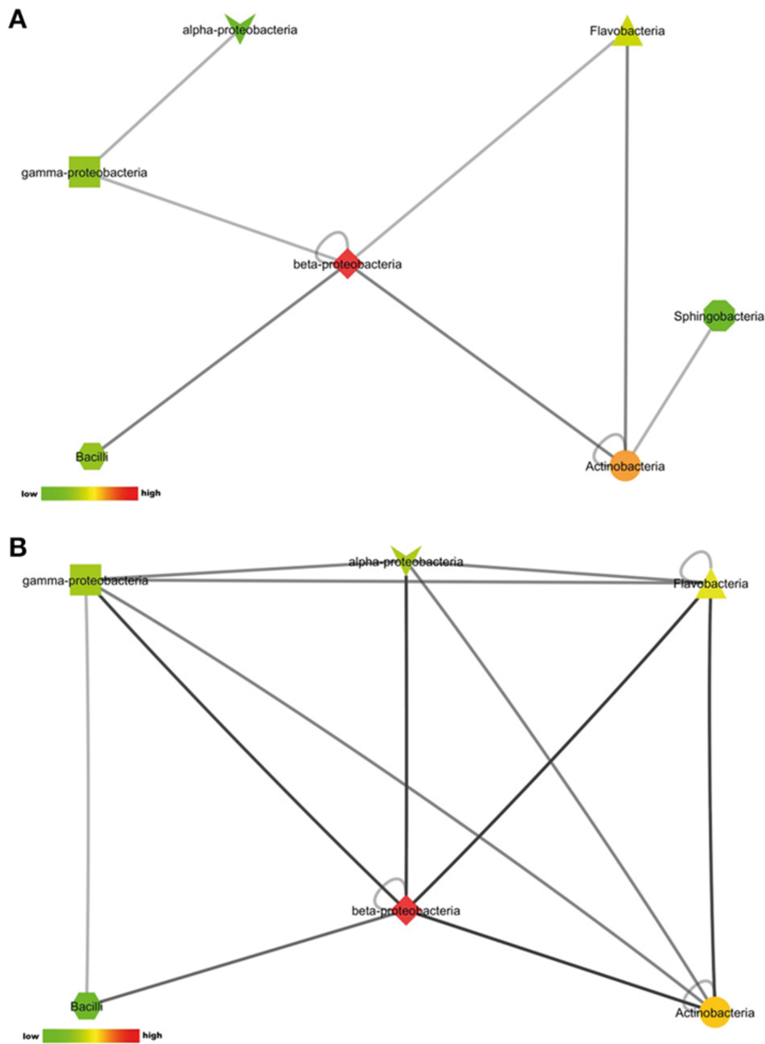

Figure 2.4: Interactions between phylogenetic classes that induced antimicrobial activity against (**A**) the Gram-negative target organism *E.coli* WA321, or (**B**) against the Gram-positive target organism *S.aureus* 533R4. Node colours are scaled to the number of interactions between the phylogenetic classes, low number of interactions in bright green, high number of interactions in dark red (see colour bar).

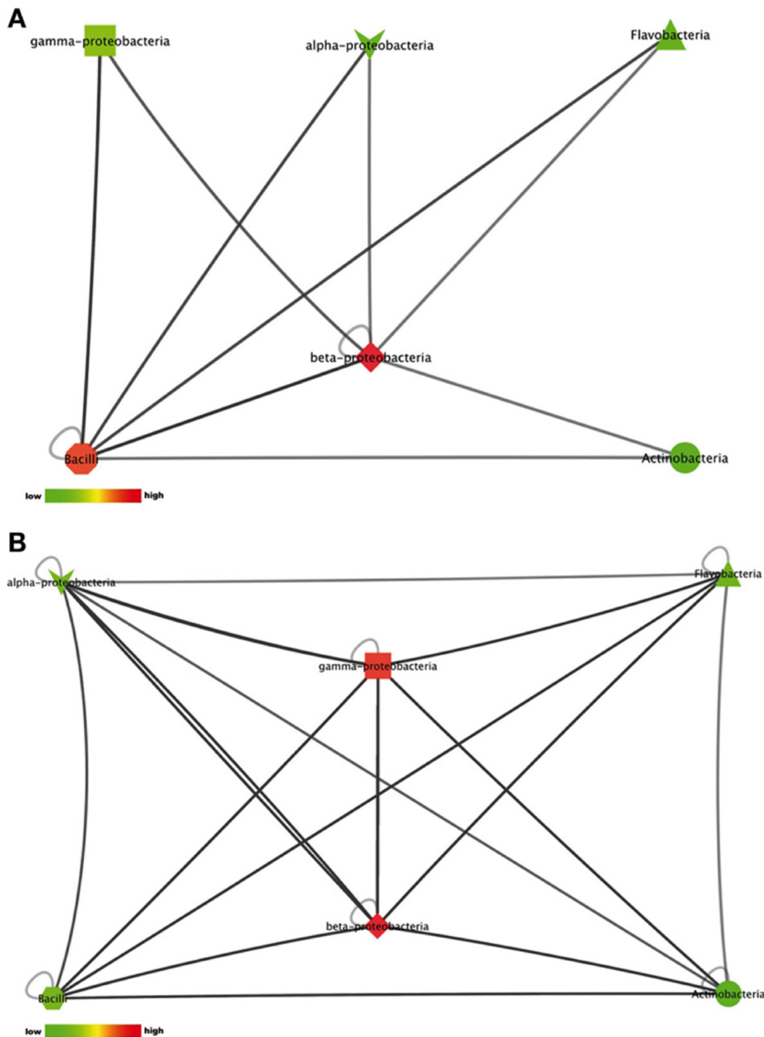

Figure 2.5: Interactions between phylogenetic classes that inhibited antimicrobial activity against (**A**) the Gram-negative target organism *E.coli* WA321, or (**B**) against the Gram-positive target organism *S.aureus* 533R4. Node colours are scaled to the respective number of interactions between the phylogenetic classes (low number of interactions in bright colours, high number of interactions in dark colours).

Discussion

Recent studies indicated the importance of interspecific bacterial interactions for triggering antibiotic production (Garbeva et al., 2011b;Seyedsayamdost et al., 2012). However, the frequency of such events in natural bacterial communities is not known. Our study focused on a collection of bacterial isolates from similar soil habitats, i.e. sandy soils covered by vegetation patches consisting of sand sedge (*Carex arenaria*). Hence, the chance that actual interactions between these bacteria can occur in their natural habitat seems plausible. Induction of antibiotic production in pair-wise combinations was not found to be an abundant phenomenon as it occurred in ~6 % of all interactions studied. Yet, 42 % of the bacterial isolates were present in combinations that showed activity against at least one of the target organisms, whereas they did not show activity in monocultures. This seems to indicate that the composition of the interacting pairs is an important factor in the induction of antibiotic production.

The observed frequency of interaction-mediated induction of antibiotic production exemplifies that a high-throughput screening as the one developed here can be an important strategy for the discovery of novel cryptic antibiotics. Many pair-wise combinations have to be screened and, subsequently, interesting pairs can be studied in more detail with respect to elucidate the mechanisms underlying the induction, signals and genes involved in the production of the antibiotic compounds (Garbeva et al., 2011b;Traxler et al., 2013). Interactions that induced antimicrobial activity often involved combinations of phylogenetically different bacteria or interactions among beta-Proteobacteria and among Actinobacteria. The present work included several bacterial genera (e.g. *Streptomyces*, *Burkholderia*, *Janthinobacterium* and *Paenibacillus*) for which multiple antibiotics have been described previously (Pantanella et al., 2007;Berdy, 2012;Cornforth and Foster, 2013;Debois et al., 2013;Zhu, 2014). Hence, there is the possibility that our screening method will reveal bacteria that produce known antibiotics but only during co-cultivation.

Few bacterial isolates of the classes Flavobacteria and alpha- Proteobacteria showed antimicrobial activity in monoculture, whereas several strains were present in antibiotic producing combinations. Hence, for these groups there is a clear potential to discover novel antibiotics. Of the 146 tested isolates, 33 % showed antimicrobial activity in monoculture. This obtained frequency is in

line with previous studies on frequencies of antimicrobial activity in *Streptomyces* spp. (Davelos et al., 2004;Kinkel et al., 2014). However, in many cases antibiotic production was lost when the strain was combined with another strain and only a small percentage (13 %) kept their antimicrobial activity in both combinations and monoculture. This suppressing effect on antibiotic production was more often found (22 % of all combinations) than the induction of antibiotic production (~6 % of all combinations). Several mechanisms can be responsible for the observed suppression of antimicrobial activity during interactions e.g. interference with the quorum sensing system or other signal transduction pathways involved in regulating antibiotic production (Gonzalez and Keshavan, 2006;Venturi and Subramoni, 2009;Christensen, 2013) or direct growth inhibition of the antibiotic producing strain (Straight et al., 2007;Hibbing et al., 2010;Schneider et al., 2012). Another possible reason for the observed inhibition of antimicrobial activity during interactions could be lower nutrient availability for each strain during co-cultivation. Growth conditions and nutrient availability are important factors affecting the production of antimicrobial compounds in bacteria (van Wezel and McDowall, 2011). Antibiotic resistance mechanisms might also play a role in the observed inhibition of antimicrobial activity during co-cultivation (Rice, 2006;Wellington et al., 2013).

Depending on the target organism there was a clear difference in antimicrobial activity with higher activity against the Gram-positive than against the Gram-negative organism (in both monocultures and interactions), which is in line with previous reports that Gram-positive bacteria are generally more sensitive to antibiotics (Rice, 2006;Giske et al., 2008;Zhu, 2014).

Soil and rhizosphere are environments where bacteria evolved the ability to produce antibiotics as competitive tool for their survival (Hibbing et al., 2010). Root-associated bacteria with antimicrobial potential play an important role in plant health (Raaijmakers and Mazzola, 2012) and understanding microbial interactions affecting antimicrobial activity may be helpful in understanding the functions and mechanisms of microbial communities contributing to plant protection.

The knowledge obtained here could help in selecting the right players in microbial consortia and as suggested by Mendes (Mendes et al., 2013) to design “a minimal microbiome” that comprises a set of microorganisms needed to fulfill a specific ecosystem services like e.g. disease suppression.

In conclusion, the high-throughput screening method developed in this work allows for a fast detection of interaction-mediated induction or suppression of antibiotic production in soil bacteria. Such screening also allows for a better insight into different interference competitive strategies that are operational in microbial communities. This knowledge in turn can be used for construction of synthetic microbial communities (Shong et al., 2012;De Roy et al., 2013;Grosskopf and Soyer, 2014).

Acknowledgment
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Supplementary material

Table S2.1: Results of antimicrobial screening in confrontation assays for all bacterial isolates used in this study.

Phylum / phylogenetic class	Species	Accession number	isolation medium	AM activity vs *E.coli*		AM activity vs *S.aureus*	
Actinobacteria				Monoculture	Interaction	Monoculture	Interaction
Actinobacteria	*Micrococcus* sp. AD12	KJ685229	TSBA	x		x	
Actinobacteria	*Rhodococcus* sp. AD22	KJ685237	TSBA			x	x
Actinobacteria	*Streptomyces* sp. AD29	KJ685244	TSBA	x		x	
Actinobacteria	*Micrococcus* sp. AD31	KJ685246	TSBA		x (1)		x (9)
Actinobacteria	*Streptomyces* sp. AD92	KJ685303	CHIT				x (4)
Actinobacteria	*Streptomyces* sp. AD94	KJ685305	CHIT			x	x
Actinobacteria	*Streptomyces* sp. AD107	KJ685318	TSBA			x	
Actinobacteria	*Streptomyces* sp. AD108	KJ685319	TSBA		x (4)		x (8)
Actinobacteria	*Mycobacterium* sp. AD110	KJ685321	TSBA	x		x	
Actinobacteria	*Microbacterium* sp. AD141	KJ685346	TSBA		x (2)		x (3)
Actinobacteria	*Tsukamurella* sp. AD106	KJ685317	TSBA				x (3)
Bacteroidetes							
Flavobacteria	*Flavobacterium* sp. AD41	KJ685256	TSBA			x	
Flavobacteria	*Flavobacterium* sp. AD42	KJ685257	TSBA				x (1)
Flavobacteria	*Flavobacterium* sp. AD43	KJ685258	TSBA				x (1)
Flavobacteria	*Flavobacterium* sp. AD44	KJ685259	TSBA				x (1)
Flavobacteria	*Flavobacterium* sp. AD45	KJ685260	TSBA				x (1)
Flavobacteria	*Flavobacterium* sp. AD47	KJ685262	TSBA		x (1)	x	x
Flavobacteria	*Chryseobacterium* sp. AD48	KJ685263	TSBA				x (3)
Flavobacteria	*Flavobacterium* sp. AD84	KJ685296	CHIT		x (1)	x	
Flavobacteria	*Flavobacterium* sp. AD85	KJ685297	CHIT				x (1)
Flavobacteria	*Flavobacterium* sp. AD86	KJ685298	CHIT	x		x	
Flavobacteria	*Flavobacterium* sp. AD91	KJ685302	CHIT				x (2)
Flavobacteria	*Flavobacterium* sp. AD131	KJ685338	TSBA				
Flavobacteria	*Flavobacterium* sp. AD134	KJ685341	TSBA				
Flavobacteria	*Flavobacterium* sp. AD142	KJ685347	TSBA				x (1)
Flavobacteria	*Flavobacterium* sp. AD146	KJ685351	CHIT				x (2)
Flavobacteria	*Flavobacterium* sp. AD149	KJ685354	TSBA				
Flavobacteria	*Flavobacterium* sp. AD151	KJ685355	TSBA		x (1)		
Flavobacteria	*Flavobacterium* sp. AD155	KJ685358	CHIT				x (1)
Flavobacteria	*Flavobacterium* sp. AD156	KJ685359	CHIT				x (1)
Sphingobacteria	*Pedobacter* sp. V48	DQ778037	TSBA		x (1)		
Firmicutes							
Bacilli	*Paenibacillus* sp. AD50	KJ685264	TSBA	x			
Bacilli	*Bacillus* sp. AD78	KJ685290	CHIT		x (1)	x	
Bacilli	*Paenibacillus* sp. AD83	KJ685295	CHIT		x (1)		x (2)
Bacilli	*Paenibacillus* sp. AD87	KJ685299	CHIT			x	
Bacilli	*Paenibacillus* sp. AD93	KJ685304	CHIT			x	
Bacilli	*Paenibacillus* sp. AD116	KJ685325	TSBA				x (1)
Bacilli	*Paenibacillus* sp. AD117	KJ685326	TSBA	x			
Proteobacteria							
alpha-proteobacteria	*Agrobacterium* sp. AD1	KJ685218	TSBA				
alpha-proteobacteria	*Phyllobacterium* sp. AD34	KJ685249	TSBA				x (1)
alpha-proteobacteria	*Mesorhizobium* sp. AD38	KJ685253	TSBA				x (1)
alpha-proteobacteria	*Mesorhizobium* sp. AD112	KJ685322	TSBA				x (1)
alpha-proteobacteria	*Bosea* sp. AD113	KJ685323	TSBA			x	
alpha-proteobacteria	*Bradyrhizobiaceae* sp. AD126	KJ685334	TSBA				
alpha-proteobacteria	*Bosea* sp. AD132	KJ685339	TSBA				x (1)
alpha-proteobacteria	*Agrobacterium* sp. AD140	KJ685345	TSBA				x (1)
alpha-proteobacteria	*Rhizobium* sp. AD148	KJ685353	TSBA				x (1)
alpha-proteobacteria	*Phyllobacterium* sp. AD136	KJ685342	TSBA				x (2)
alpha-proteobacteria	*Phyllobacterium* sp. AD152	KJ685356	TSBA		x (1)	x	
alpha-proteobacteria	*Phyllobacterium* sp. AD153	KJ685357	TSBA				x (4)
alpha-proteobacteria	*Phyllobacterium* sp. AD159	KJ685361	TSBA				x (1)
alpha-proteobacteria	*Phyllobacterium* sp. AD51	KJ685265	CHIT			x	
beta-proteobacteria	*Burkholderia* sp. AD3	KJ685220	TSBA				
beta-proteobacteria	*Burkholderia* sp. AD9	KJ685226	TSBA			x	
beta-proteobacteria	*Burkholderia* sp. AD10	KJ685227	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD15	KJ685231	TSBA	x		x	x
beta-proteobacteria	*Burkholderia* sp. AD11	KJ685228	TSBA		x (2)	x	
beta-proteobacteria	*Burkholderia* sp. AD18	KJ685234	TSBA				x (2)
beta-proteobacteria	*Collimonas* sp. AD19	KJ685235	TSBA			x	
beta-proteobacteria	*Collimonas* sp. AD23	KJ685238	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD24	KJ685239	TSBA		x (2)		x (1)
beta-proteobacteria	*Burkholderia* sp. AD25	KJ685240	TSBA	x		x	x
beta-proteobacteria	*Burkholderia* sp. AD26	KJ685241	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD27	KJ685242	TSBA	x		x	x
beta-proteobacteria	*Burkholderia* sp. AD28	KJ685243	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD30	KJ685245	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD32	KJ685247	TSBA		x (1)	x	
beta-proteobacteria	*Collimonas* sp. AD33	KJ685248	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD35	KJ685250	TSBA			x	
beta-proteobacteria	*Burkholderia* sp. AD37	KJ685252	TSBA		x (1)		x (5)
beta-proteobacteria	*Variovorax* sp. AD39	KJ685254	TSBA			x	

Table S2.1 continuation

Phylum / phylogenetic class	Species	Accession number	isolation medium	AM activity vs *E.coli*		AM activity vs *S.aureus*	
Proteobacteria				Monoculture	Interaction	Monoculture	Interaction
beta-proteobacteria	*Janthinobacterium* sp. AD54	KJ685267	TSBA			x	
beta-proteobacteria	*Janthinobacterium* sp. AD55	KJ685268	TSBA	x			
beta-proteobacteria	*Collimonas* sp. AD58	KJ685270	CHIT				
beta-proteobacteria	*Collimonas* sp. AD59	KJ685271	CHIT				
beta-proteobacteria	*Collimonas* sp. AD60	KJ685272	CHIT				
beta-proteobacteria	*Collimonas* sp. AD61	KJ685273	CHIT				x (1)
beta-proteobacteria	*Collimonas* sp. AD62	KJ685274	CHIT				x (3)
beta-proteobacteria	*Collimonas* sp. AD63	KJ685275	CHIT			x	x
beta-proteobacteria	*Collimonas* sp. AD64	KJ685276	CHIT			x	
beta-proteobacteria	*Collimonas* sp. AD65	KJ685277	CHIT				x (5)
beta-proteobacteria	*Collimonas* sp. AD66	KJ685278	CHIT			x	
beta-proteobacteria	*Collimonas* sp. AD67	KJ685279	CHIT				x (2)
beta-proteobacteria	*Collimonas* sp. AD68	KJ685280	CHIT		x (1)		x (1)
beta-proteobacteria	*Collimonas* sp. AD69	KJ685281	CHIT				x (3)
beta-proteobacteria	*Collimonas* sp. AD70	KJ685282	CHIT				x (1)
beta-proteobacteria	*Collimonas* sp. AD71	KJ685283	CHIT				x (1)
beta-proteobacteria	*Janthinobacterium* sp. AD72	KJ685284	CHIT		x (1)		x (3)
beta-proteobacteria	*Janthinobacterium* sp. AD73	KJ685285	CHIT				x (1)
beta-proteobacteria	*Janthinobacterium* sp. AD74	KJ685286	CHIT	x		x	
beta-proteobacteria	*Janthinobacterium* sp. AD75	KJ685287	CHIT				x (2)
beta-proteobacteria	*Collimonas* sp. AD76	KJ685288	CHIT			x	x
beta-proteobacteria	*Collimonas* sp. AD77	KJ685289	CHIT	x		x	
beta-proteobacteria	*Janthinobacterium* sp. AD80	KJ685292	CHIT		x (4)	x	
beta-proteobacteria	*Silvimonas* sp. AD81	KJ685293	CHIT			x	
beta-proteobacteria	*Silvimonas* sp. AD82	KJ685294	CHIT			x	
beta-proteobacteria	*Collimonas* sp. AD88	KJ685300	CHIT				x (3)
beta-proteobacteria	*Collimonas* sp. AD89	KJ685301	CHIT				x (3)
beta-proteobacteria	*Collimonas* sp. AD95	KJ685306	CHIT	x		x	
beta-proteobacteria	*Janthinobacterium* sp. AD96	KJ685307	CHIT	x		x	
beta-proteobacteria	*Collimonas* sp. AD97	KJ685308	CHIT				x (2)
beta-proteobacteria	*Collimonas* sp. AD98	KJ685309	CHIT				x (5)
beta-proteobacteria	*Collimonas* sp. AD99	KJ685310	CHIT				x (1)
beta-proteobacteria	*Collimonas* sp. AD101	KJ685312	CHIT				x (1)
beta-proteobacteria	*Collimonas* sp. AD102	KJ685313	CHIT				x (1)
beta-proteobacteria	*Collimonas* sp. AD103	KJ685314	CHIT			x	
beta-proteobacteria	*Janthinobacterium* sp. AD118	KJ685327	TSBA	x		x	x
beta-proteobacteria	*Janthinobacterium* sp. AD119	KJ685328	TSBA	x		x	
beta-proteobacteria	*Burkholderia* sp. AD123	KJ685331	TSBA				
beta-proteobacteria	*Burkholderia* sp. AD127	KJ685335	TSBA	x			
beta-proteobacteria	*Variovorax* sp. AD130	KJ685337	TSBA				
beta-proteobacteria	*Variovorax* sp. AD133	KJ685340	TSBA		x (1)	x	
beta-proteobacteria	*Collimonas* sp. AD137	KJ685343	TSBA				x (1)
beta-proteobacteria	*Burkholderia* sp. AD138	KJ685344	TSBA				x (1)
beta-proteobacteria	*Variovorax* sp. AD143	KJ685348	TSBA				x (5)
beta-proteobacteria	*Janthinobacterium* sp. AD144	KJ685349	CHIT			x	
beta-proteobacteria	*Roseateles* sp. AD145	KJ685350	CHIT				x (1)
gamma-proteobacteria	*Pseudomonas* sp. AD2	KJ685219	TSBA				
gamma-proteobacteria	*Pseudomonas* sp. AD4	KJ685221	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD5	KJ685222	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD6	KJ685223	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD7	KJ685224	TSBA			x	x
gamma-proteobacteria	*Pseudomonas* sp. AD8	KJ685225	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD14	KJ685230	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD16	KJ685232	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD17	KJ685233	TSBA			x	
gamma-proteobacteria	*Pseudomonas* sp. AD21	DQ778036	TSBA	x		x	x
gamma-proteobacteria	*Pseudomonas* sp. AD36	KJ685251	TSBA				
gamma-proteobacteria	*Pseudomonas* sp. AD79	KJ685291	CHIT			x	
gamma-proteobacteria	*Pseudomonas* sp. AD100	KJ685311	CHIT			x	x
gamma-proteobacteria	*Pseudomonas* sp. AD104	KJ685315	CHIT				x (5)
gamma-proteobacteria	*Pseudomonas* sp. AD105	KJ685316	CHIT				x (2)
gamma-proteobacteria	*Pseudomonas* sp. AD114	KJ685324	TSBA		x (1)		x (1)
gamma-proteobacteria	*Pseudomonas* sp. AD122	KJ685330	TSBA				
gamma-proteobacteria	*Pseudomonas* sp. AD124	KJ685332	TSBA				x (1)
gamma-proteobacteria	*Pseudomonas* sp. AD125	KJ685333	TSBA	x			
gamma-proteobacteria	*Pseudomonas* sp. AD157	KJ685360	TSBA				x (1)
gamma-proteobacteria	*Luteibactor* sp. AD20	KJ685236	TSBA			x	
gamma-proteobacteria	*Dyella* sp. AD40	KJ685255	TSBA			x	
gamma-proteobacteria	*Dyella* sp. AD46	KJ685261	TSBA				x (1)
gamma-proteobacteria	*Lysobacter* sp. AD52	KJ685266	CHIT			x	
gamma-proteobacteria	*Dyella* sp. AD56	KJ685269	TSBA		x (1)	x	
gamma-proteobacteria	*Frateuria* sp. AD120	KJ685329	TSBA				
gamma-proteobacteria	*Dyella* sp. AD129	KJ685336	TSBA				
gamma-proteobacteria	*Stenotrophomonas* sp. AD147	KJ685352	CHIT				x (1)
gamma-proteobacteria	*Rhodonobacter* sp. AD109	KJ685320	TSBA				

Abbreviations:

AM activity = antimicrobial activity against *E.coli* WA321 or *S.aureus* 533R4 in monoculture or in interactions. Isolation media: CHIT= chitin supplemented agar plates, TSBA= Tryptic soy broth agar plates. Numbers in brackets represent the number of interactions that triggered antimicrobial activity either against *E.coli* WA321 or *S.aureus* 533R4.

Table S2.2: Bacterial strains used as target and positive control in the high-through-put interaction assay and the agar-overlay assay to detect antimicrobial activity.

Organism	Strain number	Assay function
Streptomyces rimosus	DSMZ 40 260 (type strain)	positive control, produces oxytetracycline
Streptomyces nodosus	DSMZ 40 109 (type strain)	positive control, produces amphotericin A and amphotericin B
Streptomyces kanamyceticus	DSMZ 40 500 (type strain)	positive control, produces kanamycin A,B and C
Escherichia coli WA321	DSMZ 4509	target, model for human pathogen
Staphylococcus aureus 533R4 Serovar 3	DSMZ 20231	target, model for human pathogen

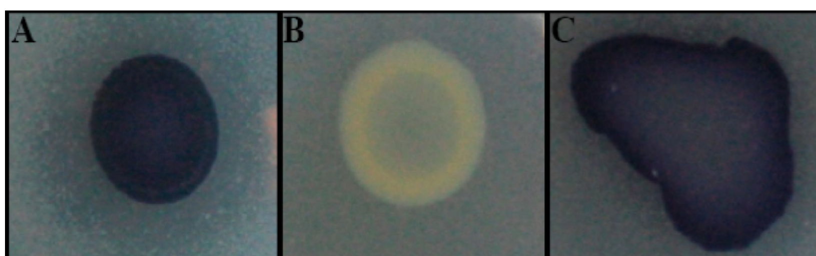

Figure S2.3: Suppression of antimicrobial activity revealed via the agar overlay assay: *Janthinobacterium* sp. AD55 monoculture (**A**), *Flavobacterium* sp. AD86 monoculture (**B**), Interaction *Janthinobacterium* sp. AD55 with *Flavobacterium* sp. AD86 (**C**) loss of antimicrobial activity against *E. coli* WA321.

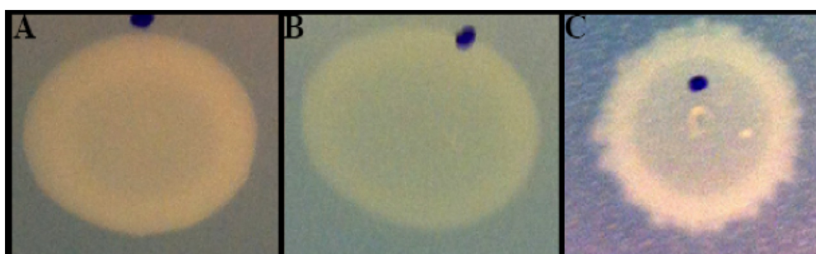

Figure S2.4: Induction of antimicrobial activity revealed via the agar overlay assay: (**A**) *Dyella* sp. AD56 monoculture, (**B**) *Janthinobacterium* sp. AD80 monoculture (**C**) interaction *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80, antimicrobial activity against *E. coli* WA321.

Table S2.5: Results of the Chi-square test for frequencies of induction of antimicrobial activity against (**A**) *E.coli* WA321 and (**B**) *S.aureus* 533R4.

A 1.6.2014 Contingency Table: Results

r × *c* Contingency Table: Results

The results of a contingency table X^2 statistical test performed at 11:29 on 1-JUN-2014

data: contingency table

	A	B	
1	6	2063	2069
2	7	673	680
3	1	48	49
	14	2784	2798

expected: contingency table

	A	B
1	10.4	2.059E+03
2	3.40	677.
3	0.245	48.8

chi-square = 8.00
degrees of freedom = 2
probability = 0.018

B 1.6.2014 Contingency Table: Results

r × *c* Contingency Table: Results

The results of a contingency table X^2 statistical test performed at 11:30 on 1-JUN-2014

data: contingency table

	A	B	
1	35	2034	2069
2	25	655	680
3	3	46	49
	63	2735	2798

expected: contingency table

	A	B
1	46.6	2.022E+03
2	15.3	665.
3	1.10	47.9

chi-square = 12.6
degrees of freedom = 2
probability = 0.002

Abbreviations: Rows: Gram-group interactions (1: Gram-/Gram- interactions, 2: Gram+/Gram- interactions, 3: Gram+/Gram+ interactions). Columns: Number of interactions with **A:** triggering of antimicrobial activity and **B:** Interactions without triggering.

Table S2.6: Results of the Chi-square test for the frequencies of silencing of antimicrobial activity against (**A**) *E.coli* WA 321 and (**B**) *S.aureus* 533R4.

A 1.6.2014 Contingency Table: Results

$r \times c$ Contingency Table: Results

The results of a contingency table X^2 statistical test performed at 11:33 on 1-JUN-2014

data: contingency table

	A	B	
1	22	2047	2069
2	36	644	680
3	2	47	49
	60	2738	2798

expected: contingency table

	A	B
1	44.4	2.025E+03
2	14.6	665.
3	1.05	47.9

chi-square = 44.5
degrees of freedom = 2
probability = 0.000

B 1.6.2014 Contingency Table: Results

$r \times c$ Contingency Table: Results

The results of a contingency table X^2 statistical test performed at 11:35 on 1-JUN-2014

data: contingency table

	A	B	
1	499	1570	2069
2	129	551	680
3	11	38	49
	639	2159	2798

expected: contingency table

	A	B
1	473.	1.596E+03
2	155.	525.
3	11.2	37.8

chi-square = 7.70
degrees of freedom = 2
probability = 0.021

Abbreviations: Rows: Gram-group interactions (1: Gram-/Gram- interactions, 2: Gram+/Gram- interactions, 3: Gram+/Gram+ interactions). Columns: Number of interactions with **A**: observed silencing of antimicrobial activity and **B**: without observed silencing.

Table S2.7: Bacterial isolates without any antimicrobial activity against *E.coli* WA 321 or *S.aureus* 533R4 either in paired combination or monoculture.

Phylum / phylogenetic class	Species	Accession number	isolation medium	AM activity vs *E.coli*		AM activity vs *S.aureus*	
Bacteroidetes				Monoculture	Interaction	Monoculture	Interaction
Flavobacteria	*Flavobacterium* sp. AD131	KJ685338	TSBA	-	-	-	-
Flavobacteria	*Flavobacterium* sp. AD134	KJ685341	TSBA	-	-	-	-
Flavobacteria	*Flavobacterium* sp. AD149	KJ685354	TSBA	-	-	-	-
Proteobacteria							
alpha-proteobacteria	*Agrobacterium* sp. AD1	KJ685218	TSBA	-	-	-	-
alpha-proteobacteria	*Bradyrhizobiaceae* sp. AD126	KJ685334	TSBA	-	-	-	-
beta-proteobacteria	*Burkholderia* sp. AD3	KJ685220	TSBA	-	-	-	-
beta-proteobacteria	*Collimonas* sp. AD58	KJ685270	CHIT	-	-	-	-
beta-proteobacteria	*Collimonas* sp. AD59	KJ685271	CHIT	-	-	-	-
beta-proteobacteria	*Collimonas* sp. AD60	KJ685272	CHIT	-	-	-	-
beta-proteobacteria	*Burkholderia* sp. AD123	KJ685331	TSBA	-	-	-	-
beta-proteobacteria	*Variovorax* sp. AD130	KJ685337	TSBA	-	-	-	-
gamma-proteobacteria	*Pseudomonas* sp. AD2	KJ685219	TSBA	-	-	-	-
gamma-proteobacteria	*Pseudomonas* sp. AD36	KJ685251	TSBA	-	-	-	-
gamma-proteobacteria	*Rhodonobacter* sp. AD109	KJ685320	TSBA	-	-	-	-
gamma-proteobacteria	*Frateuria* sp. AD120	KJ685329	TSBA	-	-	-	-
gamma-proteobacteria	*Pseudomonas* sp. AD122	KJ685330	TSBA	-	-	-	-
gamma-proteobacteria	*Dyella* sp. AD129	KJ685336	TSBA	-	-	-	-

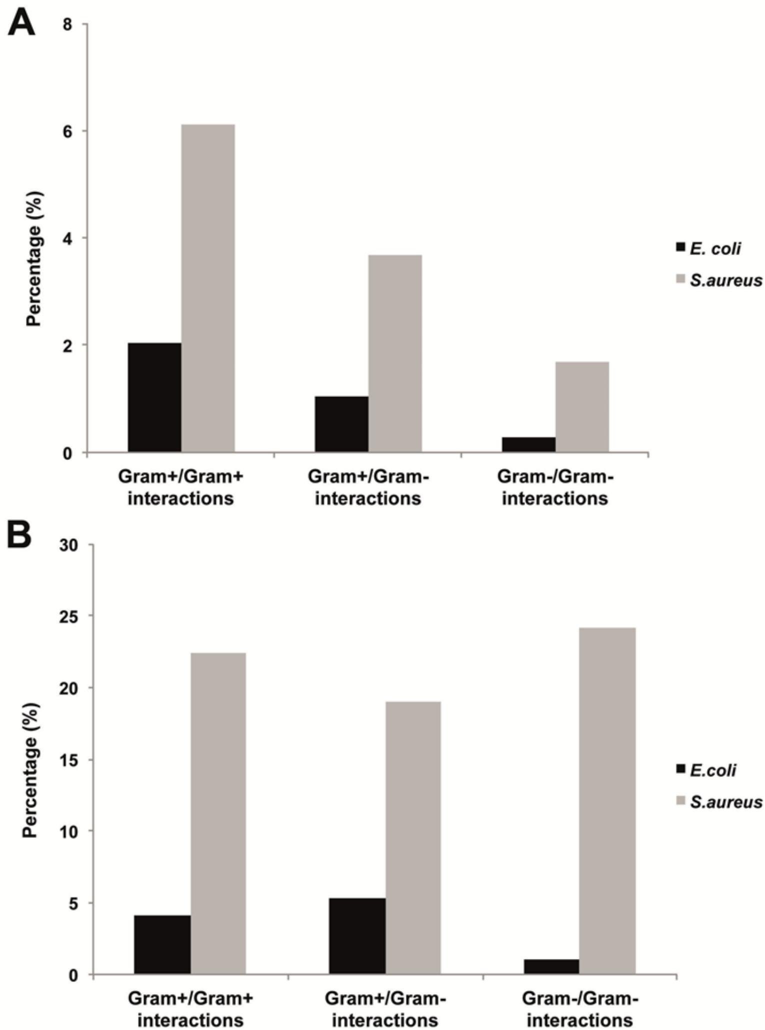

Figure S2.8: Frequencies of induction (**A**) or suppression of (**B**) antimicrobial activities for combinations of Gram- or Gram+ bacterial isolates. Antimicrobial activities were tested against *E.coli* WA321 or *S.aureus* 533R4. Number of tested interactions: Gram+/Gram+: n=49, Gram+/Gram-: n=690, Gram-/Gram-: n=2069. The results of the statistical analyses (Chi-square tests) are presented in **Table S2.5** and **S2.6**.

Table S2.9: Bacterial combination pairs which suppressed antimicrobial activity against *E.coli* WA 321. For the strain designation please see **Table S2.1**.

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
Bacilli	AD50	beta-proteobacteria	AD10
Bacilli	AD50	Actinobacteria	AD22
Bacilli	AD50	alpha-proteobacteria	AD34
Bacilli	AD50	beta-proteobacteria	AD60
Bacilli	AD50	beta-proteobacteria	AD70
Bacilli	AD50	beta-proteobacteria	AD9
Bacilli	AD50	beta-proteobacteria	AD26
Bacilli	AD50	beta-proteobacteria	AD81
Bacilli	AD50	beta-proteobacteria	AD98
Bacilli	AD50	beta-proteobacteria	AD89
Bacilli	AD50	Bacilli	AD87
Bacilli	AD50	beta-proteobacteria	AD137
Bacilli	AD50	alpha-proteobacteria	AD148
Bacilli	AD50	gamma-proteobacteria	AD157
Bacilli	AD50	Flavobacteria	AD155
Bacilli	AD50	beta-proteobacteria	AD25
Bacilli	AD50	beta-proteobacteria	AD33
Bacilli	AD50	Bacilli	AD83
Bacilli	AD50	beta-proteobacteria	AD101
Bacilli	AD50	gamma-proteobacteria	AD125
Bacilli	AD50	Flavobacteria	AD142
Bacilli	AD117	gamma-proteobacteria	AD20
Bacilli	AD117	Flavobacteria	AD42
Bacilli	AD117	beta-proteobacteria	AD68
Bacilli	AD117	beta-proteobacteria	AD24
Bacilli	AD117	beta-proteobacteria	AD95
Bacilli	AD117	Flavobacteria	AD146
Bacilli	AD117	alpha-proteobacteria	AD159
Bacilli	AD117	gamma-proteobacteria	AD14
Bacilli	AD117	gamma-proteobacteria	AD56
Bacilli	AD117	beta-proteobacteria	AD69
Bacilli	AD117	beta-proteobacteria	AD98
Bacilli	AD117	alpha-proteobacteria	AD001
Bacilli	AD117	Actinobacteria	AD12
Bacilli	AD117	gamma-proteobacteria	AD2
beta-proteobacteria	AD55	beta-proteobacteria	AD11
beta-proteobacteria	AD55	beta-proteobacteria	AD23
beta-proteobacteria	AD55	beta-proteobacteria	AD35
beta-proteobacteria	AD55	Flavobacteria	AD45
beta-proteobacteria	AD55	beta-proteobacteria	AD61
beta-proteobacteria	AD55	beta-proteobacteria	AD71
beta-proteobacteria	AD55	beta-proteobacteria	AD15
beta-proteobacteria	AD55	Actinobacteria	AD22
beta-proteobacteria	AD55	beta-proteobacteria	AD82
beta-proteobacteria	AD55	gamma-proteobacteria	AD100
beta-proteobacteria	AD55	beta-proteobacteria	AD98
beta-proteobacteria	AD55	beta-proteobacteria	AD138
beta-proteobacteria	AD55	beta-proteobacteria	AD143
beta-proteobacteria	AD55	gamma-proteobacteria	AD147
beta-proteobacteria	AD55	beta-proteobacteria	AD10
beta-proteobacteria	AD55	beta-proteobacteria	AD60
beta-proteobacteria	AD55	Flavobacteria	AD86
beta-proteobacteria	AD55	beta-proteobacteria	AD102
beta-proteobacteria	AD55	beta-proteobacteria	AD74
beta-proteobacteria	AD55	alpha-proteobacteria	AD112
beta-proteobacteria	AD55	alpha-proteobacteria	AD126
gamma-proteobacteria	AD125	beta-proteobacteria	AD66
gamma-proteobacteria	AD125	Bacilli	AD50
beta-proteobacteria	AD127	beta-proteobacteria	AD75
beta-proteobacteria	AD127	Actinobacteria	AD107

Table S2.10: Bacterial combination pairs which suppressed antimicrobial activity against *S.aureus* 533R4. For the strain designation please see **Table S2.1**.

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
Actinobacteria	AD107	gamma-proteobacteria	AD14
Actinobacteria	AD107	beta-proteobacteria	AD24
Actinobacteria	AD107	gamma-proteobacteria	AD36
Actinobacteria	AD107	beta-proteobacteria	AD62
Actinobacteria	AD107	beta-proteobacteria	AD72
Actinobacteria	AD107	beta-proteobacteria	AD32
Actinobacteria	AD107	Bacilli	AD83
Actinobacteria	AD107	beta-proteobacteria	AD99
Actinobacteria	AD107	gamma-proteobacteria	AD100
Actinobacteria	AD107	alpha-proteobacteria	AD140
Actinobacteria	AD107	Actinobacteria	AD22
Actinobacteria	AD107	Bacilli	AD87
Actinobacteria	AD107	beta-proteobacteria	AD103
Actinobacteria	AD107	beta-proteobacteria	AD127
Actinobacteria	AD107	Flavobacteria	AD146
Flavobacteria	AD41	beta-proteobacteria	AD19
Flavobacteria	AD41	beta-proteobacteria	AD67
Flavobacteria	AD41	beta-proteobacteria	AD25
Flavobacteria	AD41	Actinobacteria	AD94
Flavobacteria	AD41	beta-proteobacteria	AD96
Flavobacteria	AD41	beta-proteobacteria	AD145
Flavobacteria	AD41	Flavobacteria	AD151
Flavobacteria	AD41	gamma-proteobacteria	AD16
Flavobacteria	AD41	beta-proteobacteria	AD54
Flavobacteria	AD41	gamma-proteobacteria	AD8
Flavobacteria	AD41	beta-proteobacteria	AD68
Flavobacteria	AD41	beta-proteobacteria	AD32
Flavobacteria	AD41	beta-proteobacteria	AD58
Flavobacteria	AD41	gamma-proteobacteria	AD5
Flavobacteria	AD41	gamma-proteobacteria	AD79
Flavobacteria	AD41	Bacilli	AD83
Flavobacteria	AD41	Flavobacteria	AD134
Flavobacteria	AD41	Flavobacteria	AD142
Flavobacteria	AD41	beta-proteobacteria	AD24
Flavobacteria	AD41	Bacilli	AD93
Flavobacteria	AD41	beta-proteobacteria	AD27
Bacilli	AD87	gamma-proteobacteria	AD16
Bacilli	AD87	Actinobacteria	AD22
Bacilli	AD87	alpha-proteobacteria	AD38
Bacilli	AD87	Flavobacteria	AD44
Bacilli	AD87	beta-proteobacteria	AD64
Bacilli	AD87	beta-proteobacteria	AD70
Bacilli	AD87	gamma-proteobacteria	AD20
Bacilli	AD87	beta-proteobacteria	AD26
Bacilli	AD87	beta-proteobacteria	AD98
Bacilli	AD87	Bacilli	AD106
Bacilli	AD87	Flavobacteria	AD142
Bacilli	AD87	Flavobacteria	AD149
Bacilli	AD87	gamma-proteobacteria	AD21
Bacilli	AD87	Flavobacteria	AD48
Bacilli	AD87	alpha-proteobacteria	AD148
Bacilli	AD87	Flavobacteria	AD155
Bacilli	AD87	beta-proteobacteria	AD10
Bacilli	AD87	beta-proteobacteria	AD95
Bacilli	AD87	beta-proteobacteria	AD65
Bacilli	AD87	beta-proteobacteria	AD71
Bacilli	AD87	gamma-proteobacteria	AD14
Bacilli	AD87	gamma-proteobacteria	AD36
Bacilli	AD87	beta-proteobacteria	AD62
Bacilli	AD87	beta-proteobacteria	AD89
Bacilli	AD87	Bacilli	AD83
Bacilli	AD87	beta-proteobacteria	AD101

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
Bacilli	AD87	gamma-proteobacteria	AD100
Bacilli	AD87	alpha-proteobacteria	AD140
Bacilli	AD87	beta-proteobacteria	AD144
Bacilli	AD87	Actinobacteria	AD110
Bacilli	AD87	Bacilli	AD50
Bacilli	AD87	Actinobacteria	AD107
Bacilli	AD93	beta-proteobacteria	AD18
Bacilli	AD93	gamma-proteobacteria	AD40
Bacilli	AD93	beta-proteobacteria	AD66
Bacilli	AD93	beta-proteobacteria	AD19
Bacilli	AD93	gamma-proteobacteria	AD114
Bacilli	AD93	beta-proteobacteria	AD144
Bacilli	AD93	alpha-proteobacteria	AD152
Bacilli	AD93	gamma-proteobacteria	AD17
Bacilli	AD93	gamma-proteobacteria	AD52
Bacilli	AD93	beta-proteobacteria	AD67
Bacilli	AD93	Flavobacteria	AD41
Bacilli	AD93	beta-proteobacteria	AD25
Bacilli	AD93	Actinobacteria	AD92
Bacilli	AD93	Actinobacteria	AD94
Bacilli	AD93	beta-proteobacteria	AD96
Bacilli	AD93	beta-proteobacteria	AD145
Bacilli	AD93	Flavobacteria	AD151
Bacilli	AD93	gamma-proteobacteria	AD16
Bacilli	AD93	beta-proteobacteria	AD119
Bacilli	AD93	beta-proteobacteria	AD7
alpha-proteobacteria	AD113	gamma-proteobacteria	AD17
alpha-proteobacteria	AD113	beta-proteobacteria	AD39
alpha-proteobacteria	AD113	beta-proteobacteria	AD65
alpha-proteobacteria	AD113	gamma-proteobacteria	AD21
alpha-proteobacteria	AD113	Actinobacteria	AD92
alpha-proteobacteria	AD113	beta-proteobacteria	AD143
alpha-proteobacteria	AD113	alpha-proteobacteria	AD153
alpha-proteobacteria	AD113	beta-proteobacteria	AD19
alpha-proteobacteria	AD113	alpha-proteobacteria	AD51
alpha-proteobacteria	AD113	beta-proteobacteria	AD66
alpha-proteobacteria	AD113	Flavobacteria	AD91
alpha-proteobacteria	AD113	Flavobacteria	AD146
alpha-proteobacteria	AD113	beta-proteobacteria	AD118
gamma-proteobacteria	AD4	beta-proteobacteria	AD28
gamma-proteobacteria	AD4	gamma-proteobacteria	AD52
gamma-proteobacteria	AD4	gamma-proteobacteria	AD8
gamma-proteobacteria	AD4	gamma-proteobacteria	AD5
gamma-proteobacteria	AD4	beta-proteobacteria	AD76
gamma-proteobacteria	AD4	gamma-proteobacteria	AD104
gamma-proteobacteria	AD4	Flavobacteria	AD131
gamma-proteobacteria	AD4	alpha-proteobacteria	AD140
gamma-proteobacteria	AD4	beta-proteobacteria	AD30
gamma-proteobacteria	AD4	beta-proteobacteria	AD26
gamma-proteobacteria	AD4	Actinobacteria	AD29
gamma-proteobacteria	AD4	alpha-proteobacteria	AD51
gamma-proteobacteria	AD4	beta-proteobacteria	AD54
gamma-proteobacteria	AD4	beta-proteobacteria	AD74
gamma-proteobacteria	AD4	beta-proteobacteria	AD77
gamma-proteobacteria	AD4	gamma-proteobacteria	AD105
gamma-proteobacteria	AD4	alpha-proteobacteria	AD132
gamma-proteobacteria	AD4	beta-proteobacteria	AD145
gamma-proteobacteria	AD4	beta-proteobacteria	AD39
gamma-proteobacteria	AD4	beta-proteobacteria	AD009
gamma-proteobacteria	AD4	beta-proteobacteria	AD103
gamma-proteobacteria	AD4	gamma-proteobacteria	AD120

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
gamma-proteobacteria	AD4	alpha-proteobacteria	AD153
gamma-proteobacteria	AD4	beta-proteobacteria	AD137
gamma-proteobacteria	AD4	beta-proteobacteria	AD64
gamma-proteobacteria	AD4	alpha-proteobacteria	AD34
gamma-proteobacteria	AD4	beta-proteobacteria	AD37
gamma-proteobacteria	AD4	Bacilli	AD78
gamma-proteobacteria	AD4	gamma-proteobacteria	AD2
gamma-proteobacteria	AD4	beta-proteobacteria	AD3
gamma-proteobacteria	AD5	Actinobacteria	AD29
gamma-proteobacteria	AD5	beta-proteobacteria	AD54
gamma-proteobacteria	AD5	gamma-proteobacteria	AD4
gamma-proteobacteria	AD5	gamma-proteobacteria	AD8
gamma-proteobacteria	AD5	beta-proteobacteria	AD77
gamma-proteobacteria	AD5	gamma-proteobacteria	AD105
gamma-proteobacteria	AD5	alpha-proteobacteria	AD132
gamma-proteobacteria	AD5	beta-proteobacteria	AD145
gamma-proteobacteria	AD5	beta-proteobacteria	AD25
gamma-proteobacteria	AD5	beta-proteobacteria	AD009
gamma-proteobacteria	AD5	beta-proteobacteria	AD32
gamma-proteobacteria	AD5	Flavobacteria	AD48
gamma-proteobacteria	AD5	beta-proteobacteria	AD58
gamma-proteobacteria	AD5	beta-proteobacteria	AD73
gamma-proteobacteria	AD5	gamma-proteobacteria	AD79
gamma-proteobacteria	AD5	Bacilli	AD83
gamma-proteobacteria	AD5	Flavobacteria	AD134
gamma-proteobacteria	AD5	Flavobacteria	AD142
gamma-proteobacteria	AD5	beta-proteobacteria	AD37
gamma-proteobacteria	AD5	beta-proteobacteria	AD27
gamma-proteobacteria	AD5	beta-proteobacteria	AD24
gamma-proteobacteria	AD5	beta-proteobacteria	AD102
gamma-proteobacteria	AD5	Bacilli	AD116
gamma-proteobacteria	AD5	alpha-proteobacteria	AD152
gamma-proteobacteria	AD5	alpha-proteobacteria	AD136
gamma-proteobacteria	AD5	beta-proteobacteria	AD63
gamma-proteobacteria	AD5	Flavobacteria	AD41
gamma-proteobacteria	AD5	Bacilli	AD78
gamma-proteobacteria	AD5	beta-proteobacteria	AD81
gamma-proteobacteria	AD5	gamma-proteobacteria	AD104
gamma-proteobacteria	AD5	beta-proteobacteria	AD75
gamma-proteobacteria	AD5	beta-proteobacteria	AD3
gamma-proteobacteria	AD5	Actinobacteria	AD12
gamma-proteobacteria	AD5	Actinobacteria	AD108
gamma-proteobacteria	AD6	beta-proteobacteria	AD30
gamma-proteobacteria	AD6	gamma-proteobacteria	AD56
gamma-proteobacteria	AD6	Bacilli	AD78
gamma-proteobacteria	AD6	beta-proteobacteria	AD81
gamma-proteobacteria	AD6	beta-proteobacteria	AD133
gamma-proteobacteria	AD6	Actinobacteria	AD141
gamma-proteobacteria	AD6	beta-proteobacteria	AD28
gamma-proteobacteria	AD6	beta-proteobacteria	AD24
gamma-proteobacteria	AD6	beta-proteobacteria	AD11
gamma-proteobacteria	AD6	beta-proteobacteria	AD30
gamma-proteobacteria	AD6	gamma-proteobacteria	AD46
gamma-proteobacteria	AD6	beta-proteobacteria	AD72
gamma-proteobacteria	AD6	beta-proteobacteria	AD32
gamma-proteobacteria	AD6	beta-proteobacteria	AD99
gamma-proteobacteria	AD6	beta-proteobacteria	AD97
gamma-proteobacteria	AD6	Flavobacteria	AD151
gamma-proteobacteria	AD6	alpha-proteobacteria	AD140
gamma-proteobacteria	AD6	beta-proteobacteria	AD62
gamma-proteobacteria	AD6	beta-proteobacteria	AD39
gamma-proteobacteria	AD6	beta-proteobacteria	AD80

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
gamma-proteobacteria	AD6	beta-proteobacteria	AD103
gamma-proteobacteria	AD6	beta-proteobacteria	AD73
gamma-proteobacteria	AD6	beta-proteobacteria	AD7
gamma-proteobacteria	AD8	beta-proteobacteria	AD32
gamma-proteobacteria	AD8	beta-proteobacteria	AD58
gamma-proteobacteria	AD8	gamma-proteobacteria	AD4
gamma-proteobacteria	AD8	gamma-proteobacteria	AD5
gamma-proteobacteria	AD8	gamma-proteobacteria	AD79
gamma-proteobacteria	AD8	Bacilli	AD83
gamma-proteobacteria	AD8	Flavobacteria	AD134
gamma-proteobacteria	AD8	Flavobacteria	AD142
gamma-proteobacteria	AD8	beta-proteobacteria	AD27
gamma-proteobacteria	AD8	beta-proteobacteria	AD24
gamma-proteobacteria	AD8	beta-proteobacteria	AD28
gamma-proteobacteria	AD8	gamma-proteobacteria	AD52
gamma-proteobacteria	AD8	beta-proteobacteria	AD76
gamma-proteobacteria	AD8	gamma-proteobacteria	AD104
gamma-proteobacteria	AD8	Flavobacteria	AD131
gamma-proteobacteria	AD8	alpha-proteobacteria	AD140
gamma-proteobacteria	AD8	beta-proteobacteria	AD30
gamma-proteobacteria	AD8	Flavobacteria	AD41
gamma-proteobacteria	AD8	alpha-proteobacteria	AD34
gamma-proteobacteria	AD8	beta-proteobacteria	AD81
gamma-proteobacteria	AD8	beta-proteobacteria	AD77
gamma-proteobacteria	AD8	Actinobacteria	AD12
gamma-proteobacteria	AD8	gamma-proteobacteria	AD2
beta-proteobacteria	AD9	beta-proteobacteria	AD33
beta-proteobacteria	AD9	beta-proteobacteria	AD59
beta-proteobacteria	AD9	beta-proteobacteria	AD10
beta-proteobacteria	AD9	beta-proteobacteria	AD80
beta-proteobacteria	AD9	beta-proteobacteria	AD88
beta-proteobacteria	AD9	alpha-proteobacteria	AD136
beta-proteobacteria	AD9	Flavobacteria	AD91
beta-proteobacteria	AD9	beta-proteobacteria	AD26
beta-proteobacteria	AD9	beta-proteobacteria	AD32
beta-proteobacteria	AD9	alpha-proteobacteria	AD34
beta-proteobacteria	AD9	beta-proteobacteria	AD60
beta-proteobacteria	AD9	beta-proteobacteria	AD81
beta-proteobacteria	AD9	beta-proteobacteria	AD89
beta-proteobacteria	AD9	beta-proteobacteria	AD137
beta-proteobacteria	AD9	gamma-proteobacteria	AD157
beta-proteobacteria	AD9	beta-proteobacteria	AD25
beta-proteobacteria	AD9	Flavobacteria	AD42
beta-proteobacteria	AD9	Flavobacteria	AD43
beta-proteobacteria	AD9	beta-proteobacteria	AD82
beta-proteobacteria	AD9	Bacilli	AD83
beta-proteobacteria	AD9	Actinobacteria	AD31
beta-proteobacteria	AD9	Bacilli	AD50
gamma-proteobacteria	AD14	gamma-proteobacteria	AD36
gamma-proteobacteria	AD14	beta-proteobacteria	AD62
gamma-proteobacteria	AD14	Bacilli	AD83
gamma-proteobacteria	AD14	gamma-proteobacteria	AD100
gamma-proteobacteria	AD14	alpha-proteobacteria	AD140
gamma-proteobacteria	AD14	beta-proteobacteria	AD144
gamma-proteobacteria	AD14	Actinobacteria	AD22
gamma-proteobacteria	AD14	gamma-proteobacteria	AD20
gamma-proteobacteria	AD14	Flavobacteria	AD44
gamma-proteobacteria	AD14	Flavobacteria	AD42
gamma-proteobacteria	AD14	beta-proteobacteria	AD68
gamma-proteobacteria	AD14	beta-proteobacteria	AD24
gamma-proteobacteria	AD14	beta-proteobacteria	AD95

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
gamma-proteobacteria	AD14	Bacilli	AD117
gamma-proteobacteria	AD14	Flavobacteria	AD146
gamma-proteobacteria	AD14	alpha-proteobacteria	AD159
gamma-proteobacteria	AD14	gamma-proteobacteria	AD56
gamma-proteobacteria	AD14	Bacilli	AD87
gamma-proteobacteria	AD14	beta-proteobacteria	AD98
gamma-proteobacteria	AD14	beta-proteobacteria	AD69
gamma-proteobacteria	AD14	Actinobacteria	AD107
gamma-proteobacteria	AD14	Actinobacteria	AD12
gamma-proteobacteria	AD16	alpha-proteobacteria	AD38
gamma-proteobacteria	AD16	beta-proteobacteria	AD64
gamma-proteobacteria	AD16	gamma-proteobacteria	AD20
gamma-proteobacteria	AD16	Bacilli	AD87
gamma-proteobacteria	AD16	Bacilli	AD106
gamma-proteobacteria	AD16	Flavobacteria	AD142
gamma-proteobacteria	AD16	Flavobacteria	AD149
gamma-proteobacteria	AD16	gamma-proteobacteria	AD21
gamma-proteobacteria	AD16	beta-proteobacteria	AD19
gamma-proteobacteria	AD16	Flavobacteria	AD48
gamma-proteobacteria	AD16	Flavobacteria	AD41
gamma-proteobacteria	AD16	beta-proteobacteria	AD73
gamma-proteobacteria	AD16	beta-proteobacteria	AD67
gamma-proteobacteria	AD16	beta-proteobacteria	AD25
gamma-proteobacteria	AD16	Actinobacteria	AD94
gamma-proteobacteria	AD16	beta-proteobacteria	AD96
gamma-proteobacteria	AD16	beta-proteobacteria	AD145
gamma-proteobacteria	AD16	Flavobacteria	AD151
gamma-proteobacteria	AD16	beta-proteobacteria	AD54
gamma-proteobacteria	AD16	beta-proteobacteria	AD65
gamma-proteobacteria	AD16	beta-proteobacteria	AD89
gamma-proteobacteria	AD16	Bacilli	AD93
gamma-proteobacteria	AD16	beta-proteobacteria	AD68
gamma-proteobacteria	AD16	Actinobacteria	AD110
gamma-proteobacteria	AD16	beta-proteobacteria	AD7
gamma-proteobacteria	AD17	beta-proteobacteria	AD39
gamma-proteobacteria	AD17	beta-proteobacteria	AD65
gamma-proteobacteria	AD17	gamma-proteobacteria	AD21
gamma-proteobacteria	AD17	Actinobacteria	AD92
gamma-proteobacteria	AD17	alpha-proteobacteria	AD113
gamma-proteobacteria	AD17	beta-proteobacteria	AD143
gamma-proteobacteria	AD17	alpha-proteobacteria	AD153
gamma-proteobacteria	AD17	beta-proteobacteria	AD19
gamma-proteobacteria	AD17	beta-proteobacteria	AD18
gamma-proteobacteria	AD17	alpha-proteobacteria	AD51
gamma-proteobacteria	AD17	gamma-proteobacteria	AD40
gamma-proteobacteria	AD17	beta-proteobacteria	AD66
gamma-proteobacteria	AD17	beta-proteobacteria	AD19
gamma-proteobacteria	AD17	Bacilli	AD93
gamma-proteobacteria	AD17	gamma-proteobacteria	AD114
gamma-proteobacteria	AD17	beta-proteobacteria	AD144
gamma-proteobacteria	AD17	alpha-proteobacteria	AD152
gamma-proteobacteria	AD17	gamma-proteobacteria	AD52
gamma-proteobacteria	AD17	Flavobacteria	AD91
gamma-proteobacteria	AD17	beta-proteobacteria	AD67
gamma-proteobacteria	AD17	beta-proteobacteria	AD118
gamma-proteobacteria	AD17	beta-proteobacteria	AD119
beta-proteobacteria	AD19	Flavobacteria	AD41
beta-proteobacteria	AD19	beta-proteobacteria	AD67
beta-proteobacteria	AD19	beta-proteobacteria	AD18
beta-proteobacteria	AD19	beta-proteobacteria	AD25
beta-proteobacteria	AD19	Actinobacteria	AD94
beta-proteobacteria	AD19	beta-proteobacteria	AD96

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
beta-proteobacteria	AD19	beta-proteobacteria	AD145
beta-proteobacteria	AD19	Flavobacteria	AD151
beta-proteobacteria	AD19	gamma-proteobacteria	AD16
beta-proteobacteria	AD19	gamma-proteobacteria	AD17
beta-proteobacteria	AD19	beta-proteobacteria	AD54
beta-proteobacteria	AD19	gamma-proteobacteria	AD40
beta-proteobacteria	AD19	beta-proteobacteria	AD39
beta-proteobacteria	AD19	beta-proteobacteria	AD66
beta-proteobacteria	AD19	beta-proteobacteria	AD65
beta-proteobacteria	AD19	Bacilli	AD93
beta-proteobacteria	AD19	gamma-proteobacteria	AD114
beta-proteobacteria	AD19	beta-proteobacteria	AD144
beta-proteobacteria	AD19	alpha-proteobacteria	AD152
beta-proteobacteria	AD19	gamma-proteobacteria	AD21
beta-proteobacteria	AD19	gamma-proteobacteria	AD52
beta-proteobacteria	AD19	Actinobacteria	AD92
beta-proteobacteria	AD19	alpha-proteobacteria	AD113
beta-proteobacteria	AD19	beta-proteobacteria	AD143
beta-proteobacteria	AD19	alpha-proteobacteria	AD153
beta-proteobacteria	AD19	alpha-proteobacteria	AD51
beta-proteobacteria	AD19	beta-proteobacteria	AD68
beta-proteobacteria	AD19	Flavobacteria	AD91
beta-proteobacteria	AD19	beta-proteobacteria	AD7
beta-proteobacteria	AD19	beta-proteobacteria	AD119
beta-proteobacteria	AD19	beta-proteobacteria	AD118
beta-proteobacteria	AD35	beta-proteobacteria	AD11
beta-proteobacteria	AD35	beta-proteobacteria	AD61
beta-proteobacteria	AD35	beta-proteobacteria	AD15
beta-proteobacteria	AD35	beta-proteobacteria	AD82
beta-proteobacteria	AD35	beta-proteobacteria	AD98
beta-proteobacteria	AD35	beta-proteobacteria	AD138
beta-proteobacteria	AD35	beta-proteobacteria	AD143
beta-proteobacteria	AD35	beta-proteobacteria	AD23
beta-proteobacteria	AD35	beta-proteobacteria	AD61
beta-proteobacteria	AD35	Flavobacteria	AD45
beta-proteobacteria	AD35	Flavobacteria	AD86
beta-proteobacteria	AD35	beta-proteobacteria	AD55
beta-proteobacteria	AD39	gamma-proteobacteria	AD17
beta-proteobacteria	AD39	beta-proteobacteria	AD26
beta-proteobacteria	AD39	beta-proteobacteria	AD65
beta-proteobacteria	AD39	gamma-proteobacteria	AD21
beta-proteobacteria	AD39	alpha-proteobacteria	AD51
beta-proteobacteria	AD39	Actinobacteria	AD92
beta-proteobacteria	AD39	alpha-proteobacteria	AD113
beta-proteobacteria	AD39	beta-proteobacteria	AD143
beta-proteobacteria	AD39	alpha-proteobacteria	AD153
beta-proteobacteria	AD39	beta-proteobacteria	AD19
beta-proteobacteria	AD39	alpha-proteobacteria	AD51
beta-proteobacteria	AD39	beta-proteobacteria	AD74
beta-proteobacteria	AD39	beta-proteobacteria	AD103
beta-proteobacteria	AD39	gamma-proteobacteria	AD120
beta-proteobacteria	AD39	beta-proteobacteria	AD137
beta-proteobacteria	AD39	gamma-proteobacteria	AD4
beta-proteobacteria	AD39	beta-proteobacteria	AD64
beta-proteobacteria	AD39	gamma-proteobacteria	AD6
beta-proteobacteria	AD39	beta-proteobacteria	AD66
beta-proteobacteria	AD39	beta-proteobacteria	AD30
beta-proteobacteria	AD39	gamma-proteobacteria	AD56
beta-proteobacteria	AD39	beta-proteobacteria	AD76
beta-proteobacteria	AD39	Bacilli	AD78
beta-proteobacteria	AD39	beta-proteobacteria	AD81
beta-proteobacteria	AD39	beta-proteobacteria	AD133

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
beta-proteobacteria	AD39	Actinobacteria	AD141
beta-proteobacteria	AD39	beta-proteobacteria	AD11
beta-proteobacteria	AD39	Flavobacteria	AD91
beta-proteobacteria	AD39	gamma-proteobacteria	AD105
beta-proteobacteria	AD39	beta-proteobacteria	AD28
beta-proteobacteria	AD39	beta-proteobacteria	AD118
beta-proteobacteria	AD39	Actinobacteria	AD110
beta-proteobacteria	AD54	gamma-proteobacteria	AD5
beta-proteobacteria	AD54	beta-proteobacteria	AD19
beta-proteobacteria	AD54	Actinobacteria	AD29
beta-proteobacteria	AD54	Flavobacteria	AD41
beta-proteobacteria	AD54	gamma-proteobacteria	AD4
beta-proteobacteria	AD54	beta-proteobacteria	AD77
beta-proteobacteria	AD54	gamma-proteobacteria	AD105
beta-proteobacteria	AD54	alpha-proteobacteria	AD132
beta-proteobacteria	AD54	beta-proteobacteria	AD145
beta-proteobacteria	AD54	beta-proteobacteria	AD67
beta-proteobacteria	AD54	beta-proteobacteria	AD009
beta-proteobacteria	AD54	beta-proteobacteria	AD25
beta-proteobacteria	AD54	Actinobacteria	AD94
beta-proteobacteria	AD54	beta-proteobacteria	AD96
beta-proteobacteria	AD54	Flavobacteria	AD151
beta-proteobacteria	AD54	gamma-proteobacteria	AD16
beta-proteobacteria	AD54	beta-proteobacteria	AD37
beta-proteobacteria	AD54	beta-proteobacteria	AD68
beta-proteobacteria	AD54	Bacilli	AD78
beta-proteobacteria	AD54	beta-proteobacteria	AD3
beta-proteobacteria	AD54	beta-proteobacteria	AD7
beta-proteobacteria	AD64	gamma-proteobacteria	AD16
beta-proteobacteria	AD64	beta-proteobacteria	AD26
beta-proteobacteria	AD64	alpha-proteobacteria	AD38
beta-proteobacteria	AD64	alpha-proteobacteria	AD51
beta-proteobacteria	AD64	gamma-proteobacteria	AD20
beta-proteobacteria	AD64	Bacilli	AD87
beta-proteobacteria	AD64	Bacilli	AD106
beta-proteobacteria	AD64	Flavobacteria	AD142
beta-proteobacteria	AD64	Flavobacteria	AD149
beta-proteobacteria	AD64	gamma-proteobacteria	AD21
beta-proteobacteria	AD64	beta-proteobacteria	AD74
beta-proteobacteria	AD64	Flavobacteria	AD48
beta-proteobacteria	AD64	beta-proteobacteria	AD39
beta-proteobacteria	AD64	beta-proteobacteria	AD103
beta-proteobacteria	AD64	gamma-proteobacteria	AD120
beta-proteobacteria	AD64	alpha-proteobacteria	AD153
beta-proteobacteria	AD64	beta-proteobacteria	AD137
beta-proteobacteria	AD64	gamma-proteobacteria	AD4
beta-proteobacteria	AD64	beta-proteobacteria	AD65
beta-proteobacteria	AD64	beta-proteobacteria	AD76
beta-proteobacteria	AD64	beta-proteobacteria	AD89
beta-proteobacteria	AD64	beta-proteobacteria	AD123
beta-proteobacteria	AD64	Actinobacteria	AD110
beta-proteobacteria	AD66	beta-proteobacteria	AD18
beta-proteobacteria	AD66	gamma-proteobacteria	AD40
beta-proteobacteria	AD66	beta-proteobacteria	AD19
beta-proteobacteria	AD66	Bacilli	AD93
beta-proteobacteria	AD66	gamma-proteobacteria	AD114
beta-proteobacteria	AD66	beta-proteobacteria	AD144
beta-proteobacteria	AD66	alpha-proteobacteria	AD152
beta-proteobacteria	AD66	gamma-proteobacteria	AD17
beta-proteobacteria	AD66	gamma-proteobacteria	AD52
beta-proteobacteria	AD66	beta-proteobacteria	AD39
beta-proteobacteria	AD66	beta-proteobacteria	AD67

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
beta-proteobacteria	AD66	beta-proteobacteria	AD65
beta-proteobacteria	AD66	gamma-proteobacteria	AD21
beta-proteobacteria	AD66	Actinobacteria	AD92
beta-proteobacteria	AD66	alpha-proteobacteria	AD113
beta-proteobacteria	AD66	beta-proteobacteria	AD143
beta-proteobacteria	AD66	alpha-proteobacteria	AD153
beta-proteobacteria	AD66	alpha-proteobacteria	AD51
beta-proteobacteria	AD66	gamma-proteobacteria	AD125
beta-proteobacteria	AD66	beta-proteobacteria	AD119
gamma-proteobacteria	AD79	gamma-proteobacteria	AD8
gamma-proteobacteria	AD79	beta-proteobacteria	AD32
gamma-proteobacteria	AD79	beta-proteobacteria	AD58
gamma-proteobacteria	AD79	gamma-proteobacteria	AD5
gamma-proteobacteria	AD79	Bacilli	AD83
gamma-proteobacteria	AD79	Flavobacteria	AD134
gamma-proteobacteria	AD79	Flavobacteria	AD142
gamma-proteobacteria	AD79	beta-proteobacteria	AD27
gamma-proteobacteria	AD79	beta-proteobacteria	AD24
gamma-proteobacteria	AD79	Flavobacteria	AD41
gamma-proteobacteria	AD79	beta-proteobacteria	AD81
gamma-proteobacteria	AD79	beta-proteobacteria	AD130
gamma-proteobacteria	AD79	Actinobacteria	AD12
beta-proteobacteria	AD81	beta-proteobacteria	AD10
beta-proteobacteria	AD81	gamma-proteobacteria	AD6
beta-proteobacteria	AD81	alpha-proteobacteria	AD34
beta-proteobacteria	AD81	beta-proteobacteria	AD30
beta-proteobacteria	AD81	beta-proteobacteria	AD60
beta-proteobacteria	AD81	gamma-proteobacteria	AD56
beta-proteobacteria	AD81	beta-proteobacteria	AD9
beta-proteobacteria	AD81	Bacilli	AD78
beta-proteobacteria	AD81	beta-proteobacteria	AD89
beta-proteobacteria	AD81	beta-proteobacteria	AD137
beta-proteobacteria	AD81	gamma-proteobacteria	AD157
beta-proteobacteria	AD81	beta-proteobacteria	AD25
beta-proteobacteria	AD81	beta-proteobacteria	AD33
beta-proteobacteria	AD81	beta-proteobacteria	AD133
beta-proteobacteria	AD81	Actinobacteria	AD141
beta-proteobacteria	AD81	beta-proteobacteria	AD28
beta-proteobacteria	AD81	beta-proteobacteria	AD11
beta-proteobacteria	AD81	Flavobacteria	AD43
beta-proteobacteria	AD81	beta-proteobacteria	AD39
beta-proteobacteria	AD81	beta-proteobacteria	AD89
beta-proteobacteria	AD81	beta-proteobacteria	AD32
beta-proteobacteria	AD81	beta-proteobacteria	AD58
beta-proteobacteria	AD81	gamma-proteobacteria	AD5
beta-proteobacteria	AD81	Bacilli	AD83
beta-proteobacteria	AD81	gamma-proteobacteria	AD79
beta-proteobacteria	AD81	beta-proteobacteria	AD80
beta-proteobacteria	AD81	Flavobacteria	AD134
beta-proteobacteria	AD81	Flavobacteria	AD142
beta-proteobacteria	AD81	beta-proteobacteria	AD27
beta-proteobacteria	AD81	Bacilli	AD50
beta-proteobacteria	AD81	beta-proteobacteria	AD7
beta-proteobacteria	AD81	Actinobacteria	AD12
beta-proteobacteria	AD82	beta-proteobacteria	AD11
beta-proteobacteria	AD82	beta-proteobacteria	AD35
beta-proteobacteria	AD82	beta-proteobacteria	AD61
beta-proteobacteria	AD82	beta-proteobacteria	AD15
beta-proteobacteria	AD82	beta-proteobacteria	AD98
beta-proteobacteria	AD82	beta-proteobacteria	AD138
beta-proteobacteria	AD82	beta-proteobacteria	AD143

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
beta-proteobacteria	AD82	beta-proteobacteria	AD23
beta-proteobacteria	AD82	Flavobacteria	AD45
beta-proteobacteria	AD82	beta-proteobacteria	AD9
beta-proteobacteria	AD82	beta-proteobacteria	AD33
beta-proteobacteria	AD82	beta-proteobacteria	AD59
beta-proteobacteria	AD82	beta-proteobacteria	AD10
beta-proteobacteria	AD82	Flavobacteria	AD86
beta-proteobacteria	AD82	beta-proteobacteria	AD80
beta-proteobacteria	AD82	beta-proteobacteria	AD88
beta-proteobacteria	AD82	alpha-proteobacteria	AD136
beta-proteobacteria	AD82	Flavobacteria	AD91
beta-proteobacteria	AD82	beta-proteobacteria	AD26
beta-proteobacteria	AD82	beta-proteobacteria	AD55
beta-proteobacteria	AD82	Actinobacteria	AD31
beta-proteobacteria	AD103	beta-proteobacteria	AD26
beta-proteobacteria	AD103	alpha-proteobacteria	AD51
beta-proteobacteria	AD103	beta-proteobacteria	AD74
beta-proteobacteria	AD103	beta-proteobacteria	AD39
beta-proteobacteria	AD103	gamma-proteobacteria	AD120
beta-proteobacteria	AD103	alpha-proteobacteria	AD153
beta-proteobacteria	AD103	beta-proteobacteria	AD137
beta-proteobacteria	AD103	gamma-proteobacteria	AD4
beta-proteobacteria	AD103	beta-proteobacteria	AD64
beta-proteobacteria	AD103	beta-proteobacteria	AD76
beta-proteobacteria	AD103	beta-proteobacteria	AD24
beta-proteobacteria	AD103	gamma-proteobacteria	AD46
beta-proteobacteria	AD103	beta-proteobacteria	AD72
beta-proteobacteria	AD103	beta-proteobacteria	AD32
beta-proteobacteria	AD103	gamma-proteobacteria	AD105
beta-proteobacteria	AD103	beta-proteobacteria	AD99
beta-proteobacteria	AD103	beta-proteobacteria	AD97
beta-proteobacteria	AD103	Flavobacteria	AD151
beta-proteobacteria	AD103	alpha-proteobacteria	AD140
beta-proteobacteria	AD103	gamma-proteobacteria	AD6
beta-proteobacteria	AD103	Actinobacteria	AD110
beta-proteobacteria	AD103	Actinobacteria	AD107
beta-proteobacteria	AD144	beta-proteobacteria	AD18
beta-proteobacteria	AD144	gamma-proteobacteria	AD14
beta-proteobacteria	AD144	gamma-proteobacteria	AD40
beta-proteobacteria	AD144	gamma-proteobacteria	AD36
beta-proteobacteria	AD144	beta-proteobacteria	AD66
beta-proteobacteria	AD144	beta-proteobacteria	AD62
beta-proteobacteria	AD144	beta-proteobacteria	AD19
beta-proteobacteria	AD144	Bacilli	AD93
beta-proteobacteria	AD144	Bacilli	AD83
beta-proteobacteria	AD144	gamma-proteobacteria	AD114
beta-proteobacteria	AD144	gamma-proteobacteria	AD100
beta-proteobacteria	AD144	alpha-proteobacteria	AD140
beta-proteobacteria	AD144	alpha-proteobacteria	AD152
beta-proteobacteria	AD144	gamma-proteobacteria	AD17
beta-proteobacteria	AD144	gamma-proteobacteria	AD52
beta-proteobacteria	AD144	Actinobacteria	AD22
beta-proteobacteria	AD144	Flavobacteria	AD44
beta-proteobacteria	AD144	beta-proteobacteria	AD67
beta-proteobacteria	AD144	Actinobacteria	AD92
beta-proteobacteria	AD144	Bacilli	AD87
gamma-proteobacteria	AD20	Flavobacteria	AD42
gamma-proteobacteria	AD20	beta-proteobacteria	AD68
gamma-proteobacteria	AD20	gamma-proteobacteria	AD16
gamma-proteobacteria	AD20	beta-proteobacteria	AD24
gamma-proteobacteria	AD20	beta-proteobacteria	AD95

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
gamma-proteobacteria	AD20	Bacilli	AD117
gamma-proteobacteria	AD20	Flavobacteria	AD146
gamma-proteobacteria	AD20	alpha-proteobacteria	AD159
gamma-proteobacteria	AD20	gamma-proteobacteria	AD14
gamma-proteobacteria	AD20	beta-proteobacteria	AD15
gamma-proteobacteria	AD20	gamma-proteobacteria	AD56
gamma-proteobacteria	AD20	alpha-proteobacteria	AD38
gamma-proteobacteria	AD20	beta-proteobacteria	AD37
gamma-proteobacteria	AD20	beta-proteobacteria	AD64
gamma-proteobacteria	AD20	beta-proteobacteria	AD74
gamma-proteobacteria	AD20	beta-proteobacteria	AD63
gamma-proteobacteria	AD20	Bacilli	AD87
gamma-proteobacteria	AD20	Bacilli	AD106
gamma-proteobacteria	AD20	Flavobacteria	AD142
gamma-proteobacteria	AD20	Flavobacteria	AD149
gamma-proteobacteria	AD20	beta-proteobacteria	AD27
gamma-proteobacteria	AD20	gamma-proteobacteria	AD21
gamma-proteobacteria	AD20	Flavobacteria	AD48
gamma-proteobacteria	AD20	Flavobacteria	AD84
gamma-proteobacteria	AD20	beta-proteobacteria	AD101
gamma-proteobacteria	AD20	Actinobacteria	AD141
gamma-proteobacteria	AD20	Flavobacteria	AD47
gamma-proteobacteria	AD20	beta-proteobacteria	AD69
gamma-proteobacteria	AD20	beta-proteobacteria	AD65
gamma-proteobacteria	AD20	beta-proteobacteria	AD98
gamma-proteobacteria	AD20	beta-proteobacteria	AD89
gamma-proteobacteria	AD20	beta-proteobacteria	AD88
gamma-proteobacteria	AD20	Actinobacteria	AD12
gamma-proteobacteria	AD20	Actinobacteria	AD110
gamma-proteobacteria	AD20	Actinobacteria	AD108
gamma-proteobacteria	AD40	beta-proteobacteria	AD18
gamma-proteobacteria	AD40	beta-proteobacteria	AD66
gamma-proteobacteria	AD40	beta-proteobacteria	AD19
gamma-proteobacteria	AD40	Bacilli	AD93
gamma-proteobacteria	AD40	gamma-proteobacteria	AD114
gamma-proteobacteria	AD40	beta-proteobacteria	AD144
gamma-proteobacteria	AD40	alpha-proteobacteria	AD152
gamma-proteobacteria	AD40	gamma-proteobacteria	AD17
gamma-proteobacteria	AD40	gamma-proteobacteria	AD52
gamma-proteobacteria	AD40	beta-proteobacteria	AD67
gamma-proteobacteria	AD40	Actinobacteria	AD92
gamma-proteobacteria	AD40	beta-proteobacteria	AD119
gamma-proteobacteria	AD52	gamma-proteobacteria	AD4
gamma-proteobacteria	AD52	beta-proteobacteria	AD18
gamma-proteobacteria	AD52	beta-proteobacteria	AD28
gamma-proteobacteria	AD52	gamma-proteobacteria	AD40
gamma-proteobacteria	AD52	gamma-proteobacteria	AD8
gamma-proteobacteria	AD52	beta-proteobacteria	AD76
gamma-proteobacteria	AD52	gamma-proteobacteria	AD104
gamma-proteobacteria	AD52	Flavobacteria	AD131
gamma-proteobacteria	AD52	alpha-proteobacteria	AD140
gamma-proteobacteria	AD52	beta-proteobacteria	AD30
gamma-proteobacteria	AD52	beta-proteobacteria	AD66
gamma-proteobacteria	AD52	gamma-proteobacteria	AD8
gamma-proteobacteria	AD52	beta-proteobacteria	AD19
gamma-proteobacteria	AD52	Bacilli	AD93
gamma-proteobacteria	AD52	gamma-proteobacteria	AD114
gamma-proteobacteria	AD52	beta-proteobacteria	AD144
gamma-proteobacteria	AD52	alpha-proteobacteria	AD152
gamma-proteobacteria	AD52	gamma-proteobacteria	AD17
gamma-proteobacteria	AD52	alpha-proteobacteria	AD34
gamma-proteobacteria	AD52	beta-proteobacteria	AD67

Table S2.10 continuation

Phylogenetic class	Strain code genus A	Phylogenetic class	Strain code genus B
gamma-proteobacteria	AD52	beta-proteobacteria	AD77
gamma-proteobacteria	AD52	gamma-proteobacteria	AD2
alpha-proteobacteria	AD51	beta-proteobacteria	AD26
alpha-proteobacteria	AD51	gamma-proteobacteria	AD17
alpha-proteobacteria	AD51	beta-proteobacteria	AD74
alpha-proteobacteria	AD51	beta-proteobacteria	AD39
alpha-proteobacteria	AD51	beta-proteobacteria	AD103
alpha-proteobacteria	AD51	gamma-proteobacteria	AD120
alpha-proteobacteria	AD51	alpha-proteobacteria	AD153
alpha-proteobacteria	AD51	beta-proteobacteria	AD137
alpha-proteobacteria	AD51	gamma-proteobacteria	AD4
alpha-proteobacteria	AD51	beta-proteobacteria	AD39
alpha-proteobacteria	AD51	beta-proteobacteria	AD64
alpha-proteobacteria	AD51	beta-proteobacteria	AD65
alpha-proteobacteria	AD51	gamma-proteobacteria	AD21
alpha-proteobacteria	AD51	Actinobacteria	AD92
alpha-proteobacteria	AD51	alpha-proteobacteria	AD113
alpha-proteobacteria	AD51	beta-proteobacteria	AD143
alpha-proteobacteria	AD51	beta-proteobacteria	AD19
alpha-proteobacteria	AD51	beta-proteobacteria	AD76
alpha-proteobacteria	AD51	beta-proteobacteria	AD66
alpha-proteobacteria	AD51	gamma-proteobacteria	AD105
alpha-proteobacteria	AD51	beta-proteobacteria	AD118

Chapter 3

Volatiles in interspecific bacterial interactions

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Abstract

The importance of volatile organic compounds for functioning of microbes is receiving increased research attention. However, to date very little is known on how interspecific bacterial interactions effect volatiles production as most studies have been focused on volatiles produced by monocultures of well described bacterial genera. In this study we aimed to understand how interspecific bacterial interactions affect the composition, production and activity of volatiles. Four phylogenetically different bacterial species namely: *Chryseobacterium*, *Dyella*, *Janthinobacterium* and *Tsukamurella* were selected. Earlier results had shown that pairwise combinations of these bacteria induced antimicrobial activity in agar media whereas this was not the case for monocultures. In the current study, we examined if these observations were also reflected by the production of antimicrobial volatiles. Thus, the identity and antimicrobial activity of volatiles produced by the bacteria were determined in monoculture as well in pairwise combinations. Antimicrobial activity of the volatiles was assessed against fungal, oomycetal and bacterial model organisms.

Our results revealed that interspecific bacterial interactions affected volatiles blend composition. Fungi and oomycetes showed high sensitivity to bacterial volatiles whereas the effect of volatiles on bacteria varied between no effects, growth inhibition to growth promotion depending on the volatile blend composition. In total 35 volatile compounds were detected most of which were sulfur-containing compounds. Two commonly produced sulfur-containing volatile compounds (dimethyl disulfide and dimethyl trisulfide) were tested for their effect on three target bacteria.

Here we display the importance of interspecific interactions on bacterial volatiles production and their antimicrobial activities.

Introduction

Soil bacteria produce an astounding array of secondary metabolites. Gaseous secondary metabolites, commonly known as volatile organic compounds (VOCs) are small molecules (<300 Da) belonging to different chemical classes that can evaporate and diffuse easily through air- and water-filled pores (Schulz and Dickschat, 2007;Penuelas et al., 2014). These physiochemical properties make volatiles ideal metabolites for communication and antagonistic interactions between soil microorganisms living at a certain distance from each other. Indeed, recent studies indicate that soil microorganisms can employ volatile compounds as info-chemicals, growth stimulants, growth inhibitors and inhibitors of quorum-sensing (Kai et al., 2009;Chernin et al., 2011;Effmert et al., 2012;Kim et al., 2013). Furthermore, rhizosphere bacteria emit volatiles that can promote plant growth and elicit induced systemic resistance (ISR) and induced systemic tolerance (IST) in plants (Ryu et al., 2003;Ryu et al., 2004). However, the role of volatiles in competitive interactions between soil bacteria is so far poorly understood.

In the past few years the research on volatiles emitted by bacteria received increased attention from a more applied point of view as these compounds have intriguing properties which are of great interest for agriculture (pathogen suppression), food preparation (aroma) and cosmetics industry (perfume odors) (Krings and Berger, 1998;Wheatley, 2002;Beshkova et al., 2003;Schwab et al., 2008;Deetae et al., 2009;Effmert et al., 2012;Kanchiswamy et al., 2015).

Bacterial volatiles belong to different chemical classes like alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids and esters. However, the composition of emitted volatiles (volatile blend composition) may vary with cultivation conditions, in particular with respect to the substrate composition of the growth media (Cleason, 2006;Blom et al., 2011;Groenhagen et al., 2013;Garbeva et al., 2014a). Other factors known to influence volatile production are microbial physiological state, oxygen availability, moisture, temperature and pH (Bjurman, 2007;Insam and Seewald, 2010;Romoli et al., 2014).

The technical developments that have been made in recent years in the field of mass spectrometry have led to the improvement of volatile compounds detection. The details of these developments have recently been summarized

by Carter (Carter, 2014). However, the main challenge in volatolomics is the ability to identify and quantify the entire set of emitted volatiles. The detected volatile blends are mostly quite complex and make the identification of biologically relevant volatiles a demanding and challenging task (Farag et al., 2012;Tait et al., 2014).

To date more than over 1000 microbial volatiles are reported and described in a special database for microbial volatile organic compounds called mVOC (http://bioinformatics.charite.de/mvoc/) (Lemfack et al., 2014). Nevertheless, this number is rather low compared to the high diversity of bacterial taxa in soil, suggesting a big underestimation of the actual real number of microbial volatiles (Kai et al., 2009;Lemfack et al., 2014). Moreover, most of the studies on microbial volatile detection have dealt with monocultures of already well-described bacterial genera. Thus, very little is known on how interspecific interactions affect the volatile production. The investigation of volatiles production in more complex communities is of great interest since it could help to reveal the ecological role of these compounds. In the last years several independent studies reported that the production of secondary metabolites by soil bacteria can be influenced by interactions with microorganisms in their vicinity (Garbeva et al., 2011b;Traxler et al., 2013;Tyc et al., 2014). A high-throughput screening performed recently in our lab revealed that interactions between soil bacterial species have major effects in both directions: induction and suppression of antimicrobial activity (Tyc et al., 2014).

In this study we aimed to understand how interspecific bacterial interactions affect the emission of volatiles and their activity. For this we selected four strains belonging to different bacteria species that have been isolated from the soil bacterial community associated with sand sedge (*Carex arenaria* L.) namely *Chryseobacterium* sp. AD48, *Dyella* sp. AD56, *Janthinobacterium* sp. AD80 and *Tsukamurella* sp. AD106 (Tyc et al., 2014). In an earlier screening it was observed that these bacteria showed induced antimicrobial activity during interactions but not in monocultures. In the current study, it was examined if these observations were also reflected by the volatiles emission. To this end the effects of volatiles on growth of fungal, oomycetal and bacterial model organisms produced by the bacteria in monocultures as well in pairwise combinations were tested. Our overall hypothesis is that the blend composition volatiles produced during interactions differs from that of monocultures and consequently has different effect on model target organisms.

Materials and methods

Bacteria & culture conditions

The bacterial isolates applied in this work were selected based on a previous observations of antimicrobial activity triggered by interspecific interactions (Tyc et al., 2014). Four bacterial isolates were used: *Chryseobacterium* sp. AD48 (Class: Flavobacteriia) GenBank: KJ685263, *Dyella* sp. AD56 (Class: *Gammaproteobacteria*) GenBank: KJ685269, *Janthinobacterium* sp. AD80 (Class: *Betaproteobacteria*) GenBank: KJ685292 and *Tsukamurella* sp. AD106 (Class: *Actinobacteria*) GenBank: KJ685317. The bacterial isolates were pre-cultured from -80 °C glycerol stocks on 1/10th TSBA (5.0 gL^{-1} NaCl, 1.0 gL^{-1} KH_2PO_4; 3 gL^{-1} Oxoid Tryptic Soy Broth; 20 gL^{-1} Merck Agar, pH 6.5) (Garbeva and de Boer, 2009) and incubated for three days at 24 °C before starting the experiments.

To test the effect of bacterial volatile compounds on bacterial growth and colony morphology three indicator bacteria were used: *E. coli* WA321, *S. aureus* 533R4 (Meyer and Schleifer, 1978;Tyc et al., 2014) and *S. marcescens* P87 (Garbeva et al., 2014b). All three indicator bacteria were pre-cultured from -80 °C glycerol stocks either on LB-A media (LB-Medium Lennox, Carl Roth GmbH + Co. KG, Netherlands, art.no. X964.2, 20 gL^{-1} Merck Agar) (*E. coli* WA321 and *S. aureus* 533R4) (Sambrook and Russell, 2001) or on 1/10th TSBA (*S. marcescens* P87). The indicator organisms *E. coli* and *S. aureus* were incubated overnight at 37 °C prior application, *S. marcescens* P87 was incubated at 24 °C for four days prior usage. All bacterial isolates used in this study are listed in **Table 3. 1**.

Cultures and growth conditions of fungi and oomycetes

The fungi *Rhizoctonia solani* AG2.2IIIB and *Fusarium culmorum* PV and the oomycete *Pythium ultimum* P17 were used in this study (Garbeva et al., 2014b). The fungi and oomycete were pre-cultured on 1/5th Potato Dextrose Agar (PDA) (29 gL^{-1} Oxoid CM 139) (Fiddaman and Rossall, 1993) and incubated at 24 °C for 7 days prior usage. All fungal and oomycetal organisms are listed in **Table 3.1**.

Table3. 1: Bacterial, fungal and oomycetal organisms used in this study.

Strain	Phylum/class	Genbank	Reference	Function
volatile producing bacteria tested				
Chryseobacterium sp. AD48	Flavobacteriia	KJ685263	Tyc *et al.* 2014	used for volatile analysis
Dyella sp. AD56	γ-proteobacteria	KJ685269	Tyc *et al.* 2014	
Janthinobacterium sp. AD80	β-proteobacteria	KJ685292	Tyc *et al.* 2014	
Tsukamurella sp. AD106	Actinobacteria	KJ685317	Tyc *et al.* 2014	
fungal / oomycetal test organisms				
Rhizoctonia solani AG2.2IIIB	Basidiomycota	KT124637	Garbeva *et al*. 2011	eukaryotic model organisms for growth inhibition
Pythium ultimum P17	Oomycete	KT124638	Garbeva *et al.* 2014	
Fusarium culmorum PV	Ascomycota	-	Garbeva *et al.* 2014	
bacterial test organisms				
Serratia marcescens P87	γ-proteobacteria	-	Garbeva *et al.* 2014	bacterial model organisms for growth inhibition and colony morphology changes
Escherichia coli WA321 DSMZ 4509	γ-proteobacteria	-	Tyc *et al.* 2014	
Staphylococcus aureus 533R4 Serovar 3 DSMZ 20231	Firmicutes	LN681573	Meyer *et al.* 1978	

Experimental treatments

Ten different treatments were performed in triplicates. These treatments were: monoculture 1 (*Chryseobacterium* sp. AD48), monoculture 2 (*Tsukamurella* sp. AD106), monoculture 3 (*Dyella* sp. AD56), monoculture 4 (*Janthinobacterium* sp. AD80) and pairwise interaction of the isolates: interaction 1 (*Chryseobacterium* sp. AD48 + *Tsukamurella* AD106), interaction 2 (*Dyella* sp. AD56 + *Janthinobacterium* sp. AD80), Control 1 (glass Petri dish with TSBA media without inoculated bacteria, as background control in GC/MS measurement), Control 2 (two compartment Petri dish inoculated with model organisms without exposure to bacterial volatiles), Control 3 (top bottom Petri dish inoculated with fungal/oomycetal model organisms without exposure to bacterial volatile compounds). Control 4 (two compartment Petri dish inoculated with model organisms without exposure to the tested pure volatile compounds). The effect of the produced volatiles was tested on fungal, oomycetal and bacterial growth via determination of hypha biomass or growth inhibition assays. For the inoculation of the experiments a single colony of each test isolate was picked from a plate and inoculated in 20 mL 1/10th TSB (5.0 gL^{-1} NaCl, 1.0 gL^{-1} KH_2PO_4; 3 gL^{-1} Oxoid Tryptic Soy Broth) and incubated overnight at 24 °C, 220 rpm. On the next day the OD_{600} of each isolate was measured on a GENESYS™ 20 spectrophotometer (Thermoscientific, Netherlands, Cat# 4001-000) and a inoculation suspension

for each treatment was prepared in 20 mL of 10mM P-Buffer (pH 6.5) containing bacterial cells in a concentration of ~ 1 x 10^5 CFU/mL.

Volatile trapping

Next to the inhibition experiments, bacterial volatiles emitted in monocultures and pairwise combinations were trapped and analyzed. For trapping of volatile organic compounds emitted by bacteria a volume of 100 µl of inoculation suspension was spread on 1/10th TSBA (20 mL) in glass Petri dishes designed for headspace volatile trapping (Garbeva et al., 2014b). The Petri dishes were closed by a lid with an outlet connected to a steel trap containing 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) (**Figure S3.1**). All treatments were inoculated in triplicate. The volatiles were collected after 48 and 72 hrs. of incubation and the Tenax steel traps were stored at 4 °C until GC-Q-TOF analysis.

GC-Q-TOF analysis

The trapped volatile organic compounds were desorbed from the traps using an automated thermodesorption unit (Unity TD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (He flow 50 mL/min) and trapped on a cold trap at -10 °C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA) by heating the cold trap for 3 min to 280 °C. Split ratio was set to 1:10, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, Bellefonte, PA, USA). Temperature program used was as follows: 39 °C for 2 min, from 39 °C to 95 °C at 3.5 °C/min, then to 165 °C at 6 °C/min, to 250 °C at 15 °C/min and finally to 300 °C at 40 °C/min, hold 20 min. The MS operating at 70 eV in EI mode detected the VOCs. Mass spectra were acquired in full-scan-mode (30–400AMU, 4 scans/s). Mass-spectra's were extracted with MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, USA) using the GC-Q-TOF qualitative analysis module. The obtained mass spectra's were exported as mzData files for further processing in MZmine V2.14.2. The files were imported to MZmine V2.14.2 (Copyright © 2005-2012 MZmine Development Team) (Katajamaa et al., 2006;Pluskal et al., 2010) and compounds were identified via their mass spectra using deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 7th edition spectral libraries and by their linear retention indexes (LRI). The LRI values were calculated using an alkane

calibration mix before the measurements in combination with AMDIS 2.72 (National Institute of Standards and Technology, USA). The calculated LRI were compared with those found in the NIST and in the in-house NIOO LRI database. After deconvolution and mass identification peak lists containing the mass features of each treatment (MZ-value/Retention time and the peak intensity) were created and exported as CSV files for statistical processing. The whole volatolomic workflow is shown in **Figure S3.2**.

Bioassay for testing the effect of bacterial volatiles on fungal and oomycete growth

To test the effect of the emitted bacterial volatiles on fungal/oomycete growth the hyphal extension and biomass were measured. The assays were performed in Petri dishes containing top and bottom growth areas (**Figure S3.3**). At the bottom of the Petri dish, 100 µl of bacterial suspensions in 10 mM phosphate buffer (pH 6.5) containing ~ 1 x 10^5 CFU/mL were spread on 20 mL 1/10th Tryptic Soy Broth Agar (TSBA). At the lid of the Petri dish 12.5 mL of water-agar medium (WA) (20 gL^{-1} MERCK agar) was added and inoculated in the middle with a 6-mm-diameter PDA agar plug containing fungal (*R. solani*, *F. culmorum*) or oomycete (*P. ultimum*) hyphae. The plates were sealed with two layers of parafilm and incubated at 24 °C for five days. In this way the tested fungi were exposed (without direct physical contact) to the volatiles produced by the bacteria in the bottom compartment. On the fifth day the extension of the hyphae was measured in 4 evenly spaced directions and compared to the hyphae extension in the control plates (fungi exposed to 1/10th TSBA growth medium without bacteria).

Determination of fungal and oomycetal biomass

Fungal biomass was determined as described by Garbeva et al. (2014). The whole growth area in the lids containing water agar and fungal hyphae was cut in ~2 cm^2 pieces and transferred to a glass beaker containing 100 mL of sterile demi-water (H_2O). The agar was melted for ~2.5 min. in a microwave oven (temperature increased to about 100 °C). The melted agar containing the hyphae was filtered over a tea strainer and the remaining hyphae were rinsed with about 150 – 200 mL of hot water (~80 °C). The hyphae were picked with tweezers from the tea strainer and transferred to a micro centrifuge tube and stored at -20 °C until analysis. For determination of fungal/oomycete biomass the frozen hyphae were transferred to a glass tube with lids with small holes and subjected to freeze-drying for 48 h (Labconco Freezone 12 with Labcanco Clear Drying Chamber nr.7867000). The samples were stored in an exsiccator

with dried silica gel for 3 h (Silica Gel Orange, 2–5 mm, indicator, Roth, art.nr.P077.2) prior weighing the dry biomass.

Bioassay for testing the effect of bacterial volatiles on growth and colony morphology of target bacteria

The assays were performed in two-compartment Petri dishes (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 635102) containing two separated compartments (**Figure S3.4**). In such way the growth response of target bacteria to volatile producing bacteria could be determined without direct physical contacts. One compartment was supplemented with 12.5 mL TSBA and contained the volatile producing bacteria either in monoculture or in pairwise interactions. The second compartment contained the indicator bacteria and was supplemented either with 12.5 mL LBA (*E. coli* WA321, *S. aureus* 533R4) or with 12.5 mL TSBA (*S. marcescens* P87). The compartment for the volatile producing bacteria was inoculated with 100 µl bacterial suspensions master mix of monocultures or pairwise interactions prepared with 20 mL of 10 mM phosphate buffer (pH 6.5) containing ~1 x 10^5 CFU/mL. The compartment for the indicator organisms was inoculated with four droplets (5 µL) of each indicator bacteria. The droplets of the indicator bacteria were placed in a distance of 2 cm to each other and contained 1 x 10^5, 1 x 10^4, 1 x 10^3 and 1 x 10^2 CFU/mL of either *E. coli* WA321, *S. aureus* 533R4 or *S. marcescens* P87 (**Figure S3.4**). As controls the first compartment of the Petri dish was kept empty. After four days of incubation at 24 °C the plates were examined and digital photographs were taken. The digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany) for enumeration and surface-area determination (in pixel^2) of the bacterial colonies. All treatments were performed in triplicate.

Test of pure volatile compounds on bacterial growth and colony morphology

The effect on growth, colony morphology and pigmentation by pure dimethyl disulfide ($CH_3S_2CH_3$), dimethyl trisulfide ($CH_3S_3CH_3$) and the mixture of both compounds was tested on *E. coli* WA321, *S. aureus* 533R4 and *S. marcescens* P87. The assays were performed in two-compartment Petri dishes (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 635102). Both compartments were supplemented with either 12.5 mL LB-A (assay performed with *E. coli* WA321 and *S. aureus* 533R4) or with 12.5 mL TSBA (assay performed with *S. marcescens* P87). In one compartment a filter paper with a

diameter of ~5,5 mm (Whatman™ filter paper Cat# 1003-150, 6 µm pore size) was placed on the agar surface in the middle of the compartment. Stock solutions with a concentration of 10 µM, 1 µM and 0.1 µM of the pure volatile compounds (dimethyl disulfide or dimethyl trisulfide) and the mixture of both compounds (dimethyl disulfide + dimethyl trisulfide) were prepared by serial dilution of the pure compounds in Methanol (LiChrosolv®, Index-No: 603-001-00-X, Merck, Darmstadt, Germany). For the test a volume of 5 µl of each of the pure volatile stock solutions was added directly onto the filter paper resulting in a final concentration of 50 µM, 5 µM and 0.5 µM, respectively. The other compartment was inoculated with the target bacteria *E. coli* WA321, *S. aureus* 533R4 or *S. marcescens* P87 by inoculating four spots in a distance of 2 cm from each other containing 1 x 10^5, 1 x 10^4, 1 x 10^3 and 1 x 10^2 CFU/mL (**Figure S3.4**). As controls bacteria exposed to filter papers with no added volatile compounds were applied. The Petri dishes were sealed with a double layer of parafilm and incubated for four days at 24 °C. After incubation digital photographs were taken and the effect on colony growth, colony morphology and pigment production (prodigiosin) in *S. marcescens* P87 was examined. All digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany) for enumeration and surface-area determination (in pixel^2) of the bacterial colonies. All treatments were performed in triplicate.

Statistical analysis

Statistical analysis on volatolomic data was performed using the statistical analysis module of MetaboAnalyst V3.0, www.metaboanalyst.ca (Xia et al., 2012;Xia et al., 2015). Prior to statistical analysis data normalization was performed via log-transformation. To identify significant abundant mass features ONE-WAY-ANOVA with post-hoc Tukey test (HSD- test) was performed between the data sets. To identify important mass features in the samples PLS-D analysis was performed. Mass features were considered to be statistical relevant if p- values were ≤ 0.05. Statistical relevant mass features were further used for the compound identification. Statistical analyses on fungal dry biomass and bacterial colony sizes were performed with IBM SPSS Statistics 23 (IBM, Somers, NY, USA) using one-way ANOVA and post-hoc Tukey test between the data sets. The 5 % level was taken as threshold for significance between control and volatile treatments.

Determination of HCN, NH3 emission and pH values in the agar

All bacterial strains used in this study were tested for the emission of ammonia and HCN as well as for the ability to change the pH- value of the growth medium where the target organisms were inoculated. For these tests the bacteria were inoculated in two-compartment petri dishes (start density ~1 x 10^5 CFU/mL) on 12.5 mL 1/10th TSBA. The second compartment was supplemented with 12.5 mL water-agar (WA). After 4 days of growth the HCN and ammonia emission as well the pH-value of the target organism growth medium (WA) was determined. To test for the presence of Hydrocyanic acid the gaseous content of the petri dish headspace was sucked through a Hydrocyanic acid test tube (Dräger Safety AG & CO. KGaA, Lübeck, Germany, order number: CH25701) using the Dräger accuro® gas detection pump (Dräger Safety AG & CO. KGaA, Lübeck, Germany).

Presence of Hydrocyanic acid was determined by colour change of the test tube (formation of a red reaction product) (**Figure S3.5**).

The pH of the target organism growth medium (WA) exposed to bacterial volatiles was determined by slightly pressing a pH test-strip VWR PROLABO dosatest® (VWR international, Cat# 35309.606UK) for 30 seconds into the agar surface. The pH values were determined by colour change of the test strip and compared to the colour scale on the package (**Figure S3.6**).

The ammonia concentration was determined using the MQuant™ ammonium test kit (Merck, Darmstadt, Germany, Cat# 110024) by placing a reaction activated test-strip on the lid of the petri dish directly opposite to the bacterial culture and fixed with tape. The petri dish were closed and sealed with parafilm and incubated for 2 hours at 24 °C. After incubation the presence of ammonium was determined by colour change of the test strip (**Figure S3.7**).

Results

Detected headspace volatile compounds and GC/MS-Q-TOF analysis

GC/MS-Q-TOF based volatolomic analysis revealed a total number of 35 compounds that were not detected in the non-inoculated controls (**Table 3.2**). 27 compounds were obtained from the monocultures of *Chryseobacterium* sp. AD48, 15 compounds were obtained from the monocultures of *Tsukamurella* sp. AD106 and 26 compounds were detected in the interactions between these two bacteria (**Table 3.2**, **Figure 3.1A**). For the combinations of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 we obtained a total number of 18 compounds, whereas 16 compounds were detected in the monoculture of *Janthinobacterium* sp. AD80 and only 13 compounds in the monoculture of *Dyella* sp. AD56 (**Table 3.2, Figure 3.1B**). We were able to tentatively identify 19 volatile organic compounds belonging to seven different chemical classes including alcohols, amines, esters, indole, thiocyanates, thioesters and sulfides. However a vast number of the detected compounds (n=16) could not be assigned with certainty to a volatile organic compound and remained unknown. The most prominent detected headspace volatile organic compounds were sulfur-containing compounds (such as sulfur dioxide, methyl thioacetate, dimethyl sulfoxide, etc.). Two sulfur compounds dimethyl disulfide ($C_2H_6S_2$) and dimethyl trisulfide ($C_2H_6S_3$) were produced by all bacteria (except dimethyl trisulfide which was not detected for *Janthinobacterium* sp. AD80).

Effect of interspecific interactions on bacterial volatile blend composition

Volatolomic analysis on monocultures and pairwise combinations of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106 revealed that the volatile composition of the monocultures differed from that of the mixtures (**Figure 3.1A**, **Table 3.2**). Clear separations between controls, monocultures and pairwise combinations of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106 were obtained in PCA score plots (**Figure 3.1A**). The volatile composition of the pairwise combinations resembled that of the monocultures of *Chryseobacterium* sp. AD48 (**Figure 3.1A, Table 3.2**). The indole produced by the monoculture of *Chryseobacterium* sp. AD48 was not detected in the interactions (**Table 3.2**).

The analysis on the volatiles emitted by monocultures and pairwise combinations of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 revealed that the volatile profiles of the monocultures differed from that of the mixtures (**Figure 3.1B**, **Table 3.2**). Different PCA score plots were obtained between controls, monocultures and pairwise combinations of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 (**Figure 3.1B**). A higher number of volatile compounds were detected in the pairwise combinations of these two bacteria. However the higher number of detected volatiles is most probably due to the combination of the volatile blends of these two bacterial isolates. We did not detect any novel or different volatile compounds which production was triggered during the pairwise interaction of these two bacteria. Interestingly the volatile compound cyclopentene produced by the monocultures of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 was not detected in the interactions **(Table 3. 2)**.

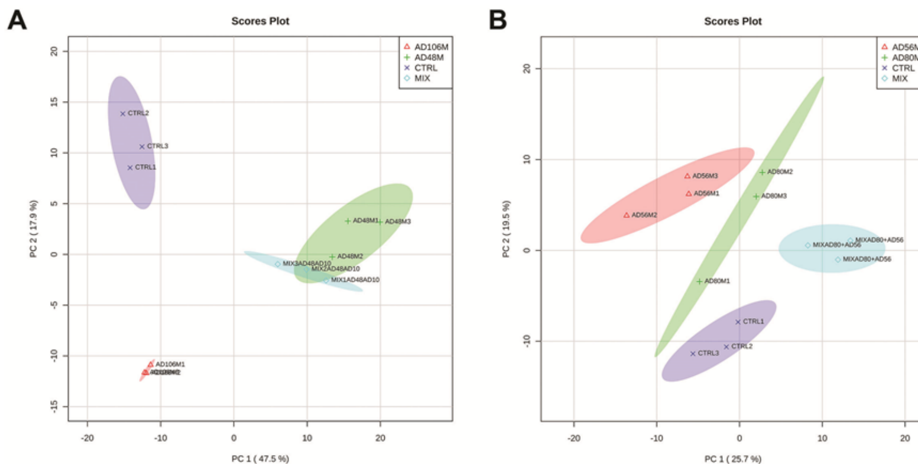

Figure 3.1: PCA 2D- plots of volatiles emitted by monocultures and pairwise combinations of bacteria including confidence intervals (in semi-transparent colors) (**A**) monocultures and mixtures of *Tsukamurella* sp. AD106 and *Chryseobacterium* sp. AD48 and (**B**) monocultures and mixtures of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80.

Table 3.2: Tentatively identified volatile organic compounds emitted by four bacterial strains cultivated either in monoculture or in pairwise combination.

				Detected in treatment					
#	**Compound name / chemical class**	**RT***	**ERI****	**Chry**	**Tsuk**	**MIX Chry + Tsuk**	**Dye**	**Jant**	**MIX Jant + Dye**
1	Sulfurdioxide	2,58	521	x		x		x	x
2	Cyclopentene	2,96	551	x		x	x	x	
3	2 -Pentene	3,29	575				x		x
4	Unknown compound 1	3,77	612	x	x	x	x	x	x
5	Methyl isobutyrate	4,70	682				x		
6	Methyl thioacetate	4,94	700	x		x		x	x
7	Methyl thiocyanate	5,28	713		x			x	x
8	1-Butanol, 3-methyl-	5,69	728	x		x			
9	Dimethyl disulfide	6,10	744	x	x	x	x	x	x
10	Methyl isovalerate	6,86	769				x		
11	S-methyl propanethioate	7,45	782	x		x		x	x
12	1,3 Dithiethane	7,64	786	x	x	x		x	x
13	Dimethyl sulfoxide	8,46	806		x				
14	2,4-Dithiapentane	10,74	865	x	x	x		x	x
15	Benzaldehyd	13,72	944	x	x	x		x	x
16	Dimethyl trisulfide	14,33	960	x	x	x	x		x
17	Unknown cycloalkane	16,86	1026	x	x	x	x	x	x
18	Unknown branched alkene	17,39	1040	x	x	x	x	x	x
19	Unknown sulfur containing compound	18,09	1058	x	x	x			
20	1,2,4-Trithiolane	19,30	1090	x	x	x		x	x
21	Unknown compound 2	19,70	1101				x	x	x
22	Unknown compound 3	19,99	1110	x	x	x	x	x	x
23	Unknwon compound 4	20,63	1131	x		x			
24	Dimethyl tetrasulfide	23,64	1227		x				
25	Indole	25,82	1298	x					
26	Butylhydroxytoluene	30,28	1540	x	x	x	x	x	x
27	Unknown terpene like compound 1	32,84	1674	x		x			
28	Unknown terpene like compound 2	33,46	1703	x		x			
29	Unknown tetralin isomer	33,75	1710	x		x			
30	Unknown aromat isomer	34,22	1721	x		x			
31	Unknown compound 5	34,34	1724	x		x			
32	Unknown di-terpene	34,78	1734	x		x			
33	Unknown terpene like compound 3	35,31	1746	x		x			
34	Unknown compound 6	38,73	2101				x		x
35	Unknown compound 7	42,04	2360	x		x			
Number of detected compounds (n)				**27**	**15**	**26**	**13**	**16**	**18**

Abbreviations:

= Compound number, Chry = *Chryseobacterium*, Dye = *Dyella*, Jant = *Janthinobacterium*, Tsuk = *Tsukamurella*, MIX Chry + Tsuk = pairwise combination of *Chryseobacterium* + *Tsukamurella*. MIX Jant + Dye = pairwise combination of *Dyella* + *Janthinobacterium*.

X = detected.

RT* = Retention time, the RT value stated is the average.

ERI** = Experimental retention index value, the RI value stated is the average.

Effect of bacterial volatiles on fungal and oomycetal growth

Volatiles produced by all treatments including monocultures and pairwise combinations of the selected bacteria revealed strong growth inhibition of the plant pathogenic fungi and oomycete.

The dry biomass of fungi and oomycete exposed to bacterial volatiles was significantly reduced as compared to the controls without bacterial volatiles (**Table 3.3, Figure 3.2, 3.3**).

Table 3.3: Effect of bacterial volatiles on fungal and oomycetal biomass production (mg/dry weight of fungal/oomycetal biomass). Data represent mean and standard deviation of three replicates.

Treatment	*F. culmorum*	*P. ultimum*	*R. solani*
Monocultures			
Chryseobacterium sp. AD48	1.63±0.25*	0.83±0.28*	1.67±0.75*
Dyella sp. AD56	1.03±0.55*	1.47±0.47*	1.1±0.71*
Janthinobacterium sp. AD80	1.05±0.77*	0.9±0.44*	1.1±0.44*
Tsukamurella sp. AD106	2.3±0.69*	1.47±0.12*	2.67±0.47*
Interactions			
Chryseobacterium sp. AD48 + *Tsukamurella* sp. AD106	1.73±0.4*	1.47±0.25*	2.53±0.37*
Janthinobacterium sp. AD80 + *Dyella* sp. AD56	1.3±1.27*	0.97±0.40*	1.23±0.15*
Controls	5.97±2.13	4.42±0.88	5.47±1.23

Asterisk indicates significant differences between the treatments and the respective control (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$).

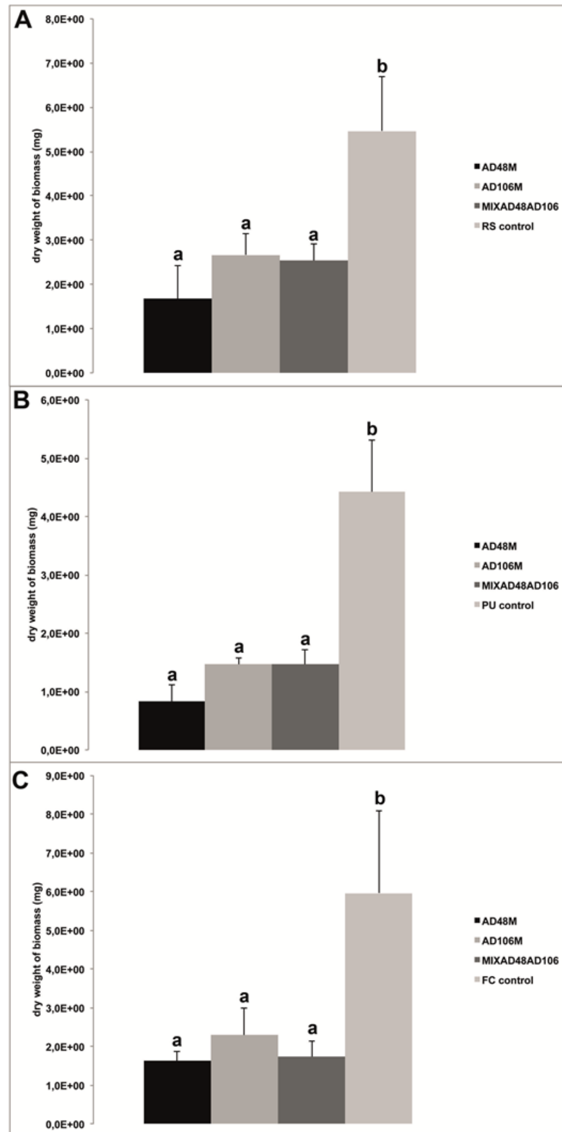

Figure 3.2: Effect of volatiles produced by monocultures and mixtures of *Tsukamurella* sp. AD106 and *Chryseobacterium* sp. AD48 on growth of eukaryotic plant-pathogens. Bars represent the average values for fungal and oomycetal biomass dry weight and error bars represent standard deviation of the mean. (**A**) Dry weight of *R. solani* (**B**) Dry weight of *P. ultimum* (**C**) Dry weight of *F. culmorum*. Significant differences between treatments and the control are indicated by different letters (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$).

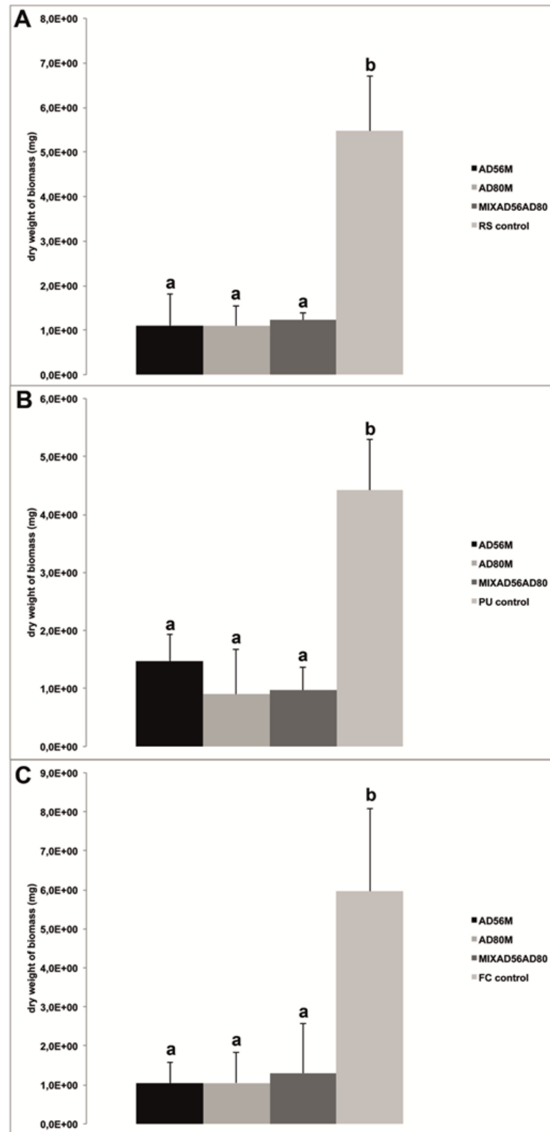

Figure 3.3: Effect of volatiles produced by monocultures and mixtures of volatile emitting *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 on growth of eukaryotic plant-pathogens. Bars represent the average values for fungal and oomycetal biomass dry weight and error bars represent standard deviation of the mean. (**A**) Dry weight of *R. solani* (**B**) Dry weight of *P. ultimum* (**C**) Dry weight of *F. culmorum.* Significant differences between treatments and the control are indicated by different letters (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$).

Effect of bacterial volatiles on the growth and behavior of target bacteria

Volatiles emitted by *Chryseobacterium* sp. AD48 and the mixture of *Chryseobacterium* sp. AD48 and *Tsukamurella* sp. AD106 inhibited the growth of *E. coli* WA321 significantly as compared to the control (**Figure 3.4A**). This observation is in agreement with the observed volatolomic profile (**Figure 3.1A**) which revealed that the volatolomic profile of the mixture is dominated by the volatiles produced by the monoculture of *Chryseobacterium* sp. AD48.

Besides growth inhibition we observed significant growth promotion of *S. aureus* 533R4 when exposed to volatiles emitted by the monocultures of *Dyella* sp. AD56 (**Figure 3.4B**). Changes in colony morphology of *S. marcescens* P87 were observed when exposed to volatiles emitted by *Chryseobacterium* sp. AD48 and to volatiles emitted by the mixtures of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80. The *S. marcescens* P87 colonies were more circular and round shaped (**Figure S3.8**). However, no significant effects of bacterial volatiles on the growth of the target bacteria were also observed (**Figure S3.9**).

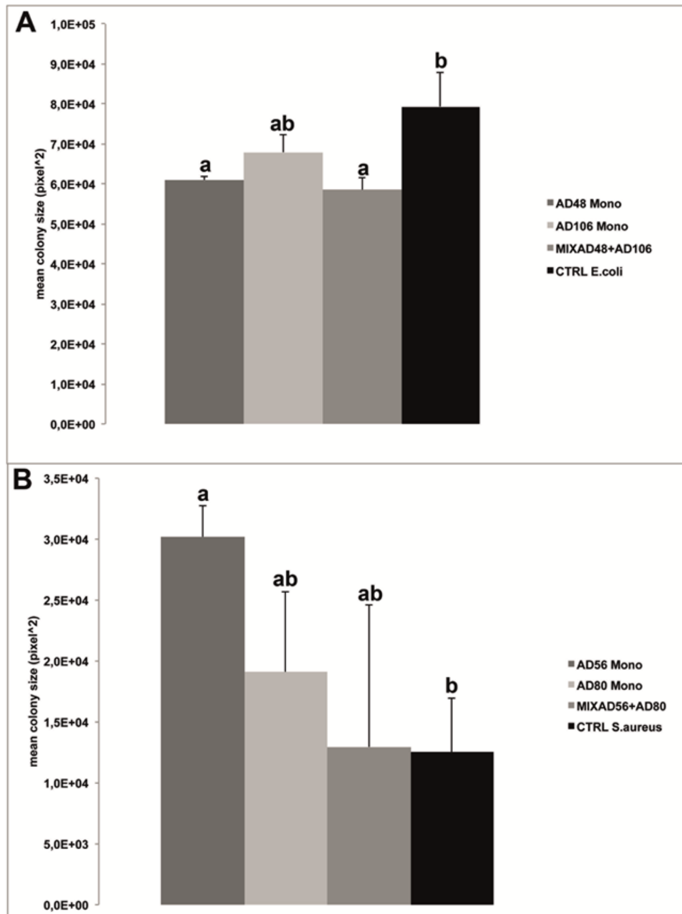

Figure 3.4: Effect of volatiles produced by monocultures and pair-wise combinations of the four selected rhizosphere bacterial strains on average colony size of the target bacteria. (**A**) Mean colony size of *E.coli* WA321 exposed to volatile compounds of *Chryseobacterium* sp. AD48 and *Tsukamurella* sp. AD106 and the mixture of both bacteria. (**B**) Mean colony sizes of *S.aureus* 533R4 exposed to volatile compounds of *Dyella* sp. AD56, *Janthinobacterium* sp. AD80 and the mixture of both bacteria. Significant differences between treatments and the control are indicated by different letters (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

Effect of pure individual volatile compounds on the growth and colony morphology of target bacteria

We applied a two-compartment Petri dish testing system (**Figure S3.4**) in which the model organisms could grow without direct physical contacts to the tested pure volatile compounds. After four days of growth *S. marcescens* P87 colonies were small and showed a white phenotype when exposed to 50 μM of dimethyl trisulfide, indicating the lack of prodigiosin production (**Figure 3.5A**). Furthermore we observed significant inhibition of growth of *S. marcescens* P87, *E. coli* WA321 and *S. aureus* 533R4 when exposed to 50 μM of dimethyl trisulfide (**Figure 3.5A, B; 3.6A, B; 3.7A, B**).

Exposure to dimethyl disulfide did not reveal any significant growth inhibiting or changes in colony morphology at all concentrations tested (500 nM, 5 μM, 50 μM). The mixture of dimethyl disulfide and dimethyl trisulfide resulted in growth inhibition of *S. marcescens* P87 and *E. coli* WA321 at 50 μM concentration. However the mixture of these compounds did not affect the pigmentation in *S. marcescens* P87.

The two lowest applied concentrations 5 μM and 0,5 μM of dimethyl trisulfide and dimethyl disulfide and the mixture of both compounds did not reveal any effect on colony morphology or growth of the tested bacteria (**Figure 3.5A, B; 3.6A, B; 3.7A, B**).

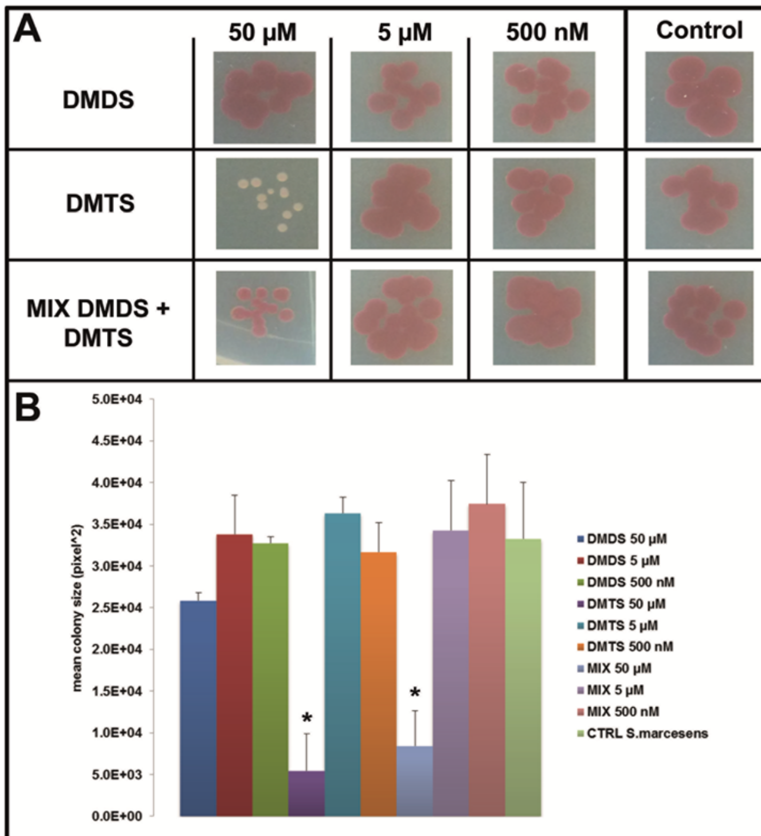

Figure 3.5: Effect of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and the mixture of both volatile compounds (DMDS + DMTS) on colony development of *S. marcescens* (**A**) colony morphology and growth of *S. marcescens* P87 after four days of incubation. The pure volatile compounds were applied in a concentration ranging from 500 nM to 50 µM. Control *S. marcescens* P87 grown without exposure to the compounds. (**B**) Mean colony sizes of *S. marcescens* P87 exposed to volatile compounds of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and the mixture of both volatile compounds (DMDS + DMTS). Asterisk indicates significant differences between the treatments and the control (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

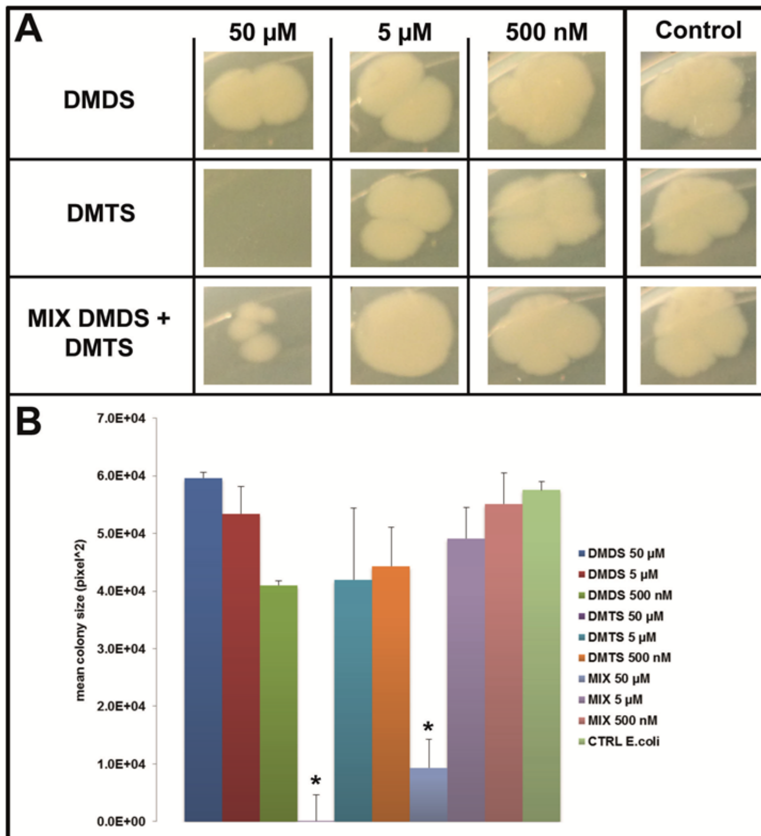

Figure 3.6: Effect of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and the mixture of both volatile compounds (DMDS + DMTS) (**A**) colony morphology and growth of *E. coli* WA321 after four days of incubation. The pure volatile compounds were applied in a concentration ranging from 500 nM to 50 µM. Control *E. coli* WA321 grown without exposure to the compounds. (**B**) Mean colony sizes of *E. coli* WA321 exposed to volatile compounds of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and the mixture of both volatile compounds (DMDS + DMTS). Asterisk indicates significant differences between the treatments and the control (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

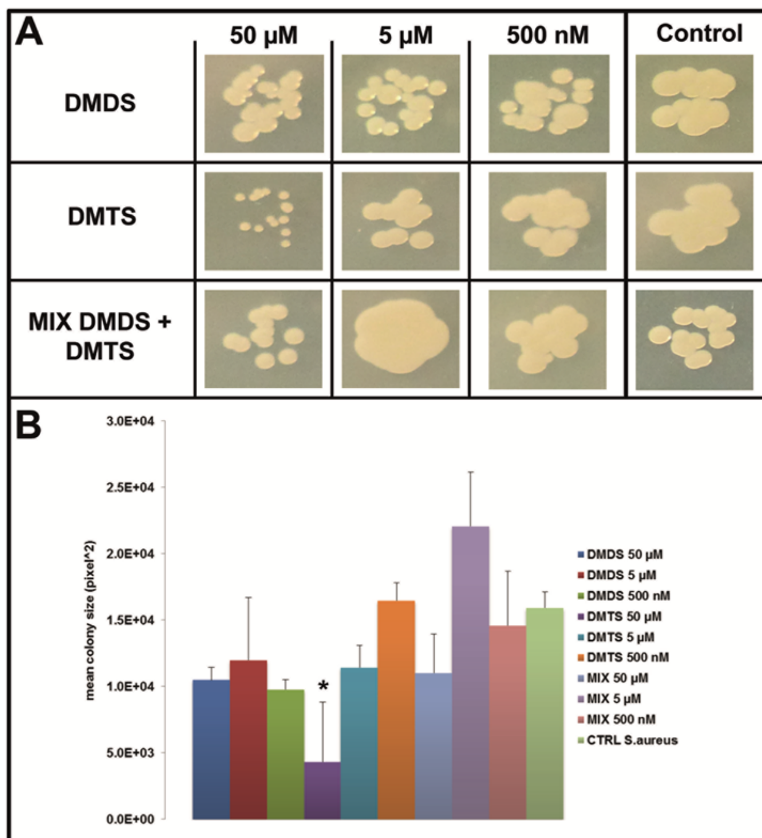

Figure 3.7: Effect of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and the mixture of both volatile compounds (DMDS + DMTS) (**A**) colony morphology and growth of *S. aureus* 533R4 after four days of incubation. The pure volatile compounds were applied in a concentration ranging from 500 nM to 50 µM. Control *S. aureus* 533R4 grown without exposure to the compounds. (**B**) Mean colony sizes of *S. aureus* 533R4 exposed to volatile compounds of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and the mixture of both volatile compounds (DMDS + DMTS). Asterisk indicates significant differences between the treatments and the control (ONE-WAY ANOVA, post-hoc Tukey test $p<0.05$). Data represented are the mean of three replicates, error bars represent standard deviation of the mean.

Discussion

Bacteria coexist with many different species in a heterogeneous and challenging soil environment (Gans et al., 2005). In this environment interspecific interactions between microorganisms are ongoing and are a key factor for their spatial distribution (Keller and Surette, 2006). To cope with the competitive conditions, bacteria developed different survival strategies such as the production of secondary metabolites with inhibitory capacity (Hibbing et al., 2010;Cornforth and Foster, 2013). Most of the studies on bacterial secondary metabolites so far were focused on non-volatile compounds (Korpi et al., 1998;Foster and Bell, 2012). However, bacteria do also release complex blends of volatile organic compounds. Yet, the effect of interspecific interactions on volatiles production and composition is still unknown (Garbeva et al., 2014a).

Here we compared the volatile blends emitted by four phylogenetically different soil-bacteria either grown in monocultures or in pairwise combinations. Our results revealed that the blend of volatiles emitted during pairwise combinations differed from the volatile blends of the respective monocultures. Yet, the volatile blend of the mixtures mostly included volatiles compounds produced by monocultures, although some compounds produced by the monocultures were not detected in mixtures. For example dimethyl sulfoxide produced by *Tsukamurella* sp. AD106 was not detected in the mixture with *Chryseobacterium* sp. AD48. Another interesting example is indole which was produced by the monocultures of *Chryseobacterium* sp. AD48 but was not detected in the presence of *Tsukamurella* sp. AD106. Indole is a very well-studied compound and has been reported to be produced by about 85 different bacterial species including *Chryseobacterium* sp. (Yamaguchi and Yokoe, 2000;Lee and Lee, 2010). Indole and its derivatives (quinolones and (*S*)-3-hydroxytridecan-4-one) are involved in intercellular and multispecies signaling controlling diverse bacterial physiological properties like sporulation, plasmid stability, biofilm formation, drug resistance and virulence (Wang et al., 2001;Di Martino et al., 2003;Diggle et al., 2006;Nikaido et al., 2008;Lee et al., 2009;Lee and Lee, 2010). In addition, indole has been shown to have inhibitory activities on fungal growth (*Aspergillus niger*) and plant growth stimulating properties (*A. thaliana*) (Kamath and Vaidyanathan, 1990;Blom et al., 2011). In general indole is known to be a stable compound in the producing bacteria, however many non-indole producing bacteria are able to modify and to degrade indole (Shimada et al.,

2013;Lee et al., 2015). The fact that indole was not detected during the interaction of *Chryseobacterium* sp. AD48 with *Tsukamurella* sp. AD106 suggests that the production of such signaling compounds in nature depends strongly on the interspecific interactions. Similar result was observed for the compound cyclopentene produced by the monocultures of *Dyella* sp. AD56 and *Janthinobacterium* sp. AD80 but not produced during the interaction of these two bacteria. With the volatolomic methods applied in this study we were able to detect 35 compounds from which 19 were tentatively identified. This discrepancy between numbers of detected and identified compounds shows that the identification of bacterial volatiles is yet a challenging and time demanding task, even with the use of sophisticated programs and software for metabolomics data analysis. Hence, the produced volatile blends are very complex and consist of a mixture of many unknown and difficult to identify compounds (Tait et al., 2014). Most of the volatile organic compounds that were tentatively identified within this study (~58 %) contained sulfur (e.g. methyl thiocyanate, dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide etc.). The high abundance of sulfur containing volatiles in this study can be related to the cultivation of the tested bacteria on 1/10th TSBA growth media. Several studies indicated that the composition of the volatile blend greatly depends on the growth media composition and the growth conditions (Schulz et al., 2004;Schulz and Dickschat, 2007;Blom et al., 2011;Garbeva et al., 2014b). The high amount of dimethyl di- and trisulfide detected in both monocultures and interactions indicate that these compounds are commonly produced. Many studies have shown that bacterial volatiles play a major role in soil fungistasis (Zou et al., 2007;Garbeva et al., 2011a;Garbeva et al., 2014b;van Agtmaal et al., 2015).

Indeed our results revealed that the fungal and oomycete tested organism are sensitive to bacterial volatiles and were inhibited significantly by all monocultures and pairwise combinations. The observed fungal and oomycetal growth inhibition is most probably related to sulfur containing volatiles. Sulfur containing volatiles like dimethyl di- and trisulfide have been shown to effect fungi and are able to inhibit the growth of different plant pathogenic fungi (Kai et al., 2009;Li et al., 2010;Huang et al., 2012;Wang et al., 2013;Garbeva et al., 2014b;Kanchiswamy et al., 2015).

While many study tested the effect of bacterial volatiles on various fungi, little is known so far on the effect of bacterial volatiles on other bacteria. In this study the volatiles emitted by *Chryseobacterium* sp. AD48 and the mixture of

Chryseobacterium sp. AD48 *with Tsukamurella sp.* AD106 inhibited *E. coli* WA321. The observed growth promotion of *S. aureus* 533R4 was caused by the volatiles emitted by *Dyella* sp. AD56. However, this growth promotion was not observed by the volatiles emitted during the interaction of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 correlating with a shift in volatile blend composition. Interestingly volatiles emitted by the monocultures of *Chryseobacterium* sp. AD48 and the mixture of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 induced changes in colony morphology of *S. marcescens* P87. Our previous high-throughput screening for production of non-volatile antimicrobial compounds revealed that all four bacteria used here, showed induced antibacterial activity during pairwise interactions as compared to monocultures (Tyc et al., 2014). This was not observed in the present study, as we didn't observed novel produced volatile compounds during the pairwise interactions. Therefore it's questionable if volatiles solely play an important role as a competitive strategy between bacteria. However, it is possible that volatiles have synergistic or additive effect to other non-volatile antibacterial compounds (Schmidt et al., 2015). Many bacteria are known to emit inorganic volatiles like CO_2, NH_3, HCN, which also have biological activities and can have an additive effect (Effmert et al., 2012). However such compounds were not detected in this study as significant volatile compounds.

Here we tested two commonly produced bacterial volatile compounds for their effect on the target bacteria. The experiments with pure dimethyl trisulfide revealed strong growth inhibition on all tested bacterial model organisms, when applied in a concentration of 50 μM. Bacterial growth suppression was already reported for dimethyl disulfide emitted by *Pseudomonas* strains against the crown-gall diseases causing *Agrobacterium* sp. (Dandurishvili et al., 2011;Popova et al., 2014). Dimethyl trisulfide effected colony morphology and pigmentation in *S. marcescens* P87 when applied in a concentration of 50 μM. Volatiles exposed colonies showed reduced growth and white coloration indicating the lack of prodigiosin production. It is plausible that this observation is related to the inhibition of quorum sensing as previously reported by Moroshi and Chernin (Morohoshi et al., 2007;Chernin et al., 2011).

However, the effective concentration of 50 μM dimethyl trisulfide is most probably very high and far away from the concentrations in which those volatile compounds are produced in nature (Groenhagen et al., 2013) as we did

not observed this effect in the experiments where *S. marcescens* P87 was exposed to the volatile blend produced by bacteria. The biological relevant concentration of volatile compounds remains to be determined in future studies.

In conclusion, this work revealed that interspecific bacterial interactions affect volatile blend composition. This observed change is most probably related to the combination of volatile compounds produced by each isolate rather than triggering the production of novel volatiles as the volatile blend was composed of the mixture of the respective interacting bacteria. Furthermore the loss of production of certain compounds during pairwise interaction suggests that the production of volatile signaling compounds (e.g. indole) in nature is influenced by interspecific interactions. While fungi and oomycetes showed to be very sensitive to bacterial volatiles the effect of volatiles on bacteria varied greatly between no effects, growth inhibition to growth promotion depending on the volatile blend composition.

Acknowledgment

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Supplementary material

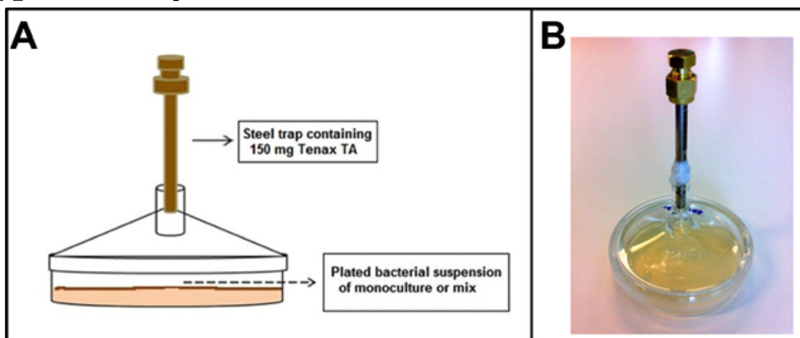

Figure S3.1: Used glass-petri dish system for bacterial volatile organic compounds (VOCs) trapping. (**A**) Illustration of the glass-petri dish; (**B**) photograph of a inoculated glass-petri dish with a connected Tenax TA steel trap for volatile organic compound trapping.

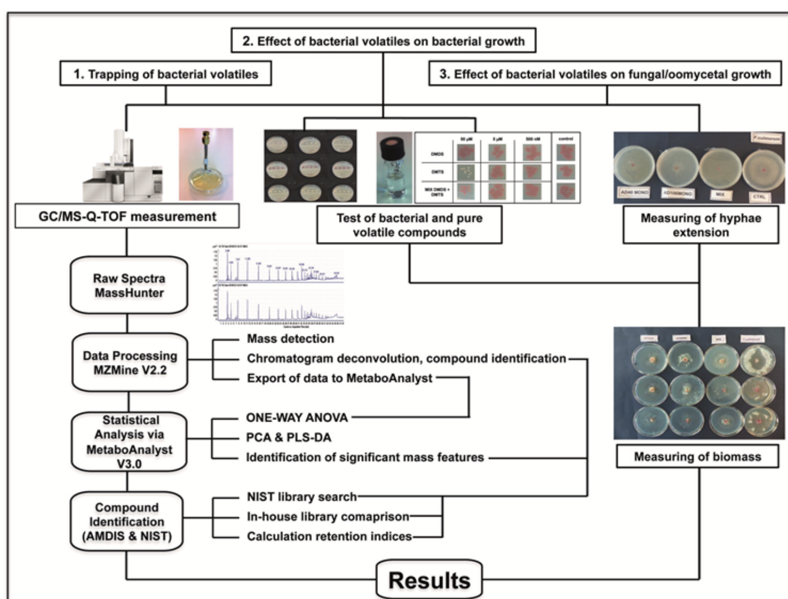

Figure S3.2: Workflow of the volatolomics analysis performed in this study. In total three experimental parts were carried out: 1. Volatile trapping and GC/MS-Q- TOF combined with statistical analysis, 2. Effect of bacterial volatiles on bacterial growth and colony morphology and 3. Effect of bacterial volatiles on fungal and oomycetal model organisms.

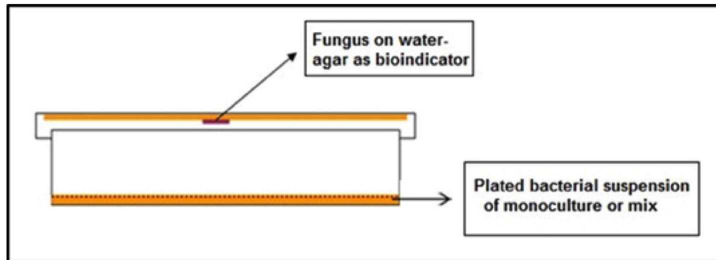

Figure S3.3: Used top-bottom-petri dish system for experiments to elucidate fungal inhibitory capacities of the produced bacterial volatiles.

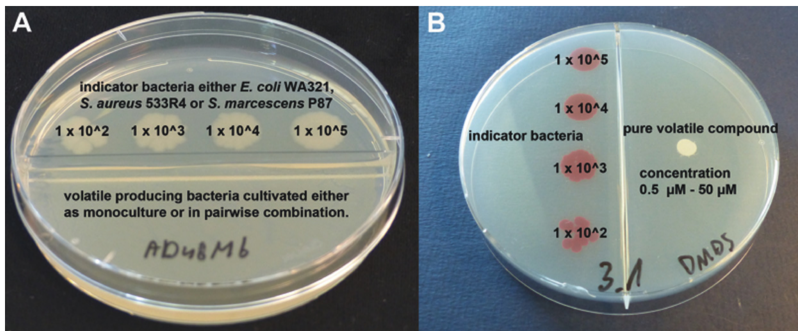

Figure S3.4: Two-compartment petri dish system used in bacterial volatile and pure volatile compound experiments. (**A**) to elucidate the effect of bacterial volatiles on growth and colony morphology of *E. coli* WA321, *S. aureus* 533R4 and *S. marcescens* P87 (**B**) to elucidate the effect of pure volatile compounds dimethyl di- and tri- sulfide on growth and colony morphology on *E. coli* WA321, *S. aureus* 533R4 and *S. marcescens* P87.

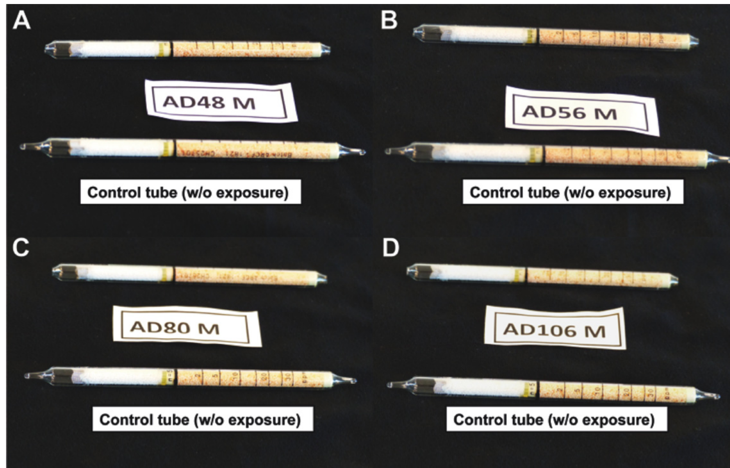

Figure S3.5: Hydrocyanic-acid tests using Dräger Hydrocyanic acid test tubes in combination with the Dräger accuro® gas detection pump. All bacterial strains were tested negative for the production of Hydrocyanic acid (< 2 ppm). Test result for: (**A**) *Chryseobacterium* sp. AD48. (**B**) *Dyella* sp. AD56 (**C**) *Janthinobacterium* sp. AD80. (**D**) *Tsukamurella* sp. AD106.

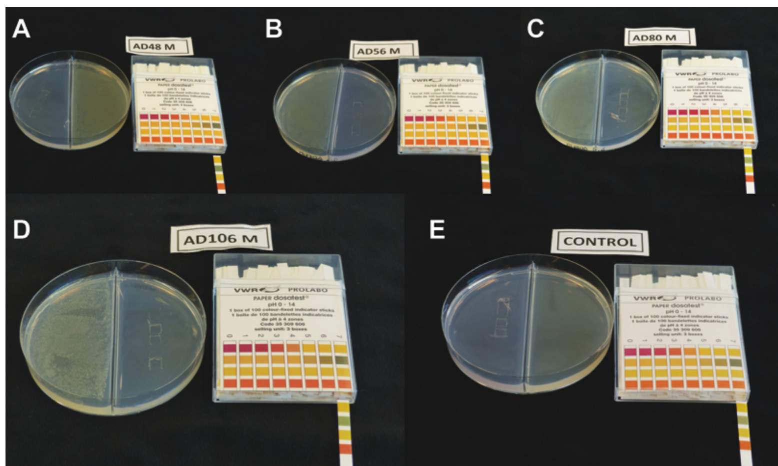

Figure S3.6: pH- tests using VWR PROLABO dosatest® pH- test strips. All bacterial strains did not change the pH- value of the growth medium where the target organisms were grown (pH = 7). Test result for: (**A**) *Chryseobacterium* sp. AD48. (**B**) *Dyella* sp. AD56 (**C**) *Janthinobacterium* sp. AD80. (**D**) *Tsukamurella* sp. AD106. (**E**) The control, two compartment petri dish without exposure to bacterial volatiles.

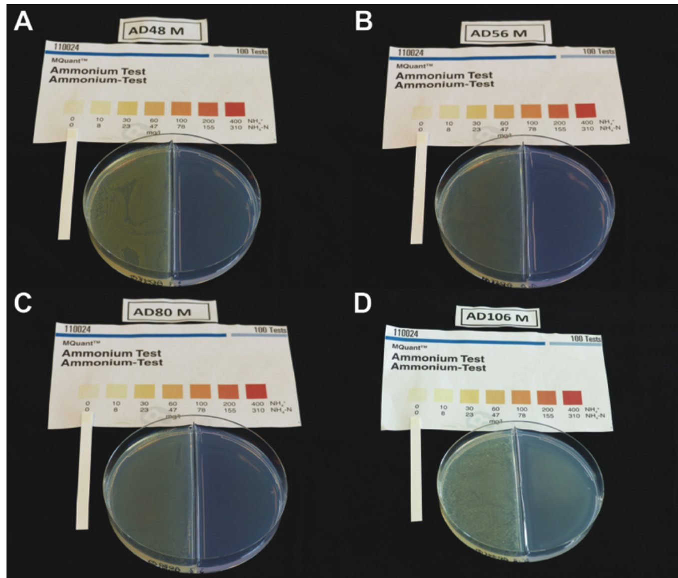

Figure S3.7: Measurements of ammonia emission using the MQuant™ ammonium test kit. All bacterial strains were tested negative for the production of ammonium. Test result for: (**A**) *Chryseobacterium* sp. AD48. (**B**) *Dyella* sp. AD56 (**C**) *Janthinobacterium* sp. AD80. (**D**) *Tsukamurella* sp. AD106.

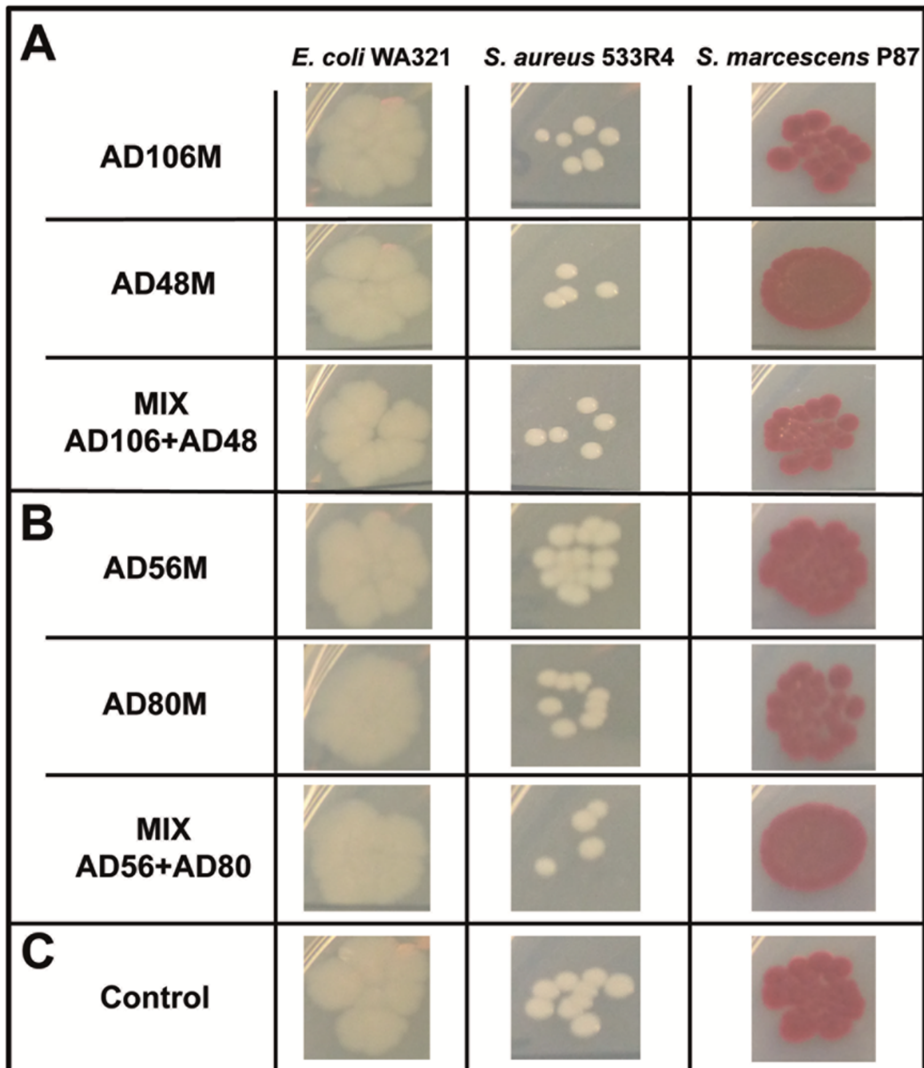

Figure S3.8: Effect of bacterial volatile compounds on colony morphology on the three model organisms: *E.coli* WA321, *S.aureus* 533R4 and *S. marcescens* P87. (**A**) Effect of the produced volatile blend of *Tsukamurella* sp. AD106, *Chryseobacterium* sp. AD48 and the interaction of both bacteria (**B**) Effect of the produced volatile blend of *Dyella* sp. AD56, *Janthinobacterium* sp. AD80 and the interaction of both bacteria. (**C**) Control without exposure to bacterial volatiles.

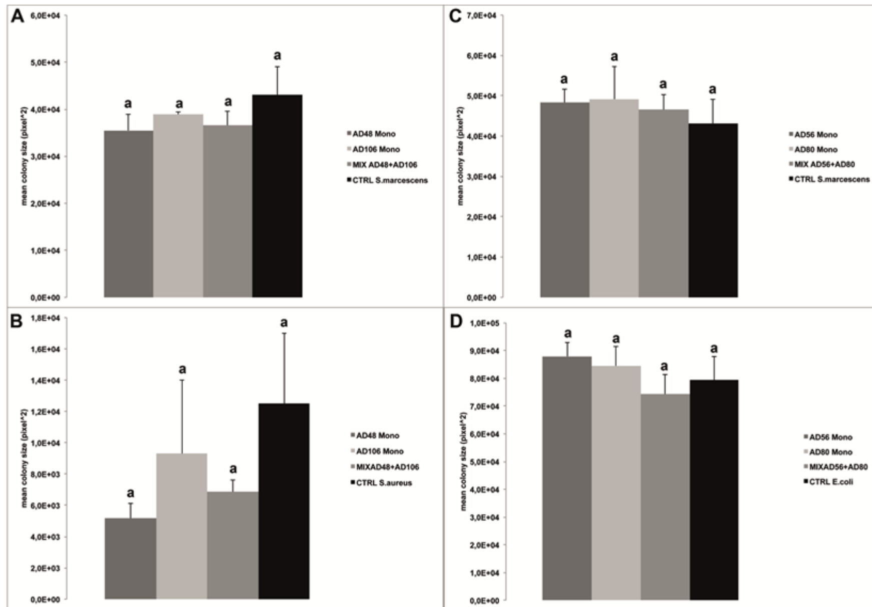

Figure S3.9: Average colony size of the target organisms. (**A**) Mean colony size of *S. marcescens* P87 exposed to volatile compounds of *Chryseobacterium* sp. AD48 and *Tsukamurella* sp. AD106 and the interaction of both bacteria. (**B**) Mean colony size of *S. aureus* 533R4 exposed to volatile compounds of *Chryseobacterium* sp. AD48 and *Tsukamurella* sp. AD106 and the interaction of both bacteria. (**C**) Mean colony size of *S. marcescens* P87 exposed to volatile compounds of *Dyella* sp. AD56, *Janthinobacterium* sp. AD80 and the interaction of both bacteria. (**D**) Mean colony size of *E. coli* WA321 exposed to volatile compounds of *Dyella* sp. AD56, *Janthinobacterium* sp. AD80 and the interaction of both bacteria. Data represented are the mean of three replicates, error bars represent standard deviation (SD).

Chapter 4

Exploring bacterial interspecific interactions for discovery of novel antimicrobial compounds

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Abstract

In terrestrial ecosystems bacteria live in close proximity with many species and form complex communities. Recent studies indicated that the production of secondary metabolites by soil bacteria can be triggered by interspecific interactions. However, little is known about the mechanisms involved in interspecific interactions, in particular for interactions between Gram-positive and Gram- negative bacteria.

In this study we aimed to understand how the interspecific interaction between *Paenibacillus* sp. AD87 and *Burkholderia* sp. AD24 affects fitness, gene expression and metabolism. To facilitate the transcriptomic and metabolomics analysis both bacteria were subjected to genome and RNA-sequencing.

Our results revealed that the interaction between the two bacteria affected their gene expression, fitness and the production of secondary metabolites. The growth of *Burkholderia* was more affected during the interspecific interaction than that of *Paenibacillus.* One volatile and a soluble compound were produced in higher amounts during interaction but not in the monocultures. The identified volatile compound was subjected to bioassays and showed strong inhibitory activity against a range of plant and human pathogens.

Our results highlight the importance of interspecific interactions for triggering secondary metabolites production for the discovery of novel useful bioactive compounds important for agriculture as well for medical purposes.

Introduction

Recent studies have shown that interspecific interactions between soil bacteria strongly affect their behavior such as the induced secretion of secondary metabolites (Seyedsayamdost et al., 2012;Traxler et al., 2013;Tyc et al., 2014). The soil and rhizosphere are environments characterized by high complexity, diversity and density of microorganisms (Gans et al., 2005;Uroz et al., 2010). In these environments microorganisms can interact in different ways. These interactions are lively and range from competition to cooperation (Czaran and Hoekstra, 2009;Foster and Bell, 2012;Allen and Nowak, 2013). Many soil bacterial species have overlapping metabolic niches i.e. they use similar substrates as an energy source for their growth and persistence (Yin, 2000;Demoling et al., 2007;Strickland, 2009). Consequently, competition for nutrients is one of the most abundant forms of interaction occurring in soil and rhizosphere bacterial communities (Demoling et al., 2007;Rousk et al., 2009). To sustain in such demanding environmental conditions bacteria evolved the ability to produce a wide range of secondary metabolites with antimicrobial properties (e.g. antibiotics, siderophores, bacteriocins, volatiles and others) as competitive tools (Hibbing et al., 2010). Bacteria with the ability to produce antimicrobial compounds can also contribute to crop health by suppression of plant-pathogenic micro-organisms (Raaijmakers and Mazzola, 2012;Mendes et al., 2013). Hence, comprehensive knowledge of bacterial interspecific interactions is important for better understanding of soil microbial community composition and soil functions such as disease suppression and plant growth promotion.

Previously we have performed a high-throughput screening of interaction-mediated production of antimicrobial compounds by rhizobacterial strains (Tyc et al., 2014). A clear case of such interaction-mediated triggering of antibiotic production was observed when a *Burkholderia* and a *Paenibacillus* strain were co-cultured. So far, very little is known on interactions between Gram-positive and Gram-negative bacteria and the triggering of secondary metabolite production during such interactions. Bacteria belonging to the genus *Burkholderia* are Gram-negative, non-spore forming and belong to the phylum *Proteobacteria*. These bacteria are able to occupy a diverse range of ecological niches (Salles et al., 2002;van Elsas et al., 2002;Coenye and Vandamme, 2003;Compant et al., 2008). The lifestyle of *Burkholderia* spp. can range from free living in soil and rhizosphere to endo- and epiphytic, including obligate endosymbionts and plant pathogens (Coenye and Vandamme, 2003;Compant et al., 2005;Vial et al., 2011). In recent years the

interest on *Burkholderia* strains has increased as these bacteria have shown to have compelling properties for agriculture like plant growth promotion, increasing of diseases resistance, improvement of nitrogen fixation and phosphorus utilization (Nowak and Shulaev, 2003;Sessitsch et al., 2005;Schmidt et al., 2009;Groenhagen et al., 2013;Zhao et al., 2014).

Soil bacteria belonging to the genus *Paenibacillus* are Gram positive, facultative anaerobe and endo-spore forming bacteria (von der Weid et al., 2000;da Mota et al., 2005). Many paenibacilli are known to act as PGPR in agricultural systems (Anand et al., 2013). Bacteria of this genus are able to colonize diverse habitats like water, soil and insects (Berge et al., 2002;Bosshard et al., 2002;Daane et al., 2002;Peters et al., 2006;Timmusk et al., 2009). Many studies have shown that paenibacilli play an important role in soil for plant health and growth (e.g. nitrogen fixing, pest control) (McSpadden Gardener, 2004;Ryu et al., 2005;Debois et al., 2013). Furthermore members of the genus *Paenibacillus* are known as a rich source for chemical compounds useful in the field of biotechnology and agriculture such as antibiotics, enzymes and other bioactive molecules (Wu et al., 2010;Debois et al., 2013;Cochrane and Vederas, 2016).

The main goal of our study was to obtain insight in the mechanisms and consequences of interspecific interaction between *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87. The major research questions were how this interspecific interaction will affect bacterial cell numbers and fitness, gene expression, the production of soluble and volatile secondary metabolites and their activity.

Materials and methods

Bacteria and culture conditions

Based on a previous screening (Tyc et al., 2014) a Gram-negative strain, *Burkholderia* sp. AD24 (*Beta-proteobacteria*), and a Gram-positive strain, *Paenibacillus* sp. AD87 (*Firmicutes*), were selected for this study. The bacterial isolates were pre-cultured from -80 °C glycerol stocks on 1/10th TSBA (Garbeva and de Boer, 2009) and incubated for three days at 24 °C.

Two indicator bacteria, *E. coli* WA321 and *S. aureus* 533R4, were used as target bacteria to detect the production of compounds with antibacterial activity (Meyer and Schleifer, 1978;Tyc et al., 2014). The bacteria were pre-cultured from -80 °C glycerol stocks on LB-A media (LB-Medium Lennox, Carl Roth GmbH + Co. KG, The Netherlands, solidified with 20 gL^{-1} Merck Agar). The indicator bacteria were incubated overnight at 37 °C prior application. All bacterial isolates used in this study are listed in **Table 4.1**.

Eukaryotic model organisms and culture conditions

The plant pathogens *Rhizoctonia solani* AG2.2IIIB and *Fusarium culmorum* PV were used as fungal model organisms (Garbeva et al., 2014b). The fungi were pre-cultured on 1/5th Potato Dextrose Agar (PDA) (29 gL^{-1} Oxoid CM 139) (Fiddaman and Rossall, 1993) and incubated at 24 °C for 7 days. As a model organism for yeast-like fungi *Candida albicans* BSMY 212 (DSMZ # 10697) was used. *C. albicans BSMY 212* (Schmidt, 1996) was pre-cultured from -80 °C glycerol stocks on YEPD plates (20 gL^{-1} Merck Dextrose, 20.0 gL^{-1} BACTO™ Peptone, 10.0 gL^{-1} BACTO™ Yeast extract, 20 gL^{-1} Merck Agar). All eukaryotic model organisms are listed in **Table 4.1**.

Experimental treatments

For all experiments three different treatments were performed in triplicates at three time points. These treatments were: monoculture AD24M (*Burkholderia*), monoculture AD87M (*Paenibacillus*) and the interaction of *Burkholderia* + *Paenibacillus*.

Table4. 1: Bacterial and fungal strains used in this study.

Strain / isolate / organism	Phylum/class	Genbank	Reference	Function
Bacteria tested during this study				
Burkholderia sp. AD24	beta-proteobacteria	KJ685239	Tyc *et al.* 2014	used in interaction assays, RNA isolation and in LC/MS & GC/MS measurements
Paenibacillus sp. AD87	Firmicutes	KJ685299	Tyc *et al.* 2014	
Eukaryotic model organisms				
Candida albicans BSMY 212 DSMZ 10697	Saccharomycetes		Schmidt 1996	eukaryotic model organisms for growth inhibition
Fusarium culmorum PV	Ascomycota	-	Garbeva *et al.* 2014	
Rhizoctonia solani AG2.2IIIB	Basidiomycota	KT124637	Garbeva *et al*. 2011	
Bacterial model organisms				
Escherichia coli WA321 DSMZ 4509	gamma-proteobacteria	-	Tyc *et al.* 2014	bacterial model organisms for growth inhibition
Staphylococcus aureus 533R4 Serovar 3 DSMZ 20231	Firmicutes	LN681573	Meyer *et al.* 1978	

Bacterial interaction assay

After three-days of pre-cultivation a single colony of each bacterial isolate was picked and inoculated in 20 mL 1/10th TSB broth and grown overnight at 24 °C, 220 rpm to an optical density of OD_{600} 0.630 (*Burkholderia*) and OD_{600} 0.680 (*Paenibacillus*). An inoculation mix for each treatment was prepared by diluting the bacterial isolates in 20 mL 10 mM Phosphate-buffer (pH 6.5) to an starting OD_{600} of 0.002 (*Burkholderia*) and 0.005 (*Paenibacillus*) corresponding to ~ 5 x 10^5 CFU/mL. Each inoculation mix was pulse- vortexed for 30 sec. and a volume of 100 µl (monoculture or mixture) was spread on 1/10th TSBA plates (20 mL of TSBA). Plates were incubated four three days at 24 °C and sampling for bacterial cell counts, total RNA and secondary metabolite extraction was performed after 24 hours, 48 hours and 72 hours of incubation.

Enumeration of bacteria growth

The growth of the two bacterial strains was tracked by selective plate counting. After 24 hours, 48 hours and 72 hours of incubation a volume of 1 mL of 10 mM phosphate buffer (pH 6.5) was added to the surface of the 1/10th TSBA plates and cells were suspended from the plate surface with a disposable cell scratcher (VWR international B.V., Amsterdam, The Netherlands Cat# 50806-404). The cell suspension was transferred to a 15 mL Greiner tube containing 9 mL of 10 mM phosphate buffer (pH 6.5) and homogenized by pulse vortex for 30 sec. Dilution series of each treatment were prepared in triplicates. A volume of 100 µl of each serial dilution were plated in three replicates with a disposable drigalski spatula on 1/10th TSBA plates supplemented with either Streptomycin 25 µg/mL (plates for selection of

Paenibacillus) or Vancomycin 50 μg/mL (plates for selection of *Burkholderia*). The plates were incubated for three days at 20 °C. Bacterial enumeration was carried out on an aCOlyte Colony Counter (Don Whitley Scientific, Meintrup DWS Laborgeräte GmbH, Germany).

RNA sampling and isolation

For RNA- extraction 0.5 mL of each sample was transferred to an 2 mL tube containing 1 mL RNA protect Bacteria Reagent (Qiagen B.V., Venlo, The Netherlands, cat# 76506) and centrifuged for 10 min. at 10,000 x g, and 4 °C (Sigma 3K-14 centrifuge, SIGMA Laborzentrifugen GmbH, Germany). The supernatant was discarded and cells were directly frozen in liquid N_2 and immediately stored at -80 °C. Total RNA was extracted with the Aurum Total RNA Mini Kit (BIO- RAD cat# 732-6820) according to the manufacturer's protocol. Samples were treated with the TURBO DNA free Kit (AMBION cat# 1907) according to the manufacturer's protocol. The RNA concentration and quality was checked on a NanoDrop Spectrophotometer (Isogen Life Science, IJssestein, The Netherlands) and on a 1.0 % agarose gel. Samples were subjected to RNA- sequencing at Baseclear (BASECLEAR, Leiden, The Netherlands) using the Illumina Sequencing platform.

Whole genome DNA isolation and genome sequencing

Genomic DNA of *Burkholderia* and *Paenibacillus* was extracted from overnight cultures by using the QIAGEN genomic DNA Mini Kit (Qiagen B.V., Venlo, The Netherlands, cat# 13323) according to the manufacturer's protocol (for Illumina Sequencing) or by using the QIAGEN MagAttract HMW DNA Kit (Qiagen B.V., Venlo, The Netherlands, cat# 67563) from exponentially growing overnight cultures for PacBio Sequencing. The extracted DNA was dissolved in 100 μl sterile nuclease free water and quantified with a NanoDrop Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). Additionally a 1.0 % agarose gel in 0.5 % TBE buffer was run to check the size and integrity of the isolated DNA. The extracted genomic DNA was stored at -20 °C and subjected for genomic DNA sequencing at Baseclear (BASECLEAR, Leiden, The Netherlands) using the Illumina Sequencing platform and to the Institute for Genome Sciences (IGS), Baltimore, Maryland, USA for Pacbio real-time DNA sequencing.

De Novo assembly of *Paenibacillus* sp. AD87 and *Burkholderia* sp. AD24 genomes

From the paired-end Illumina sequencing platform an average read length of 101 bp was obtained and from the Pacbio RS platform (Pacific Biosciences,

Menlo Park, CA, USA) using the P4-C2 chemistry an average read length of 8184 nucleotides for *Paenibacillus* sp. AD87 and 8334 for *Burkholderia* was obtained. The Pacbio raw sequences were analyzed using SMRT portal V2.3.0.140936. p.4150482. The sequences were assembled with RS_HGAP_assembly 3 protocol (© Copyright 2010 - 2014, Pacific Biosciences, Menlo Park, CA, USA) at default settings with estimated genome sizes of 7 MBp for *Paenibacillus* and 8 MBp for *Burkholderia*. The resulting assemblies were subjected to scaffolding using the RS_AHA_scaffolding 1 protocol. The Illumina reads were filtered using Fastq MCF with default settings and aligned against the scaffolds using BWA V0.7.12. The aligned reads were re-aligned with GATK V3.5.0. The scaffolds of both genomes were corrected using the re-aligned reads with Pilon (Walker et al. 2014). The resulting improved contigs were subjected to another round of scaffolding in SMRT-portal and further corrected. The whole genome assembly properties are shown in **Table S4.1**. The final contigs were annotated using a modified version of PROKKA V1.11 (Seemann, 2014) and InterproScan 5.16 55.0 (Jones et al., 2014). Both genomes were submitted to the NCBI genome database under accession numbers NCBI PRJNA320371 (*Burkholderia* sp. AD24) and NCBI: LXQN00000000 (*Paenibacillus* sp. AD87).

Transcriptome and in-silico secondary metabolite analysis

The obtained Illumina reads from the RNA- sequencing were filtered using Fastq MCF and aligned against the cDNA sequences of both *Burkholderia* and *Paenibacillus* combined using Bowtie 2 (Langmead and Salzberg, 2012) with the following settings: - no-mixed - no-discordant - gbar 1000 – end-to-end. Transcript abundance was estimated using RSEM V1.1.26 (Li and Dewey, 2011) and differential expression between the treatments was analyzed using edgeR V3.2 (Robinson et al., 2010;Zhou et al., 2014). Data were filtered with a p-value of 0.001. For in silico analysis of secondary metabolite gene clusters the antiSMASH website (http://antismash.secondarymetabolites.org/) (Medema et al., 2011) was used.

COG annotation

COG annotations were determined for both *Burkholderia* and *Paenibacillus* by using custom scripts based on a modified version of methods described by Snel (Snel et al., 2002) with COG annotations from Galperin (Galperin et al., 2015).

Reverse transcription and quantitative real time PCR

To confirm the RNA- Sequencing results the gene expression of two gene clusters related to secondary metabolite production (a Pederin like compound in *Paenibacillus* and a polyketide synthase in *Burkholderia*) were targeted and quantified via quantitative real time PCR. For this purpose the previously extracted RNA was used to synthesize first strand cDNA by using the SuperScript® VILO™ MasterMix (Invitrogen, cat#11755050). The concentration and quality of the cDNA was determined using a NanoDrop™ spectrophotometer (Isogen Life Science, IJssestein, the Netherlands), additionally all cDNA samples were run on a 1.0 % agarose gel to check the size and integrity.

The selected gene cluster in *Burkholderia* was targeted with the following primer combinations bAD24_10391_IG_F (5'GTATTGGCCGTATCCGTCAG) and bAD24_10391_IG_R (5'AGCCACTCTTCGACGATCAC) amplifying 322 bp from gene bAD24_10391 encoding snoaL-like polyketide cyclase family protein. For normalization the two primers Eub338F (5'ACTCCTACGGGAGGCAGCAG) with Eub518R (5'ATTACCGCGGCTGCTGG) amplifying 180 bp from the 16S rRNA gene (Fierer et al., 2005) and primers RecA_bAD24_1_F (5' GGTGAGGCAATCGAAGACAT) with RecA_bAD24_1_R (5'AGCTTGCTTGCGTACTGGAT) amplifying 230 bp from gene recA encoding DNA recombination and repair protein were used.

The gene clusters in *Paenibacillus* were targeted with the primer combination gpAD87_304F (5' GTACTTCCCGCACCTGACAT) and gpAD87_304R (5'TGGCGAGAAACTCCACTTCT) amplifying 592 bp from gene encoding a dimodular nonribosomal peptide synthase. For normalization the two primers BacF (5'GGGAAACCGGGGCTAATACCGGAT) (Garbeva et al., 2003) with Eub518R (5'ATTACCGCGGCTGCTGG) amplifying about 440 bp from the 16S rRNA gene and primers RecA_gpAD87_3_F (5'CTTGCCTAAAGGCCGTATTG) and RecA_gpAD87_3_R (5'GACAATGTCCACAGCACCAC) amplifying 259 bp from gene recA encoding DNA recombination and repair protein were used. From each treatment 5µl cDNA were subjected to quantitative RT- PCR using QuantiNova SYBR Green I PCR master mix (Qiagen B.V., Venlo, The Netherlands, Cat# 208056) or with iTaq™ Universal SYBR® green (BIORAD Cat# 1725122). For quantification two-step quantitative RT- PCRs were performed on a Qiagen Research Rotor- Gene Q thermal cycler (Qiagen B.V., Venlo, The Netherlands, Cat# 9001550), with the following settings: initial cycle 95°C for 2 min., followed by 40 cycles of 95°C for 5 sec. (denaturation) and 60°C for 10 sec. (combined annealing/extension). All analyses were performed in triplicate. Five standard curves were established to calculate the

expression (CT-value). Gene expression was calculated relative to the 16S rDNA and recA gene of both, *Burkholderia* and *Paenibacillus* by using the 2-ΔΔCt method (Livak and Schmittgen, 2001). All used primers for qPCR analysis are listed in **Table S4.2**.

Trapping of volatile organic compounds

For analysis of volatile organic compounds glass Petri dishes designed for trapping headspace volatiles (Garbeva et al., 2014b) were used. The Petri dishes were closed by a lid with an outlet connected to a steel trap containing 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK). The Tenax steel traps containing the volatile compounds were collected after 72 hours of incubation and stored at 4 °C until GC-Q-TOF analysis. As controls glass Petri dishes containing 1/10th TSBA media without inoculated bacteria were used as background control in GC/MS measurements.

GC-Q-TOF analysis

The trapped volatile organic compounds were desorbed from the traps using an automated thermos desorption unit (Unity TD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (He flow 50 mL/min) and trapped on a cold trap at -10 °C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA) by heating the cold trap for 12 min to 250 °C. Split ratio was set to 1:10, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 μm (Restek 13424-6850, Bellefonte, PA, USA). Temperature program used was as follows: 39 °C for 2 min, from 39 °C to 95 °C at 3.5 °C/min, then to 165 °C at 4 °C/min, to 280 °C at 15 °C/min and finally to 320 °C at 30 °C/min, hold 7 min. A constant gas flow of 1.2 ml/min was used. Volatile organic compounds were ionized in EI mode at eV. Mass spectra were acquired in full-scan-mode (30–400 U @ 5 scans/s). Mass-spectra were extracted with MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, USA). The obtained mass spectra's were exported as mzXML files for further processing in MZmine V2.14.2. The files were imported to MZmine V2.14.2 (Copyright © 2005-2012 MZmine Development Team) (Pluskal et al., 2010) and compounds were identified via their mass spectra using deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA http://www.nist.gov) and Wiley 7th edition spectral

libraries and by their linear retention indexes (LRI). The LRI values were calculated using AMDIS 2.72 (National Institute of Standards and Technology, USA). After deconvolution and mass identification peak lists containing the mass features of each treatment (MZ-value/Retention time and peak intensity) was saved in csv file format and uploaded to Metaboanalyst V3.0 (www.metaboanalyst.ca).

Extraction of secondary metabolites

Secondary metabolites were extracted from the samples used for bacterial enumeration and RNA isolation. The agar was cut into pieces of about 2 cm^2 and transferred to 50 mL Greiner tubes. After sample collection the tubes were immediately stored at -80 °C. All samples were afterwards transferred to a freeze drier (Labconco Freezone 12 with Labcanco Clear Drying Chamber nr.7867000) and freeze-dried for ~ 48 hours. Subsequently samples were transferred to a ceramic mortar and a volume of ~ 100 mL liquid N_2 was added and the agar pieces were crushed using a pestle. The resulting powder was transferred to 1.2 mL cryo tubes (Nalgene® cryogenic tubes, Sigma-Aldrich, Zwijndrecht, Netherlands, Cat# V4757). A amount of ~ 125 mg agar powder was supplemented with 75 % Methanol + 0.1 % formic acid (Merck Methanol, Cat# 106 009, Merck formic acid Cat# 100 253, Merck, Darmstadt, Germany) after solvent addition tubes were vortexed for 30 sec.. The tubes were afterwards transferred to a sonicator (Bransonic 2510, Branson Ultrasonics Corporation, Danbury, USA) and sonicated for 30 min. After sonication the tubes were centrifuged @ 5500 rpm for 20 min (Sigma 3-16KL, Sigma Laborzentrifugen GmbH, Germany) and the resulting supernatant was transferred to 1.2 mL tubes and stored at -20 °C for further analysis.

UHPLC-ESI-MS analysis of the extracted secondary metabolites

One microliter of sample (1 µl) was analyzed on a UHPLC system of the Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA, USA) connected to a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Separation in the UHPLC system was achieved on an Acclaim C18 column (150×2.1 mm, 2.2 µm, Dionex) with a flow rate of 300 µl min^{-1} in a binary solvent system of water and acetonitrile (hypergrade for LC MS, Merck, Darmstadt, Germany), both containing 0.1 % (v/v) formic acid (eluent additive for LC-MS, Sigma Aldrich, Steinheim, Germany). ESI source parameters were set to 3 kV for spray voltage at a sheath gas flow of 35 and Aux gas flow of 7 l/h. The voltage in the transfer capillary was set to 35 V at a capillary temperature of 325 °C. The samples were measured in positive

ion mode in the mass range of m/z 100–1200 using 140,000 m/Δm resolving power in the Orbitrap mass analyzer. Data were interpreted using XCALIBUR software (Thermo Fisher Scientific, Waltham, MA, USA). For statistical analysis the raw spectra were converted to mzXML format using the MS Convert feature of ProteoWizard 3.0.3750. Subsequently, data processing was carried out with R Studio 0.96.316 using the Bioconductor XCMS package. This resulting list (mz, mzmin, mzmax, rt, rtmin, rtmax, and peak intensities/areas) was saved in csv file format and uploaded into Metaboanalyst V3.0 (www.metaboanalyst.ca).

Ambient mass-spectrometry analysis (LAESI-MS)

For LAESI-MS analysis a single colony of each bacterial isolate was picked and inoculated in 20 mL 1/10th TSB broth and grown overnight at 24 °C, 220 rpm. An inoculation mix of each treatment (AD24 monoculture, AD87 monoculture and mixed strains) was prepared by diluting the bacterial isolates in 1 mL 10 mM Phosphate-buffer (pH 6.5) as described above. A volume of 5 µl of monocultures or mixture was spotted in duplicates on 1/10th TSBA plates at a distance of approximately 6 cm from each other, resulting in two single colonies per petri dish. Plates were incubated for 24 hours, 48 hours and 72 hours and subjected to LAESI-MS measurement. The bacterial colonies were cut out of the agar after 24 hours, 48 hours and 72 hours of incubation (size approximately 1cm^2) and transferred to a glass slide. After sample preparation the glass slide containing the samples was mounted to the sample stage. The LAESI-MS analysis was carried out on a Protea Biosciences DP-1000 LAESI system (Protea Bioscience Inc., Morgantown, WV, USA) that was coupled to a Waters model Synapt G2S (Waters Corporation, Milford, MA, USA) mass spectrometer. The LAESI system was equipped with a 2940-nm mid-infrared laser yielding a spot size of 100 µm. The laser was set to fire 10 times per x-y location (spot) at a frequency of 10 Hz and 100 % output energy. The system was set to shoot at 100 locations per bacterial colony (in a grid of 10 x 10 positions). A syringe pump was delivering the solvent mixture of methanol/water/formic-acid (50:50:0.1 % v/v) at 2 mL/min to a PicoTip (5cm x 100 µm diameter) stainless steel nanospray emitter operating in positive ion mode at 3800 V. The laser focus was tuned for each sample with the help of a camera. The LAESI was operated using LAESI Desktop Software V2.0.1.3 (Protea Biosciences Inc., Morgantown, WV, USA). The Time of Flight (TOF) mass analyzer of the Synapt G2S was operated in the V-reflectron mode at a mass resolution of 18.000 to 20.000. The source temperature was 150 °C, and

the sampling cone voltage was 30 V. The positive ions were acquired in a mass range of 50 to 1200 *m/z*. The MS data were lock mass corrected post data acquisition using leucine encephalin ($C_{28}H_{37}N_5O_7$, *m/z*= 556.2771), which was added as internal standard to the electrospray solvent. Ions of potential interest for the generation of accurate ion maps (±1 ppm) were identified via background subtraction using MassLynx software (Waters Corporation, Milford, MA, USA). Ion maps were created using Protea Plot V2.0.1.3 (Protea Biosciences Inc., Morgantown, WV, USA).

Antibacterial assays

The secondary metabolite extracts were used in agar disk-diffusion test in 9 cm^2 petri dishes (Balouiri et al., 2016). Single colonies of each of the bacterial target organisms *Escherichia coli* WA321 and *Staphylococcus aureus* 533R4 were picked from plate and grown overnight in liquid LB broth at 37 °C, 220 rpm. Fresh LB- agar (1.5 % Merck Agar) was prepared, cooled down to ~45 °C and the target organisms were added to a final OD_{600} of 0.002 corresponding to approximately 6 x 10^5 CFU/mL (*E.coli* WA321) or 4 x 10^5 CFU/mL (*S. aureus* 533R4). To each plate 15 mL of the seeded agar was added and after solidification six filter papers with a diameter of ~5,5 mm (Whatman™ filter paper Cat# 1003-150, 6 µm pore size) were placed on the top of the agar surface. A volume of 5µl of each of the extracts was added in triplicates onto the filter papers. As control a volume of 5 µl of the solvent (75 % Methanol) was added. As positive control 5 µl of appropriate antibiotic (Ampicillin 100 mg/mL for *E.coli* WA321 or Tetracycline 15 mg/mL for *S.aureus* 533R4) was added onto one filter paper. As negative control filter paper with no supplemented antibiotics or secondary metabolite extracts were applied. The plates were incubated overnight at 37 °C. The next day plates were examined for visible zones of inhibition (ZOI) and digital photographs were taken. The digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany) for surface-area determination (in pixel^2). All treatments were performed in six replicates.

Bioassays to test the pure volatile compound 2,5-bis(1-methylethyl)-pyrazine

The effect on growth by pure 2,5-bis(1-methylethyl)-pyrazine was tested on *E. coli* WA321, *S. aureus* 533R4 and on *Rhizoctonia solani* AG2.2 IIIB, *Fusarium culmorum* PV and *Candida albicans* BSMY 12 (Schmidt, 1996;Garbeva et al., 2014b;Tyc et al., 2014). The assays were performed in 12 well plates (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 665180). Stock solutions

of 50 µl pure 2,5-bis(1-methylethyl)-pyrazine (Sigma-Aldrich, Zwijndrecht, Cas# 24294-83-5) were prepared. The model organisms *E.coli* WA321 and *S. aureus* 533R4 and *Candida albicans* BSMY212 were grown overnight either in liquid LB broth or liquid YEPD broth at 37 °C, 220 rpm. Fresh LB- and YEPD agar (1.5 % Merck Agar) was prepared and cooled down to ~45 °C, the target organisms were added to the liquid agar at a final OD_{600} of 0.002. A volume of 1 mL liquid agar seeded with the test organisms was added to each well. For the test on mycelial growth fresh PD- agar (1.0 % Merck Agar) was prepared and a volume of 1 mL was added to each well. The fungi were added by placing a 5mm diameter fungal plug of *Rhizoctonia solani* AG2.2 IIIB, *Fusarium culmorum* PV at the top edge of each well. To test the compound a filter paper with a diameter of ~5,5 mm (Whatman™ filter paper Cat# 1003-150, 6 µm pore size) was placed on the agar surface at the lower edge of each compartment. A droplet of 2 µl pure 2,5-bis(1-methylethyl)-pyrazine (=1.84 mg) was added onto the filter paper. As controls 2 µl of the solvent ($CHCl_3$) was applied (Merck Chloroform, Cat# 102 445 Merck, Darmstadt, Germany). The plates were incubated overnight at 37 °C (*E. coli, S. aureus* & *C. albicans*) or at 24 °C for 4 days (*R. solani, F. culmorum*). After incubation plates were examined for visible zones of inhibition (ZOI) or inhibition of fungal growth (mycelial extension) and digital photographs were taken. The digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany).

Test for synergistic effects between secondary metabolite extracts and 2,5-bis(1-methylethyl)-pyrazine

To test for synergistic effects of the secondary metabolite extracts and volatile compounds agar diffusion tests with secondary metabolite extracts of the interaction *Burkholderia* with *Paenibacillus* in combination with the pure volatile compound 2,5-bis(1-methylethyl)-pyrazine were performed. The assays were carried out in 12 well plates (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 665180). A stock solution of 25 µl pure 2,5-bis(1-methylethyl)-pyrazine was prepared in a 1.7 mL eppendorf tube. The model organisms *E.coli* WA321 and *S. aureus* 533R4 were grown overnight in liquid LB broth at 37 °C, 220 rpm. Fresh LB- agar (1.5 % Merck Agar) was prepared and cooled down to ~45 °C, the target organisms were added to the liquid agar at a final OD_{600} of 0.002. A volume of 1 mL liquid agar seeded with the test organisms was added to each well. To test the secondary metabolite extracts for synergy with the pure volatile compound a filter paper with a diameter of

~5,5 mm (Whatman™ filter paper Cat# 1003-150, 6 µm pore size) was placed in the middle of the agar surface in each compartment. To test for synergistic effects a droplet of 7 µl containing 2 µl pure 2,5-bis(1-methylethyl)-pyrazine (=1.84 mg) and 5 µl of the secondary metabolite extracts were added onto the filter paper. As controls filter papers with 7 µl of the solvents (5 µl Merck Methanol, Cat# 106 009 and 2µl Merck Chloroform, Cat# 102 445 Merck, Darmstadt, Germany) were applied. As positive assay control 2 µl of appropriate antibiotic (Ampicillin 100 mg/mL for *E.coli* WA321 or Tetracycline 15 mg/mL for *S.aureus* 533R4) were applied. As negative assay control filter paper with no supplemented antibiotics or secondary metabolite extracts were applied. The plates containing the treatments were incubated overnight at 37 °C, control plates were incubated at 37 °C in a separate incubator. After overnight incubation plates were examined for visible zones of inhibition (ZOI) and digital photographs were taken. The digital images were analyzed using the AXIO VISION v4.8 imaging Software (Carl Zeiss Imaging Solutions GmbH, Germany) for surface-area determination (in pixel^2). All treatments were performed in six replicates.

Bacterial luciferase reporter assays for antimicrobial mode of action

Reporter construction

All reporter constructs were made by cloning the respective promoter, and in some cases the associated transcription factor, at the *Bam*HI/*Xho*I cloning site of the medium copy number plasmid pCS26Pac (Bjarnason et al., 2003). The target fragments were amplified with PCR primers containing the appropriate restriction sites by making use of the Phusion polymerase (New England BioLabs Cat# M0530L). All primers used for bioreporter construction are listed in **Table S4.3**. The redox-cycling reporter plasmid pPHZlux-1 was constructed by amplifying the *PA35160* promoter and the *soxR* gene from the *P. aeruginosa* PA14 genome with the primer pair 1 and 2. The pBLAlux-2 was based on the inducible *ampC* promoter of *P. protegens* Pf-5 and included also the divergently oriented the *ampR* regulator gene and amplified with primer pair 3 and 4 and cloned in pCS26Pac to result in pBLAlux-1. Because of the higher transcriptional induction in a Δ*ampD* genetic background (data not shown), which was created by a kanamycin resistance-bearing transposon cassette, (Baba et al., 2006), a different antibiotic resistance on the plasmid was needed. Therefore a different backbone was prepared from the chloramphenicol resistance-containing pBAD33 plasmid (Guzman et al., 1995) with the primer combination 5 and 6, containing a 5'-tail with *Pac*I restriction sites, in which the initial regulatory unit cloned in front of the *luxCDABE*

operon was subcloned by digestion from the pBLAlux-1 plasmid with *Pac*I. The pSOSlux-2 plasmid was created by introducing the colicin D promoter, subcloned from the pJAMA8-cda plasmid (Tecon et al., 2010) with primer-pair 7 and 8 in the pCS26Pac plasmid by using ClonTech In-Fusion HD Cloning Kit (#CL 639650). Finally, a non-specific toxicity reporter was made by using a high level constitutive promoter, (Braatsch et al., 2008), which was made by annealing the complementary oligopeptide pair 9 and 10 at 1 pmole/µl in annealing buffer (10 mM Tris/HCl pH = 7,5, 1 mM EDTA and 50mM NaCl) following 5 minutes of boiling, gradual cooling to room temperature and subsequent ligation in *Bam*HI/*Xho*I linearized pCS26Pac. All bacterial strains and plasmids used for bioreporter construction are listed in **Table S4.4**.

Bacterial luciferase reporter assays

The reporter assays were prepared in 15 % glycerol batches with a final OD_{600} of ~0,5. These were diluted 20-fold in MOPS-buffered minimal medium (8,5 mM NaCl, 18,7 mM NH_4Cl, 47 mM MOPS, 0,3 mM $Na_2HPO_4.2H_2O$, 0,3 mM KH_2PO_4, 2 mM of $MgCl_2$; 0.1 mM of $CaCl_2$; 0.2 % glucose, pH=7) and exposed at 1 % DMSO in a final volume of 150 µl in Greiner Bio-One white polystyrene 96 wells plates (Greiner bio-one B.V., Alphen a/d Rijn, The Netherlands, Cat# 655075). Aqueous extracts of the tested bacteria were prepared by removing 4 days grown colonies of *Burkholderia* and *Paenibacillus* (monoculture) and colonies of the interaction of both bacteria (*Burkholderia* + *Paenibacillus*) from 1/10th TSBA plates with a disposable inoculation loop (VWR international B.V., Amsterdam, The Netherlands Cat# 50806-404). Scratched colonies were suspended in sterile MQ- water, homogenized by pulse- vortex for approximately 1 min. and afterwards centrifuged for 10 min. @ 5500 rpm. the resulting supernatant was filter sterilized (0.2 µM Whatman filter) and stored at -20 °C for further analysis. Reporter were exposed at 50 % and taking an *E. coli* DH5α as a blank control. The volatile compound 2,5-bis(1-methylethyl)-pyrazine was diluted in DMSO. The exposures of pBLAlux-2, pPHZlux-1 and pSOSlux-2 were incubated at 37 °C at 400 rpm for respectively 6, 1 and 1 hours and a standard curve of the model compounds penicillin G, pyocyanin and mitomycin D was taken along in the same plate. Subsequently the autonomous luminescence in every well was measured during 4 s with a TriStar luminometer (Berthold). Samples with luminescence levels above the detection limit, i.e. average $(RLU_{blank})+3*SD(RLU_{blank})$ were normalized for the cytotoxicity as measured by reduction of luminescence by the control reporter

with the same plasmid backbone (pBPlux-1, or -2) measurements, analogous to (Leedjarv et al., 2006).

Mammalian luciferase reporter assays (CALUX®) for human toxicological assessment of 2,5-bis(1-methylethyl)-pyrazine

The mammalian CALUX® reporter assays, DR-CALUX (dioxin-like activity), Nrf2-CALUX, ERα-CALUX (estrogenic activity), AR-CALUX (androgenic activity) and p53-CALUX (genotoxicity) were conducted as describe in (van der Burg et al., 2013;van der Burg et al., 2015). The 2,5-bis(1-methylethyl)-pyrazine formulation was dissolved at 0.8 % (DR), 0.1 % (ERα, AR and cytotoxicity) and 1 % (p53 and Nrf2) in 6 well plates in DMEM/F-12 medium (Life Technologies), while shaking at room temperature for 10 minutes and 200 rpm at room temperature and diluted accordingly. The DMSO concentration in the wells was also adjusted to 0.8 % (DR), 0.1 % (ERα, AR and cytotoxicity) and 1 % (p53 and Nrf2).

Statistical analysis

Statistical analyses on cell counts were performed with IBM SPSS Statistics 23 (IBM, Somers, NY, USA) using one-way ANOVA with post-hoc TUKEY (HSD-test). Significant differences between treatments and the controls are indicated by different letters ($p<0.05$). Statistical analysis on volatile and non-volatile metabolites data was performed using Metaboanalyst V3.0, www.metaboanalyst.ca (Xia et al., 2015). Prior to statistical analysis data normalization was performed via log-transformation. To identify significant abundant masses ONE-WAY-ANOVA with post-hoc TUKEY test (HSD- test) was performed between the data sets. To identify important masses in the samples PLS-D analysis was performed. Masses were considered to be statistical relevant if p- values were ≤ 0.05 and were further used for compound identification.

Results

Effect of interspecific interaction on *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 cell numbers

The bacterial colony forming units obtained from monocultures and interactions are summarized in **Figure 4.1**. *Burkholderia* reached the highest density in monoculture after 72 hours of incubation (2.17 x 10^9 CFU/mL). The growth of *Burkholderia* was negatively affected when confronted with the Gram-positive *Paenibacillus* strain resulting in significantly lower cell counts compared to the monoculture at 48 hours and 72 hours of incubation (**Figure4. 1**).

In monocultures *Paenibacillus* reached 1.69 x 10^9 CFU/mL after 48 hours and 7.92 x 10^8 CFU/mL) after 72 hours of incubation. During interactions the growth of *Paenibacillus* was negatively affected when confronted with *Burkholderia* only at 24 hours of incubation by reaching 2.17 x 10^8 CFU/mL as compared to 1.10 x 10^9 CFU/mL in monocultures (**Figure 4.1**). Overall the results revealed that growth of *Burkholderia* was more affected during the interspecific interaction than that of *Paenibacillus*.

Genomic features of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87

The genome features (genome size, number of chromosomes and plasmids, GC content, predicted number of coding sequences and the number of rRNAs) of both bacteria are summarized in **Table 4.2**. Considerable differences in genome size and differences in chromosome and plasmid content were observed. The size of the two genomes differed by approximately 1.2 MB and the number of coding sequences (CDSs) varied by 1069 between *Burkholderia* and *Paenibacillus*. Overall the genome analysis revealed that the genome of *Burkholderia* was larger and more complex as compared to the genome of *Paenibacillus*. The analysis of genes probably encoding for secondary metabolites based on antiSMASH revealed a total of 14 gene clusters for *Burkholderia* from which 5 gene clusters belonged to the class of Bacteriocins, 3 to the class of Terpenes, 2 to Non-Ribosomal Peptides, 2 to NRPS-Hserlactones, 1 to the class of type-3 Polyketide Synthase and 1 to the class of Phosphonates. For *Paenibacillus* the *in silico* analysis revealed in total 10 gene clusters from which 2 gene clusters belonged to the class of Terpenes, 1 to Bacteriocins, 1 to Lassopeptides, 2 to the class of Lantipeptides, 1 to Non-Ribosomal Peptides, 1 to others, 1 to the class of type-3 Polyketide Synthase and one gene cluster belonging to the class of Siderophores.

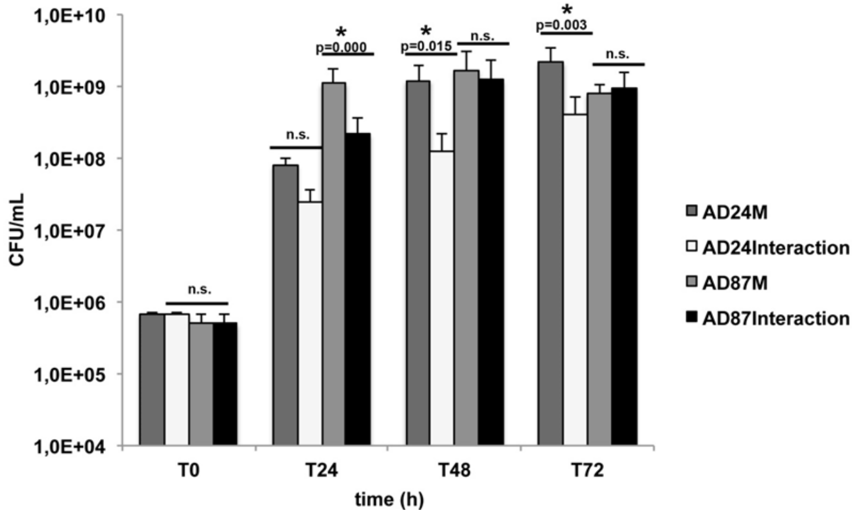

Figure 4.1: Effect of interspecific interaction on colony forming units (CFU) of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 grown on 1/10th TSBA plates. Significant differences between treatments (pairwise combinations) and the control (monocultures) are indicated by asterisks (ONE-WAY ANOVA, post-hoc TUKEY test $p<0.05$).

Abbreviations: AD24M: *Burkholderia* sp. AD24 monoculture, AD24Interaction: *Burkholderia* sp. AD24 in interaction with *Paenibacillus* sp. AD87. AD87M: *Paenibacillus* sp. AD87 monoculture, AD87Interaction: *Paenibacillus* sp. AD87 in interaction with *Burkholderia* sp. AD24.

Table 4.2: Genome assembly results for *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87.

	***Burkholderia* sp. AD24**	***Paenibacillus* sp. AD87**
contigs	3	30
bases	8243440	7086713
number of chromosomes	2	1
size chromosome 1	4476936	7086713
size chromosome 2	3444013	-
number of plasmids	1	-
size of plasmid	322651	-
CDS	7285	6216
rRNA	23	40
tmRNA	0	1
tRNA	74	118
genes	7383	6375
signal peptide	742	547
in silico detected secondary metabolite clusters (antiSMASH)	14	10
Total genome size (bases)	**8243440**	**7086713**

Effect of interspecific interactions on gene expression

Transcriptome analysis revealed that the interaction between *Burkholderia* and *Paenibacillus* caused significant transcriptional changes in both bacteria as compared to the monocultures. During interaction 45 genes of *Burkholderia* were significantly affected after 24 hours (16 up- and 29 down-regulated). At this time point no differential expression of genes was seen for *Paenibacillus* (**Figure 4.2A**). At 48 hours of incubation the expression of 38 genes (14 up- and 24 down-regulated) was significantly affected in *Burkholderia* whereas 531 genes were significantly differentially expressed in *Paenibacillus* (310 up- and 221 down-regulated) (**Figure 4.2A Table 4.3, 4.4, 4.5**). The highest number of differentially expressed genes was observed after 72 hours of incubation with 62 genes differentially expressed in *Burkholderia* (33 up- and 29 down-regulated) and 1114 genes in *Paenibacillus* (381 up- and 733 down-regulated) (**Figure 4.2A, Table 4.3, 4.4, 4.5**). Analysis based on orthologous gene categories (COG) revealed that after 48 and 72 hours most of the up-regulated genes in both bacteria belonged to the categories S (function unknown), K (transcription) and NA (not assigned).

Additionally for *Paenibacillus* genes belonging to the categories G (carbohydrate transport/metabolism) and for *Burkholderia* genes belonging to the category E (amino acid transport/metabolism) were significantly up-regulated (**Figure 4.2B, C**). Interestingly 22 genes related to defense mechanisms (category V) were up regulated in *Paenibacillus* at these two time points.

At time points 48 and 72 hours most of the down regulated genes in *Burkholderia* belonged to the categories NA (not assigned), C (Energy production and conversion), S (function unknown), I (Lipid transport/metabolism) and E (amino acid transport/metabolism) (**Figure 4.2B**). While in *Paenibacillus* most of the down-regulated genes belonged to the categories S (function unknown), G (carbohydrate transport/metabolism) and K (transcription) (**Figure 4.2C**).

Table 4.3: List of the top 50 differentially expressed genes (FDR ≥0.05) in *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD87 over the time points t=48h to t=72h.

Sequence ID	Gene description	fold change with *Paenibacillus sp. AD87*	FDR value	possible function
bAD24_p01665	Type IV secretion system protein virB1	3,94	0,00	Cell wall/membrane/envelope biogenesis
bAD24_II12395	Aminopeptidase N	3,88	0,00	Amino acid transport and metabolism
bAD24_p01670	Flagellar biosynthetic protein FliP	3,46	0,00	Intracellular trafficking, secretion, and vesicular transport
bAD24_II08090	Transcriptional activator protein NhaR	3,12	0,00	Transcription
bAD24_II11090	hypothetical protein	3,07	0,03	Function unknown
bAD24_p01610	hypothetical protein	2,92	0,05	Function unknown
bAD24_II05560	hypothetical protein	2,89	0,00	Cell wall/membrane/envelope biogenesis
bAD24_II08070	putative lipoprotein YiaD	2,69	0,01	Cell motility
bAD24_II08565	hypothetical protein	2,57	0,01	Function unknown
bAD24_II15035	23S ribosomal RNA	2,56	0,00	NA
bAD24_I13295	23S ribosomal RNA	2,56	0,00	NA
bAD24_II03345	23S ribosomal RNA	2,54	0,01	NA
bAD24_I00140	Adenylyl-sulfate kinase	2,47	0,00	Amino acid transport and metabolism
bAD24_II03425	hypothetical protein	2,47	0,01	Function unknown
bAD24_I05570	23S ribosomal RNA	2,42	0,01	NA
bAD24_II08075	hypothetical protein	2,38	0,01	Function unknown
bAD24_p01560	hypothetical protein	2,36	0,02	Function unknown
bAD24_II13810	23S ribosomal RNA	2,30	0,01	NA
bAD24_I00825	Transcriptional activator protein LasR	2,29	0,01	Transcription
bAD24_p01565	hypothetical protein	2,29	0,01	Mobilome: prophages, transposons
bAD24_II06980	Carboxymethylenebutenolidase	2,27	0,04	Secondary metabolites biosynthesis, transport and catabolism
bAD24_II02950	16S ribosomal RNA	2,24	0,02	NA
bAD24_II10295	hypothetical protein	2,23	0,02	Function unknown
bAD24_I12375	23S ribosomal RNA	2,21	0,02	NA
bAD24_I05585	16S ribosomal RNA	2,19	0,02	NA
bAD24_II12400	Disulfide-bond oxidoreductase YfcG	2,17	0,03	Posttranslational modification, protein turnover, chaperones
bAD24_p01570	Cyclic di-GMP phosphodiesterase response regulator RpfG	2,15	0,02	Signal transduction mechanisms
bAD24_p01660	hypothetical protein	2,14	0,03	Posttranslational modification, protein turnover, chaperones
bAD24_II08380	HTH-type transcriptional regulator PgrR	2,12	0,02	Transcription
bAD24_II02935	23S ribosomal RNA	2,09	0,03	NA
bAD24_II15050	16S ribosomal RNA	2,07	0,03	NA
bAD24_II00020	16S ribosomal RNA	2,01	0,04	NA
bAD24_II13825	16S ribosomal RNA	1,93	0,05	NA
bAD24_II06535	Bicarbonate transport ATP-binding protein CmpD	-6,83	0,02	Inorganic ion transport and metabolism
bAD24_I04185	putative acetoacetate decarboxylase	-6,74	0,03	Secondary metabolites biosynthesis, transport and catabolism
bAD24_I07975	Hydroxymethylglutaryl-CoA lyase YngG	-6,74	0,03	Amino acid transport and metabolism
bAD24_I18450	hypothetical protein	-6,53	0,04	Lipid transport and metabolism
bAD24_II14900	HTH-type transcriptional regulator McbR	-6,36	0,05	Transcription
bAD24_I01620	Glycerate dehydrogenase	-4,31	0,00	Energy production and conversion
bAD24_II01060	Alpha-D-ribose 1-methylphosphonate 5-triphosphate synthase subunit PhnH	-3,86	0,02	Inorganic ion transport and metabolism
bAD24_II11545	hypothetical protein	-3,86	0,02	Function unknown
bAD24_II01170	Mycothiol acetyltransferase	-3,79	0,03	General function prediction only
bAD24_II04610	Glycine cleavage system transcriptional activator	-3,79	0,03	Transcription
bAD24_II03270	hypothetical protein	-3,72	0,00	Function unknown
bAD24_II00005	23S ribosomal RNA (partial)	-3,61	0,00	NA
bAD24_I05585	16S ribosomal RNA	-3,57	0,00	NA
bAD24_II11260	hypothetical protein	-3,53	0,00	Function unknown
bAD24_II07555	tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein MnmC	-3,47	0,01	General function prediction only
bAD24_I05445	hypothetical protein	-3,21	0,00	Function unknown
bAD24_II02950	16S ribosomal RNA	-3,12	0,00	NA
bAD24_II14670	Cytochrome bd-I ubiquinol oxidase subunit 1	-3,11	0,00	Energy production and conversion

Differentially expressed genes related to secondary metabolite production

In *Burkholderia* the gene bAD24_II06980 (Carboxymethylenebutenolidase) (secondary metabolite biosynthesis) was differentially expressed at 48 and 72 hours. The gene was 2.27 fold up-regulated as compared to the control (**Table 4.3**). In *Paenibacillus* the gene gpAD87_01890 (Dienelactone hydrolase) was 3.9 fold up-regulated compared to the monoculture at 48 hours of incubation. At 72 hours in total 3 genes were highly expressed namely: gpAD87_13790 (Dienelactone hydrolase), gpAD87_02615 (Imidazolonepropionase), gpAD87_01890 (Dienelactone hydrolase). These genes were 3.4 fold and 1.9 fold respectively higher expressed during interaction as compared to the monoculture (**Table 4.4**).

Differentially expressed genes related to signal transduction

During the interaction of *Burkholderia* and *Paenibacillus* several genes related to the signal transduction systems (category T) were affected. In *Burkholderia* the gene bAD24_p01570 (Cyclic di-GMP phosphodiesterase response regulator RpfG) was 2.15 fold higher expressed as compared to the control (**Table 4.3**). Interestingly this gene was found on a mobile genetic element. In *Paenibacillus* 57 genes related to signal transduction were affected, at 48 hours 22 genes were up regulated and 35 genes down regulated. The genes gpAD87_21325 (Transcriptional regulatory protein LiaR) and gpAD87_23700 (HPr-like protein Crh) were the most affected with 6.84 up and 3.73 fold change down regulated respectively (**Table 4.4, 4.5**). At time point t=72h 30 genes were up regulated and 41 genes were down-regulated (**Figure 4.2A**) from which gene gpAD87_26840 (Methyl-accepting chemotaxis protein McpB) and gpAD87_07465 (Low-molecular weight protein-tyrosine-phosphatase YfkJ) were the most affected with 7.41 up and 8.46 down regulated, respectively (**Table 4.4, 4.5**).

Table 4.4: Top 50 up-regulated genes (FDR ≥0.05) in *Paenibacillus* sp. AD87 during interaction with *Burkholderia* sp. AD24 over the time points t=48h to t=72h.

Sequence ID	Gene description	fold change with *Burkholderia sp. AD24*	FDR value	possible function
gpAD87_28110	Vancomycin B-type resistance protein VanW	9,36	0,00	Defense mechanisms
gpAD87_16925	L-erythro-3,5-diaminohexanoate dehydrogenase	7,66	0,00	Cell wall/membrane/envelope biogenesis
gpAD87_27745	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	7,47	0,00	Cell wall/membrane/envelope biogenesis
gpAD87_26840	Methyl-accepting chemotaxis protein McpB	7,41	0,01	Signal transduction mechanisms
gpAD87_09100	Pesticidal crystal protein cry22Aa	7,37	0,00	Mobilome: prophages, transposons
gpAD87_01885	KHG/KDPG aldolase	7,36	0,00	Carbohydrate transport and metabolism
gpAD87_18840	putative ABC transporter permease	7,36	0,00	Defense mechanisms
gpAD87_19800	Autoinducer 2 import ATP-binding protein LsrA	7,13	0,00	General function prediction only
gpAD87_20500	hypothetical protein	7,13	0,00	Function unknown
gpAD87_26670	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase	6,99	0,01	General function prediction only
gpAD87_07910	Esterase EstB	6,84	0,01	Defense mechanisms
gpAD87_14330	NADH oxidase	6,84	0,01	Energy production and conversion
gpAD87_17325	Magnesium and cobalt efflux protein CorC	6,84	0,01	General function prediction only
gpAD87_21325	Transcriptional regulatory protein LiaR	6,84	0,01	Signal transduction mechanisms
gpAD87_24045	Potassium-transporting ATPase C chain	6,73	0,00	Inorganic ion transport and metabolism
gpAD87_18375	hypothetical protein	6,67	0,01	Function unknown
gpAD87_20865	hypothetical protein	6,67	0,01	Function unknown
gpAD87_18365	hypothetical protein	6,50	0,00	Function unknown
gpAD87_13865	hypothetical protein	6,48	0,03	General function prediction only
gpAD87_19825	Urease subunit beta	6,48	0,03	Amino acid transport and metabolism
gpAD87_16035	Arabinogalactan endo-beta-1,4-galactanase	6,41	0,00	Carbohydrate transport and metabolism
gpAD87_00220	putative oxidoreductase YcjS	6,26	0,04	General function prediction only
gpAD87_12085	hypothetical protein	6,26	0,04	Translation, ribosomal structure and biogenesis
gpAD87_13630	Response regulator protein VraR	6,26	0,04	Transcription
gpAD87_25705	3-oxoacyl-[acyl-carrier-protein] reductase FabG	6,26	0,04	General function prediction only
gpAD87_25320	hypothetical protein	6,25	0,00	Function unknown
gpAD87_16040	Inner membrane ABC transporter permease protein YcjP	6,16	0,00	Carbohydrate transport and metabolism
gpAD87_18370	RNA-splicing ligase RtcB	5,85	0,00	Translation, ribosomal structure and biogenesis
gpAD87_23460	23S ribosomal RNA (partial)	5,74	0,00	NA
gpAD87_16030	Beta-galactosidase BglY	5,67	0,00	Carbohydrate transport and metabolism
gpAD87_16020	hypothetical protein	5,64	0,00	Translation, ribosomal structure and biogenesis
gpAD87_06185	Chorismate synthase	5,58	0,00	Amino acid transport and metabolism
gpAD87_06685	Sensory transduction protein regX3	5,49	0,00	Signal transduction mechanisms
gpAD87_12310	2-hydroxy-6-oxononadienedioate/2-hydroxy-6-oxononatrienedioate hydrolase	5,48	0,00	Coenzyme transport and metabolism
gpAD87_05105	putative MFS-type transporter YhjX	5,35	0,00	Inorganic ion transport and metabolism
gpAD87_30715	hypothetical protein	5,31	0,00	Function unknown
gpAD87_12650	Oleandomycin glycosyltransferase	5,30	0,00	Carbohydrate transport and metabolism
gpAD87_22925	Inner membrane protein YqjA	5,27	0,00	Function unknown
gpAD87_21995	Cyclopentanol dehydrogenase	5,22	0,00	Lipid transport and metabolism
gpAD87_06680	Alkaline phosphatase synthesis sensor protein PhoR	5,14	0,00	Signal transduction mechanisms
gpAD87_00465	3-oxoacyl-[acyl-carrier-protein] reductase FabG	5,05	0,00	Lipid transport and metabolism
gpAD87_02880	K(+)/H(+) antiporter subunit KhtT	4,97	0,00	Inorganic ion transport and metabolism
gpAD87_11435	putative metallo-hydrolase YflN	4,97	0,00	General function prediction only
gpAD87_16015	hypothetical protein	4,93	0,00	Function unknown
gpAD87_01305	hypothetical protein	4,92	0,00	Function unknown
gpAD87_04025	GTPase Era	4,88	0,00	Translation, ribosomal structure and biogenesis
gpAD87_10330	Arabinose operon regulatory protein	4,88	0,00	Transcription
gpAD87_13790	Acetyl esterase Axe7A	3,49	0,00	Secondary metabolites biosynthesis, transport and catabolism
gpAD87_02615	Imidazolonepropionase	1,97	0,03	Secondary metabolites biosynthesis, transport and catabolism
gpAD87_01890	Homoserine O-acetyltransferase	1,96	0,03	Secondary metabolites biosynthesis, transport and catabolism

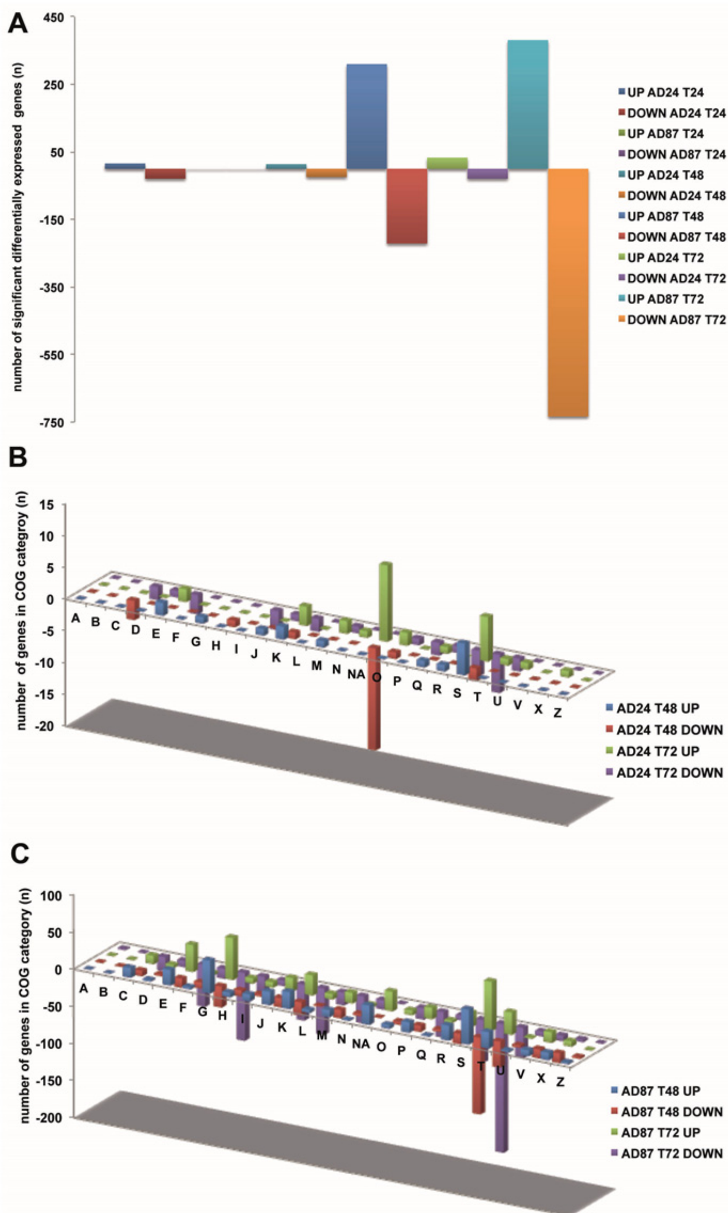

Figure 4.2: Overview of the transcriptome analysis outcome for *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 during co-culturing on 1/10th TSB agar. (**A**) overview of the number (n) of significantly (FDR <0.05) differently expressed genes over the time points T24h – T72h. (**B** and **C**) Overview of the COG classification of differentially expressed genes during interspecific

interaction between *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 over the time points T24 (t=24h) till T72 (t=72h) relative to the monoculture condition. Differentially expressed genes based on COG classification for b: *Burkholderia* sp. AD24 in interaction with *Paenibacillus* sp. AD87 and c: *Paenibacillus* in interaction with *Burkholderia* sp. AD24. The one-letter codes represent the following functional categories: A: RNA processing and modification, B: Chromatin structure and dynamics, C: energy production and conversion; D: cell cycle control, cell division, chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme transport and metabolism; I: lipid transport and metabolism; J: translation, ribosomal structure and biogenesis; K: transcription; L: replication, recombination and repair; M: cell wall/membrane/envelope biogenesis; N: cell motility; NA: not assigned; O: posttranslational modification, protein turnover; chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: function unknown; T: signal transduction mechanisms; U: intracellular trafficking, secretion, and vesicular transport; V: defense mechanisms X: mobilome: prophages, transposons.

Differentially expressed genes related to defense mechanisms

In total 22 genes belonging to defense mechanisms were affected in *Paenibacillus* after 48 hours (8 up-regulated, 14 down-regulated) and 19 genes were affected at 72 hours (12 up-regulated and 7 down-regulated) (**Figure 4.2B, C**). At both time points the most affected genes in *Paenibacillus* were gpAD87_28110 (Vancomycin B-type resistance protein (VanW) and gpAD87_18840 (putative ABC transporter permease) (**Table 4.4**).

The two most down-regulated genes at time point t=48h were gene gpAD87_12115 (Multidrug resistance protein (YkkD)) and gene gpAD87_09055 (Multidrug resistance protein (NorM)) with 6.73 and 4.67 fold changes respectively (**Table 4.5**). At t=72h the genes gpAD87_14640 (Putative penicillin-binding protein) and gpAD87_06980 (RutC family protein) were down regulated with fold changes of 6.94 and 5.96 respectively (**Table 4.5**).

Table 4.5: Top 50 down-regulated genes (FDR ≥0.05) in *Paenibacillus* sp. AD87 during interaction with *Burkholderia* sp. AD24 over the time points t=48h to t=72h.

Sequence ID	Gene description	fold change with *Burkholderia sp. AD24*	FDR value	possible function
gpAD87_04615	hypothetical protein	-10,27	0,00	Function unknown
gpAD87_07985	hypothetical protein	-9,88	0,00	Function unknown
gpAD87_30040	Putative monooxygenase YcnE	-9,81	0,00	Energy production and conversion
gpAD87_09355	putative methyltransferase YcgJ	-9,73	0,00	Coenzyme transport and metabolism
gpAD87_14585	Acetyltransferase YpeA	-9,56	0,00	Translation, ribosomal structure and biogenesis
gpAD87_04655	hypothetical protein	-9,38	0,00	Function unknown
gpAD87_29715	Inositol-1-monophosphatase	-9,23	0,00	Carbohydrate transport and metabolism
gpAD87_17060	hypothetical protein	-9,20	0,00	Function unknown
gpAD87_14605	Acetyltransferase YpeA	-9,01	0,00	Translation, ribosomal structure and biogenesis
gpAD87_22100	Alanine--tRNA ligase	-8,98	0,00	Translation, ribosomal structure and biogenesis
gpAD87_31275	Endonuclease YhcR	-8,92	0,00	RNA processing and modification
gpAD87_12550	hypothetical protein	-8,86	0,00	Function unknown
gpAD87_23185	Serine/threonine-protein kinase AfsK	-8,73	0,00	Function unknown
gpAD87_14475	hypothetical protein	-8,72	0,00	Function unknown
gpAD87_14595	hypothetical protein	-8,71	0,00	Function unknown
gpAD87_14750	Putative mutator protein MutT4	-8,71	0,00	Nucleotide transport and metabolism
gpAD87_20950	Proline dehydrogenase 1	-8,63	0,00	Amino acid transport and metabolism
gpAD87_28445	Elongation factor G	-8,63	0,00	Translation, ribosomal structure and biogenesis
gpAD87_27985	CDP-glucose 4,6-dehydratase	-8,58	0,00	Cell wall/membrane/envelope biogenesis
gpAD87_08715	Monomeric sarcosine oxidase	-8,56	0,00	Amino acid transport and metabolism
gpAD87_31365	hypothetical protein	-8,56	0,00	Amino acid transport and metabolism
gpAD87_07465	Low molecular weight protein-tyrosine-phosphatase YfkJ	-8,46	0,00	Signal transduction mechanisms
gpAD87_12005	Aldose 1-epimerase	-8,44	0,00	Carbohydrate transport and metabolism
gpAD87_16995	Chaperone protein HtpG	-8,39	0,00	Posttranslational modification, protein turnover, chaperones
gpAD87_24970	Single-stranded DNA-binding protein A	-8,37	0,00	Replication, recombination and repair
gpAD87_27970	N-acylneuraminate cytidylyltransferase	-8,37	0,00	Cell wall/membrane/envelope biogenesis
gpAD87_09250	hypothetical protein	-8,35	0,00	Function unknown
gpAD87_15590	Ribosome biogenesis GTPase A	-8,35	0,00	Translation, ribosomal structure and biogenesis
gpAD87_00840	Glutathione transport system permease protein GsiC	-8,33	0,00	Amino acid transport and metabolism
gpAD87_29710	hypothetical protein	-8,28	0,00	Function unknown
gpAD87_19650	putative endonuclease 4	-8,23	0,00	Replication, recombination and repair
gpAD87_00905	Oligopeptide transport ATP-binding protein OppD	-8,21	0,00	Amino acid transport and metabolism
gpAD87_09130	Sulfoacetaldehyde reductase	-8,18	0,00	General function prediction only
gpAD87_05250	hypothetical protein	-8,10	0,00	Function unknown
gpAD87_31865	hypothetical protein	-8,10	0,00	General function prediction only
gpAD87_10400	hypothetical protein	-8,08	0,00	Function unknown
gpAD87_28520	50S ribosomal protein L5	-8,05	0,00	Translation, ribosomal structure and biogenesis
gpAD87_00470	Fe/S biogenesis protein NfuA	-7,99	0,00	Posttranslational modification, protein turnover, chaperones
gpAD87_29875	hypothetical protein	-7,99	0,00	Function unknown
gpAD87_11325	Sensor histidine kinase YehU	-7,91	0,00	Signal transduction mechanisms
gpAD87_14755	hypothetical protein	-7,91	0,00	Function unknown
gpAD87_14315	hypothetical protein	-7,87	0,00	Function unknown
gpAD87_22410	putative metallo-hydrolase	-7,78	0,00	General function prediction only
gpAD87_23025	hypothetical protein	-7,78	0,00	Function unknown
gpAD87_23175	hypothetical protein	-7,78	0,00	Function unknown
gpAD87_07425	Sulfur carrier protein ThiS	-7,71	0,00	Coenzyme transport and metabolism
gpAD87_00725	CheY-P phosphatase CheX	-7,67	0,00	Cell motility
gpAD87_08830	putative response regulatory protein	-7,67	0,00	Signal transduction mechanisms
gpAD87_14640	Putative penicillin-binding protein PbpX	-6,94	0,01	Defense mechanisms
gpAD87_06980	RutC family protein	-5,96	0,00	Defense mechanisms

Q-PCR results

The results from the transcriptome analysis were confirmed by performing qRT-PCR. Comparison of the fold changes in gene expression of gene bAD24_10391 in *Burkholderia* and of gene gpAD87_304 in *Paenibacillus* confirmed that the expression of the targeted genes were approximately at the same level as in the transcriptome analysis (**Table S4.5**).

Effect of interspecific interaction on secondary metabolite production
Soluble metabolites

Metabolome analysis performed on extracts of monocultures and interactions of *Burkholderia* and *Paenibacillus* revealed that the metabolite composition of the monocultures differed from that of the mixtures (**Figure 4.3A**). Clear separations of metabolite composition between controls, monocultures and interactions were obtained in PLS-DA score plots (**Figure 4.3A**). One of the compounds observed in a higher concentration during interaction was identified as a Pederin-like compound ($C_{25}H_{45}NO_9$, *m/z*= 504.316) [$M+H^+$] (**Figure S4.6**).

Volatile metabolites

The comparison of volatile organic compounds emitted by the bacteria revealed that the volatile composition of the monocultures differed significantly from that of the mixtures (**Figure 4.3B**). Clear separations between the monocultures, controls and the interaction were obtained in PLSDA score plots (**Figure 4.3B**). The analysis revealed 22 volatile organic compounds produced by bacteria that were not detected in the non-inoculated controls (**Table 4.6**). Of these volatile organic compounds 17 could be tentatively identified and categorized in 6 different chemical classes (Alkenes, Benzoids, Sulfides, Terpenes, Furans, and Pyrazines). However, five compounds could not be assigned with certainty to a volatile organic compound. The most prominent headspace volatile organic compounds were sulfur-containing compounds (such as dimethyl disulfide, dimethyl trisulfide etc.). Two sulfur compounds dimethyl disulfide ($C_2H_6S_2$) and dimethyl trisulfide ($C_2H_6S_3$) were produced by both *Burkholderia* and *Paenibacillus*. Interestingly two compounds produced by the monoculture of *Burkholderia* (S-Methyl methanethiosulfonate and unknown compound 4) were not detected during the interaction with *Paenibacillus* (**Table 4.6**). One volatile organic compound was produced in very high concentration during the interaction of the two bacteria. This compound was identified as 2,5-bis(1-methylethyl)-pyrazine ($C_{10}H_{16}N_2$, *m/z*= 164.247, RT= 19.7) and is most probably

produced by *Burkholderia* (**Figure S4.7**, **Table 4.6**). For confirmation of the structure and for bioassays, the 2,5-bis(1-methylethyl)-pyrazine was commercially synthesized.

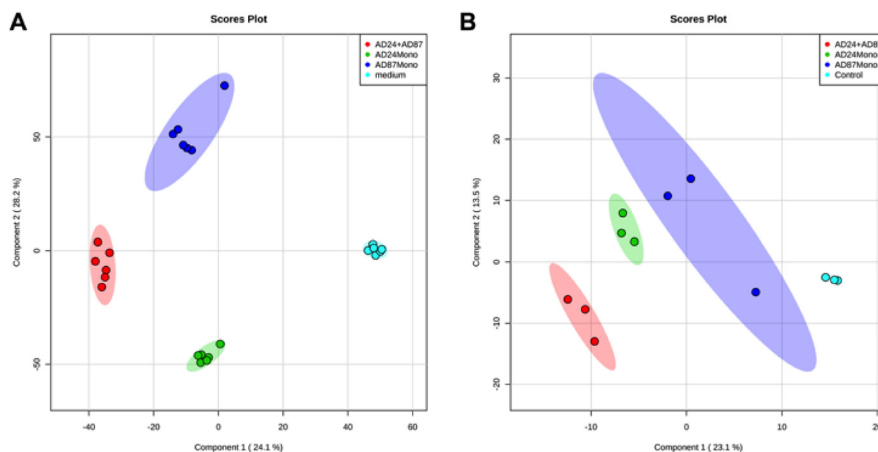

Figure 4.3: PLSDA plots of metabolomics data of monocultures and interactions of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 (**A**) PLSDA 2D- plots of the analyzed LC-MS data of soluble compounds after three days of incubation) (**B**) PLSDA 2D- plots of GC-MS data of volatiles emitted after three days of inoculation.

LAESI- mass spectrometry (ambient imaging mass spectrometry)

The LAESI-DP1000 system was used to visualize the production of secondary metabolites by living bacterial colonies. With the LAESI-mass spectrometry system we confirmed the production of the two identified compounds 2,5-bis(1-methylethyl)-pyrazine (m/z= 164.247) and the unknown Pederin-like compound (m/z= 526.298 [$M+Na^+$]) in the micro colonies of *Burkholderia* and *Paenibacillus* (**Figure 4.4A, B**).

Table 4.6: Tentatively identified volatile organic compounds (VOCs) produced by a *Burkholderia* and *Paenibacillus* strain in mono- and co-culture on 1/10th TSB agar.

#	Compound name	RT*	ELRI**	p-value***	chemical class	Detected in treatment		
						Burk	**Paen**	**MIX Burk+Paen**
1	1,3-butadiene, 2-methyl-	2,11	525	0,018	Alkenes	X	X	X
2	2-methylfuran	2,53	586	0,030	Furan	X	X	X
3	dimethyl disulfide	4,20	741	0,001	Sulfides	X	X	X
4	toluene	4,63	762	0,000	Benzenoids	X	X	X
5	unknown compound 1	5,25	786	0,000	-	X	X	X
6	1,3-dithiethane	5,44	793	0,001	Sulfides	X		X
7	2,4 dithiapentane	7,96	887	0,009	Sulfides	X		X
8	alpha-pinene	9,59	930	0,011	Terpenes	X	X	X
9	benzaldehyde	10,53	956	0,016	Aldehydes	X	X	X
10	unknown compound 2	10,63	959	0,017	-	X	X	X
11	dimethyl trisulfide	10,86	964	0,027	Sulfides	X	X	X
12	C10-decane	12,19	998	0,000	Alkenes	X	X	X
13	unknown alkene	12,41	1003	0,018	Alkenes	X	X	X
14	unknown compound 3	13,87	1040	0,013	-	X	X	X
15	S-Methyl methanethiosulfonate	14,65	1059	0,010	Sulfides	X		
16	1,2,4-Trithiolane	15,71	1082	0,012	Sulfides	X		X
17	unknown compound 4	15,89	1087	0,000	-	X	X	
18	C11-alkene	16,47	1102	0,000	Alkenes	X	X	X
19	naphthalene	19,43	1178	0,000	Benzenoids	X	X	X
20	2,5-bis(1-methylethyl)-pyrazine	19,73	1186	0,001	Pyrazines	X		X
21	branched alkene	23,19	1284	0,008	Alkenes	X	X	X
22	unknown compound 5	30,81	1471	0,008	-	X	X	X
Number of detected compounds (n)						**22**	**17**	**20**

Abbreviations:
\# = Compound number, Burk = *Burkholderia* sp., AD24, Paen = *Paenibacillus* sp. AD87, MIX Burk+Paen = *Burkholderia* sp., AD24 + *Paenibacillus* sp. AD87.
RT* = Retention time, the RT value stated is the average of three replicates.
ELRI** = Experimental linear retention index value, the RI value stated is the average of three replicates.
p-value***= statistical significance (peak area and peak intensity).

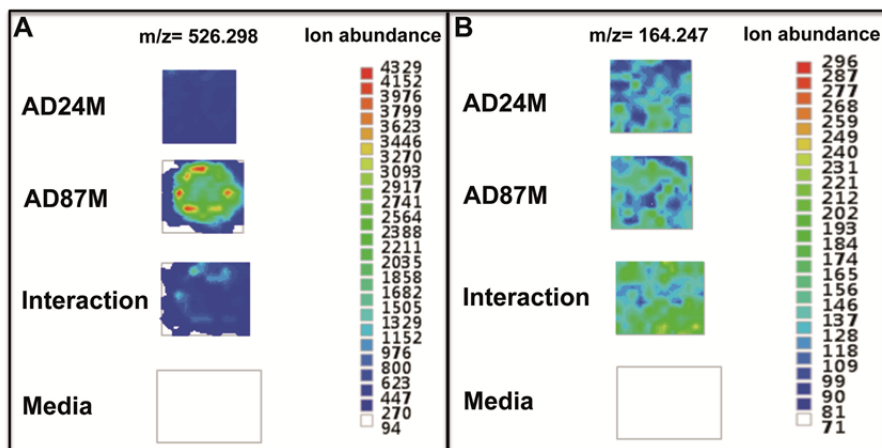

Figure 4.4: Results of the LAESI-MS imaging (**A**) heat map targeting the Pederin-like compound with an *m/z* of 526.298 [M+Na$^+$] showing specific accumulation of ions related to this compound in monocultures of *Paenibacillus* sp. AD87 (AD87M) and the interaction of *Paenibacillus* sp. AD87 and *Burkholderia* sp. AD24 (**B**) heat map targeting 2,5-bis(1-methylethyl)-pyrazine with an *m/z* of 164.247 showing specific accumulation of ions related to this compound in interaction samples of *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD87 (Interaction). The color associated with the ion map represents the base peak intensity (BPI) of [M+H$^+$] masses at a 1-ppm window, scale bar of ion abundance on the right side.

Biological activity of 2,5-bis(1-methylethyl)-pyrazine

After overnight incubation significant growth inhibition of *E. coli* WA321, *S. aureus* 533R4 and *C. albicans* BSMY212 was observed by exposure to 1.84 mg of pure 2,5-bis(1-methylethyl)-pyrazine. The bacterial and the yeast-like model organisms exposed to this pure volatile compound showed significant zones of inhibition (ZOI) around the filter paper compared to the controls (**Figure 4.5A**). Strong growth inhibition on the two plant pathogenic fungi was also observed by application of 1.8 mg 2,5-bis(1-methylethyl)-pyrazine. The mycelial extension of *Rhizoctonia solani* AG2.2IIIB and *Fusarium culmorum* PV exposed to the pure volatile compound was significantly reduced as compared to the controls (**Figure 4.5B**).

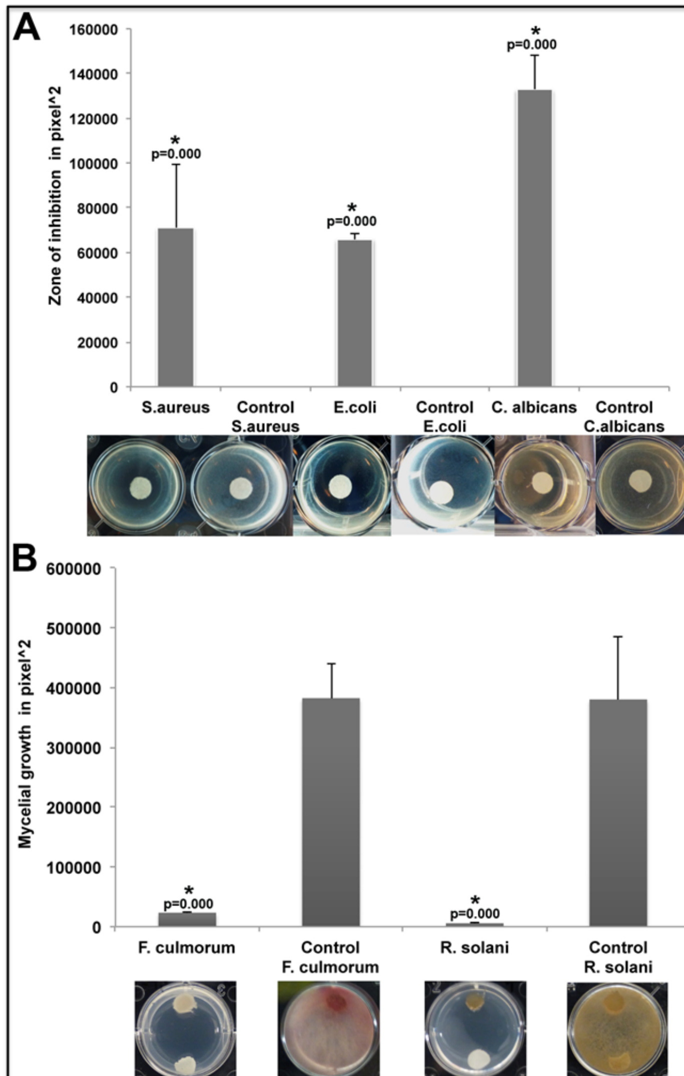

Figure 4.5: Effect of 2,5-bis(1-methylethyl)-pyrazine on growth of target organisms. (**A**) effect on *E.coli* WA321, *S.aureus* 533R4 and *C. albicans* BSMY212 growth. Bars represent the mean sizes of the Zone of inhibition (ZOI) in pixel^2 (**B**) effect of 2,5 2,5-bis(1-methylethyl)-pyrazine on mycelial extension of *Rhizoctonia solani* and *Fusarium culmorum*. Bars represent the mean of mycelial extension in pixel^2. Error bars represent standard deviation (SD). Significant differences between the non exposed control and the treatment are indicated by an asterisk (ONE-WAY ANOVA, post-hoc TUKEY test $p<0.05$).

Antibacterial activity of diffusible secondary metabolites

Agar diffusion tests performed with secondary metabolite extracts from monocultures and interactions of *Burkholderia*, *Paenibacillus* revealed antimicrobial activity against both tested model organisms, namely *E.coli* WA321 (**Figure 4.5A**) and *S. aureus* 533R4 (**Figure S4.8**).

The growth of the model bacteria in the seeded agar around the filter papers supplemented with 5 µl of the secondary metabolite extracts was significantly inhibited however without difference in inhibition between extracts obtained from monocultures or interactions (**Figure 4.5A, Figure S4.8**).

Synergistic effect of diffusible and volatile secondary metabolites

The agar diffusion tests performed with extracts from the interacting bacteria, in combination with 2 µl (=1.84 mg) of the pure volatile compound 2,5-bis(1-methylethyl)-pyrazine revealed strong synergistic effects between diffusible secondary metabolite extracts and the pure volatile compound against *E.coli* WA321. The exposure to the secondary metabolite extract in combination with pure 2,5-bis(1-methylethyl)-pyrazine led to significant bigger zones of inhibition (ZOI) around the filter paper compared to the controls (secondary metabolite extracts without added pure volatile compound) (**Figure 4.5B**). There was no such significant synergistic effect against the Gram-positive model organism *S. aureus* 533R4 by the combination of these compounds (**Figure S4.8**).

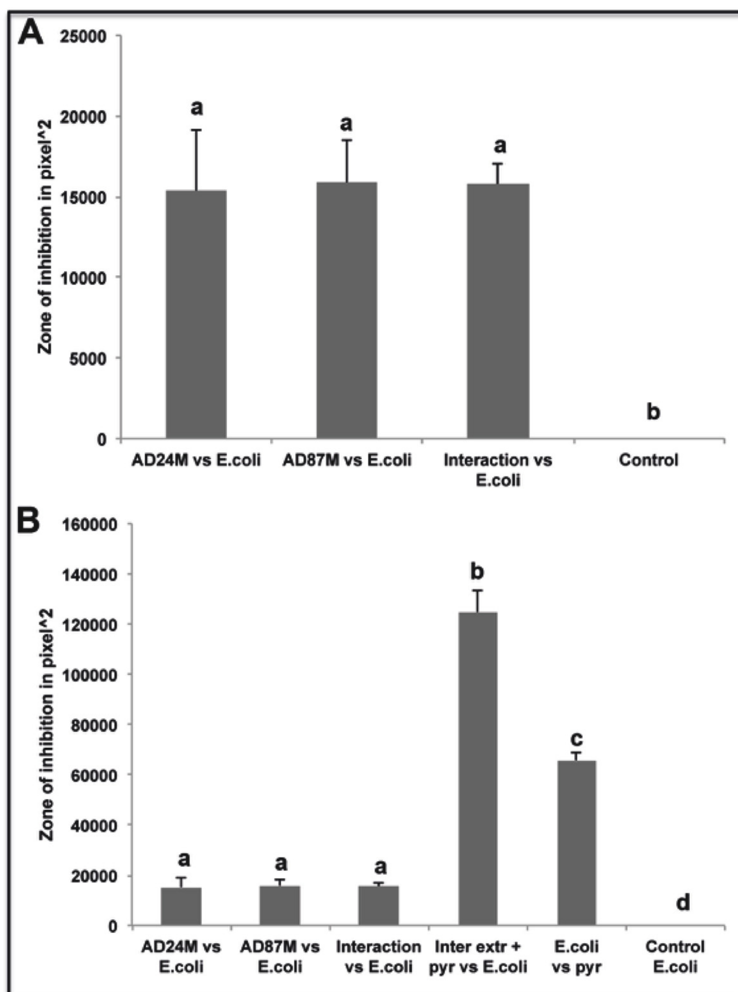

Figure 4.6: Effect of the secondary metabolite extracts of monocultures and interaction of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 in combination with 2,5-bis(1-methylethyl)-pyrazine on model organism growth. (A) Effect of the secondary metabolite extracts on *E.coli* WA321. (B) Effect of the secondary metabolite extracts in combination with 2 µl 2,5-bis(1-methylethyl)-pyrazine revealing a synergistic effect between the secondary metabolite extracts and the pure pyrazine compound. Bars represent the mean sizes of the Zone of inhibition (ZOI) in pixel^2. Error bars are indicating standard deviation (SD) between the replicates. Significant differences between the treatments are indicated by different letters (ONE-WAY ANOVA, post-hoc TUKEY test $p<0.05$).

Bacterial luciferase reporter assays

The reporter assays on aqueous extracts from cell material of monocultures and interactions confirmed the elevated inhibition of target strain growth, as measured by the pBPlux reporters for general toxicity. On these samples we deployed also a specific reporter assays for redox cycling compounds, such as phenazines. This reporter gave a clear signal above the detection limit which, upon normalization for the relative toxicity by pBPlux reporters, resulted in a transcriptional signal that reached up to 15 % of the maximum signal induced by the model compound pyocyanin (**Figure S4.9**) in the interaction extract, but not in one of the monoculture extracts.

The volatile compound that exhibited an elevated biosynthesis in the interaction, 2,5-bis(1-methylethyl)-pyrazine was used for bioactivity profiling by bacterial and mammalian reporter assays. Dilutions up to ~3.10^{-4} M caused a major reduction in the general toxicity reporter signal (pBPlux-1 in DH5α and MG1655), while the pBPlux-2 reporter in Δ*ampD* showed a clear decrease from ~ 1.10^{-3} M. The normalized transcriptional signal of the reporter for cell wall synthesis interference (pBLAlux-2 Δ*ampD*) was induced up to 19.5 % of the maximal transcriptional induction by the model compound penicillin G at ~3.10^{-4} M (**Figure S4.10A, B**). The redox cycling (pPHZux-1 DH5α) reporter assays did not showed a signal above the detection limit (**Figure S4.10C**). The DNA damage reporter pSOSlux-2 MG1655 was induced at nearly maximal induction levels (after normalization), well into the highly toxic concentration range (**Figure S4.10D**).

CALUX® reporter assays for human toxicological assessment of 2,5-bis(1-methylethyl)-pyrazine

The mammalian transcriptional reporter assays (CALUX®) revealed a cytotoxic response at concentration above 10^{-6} M, higher concentrations revealed minor induction of the DR-CALUX (induction of the AhR by aromatics such as dioxin-like compounds), ERα-CALUX (estrogen-like behavior), Nrf2-CALUX (anti-oxidant response) and p53-CALUX (genotoxicity) (**Figure S11A-F**). These transcriptional activations occurred at elevated concentrations and can be the cause of the stochastic transcriptional behavior during cytotoxicity). The p53 induction suggests genotoxic mode of action and is confirmed by the pSOSlux-2 (MG1655) data from the bacterial reporter assays.

Discussion

Phenotypic changes occurring during microbial interspecific interactions are receiving increased attention as they are the basis for explaining functioning of microbes in complex communities (Seyedsayamdost et al., 2012;Traxler et al., 2013). For example, interspecific interactions between soil bacteria were shown to have a major impact on production of antimicrobial compounds, with both induction and suppression of antimicrobial activity (Tyc et al., 2014). Yet, detailed information on the mechanisms of phenotypic changes during interspecific interactions is still scarce. Here we studied the interspecific interaction between a Gram-positive (*Paenibacillus*) and a Gram-negative bacterium (*Burkholderia*), in order to explore how this interspecific interaction will affect bacterial cell numbers, gene expression and the production of antimicrobial secondary metabolites.

The results of the present study revealed that the interaction between both bacteria had a negative effect on *Burkholderia* cell numbers whereas cell numbers of *Paenibacillus* appeared not to be affected. Hence it seems that *Paenibacillus* is a better competitor than *Burkholderia* under the conditions tested. Similar observations were previously reported for the interspecific interaction between Gram-negative and Gram-positive bacteria (Garbeva et al., 2011b;Tyc et al., 2015). Besides significant reduction of *Burkholderia* cell counts, we observed also up- and down- regulation of ribosomal proteins. Ribosomal proteins may have various functions apart from protein synthesis. The observed differential expression of ribosomal proteins can point at a general stress response (Ishige et al., 2003;Silberbach and Burkovski, 2006;Picard et al., 2013). Furthermore, ribosomal proteins may be important for antimicrobial activity as reported by de Carvalho (de Carvalho et al., 2010).

Up-regulation of several genes related to signal transduction, secondary metabolite production and to cell motility was observed for *Burkholderia* during the interaction with *Paenibacillus*. The elevated expression of gene bAD24_II08070 YiaD which is associated with the flagellar biogenesis and the cellular motility apparatus (Hu et al., 2009) indicates that motility can be an important feature during bacterial interspecific interactions. This observation could imply that *Burkholderia* is trying to move away from *Paenibacillus* micro colonies. Elevated expression of genes related to cell motility during interspecific bacterial interactions was already reported for *Pseudomonas fluorescens* Pf0-1 (Garbeva et al., 2011b;Garbeva et al., 2014a). Interestingly the highest fold change in gene expression in *Burkholderia* was found for the gene

bAD24_p01665 which is related to type IV secretion system. This secretion system plays an important role for the virulence of *Burkholderia* spp. (Zhang et al., 2009). The gene encoding for this secretion system was found on the mobile genetic element of *Burkholderia* which is in line with previous reports on type IV secretion systems in *Burkholderia* spp. (Engledow et al., 2004).

For *Paenibacillus*, genes encoding antibiotic resistance were upregulated. In particular gene gpAD87_28110 encoding the Vancomycin B-type resistance gene VanW was 9.36 fold up regulated, suggesting protection against antimicrobial compounds produced by *Burkholderia*. So far, the exact function of gene VanW is yet not completely understood (Evers and Courvalin, 1996;McGregor and Young, 2000). Recently, Letoffe and co-workers (Letoffe et al., 2014) reported the increase of antibiotic resistance due to possible synergistic effects between volatile compounds. Other studies also indicate that VOCs can act as modulator of antibiotic resistance (Kova et al., 2015). Thus it is possible that here, the produced volatile compounds induce increased expression of genes related to antibiotic resistance in *Paenibacillus*.

Several of the differentially expressed genes in both the *Burkholderia* and *Paenibacillus* strains are hypothetical proteins with unknown functions e.g. gpAD87_25000, gpAD87_13180, bAD24_II11090, bAD24_p01610. Despite the advantages made in the field of genome sequencing and genome annotation a vast percentage of bacterial genome sequences remains with unknown function (Galperin and Koonin, 2004;Song et al., 2015). Independent studies also indicated that such "unknown genes" are differentially expressed during interspecific interactions between bacteria (Garbeva et al., 2011b;Garbeva et al., 2014a). It will be important in further studies to determine the function and the products of such unknown genes that are apparently prominent during bacterial interactions.

The metabolomic analysis revealed that the interspecific interaction between *Burkholderia* and *Paenibacillus* increased the production of specific antimicrobial compounds such as 2,5-bis(1-methylethyl)-pyrazine and an unknown Pederin-like compound. These two compounds were detected in higher concentrations during interspecific interaction by using three independent approaches namely Orbitrap-XL-MS analysis, GC/MS-Q-TOF analysis and ambient imaging mass spectrometry (LAESI- MS) from living bacterial colonies. The performed bioassays with the volatile compound 2,5-bis(1-methylethyl)-pyrazine revealed significant antibacterial and antifungal

activity against the used microbial strains. Previous studies have already shown that pyrazine compounds can exhibit antimicrobial activities (Beck et al., 2003;Kucerova-Chlupacova et al., 2015). The detected pyrazine is most probably produced by the *Burkholderia* isolate, as small amounts of this compound were detected in the monoculture of these bacteria but not in monocultures of *Paenibacillus*. The bacterial production of the volatile 2,5-bis(1-methylethyl)-pyrazine was ,so far, only reported for few bacteria (Beck et al., 2003;Dickschat et al., 2005;Rajini et al., 2011).

The bioreporter assays performed with this compound revealed that the antimicrobial activity of 2,5-bis(1-methylethyl)-pyrazine is exerted by generating stress at the level of cell wall integrity, which is in line with results of the RNA-sequencing analysis were we observed higher expression of genes related to cell-wall synthesis in both *Burkholderia* and *Paenibacillus*. The toxicity of 2,5-bis(1-methylethyl)-pyrazine occurs below the toxic range observed in direct exposure of the CALUX® reporter panel.

Increased antimicrobial effects were seen when the extracts of the interaction and the pure volatile compound 2,5-bis(1-methylethyl)-pyrazine were combined. This might be due to synergetic effects between non-volatile and volatile compounds enhancing the overall antimicrobial activity (Schmidt et al., 2015). Such synergistic effects between volatile and non-volatile compounds were previously observed for hydrophilic antibiotics like e.g. beta-lactam antibiotics, which showed only negligible antimicrobial effects on Gram-negative bacteria if applied as soluble compound alone (Hemaiswarya and Doble, 2010). However, if these antibiotics were applied together with the volatile organic compound eugenol, increased antimicrobial activity towards Gram-negative and Gram- positive bacteria was observed (Hemaiswarya and Doble, 2010). In addition, combinations of soluble compounds non-volatile and volatile organic compounds have shown to inhibit the growth of multi-resistant *E. coli* and multi-resistant *S. aureus* isolates (Gallucci et al., 2009). Another compound produced in higher concentration during the interspecific interaction of *Burkholderia* and *Paenibacillus* was a soluble compound with an *m/z* of 504.316 $[M+H^{+}]$. So far, we were able to identify this compound as a Pederin like compound (exact mass difference < 0.05 ppm), however not all gene clusters related to Pederin production were detected in the genome of *Paenibacillus*. Pederin is a toxic amid with anti-tumoral and mitosis inhibiting properties found in endo-symbiotic *Pseudomonads* of *Paederus* beetles (Frank and Kanamitsu, 1987;Piel, 2002; 2009). Bioinformatics analysis revealed that

only three gene clusters (pedB, pedC and pedF) related to the known Pederin synthesis in *Pseudomonas fluorescens* and *Bacillus subtilis* with similarities of 37 % to pedB and 29 % to pedC and pedF were present in the genome of *Paenibacillus*. Additional bioinformatics analysis targeting KS (keto-synthase) domains revealed that only two KS domains are present in the genome of *Paenibacillus*, however nine KS domains would be needed for the full synthesis of Pederin (Piel, 2002; 2009). This suggests that the detected compound is most probably a novel compound from the same group/chemical class as Pederin. Soil is a very heterogeneous and complex environment consisting of aggregated particles harboring a high density and diversity of bacteria. In this environment bacteria can encounter several different competitors at the same time (Hibbing et al., 2010;Cornforth and Foster, 2013). Thus, production of secondary metabolites with antimicrobial properties such as the volatile 2,5-bis(1-methylethyl)-pyrazine during interspecific interactions may offer advantages during interspecific interactions for the producing strain by inhibition of surrounding competitors.

Our present study on interspecific bacterial interactions has been performed on nutrient rich agar media, these growth conditions are different from the nutritional conditions in natural soils (Torsvik et al., 1990b;Demoling et al., 2007). However, in a previous study a synthetic microbial community consisting out of five bacterial species, including *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 the volatile compound 2,5-bis(1-methylethyl)-pyrazine was also found to be produced in a soil-microcosm system (Schulz-Bohm et al., 2015). Thus, it is likely that this compound will be also produced during interspecific interactions of *Burkholderia* in natural systems such as soil. This observation makes this bacterium a promising candidate for pro-biotic treatments in soil. Based on all results, we cannot exclude the application of 2,5-bis(1-methylethyl)-pyrazine for applications like fumigation in logistics, food, or for plant protection against plant-pathogenic fungi and bacteria in agricultural production systems (Audrain et al., 2015).

In conclusion, the present study revealed that interspecific bacterial interactions affected fitness, gene expression and secondary metabolite profiles (volatile and non-volatile compounds). A graphical summary of the transcriptome and metabolome analysis and the mechanisms involved during the interspecific interaction between *Paenibacillus* sp. and *Burkholderia* is presented in **Figure 4.7**.

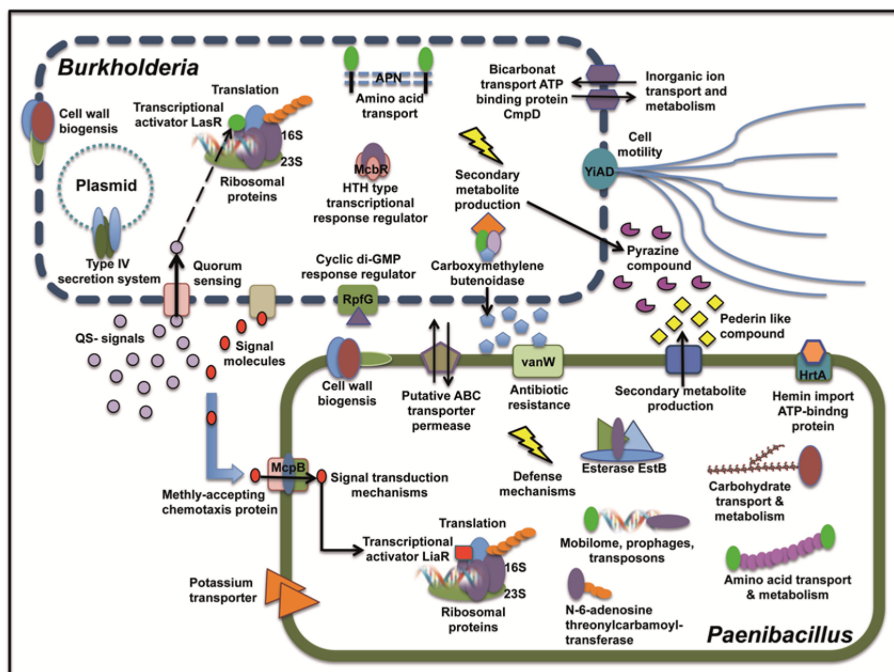

Figure 4.7: Schematic overview of the most important changes in gene expression and metabolite production in *Burkholderia* sp. AD24 (top) and *Paenibacillus* sp. AD87 (bottom) during interspecific interaction. This overview additionally includes the two secondary metabolites 2,5-bis(1-methylethyl)-pyrazine and the Pederin like compound which were detected in higher concentrations during interspecific interaction using mass spectrometric technologies.

The knowledge obtained here can be beneficial for the construction of synthetic microbial communities that consists of a minimal set of microorganisms needed to fulfil specific ecosystem services like e.g. disease suppression in agricultural systems (Shong et al., 2012;De Roy et al., 2013;Grosskopf and Soyer, 2014).

The observed triggering of a volatile pyrazine compound production during interspecific interaction indicates that screening of interspecific interactions may lead to the discovery of novel volatile compounds with antimicrobial activities.

Acknowledgments
This work was supported by the BE-Basic Foundation (http://www.be-basic.org/). PG is financed by The Netherlands Organization for Scientific Research (NWO) VIDI personal grant 864.11.015. The authors want to thank Kristin Schulz-Bohm for her useful advices on the qPCR assays, Prof. Dr. Harald Gross and Judith Bauer for their advices on the Pederin like compound identification. This is publication XXXX of the NIOO-KNAW.

Supplementary material

Table S4.1: De-Novo genome assembly statistics of the two sequenced bacterial isolates.

	***Burkholderia* sp. AD24**	***Paenibacillus* sp. AD87**
Number of PacBio subreads	57683	116628
N50 subreads	6956	7032
PacBio coverage	31	36,65
Illumina coverage	79	200
number of Illumina read pairs	3515383	5018466
contigs HGAP 3	10	36
contigs scaffolding	4	7
Final number of scaffolds	4	30
Final number of contigs	3	37
Number of gaps	17	7
N50	4476846	5194573
Total genome size	**8243440**	**7086713**

Table S4.2: Primers used for quantitative real-time PCRs.

Primer code	Sequence (5' - 3') of primers	target	reference
gpAD87_304F	GTACTTCCCGCACCTGACAT	dimodular nonribosomal peptide synthase	this study
gpAD87_304R	TGGCGAGAAACTCCACTTCT	dimodular nonribosomal peptide synthase	this study
bAD24_10391_IG_F	GTATTGGCCGTATCCGTCAG	snoaL-like polyketide cyclase family protein	this study
bAD24_10391_IG_R	AGCCACTCTTCGACGATCAC	snoaL-like polyketide cyclase family protein	this study
RecA_bAD24_1_F	GGTGAGGCAATCGAAGACAT	DNA recombination and repair protein	this study
RecA_bAD24_1_R	AGCTTGCTTGCGTACTGGAT	DNA recombination and repair protein	this study
RecA_gpAD87_3_F	CTTGCCTAAAGGCCGTATTG	DNA recombination and repair protein	this study
RecA_gpAD87_3_R	GACAATGTCCACAGCACCAC	DNA recombination and repair protein	this study
BacF	GGGAAACCGGGGCTAATACCGGAT	16S rDNA specific for *Bacilli*	Garbeva *et al.* (2003)
Eub338F	ACTCCTACGGGAGGCAGCAG	16S rDNA	Fierer *et al.* (2005)
Eub518R	ATTACCGCGGCTGCTGG	16S rDNA	Fierer *et al.* (2005)

Table S4.3: Primers used in bioreporter construction.

Primer code	Sequence (5' - 3') of primers
Primer 1	AAAAAGGATCCGGCGGGTTACTCCGGG
Primer 2	AAAAACTCGAGCCGCTGCCTAGCCGTCG
Primer 3	AAAAAGAATCCGATTGGTTGGTTTCCCTGGGTTCTGG
Primer 4	AAAAACTCGAGCTAGCCGGCCACCGCTTCC
Primer 5	AAAAATTAATTAAATCAGAACGCAGAAGCGG
Primer 6	AAAAATTAATTAACCTATATCGCCGACATCACC
Primer 7	TCGTCTTCACCTCGAGTGCCAGTCGGCGCTCTTC
Primer 8	CGCAACTAGAGGATCCAAACACCTCTTTGAC
Primer 9	TCGAAATACTTGACATATCACTGTGATTCACATATAATATGCG
Primer 10	GATCCGCATATTATATGTGAATCACAGTGATATGTCAAGTATT

Table S4.4: Bacterial strains and plasmids used for bioreporter construction and the performed bioreporter assays on aqueous and pure volatile extracts.

	Description	Reference
Strain		
E. coli DH5α	F-,Δ(argF-lac)169, φ80dlacZ58(M15), ΔphoA8, glnX44(AS), λ-, deoR481, rfbC1?, gyrA96(NalR), recA1, endA1, thiE1, hsdR17	
E. coli MG1655	F-, λ-, rph-1	
E. coli Δ*ampD*	F-,Δ(araD-araB)567, ΔampD728::kan, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514	Baba et al (2006)
P. aeruginosa PA14	Wild type, as PCR template	
P. protegens Pf-5		USDA-ARS
Plasmid		
pCS26Pac	Km^r, pSC101, *luxCDABE*	Bjarnason et al (2003)
pBAD33	Cm^r, pACYC184	Guzman et al (1995)
pJAMA8-cda	Ap^r, ColE1, *lux*AB promoter-probe vector with the cda (colicin D) promoter.	Tecon et al (2010)
pBPlux-1		This study
pBPlux-2		This study
pPHZlux-1		This study
pSOSlux-2		This study
pBLAlux-1		This study
pBLAlux-2		This study

Table S4.5: Outcome of the qRT-PCR analysis and comparison to the outcome of the RNA-seq analysis. The fold change values for the qRT-PCR are analysis are calculated with the ΔΔ-CT method.

Gene	function	time point	fold change qPCR ΔΔ–CT	SD qPCR	fold change RNA-seq data
bAD24_10391	snoaL-like polyketide cyclase family protein	t48	1,30	±0.73	1,61
bAD24_10391	snoaL-like polyketide cyclase family protein	t72	1,20	±0.50	1,21
gpAD87_304	dimodular nonribosomal peptide synthase 29% similiarity with pedF	t48	0,75	±0.33	0,51
gpAD87_304	dimodular nonribosomal peptide synthase 29% similiarity with pedF	t72	1,35	±0.81	1,34

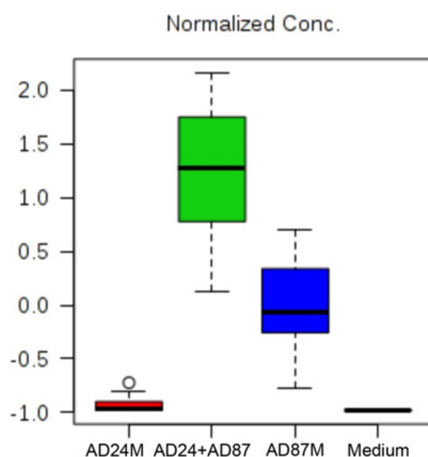

Figure S4.6: Normalized concentration of the unknown Pederin like compound with a mass of 504.316 ($M+H^+$). The compound was detected in a higher concentration during interaction of *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD87. The compound was not detected in samples of *Burkholderia* sp. AD24 monoculture and in the medium control (extracts of $1/10^{th}$ TSBA).

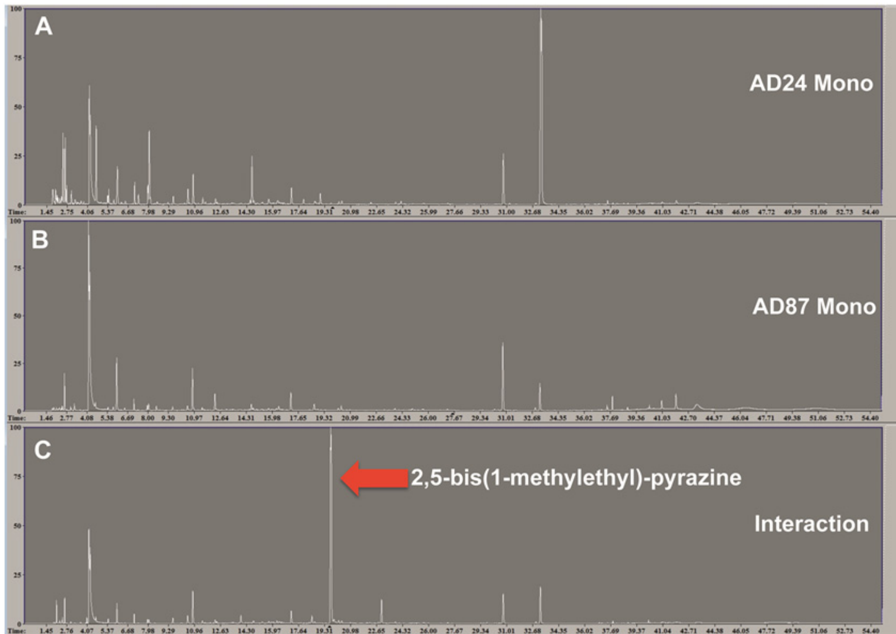

Figure S4.7: Representation of GC/MS chromatograms of (**A**) *Burkholderia* sp. AD24 monoculture (top) (**B**) *Paenibacillus* sp. AD87 monoculture (middle) and (**C**) interaction of both bacteria (bottom). The compound 2,5-bis(1-methylethyl)-pyrazine (RT 19.7 *m/z*= 164.247) was detected in a higher concentration in samples of the pairwise combination of *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD87.

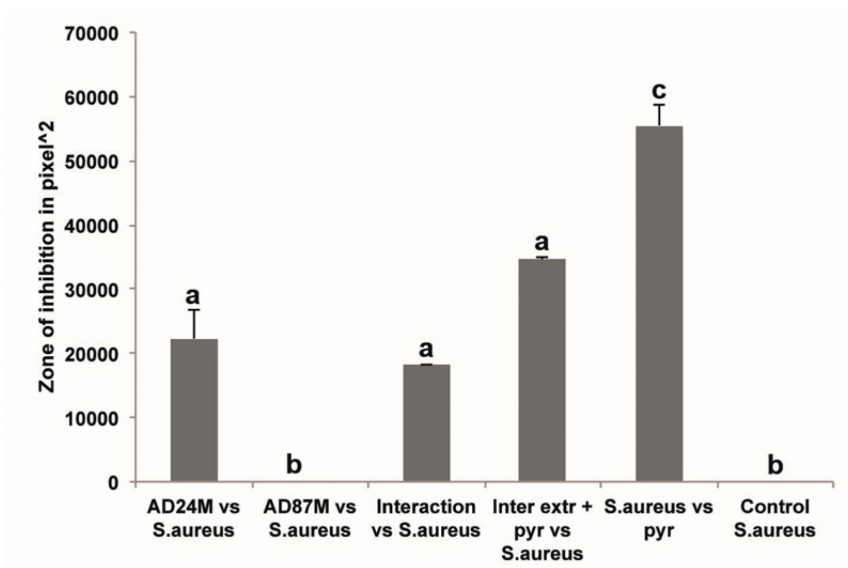

Figure S4.8: Effect of the secondary metabolite extracts and the interaction extract in combination with 2,5-bis(1-methylethyl)-pyrazine on growth of *S.aureus* 533R4. Bars represent the mean sizes of the Zone of inhibition (ZOI) in pixel^2. Error bars are indicating standard deviation (SD) between the replicates. Significant differences between the control and the treatments are indicated by different letters (ONE-WAY ANOVA, post-hoc TUKEY test $p<0.05$).

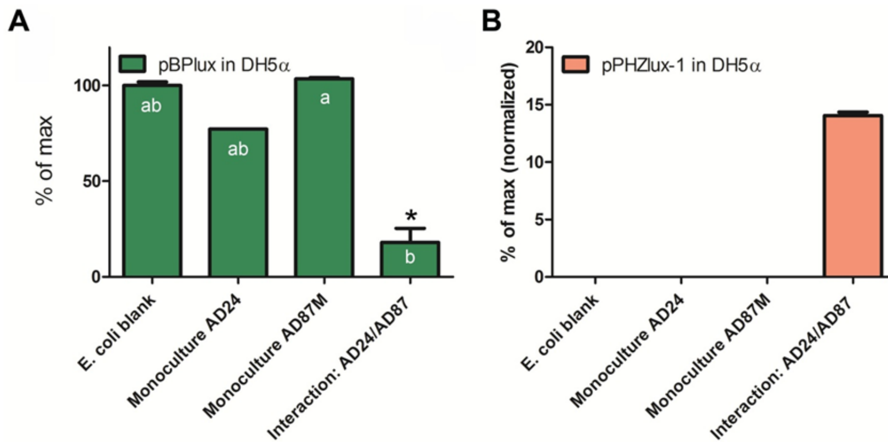

Figure S4.9: Aqueous extracts were used for exposure of the bacterial reporters for toxicity (pBPlux), measured as a reduction luminescence, and redox cycling by induction of pPHZlux-1. The resulting signals were represented as % of the maximum in the blank sample (**A**) or the reference compound pyocyanine (**B**) respectively. The interaction showed a significant toxicity relative to the blank and the monocultures. The extracts from the interaction revealed a redox cycling activity, as measured by the pPHZlux-1 reporter. Above detection limit measurements of the latter assay were corrected for toxicity. Significance levels * $p<0.05$ after Kruskal-Wallis test (Dunn post-hoc multiple comparison) in GraphPad Prism.

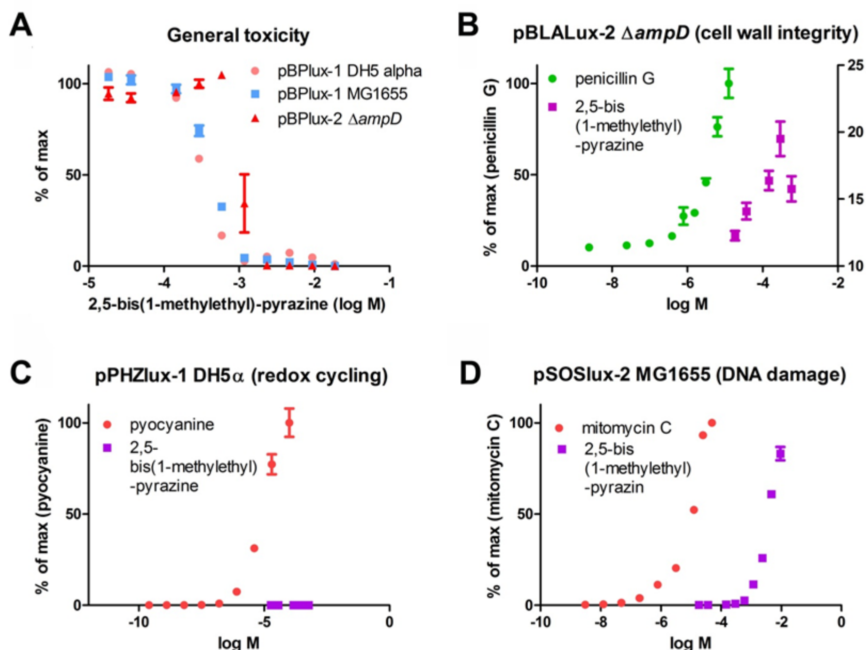

Figure S4.10: Overview of the dose response curves of the bacterial reporter assays upon 2,5-bis(1-methylethyl)-pyrazine (**A**) The synthesized pyrazine proved to the E. coli host as revealed by the three control reporter strains. (**B**) The reporter that detects interference with cell wall synthesis and integrity, pBLAlux-2 (ΔampD) was induced after normalization to toxicity up to 19.5 % of the maximum response of the model compound penicillin G (at ~3.10^{-4} M). b: pPHZlux-1 DH5α. (**C**) The reporter for redox cycling compounds was not induced at all by this compound. (**D**) pSOSlux-2 MG1655 exhibited a normalized response of up 83 % of the reference compound at elevated concentrations, within the toxic range.

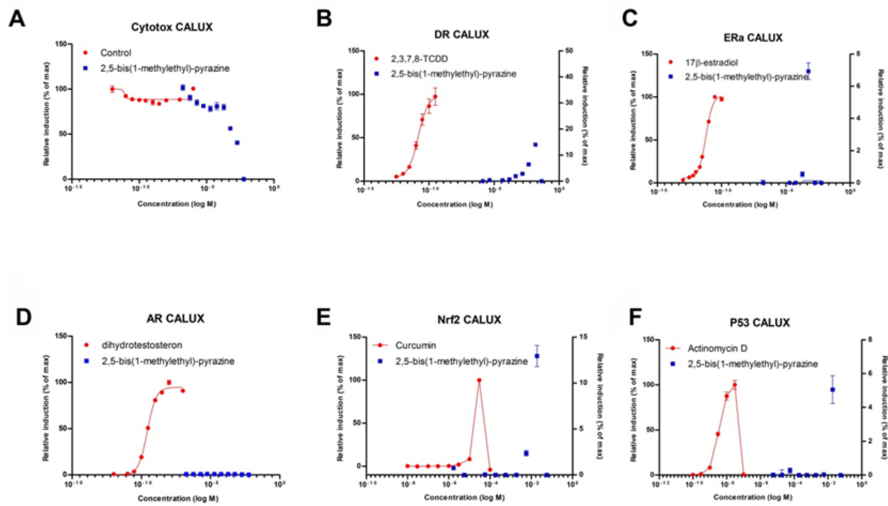

Figure S4.11: Overview of the selection of CALUX® mammalian reported assays exposed to the 2,5-bis(1-methylethyl)-pyrazine as compared to the response of the model compounds. The dose responses are represented as concentrations in the wells. Despite the cytotoxicity above $10E^{-4}$ M (**A**), adverse toxicological endpoints, such as dioxin-like (**B**), estrogen-like (**C**), Nrf2-like, (**D**) Androgenic activity was not observed with this compound. (**E**) and genotoxic activity (**F**). Responses are observed at elevated levels up to 10^{-2}M. When present, the right axis represent the relative response of 2,5-bis(1-methylethyl)-pyrazine.

Chapter 5

The effect of phylogenetically different bacteria on the fitness of *Pseudomonas fluorescens* in sand microcosms

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The effect of phylogenetically different bacteria on the fitness of *Pseudomonas fluorescens* in sand microcosms

Abstract

In most environments many microorganisms live in close vicinity and can interact in various ways. Recent studies suggest that bacteria are able to sense and respond to the presence of neighboring bacteria in the environment and alter their response accordingly. This ability might be an important strategy in complex habitats such as soils, with great implications for shaping the microbial community structure.

Here, we used a sand microcosm approach to investigate how *Pseudomonas fluorescens* Pfo-1 responds to the presence of monocultures or mixtures of two phylogenetically different bacteria, a Gram-negative (*Pedobacter* sp. V48) and a Gram-positive (*Bacillus* sp. V102) under two nutrient conditions. Results revealed that under both nutrient poor and nutrient rich conditions confrontation with the Gram-positive *Bacillus* sp. V102 strain led to significant lower cell numbers of *Pseudomonas fluorescens* Pfo-1, whereas confrontation with the Gram-negative *Pedobacter* sp. V48 strain did not affect the growth of *Pseudomonas fluorescens* Pfo-1. However, when *Pseudomonas fluorescens* Pfo-1 was confronted with the mixture of both strains, no significant effect on the growth of *Pseudomonas fluorescens* Pfo-1 was observed. Quantitative real-time PCR data showed up-regulation of genes involved in the production of a broad-spectrum antibiotic in *Pseudomonas fluorescens* Pfo-1 when confronted with *Pedobacter* sp. V48, but not in the presence of *Bacillus* sp. V102.

The results provide evidence that the performance of bacteria in soil depends strongly on the identity of neighboring bacteria and that interspecific interactions are an important factor in determining microbial community structure.

Introduction

Culture-independent technologies have given us insight in the tremendous phylogenetic and functional diversity of microbial communities (Gans et al., 2005;Uroz et al., 2010). Recently, the role of interactions between the members of microbial communities and how these shape community composition and dynamics is receiving increasing interest (Hibbing et al., 2010;Foster and Bell, 2012;Cornforth and Foster, 2013;Mitri and Foster, 2013). Both theoretical models and empirical studies are used to explain the coexistence of competing microbial species and consequently microbial community assembly (D'Onofrio et al., 2010;Foster and Bell, 2012).

In soil and in the rhizosphere, many microbial species live in close vicinity and interact with each other in various ways ranging from competition to cooperation (Czaran and Hoekstra, 2009;Foster and Bell, 2012;Allen and Nowak, 2013). Bacteria can recognize cues from their environment to modulate behavior in order to increase their chance of survival.

Using recently developed techniques (NanoDESI and MALDI-TOF imaging mass spectrometry) Traxler and co-authors indicated the importance of interspecific interactions for triggering the production of different secondary metabolites in a single strain (Traxler et al., 2013). Recent studies in our group also indicate that bacteria are respond differently to the presence of different microbial species (de Boer et al., 2007;Garbeva and de Boer, 2009;Garbeva et al., 2011b). Studies on behavior and the transcriptional responses of the soil bacterium *Pseudomonas fluorescens* Pf0-1 on nutrient-poor agar in confrontation with taxonomically different bacterial species revealed significant differences in the responses of *Pseudomonas fluorescens* Pf0-1 to different bacteria. In particular, the expression of genes involved in signal transduction and antibiotic production was strongly affected by the identity of the interacting strains (Garbeva et al., 2011b).

So far the response of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacteria has only been studied during one-to-one confrontations on agar media (Garbeva and de Boer, 2009;Garbeva et al., 2011b). However, these conditions are very artificial compared to the situation in the natural soil environment, which is a heterogeneous and complex habitat consisting of aggregated particles with huge bacterial diversity (Torsvik et al., 1990a;Gans et al., 2005;Demoling et al., 2007). It is thus plausible that bacteria can sense more easily the presence of neighbors in their vicinity on an agar plate than in soil.

Furthermore, in natural environments bacteria are likely to encounter several different competitors at the same time or in sequential events (Hibbing et al., 2010). In the present study, we made a first attempt to study bacterial interactions in soil-like systems.

To this end we investigated the interaction between *Pseudomonas fluorescens* Pf0-1 with monocultures and mixtures of *Pedobacter* sp. V48 and *Bacillus* sp. V102 in sand microcosms under two different nutrient conditions. We hypothesized that both nutrient conditions and the identity of the competitor would have an effect on the performance *of Pseudomonas fluorescens* Pf0-1.

Materials and methods

Bacterial strains and growth conditions

Three different bacterial species, *Pseudomonas fluorescens* Pfo-1 (γ-Proteobacteria) (Compeau et al., 1988), *Pedobacter* sp. V48 (Sphingobacteria) and *Bacillus* sp. V102 (Bacilli) (de Boer et al., 2007) were used in this study (**Table 5.1**). The strains were pre-cultured from frozen -80 °C glycerol stocks on 1/10th TSB agar (5.0 gL^{-1} NaCl (Merck), 1.0 gL^{-1} KH_2PO_4; 3 gL^{-1} Tryptic Soy Broth (OXOID); 20 gL^{-1} Agar (Merck), pH 6.5) (Garbeva and de Boer, 2009) for three days at 20 °C.

Table 5.1: Bacterial strains and used antibiotics / selection method.

Bacterial strain	Description	Reference	Selective antibiotic
P. fluorescens Pfo-1	Wild type, soil isolate, Gram-negative, Class: *Gamma-proteobacteria*	Compeau *et al.*, (1988)	Ampicillin
Pedobacter sp. V48	Wild type, Gram-negative, Phylum: *Bacteroidetes*, Class: *Sphingobacteria*	de Boer *et al.*, (2007)	Kanamycin
Bacillus sp. V102	Wild type, Gram-positive, Class: *Bacilli*	de Boer *et al.*, (2003)	n/a pasteurization

Microcosm setup

Microcosms were established in 100 mL glass vials with a plastic screw cap lid (**Figure S5.1**) containing sterile acid washed sea sand with pore size fractions ranging from 0.075 to 0.425 mm (Honeywell Specialty Chemicals Seelze GmbH, Germany). The amount of sand was either 25 g (Microcosms supplemented with 1.5 mL 1/10th strength Tryptic Soy Broth (nutrient rich media) (5.0 gL^{-1} NaCl (Merck), 1.0 gL^{-1} KH_2PO_4; 3 gL^{-1} Tryptic Soy Broth (OXOID)) or 30 g (Microcosms supplemented with 1.5 mL nutrient poor media (5.0 gL^{-1} NaCl (Merck), 1.0 gL^{-1} KH_2PO_4; 0.1 gL^{-} $(NH_4)_2SO_4$; 0.5 gL^{-1} Tryptic Soy Broth (OXOID)). The sand was weighed directly into the glass vials and afterwards sterilized by autoclaving for 20 minutes. The sterilized microcosms were dried overnight in a 60 °C oven prior to inoculation. All treatments were performed in triplicates over a time of 6 days. A detailed overview of all treatments and controls is given in **Table 5.2**.

Table 5.2: Treatment overview of the microcosm treatments.

Treatment codec	Involved bacterial strains	Number of tested interactions	Supplied growth media
1	*P. fluorescens* Pfo-1, *Pedobacter* sp. V48, *Bacillus* sp. V102	3	1/10th TSB
2	*P. fluorescens* Pfo-1, *Pedobacter* sp. V48	2	1/10th TSB
3	*P. fluorescens* Pfo-1, *Bacillus* sp. V102	2	1/10th TSB
4	*P. fluorescens* Pfo-1, *Pedobacter* sp. V48, *Bacillus* sp. V102	3	Nutrient poor media
5	*P. fluorescens* Pfo-1, *Pedobacter* sp. V48	2	Nutrient poor media
6	*P. fluorescens* Pfo-1, *Bacillus* sp. V102	2	Nutrient poor media
	Controls		
7	*P. fluorescens* Pfo-1 Monoculture	-	1/10th TSB
8	*Pedobacter* sp. V48 Monoculture	-	1/10th TSB
9	*Bacillus* sp. V102 Monoculture	-	1/10th TSB
10	*P. fluorescens* Pfo-1 Monoculture	-	Nutrient poor media
11	*Pedobacter* sp. V48 Monoculture	-	Nutrient poor media
12	*Bacillus* sp. V102 Monoculture	-	Nutrient poor media

Microcosm inoculation

Sand microcosms were inoculated with either each strain as monoculture, pairwise combinations, or with all three strains together (**Table 5.2**). To inoculate the microcosms a single colony of the respective strain was transferred into 10 ml of $1/10^{th}$ TSB and grown overnight at 20 °C, 220 rpm to an optical density (OD_{600}) of: ~ 0.700 (*Pseudomonas fluorescens* Pfo-1), ~0.600 (*Pedobacter* sp. V48) and ~0.650 (*Bacillus* sp. V102). The bacterial strains were diluted in a nutrient rich or nutrient poor inoculation master mix to a density of ~1 * 10^5 CFU/mL. Each microcosm was inoculated with a volume of 1.5 mL of the respective inoculum master mix in the middle of the sterilized sand and mixed well.

To verify bacterial cell numbers in the inoculum, dilution plating was done in duplicates on selective agar plates (*Pseudomonas fluorescens* Pfo-1: $1/10^{th}$ TSBA plates supplemented with 100µg/mL Ampicillin, *Pedobacter* sp. V48: $1/10^{th}$ TSBA plates supplemented with 50µg/mL Kanamycin, *Bacillus* sp. V102: samples were pasteurized by heat treatment for 10 min. @ 80 °C).

Bacterial enumeration

The growth of the three bacterial strains in the different treatments was tracked by plate counting of all culturable cells (*Pseudomonas fluorescens* Pfo-1 and *Pedobacter* sp. V48) or by counting of spores and heat-stable cells (*Bacillus* sp. V102) (**Table 5.1**). The enumeration was performed as follows: after one and six days of incubation a sterilized stainless steel spoon was used for sampling by mingling the sand by a full clock- and one counter- clockwise turn. After mixing 1 g sand was taken from the center of each microcosm and transferred into a 15 mL Greiner tube. A volume of 10 ml 10 mM phosphate buffer (pH 6.5) was added and the tubes were shaken in a rotary shaker at 350 rpm for 30 minutes at 20 °C. Subsequently, serial dilutions were prepared and plated in triplicates on selective media (antibiotics used are indicated in **Table 5.1**). For the enumeration of the *Bacillus* sp. V102, samples were pasteurized by heating the tubes to 80 °C for 10 min in a pre-warmed heating block. The plates were incubated for two to four days at 20 °C and the CFUs of the respective strains were determined.

RNA extraction and quantitative real time PCR

The expression of gene cluster Pfl01_3463-3466, which is involved in the production of a broad-spectrum antibiotic (Garbeva et al., 2011b) was quantified via quantitative real time PCR. Total RNA was extracted at day 6 from nutrient rich microcosms (1/10th TSB) containing *Pseudomonas fluorescens* Pfo-1 as monoculture or in interaction as follows: the double volume (2 mL) of RNA protect Bacteria Reagent (QIAGEN cat# 76506) was added to 1 g sand sample and centrifuged at 10,000 x g for 10 min (Sigma 3K-14 centrifuge, SIGMA Laborzentrifugen GmbH, Germany). The supernatant was discarded and the pellets were stored at -80 °C until further analysis. Total RNA was extracted with the MO-BIO PowerSoil Total RNA Isolation Kit (MO-BIO cat# 12866-25) according to the manufacturer's protocol. The RNA extracts were treated with the TURBO DNA free Kit from AMBION (cat# 1907) according to the manufacturer's protocol to remove any remaining DNA. The RNA concentration and quality was checked on a NanoDrop Spectrophotometer (Isogen Life Science, IJssestein, The Netherlands). cDNA was synthesized from the extracted RNA with random hexamer primers from Invitrogen (cat# 48190-011) by using reverse transcriptase of the Fermentas RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas cat#K1651) according to manufacturer's protocol. The concentration and quality of the cDNA was determined using a NanoDrop spectrophotometer by measuring the A260/A280 ratio and samples were run on a 1.5 % agarose gel in 0.5 % TBE

buffer to check size and integrity of the synthesized cDNA. The selected gene cluster was targeted with primer combination 3463F835 (5'ATTTTTACGCGGTCTACGC) and 3463R1036 (5'TGATCAGGTTGCTGTTTCAGG) (Garbeva et al., 2011b) amplifying 202bp from gene Pfl01_3463 encoding the two branched-chain alpha-keto acid dehydrogenase E1 component. From each treatment, 50 ng cDNA was subjected to quantitative RT- PCR using SYBR Green PCR master mix (Applied Biosystem, Warrington, UK). Quantitative RT- PCR was performed on a Corbett Research Rotor- Gene 3000 thermal cycler (Westburg, Leusden, The Netherlands) with the following settings: initial cycle 95 °C for 15min, followed by 40 cycles of 95 °C for 15 sec, 56 °C for 50 sec and 72 °C for 50 sec. All analysis was performed in triplicate. Five standard curves (9.5 ng/µl, 0.95 ng/µl, 0.095 ng/µl, 0.0095 ng/µl and 0.00095 ng/µl) were established. Gene expression data was analyzed with a post-hoc LSD- test and differences between the means of data of different *Pseudomonas* interactions were considered to be statistically different at $p \leq 0.05$.

Malthusian parameter

As an estimate for fitness of the *Pseudomonas fluorescens* Pf0-1 as monoculture or in competition with the two other strains was calculated by applying the Malthusian parameter (M) growth model (Vasi et al., 1994;van den Berg et al., 2008).

The Malthusian parameter was calculated for both monocultures and mixed cultures by comparing the number *of Pseudomonas fluorescens* Pf0-1 individuals at an initial time (t_0), N_0, to the number of *Pseudomonas fluorescens* Pf0-1 individuals at a future time (t_N): $M = \ln (N_t/N_0) / t$.

Statistical analysis

Statistical analyses of the cell counts were performed with IBM SPSS Statistics 20 (IBM, Somers, NY, USA) using one-way ANOVA and post-hoc TUKEY LSD test. Significant differences between the controls (monocultures of the respective bacterial strain) and the treatments are marked with an asterisk ($p \leq 0.05$).

Results and discussion

In the present study, we investigated how the interactions between phylogenetically different bacterial strains affect the growth of *Pseudomonas fluorescens* Pfo-1 in sand microcosms under two different nutrient conditions. Our interests were particularly focused on *Pseudomonas fluorescens* Pfo-1, as our previous research had shown that *Pseudomonas fluorescens* Pfo-1 responded differently (behavior and gene expression profile) to phylogenetically different bacteria on nutrient poor agar (Garbeva et al., 2011b).

The growth of *Pseudomonas fluorescens* Pfo-1 in microcosms supplemented with either nutrient rich or nutrient poor growth media are presented in **Figure 5.1A and 5.1B**. Bacterial enumeration revealed that all tested bacterial strains used in this study were able to grow in the sand microcosms although with lower numbers under nutrient poor conditions (**Figure 5.2A and 5.2B**). In microcosms supplemented with nutrient rich media *Pseudomonas fluorescens* Pfo-1 reached approximately $5.5 * 10^5$ cells/g of sand as a monoculture, while in microcosms supplemented with nutrient poor media reached only $9.2 * 10^4$ cells/g of sand.

The *Bacillus* sp. V102 cell numbers in monocultures reached $3.6 * 10^4$ cells/g of sand in nutrient rich microcosms and $7 * 10^3$ cells/g of sand in nutrient poor microcosms. The cell counts of *Pedobacter* sp. V48 as monoculture were approximately $2.3 * 10^6$ cells/g of sand in nutrient rich and $2.8 * 10^4$ cells/g of sand in nutrient poor microcosms (**Figure 5.2A and 5.2B**).

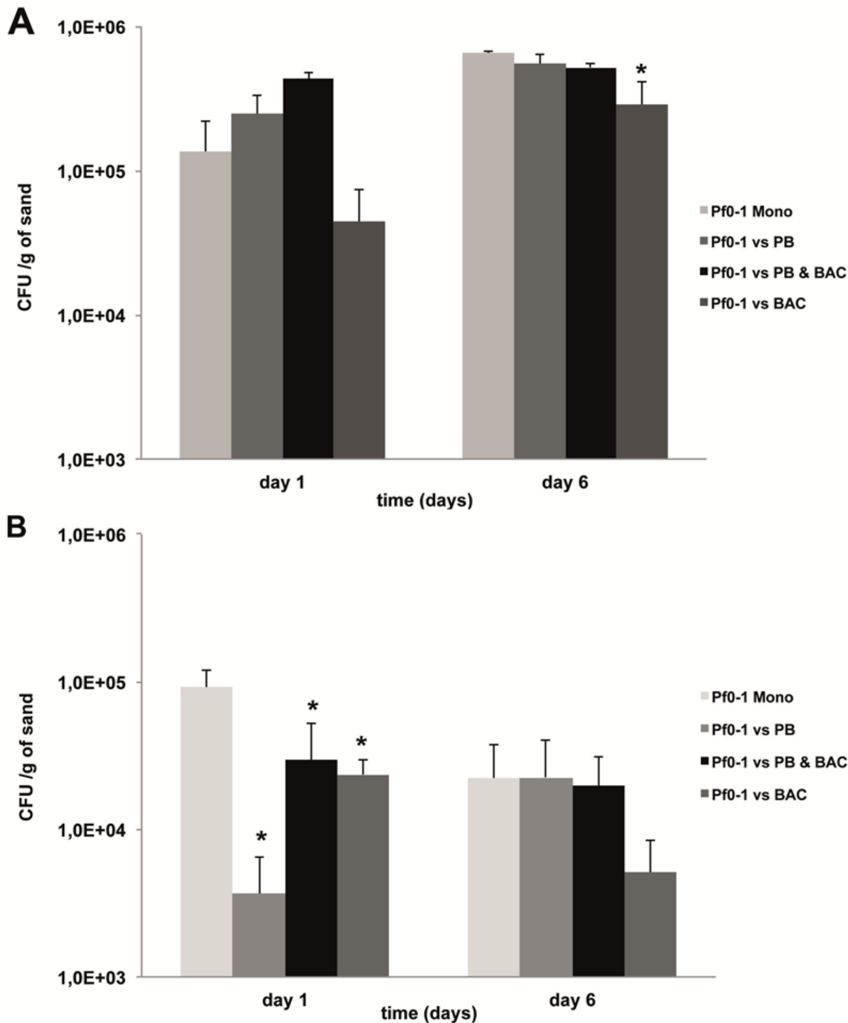

Figure 5.1: Cell counts of *Pseudomonas fluorescens* Pf0-1 at day 1 and day 6 under (**A**) nutrient rich and (**B**) nutrient poor conditions. Significant differences between the numbers of Pf0-1 in monoculture and in mixed cultures are indicated with an asterisk ($p \leq 0.05$). Error bars are indicating standard deviation (SD) between the triplicates. Abbreviations: Pf0-1: *Pseudomonas fluorescens* Pf0-1, PB: *Pedobacter* sp. V48, BAC: *Bacillus* sp. V102.

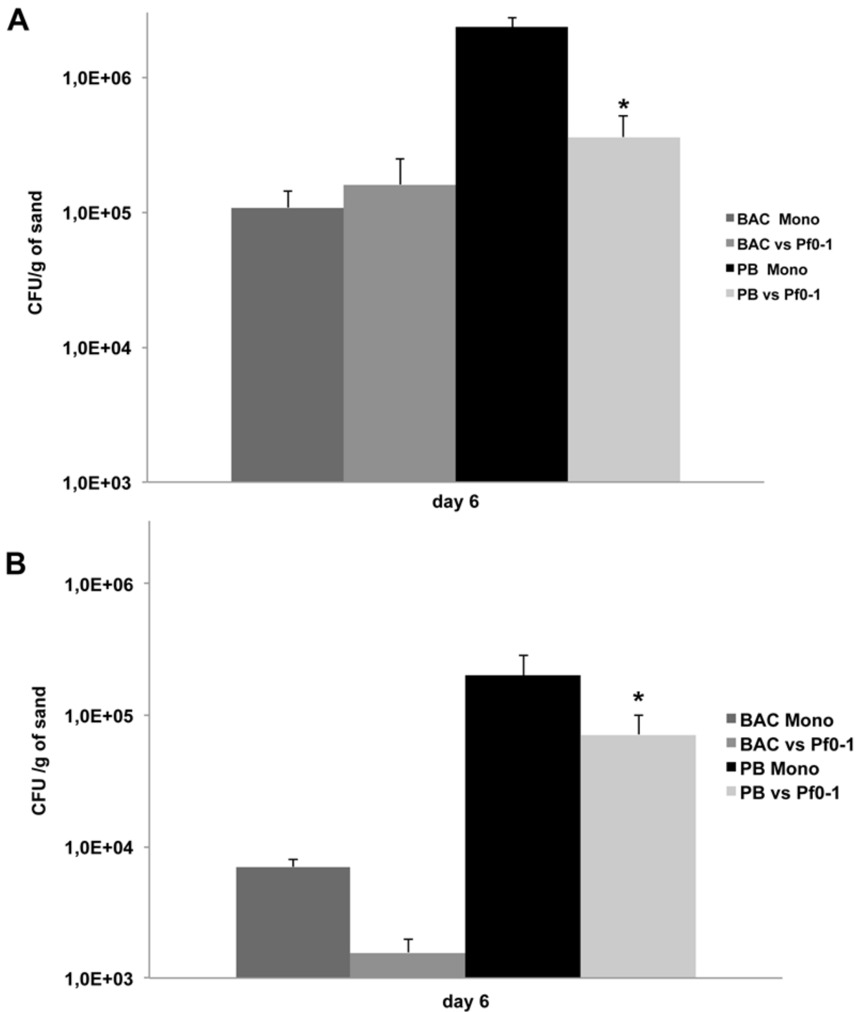

Figure 5.2: Numbers of CFUs of *Bacillus* sp. V102 and *Pedobacter* sp. V48 in monoculture and in mixed cultures (with strain Pf0-1) at day 6 under nutrient rich conditions (**A**) and under nutrient poor conditions (**B**). Significant differences between the CFUs in monoculture and in mixed cultures are indicated with an asterisk ($p \leq 0.05$). Error bars are indicating standard deviation (SD) between the triplicates. Abbreviations: Pf0-1: *Pseudomonas fluorescens* Pf0-1, BAC: *Bacillus* sp. V102, PB: *Pedobacter* sp. V48.

The effect of phylogenetically different bacteria on the fitness of *Pseudomonas fluorescens* in sand microcosms

The growth of *Pseudomonas fluorescens* Pfo-1 was negatively affected when confronted with the Gram-positive *Bacillus* sp. V102 strain resulting in significantly lower cell counts at day 6 in nutrient rich microcosms (p=0.012) and at day 1 in nutrient poor microcosms (p=0.008). When co-cultivated with *Bacillus* sp. V102 *Pseudomonas fluorescens* Pfo-1 reached a maximum of approximately 4.8 * 10^5 cells/g of sand (**Figure 5.1A and 5.1B**). Strong reduction of *Pseudomonas fluorescens* Pfo-1 growth during confrontation with *Bacillus* sp. V102 was observed previously on nutrient-poor agar even without direct cell-cell contact (Garbeva et al., 2011b). When co-cultivated with the Gram-negative *Pedobacter* sp. V48 strain, no significant effect on the growth of *Pseudomonas fluorescens* Pfo-1 was observed at day 6 (p=0.988), whereas there was a significant reduction at day 1 in nutrient-poor microcosms (p=0.000). Based on the cell enumeration we applied the Malthusian growth model (population growth) as an estimate for fitness (**Figure S5.2**). This revealed that the population growth of *Pseudomonas fluorescens* Pfo-1 was significantly negative affected only during co-cultivation with *Bacillus* sp. V102 on both day 1 and day 6 (p=0.026 and p=0.014).

The observed difference in response of strain *Pseudomonas fluorescens* Pfo-1 to co-cultivated bacteria was not due to the difference in bacterial growth as both *Pedobacter* sp. V48 and *Bacillus* sp. V102 were growing in the sand microcosms with *Pedobacter* sp. V48 reaching higher cell counts per gram of sand than *Bacillus* sp. V102 (**Figure 5.2A and 5.2B**). However, when co-cultivated with both *Bacillus* sp. V102 and *Pedobacter* sp. V48 simultaneously, there was no significant effect on the growth of *Pseudomonas fluorescens* Pfo-1 in both nutrient rich (p=0.650) and nutrient poor microcosms (p=0.995) (**Figure 5.1A and 5.1B**).

From the interspecific interactions investigated in the present study, it is clear that *Bacillus* sp. V102 acts as “bad” neighbor that can negatively affect the fitness of *Pseudomonas fluorescens* Pfo-1 as compared to the “good” neighbor *Pedobacter* sp. V48 that did not show any negative effect on the growth of *Pseudomonas fluorescens* Pfo-1. However, when co-cultivated with both strains simultaneously, *Pseudomonas fluorescens* Pfo-1 growth was better than when confronted only with *Bacillus* sp. V102 (**Figure 5. 3**).

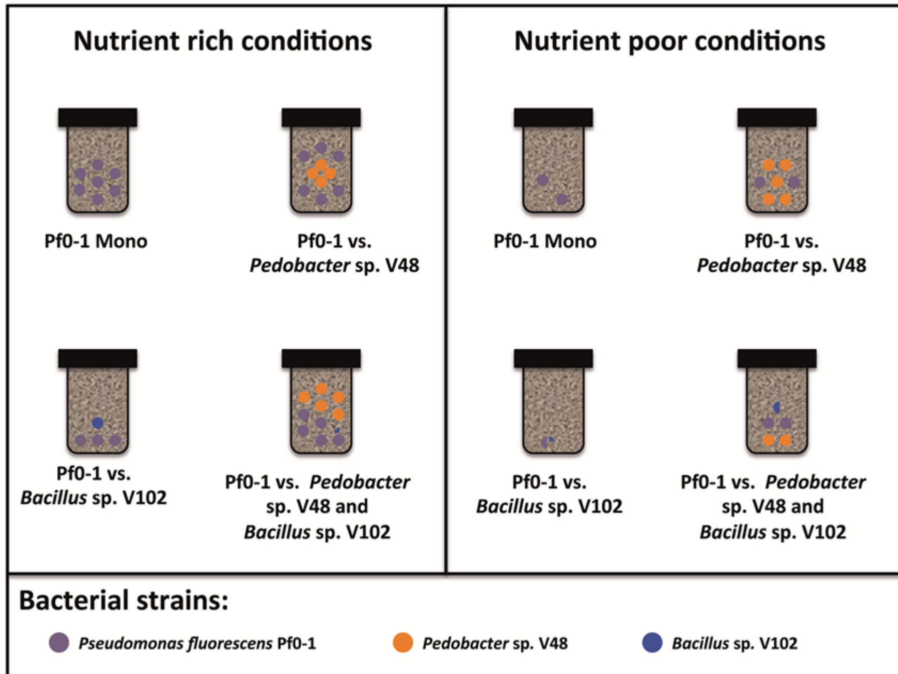

Figure 5.3: Schematic representation of the fitness of *Pseudomonas fluorescens* Pfo-1 during interspecific interactions with either *Pedobacter* sp. V48 or *Bacillus* sp. V102 (2-way interaction) or with both strains together (3-way interaction). The number of the coloured circles indicates the effects of the respective interaction on the fitness of *Pseudomonas fluorescens* Pfo-1. Each full circle represents 1.0 * 10^5 CFU/mL (nutrient rich media) or 1.0 * 10^4 CFU/mL (nutrient poor media).

From previous studies in our group it is known that *Pseudomonas fluorescens* Pfo-1 can be triggered to produce a broad-spectrum antibiotic when co-cultivated with *Pedobacter* sp. V48, but not in co-cultivation with *Bacillus* sp. V102 (Garbeva and de Boer, 2009;Garbeva et al., 2011b). It was hypothesized that this facultative- rather than the constitutive production of antibiotic compound represent a cost-effective strategy, as the antibiotic compound is only produced in situation when it is needed (Garbeva et al., 2011c).

It is plausible that in a more complex habitat, the production of a broad-spectrum antibiotic triggered by *Pedobacter* sp. V48 gives *Pseudomonas fluorescens* Pf0-1a advantage when confronted with phylogenetically different strains simultaneously.

To confirm that the observed results are related to antibiotic production, we performed quantitative RT-PCR with primers targeting genes Pfl01_3463 known to be involved in the production of a broad-spectrum antimicrobial compound (Garbeva et al., 2011b). Results revealed that indeed genes Pfl01_3463 were highly expressed at day 6 in the microcosms where *Pseudomonas fluorescens* Pf0-1 was interacting with *Pedobacter* sp. V48 ($p=0.014$). Gene expression was slightly higher in treatments were *Pseudomonas fluorescens* Pf0-1 was confronted with both *Pedobacter* sp. V48 and *Bacillus* sp. V102, although not significantly ($p=0.750$) (**Figure 5.4**). Unfortunately, due to the low cell number in the microcosms supplemented with nutrient poor growth media, our attempts to extract good quality and quantity of RNA for cDNA synthesis and quantitative RT-PCR failed.

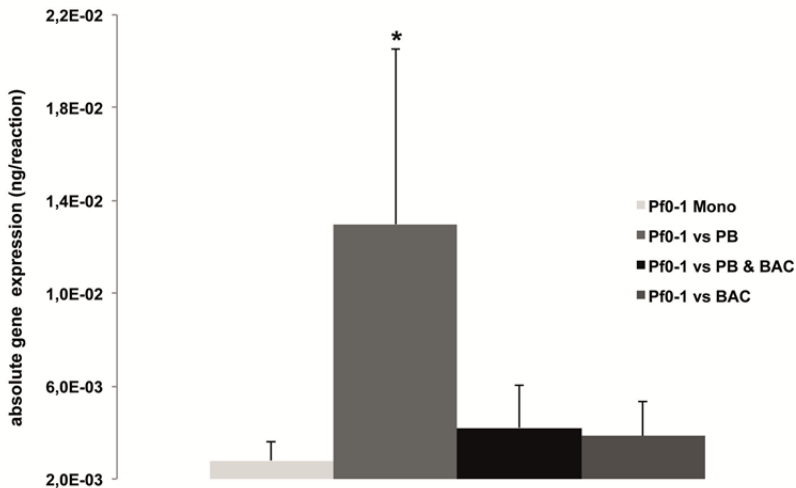

Figure 5.4: qRT-PCR results representing absolute gene expression of gene cluster Pf0-1_3463 obtained at day 6 (nutrient rich media). Error bars are indicating standard deviation (SD) between the triplicates. Significant differences between the qRT-PCR based gene expression by Pf0-1 in monocultures and mixed cultures is indicated by an asterisk ($p \leq 0.05$).

Interspecific interactions may trigger the production of antimicrobial compounds in complex microbial communities where this so-called chemical warfare may offer comparative advantage for the producing strains (Hibbing et al., 2010;Foster and Bell, 2012). A recent study showed that interspecific interactions between soil bacteria can have a major impact on antimicrobial compound production with effects in both directions, i.e. induction or suppression of antimicrobial compound production (Tyc et al., 2014). In soil and in the rhizosphere environment *Pseudomonas* species coexist with many other bacterial species and compete for the same nutrient resources (Demoling et al., 2007;Jousset et al., 2011;Becker et al., 2012;Inglis et al., 2012). The ability to cope with the presence of a range of competing microbial species is essential for growth and survival in soil ecosystems and the performance of soil bacteria may strongly depend on the neighboring competitors.

Overall, our data suggests that the performance of *Pseudomonas fluorescens* Pfo-1 in sand microcosms depends greatly on the presence and identity of neighboring microorganisms. Although *Pseudomonas fluorescens* Pfo-1 cell counts were lower in the nutrient poor sand microcosms than in the nutrient rich microcosms, similar growth patterns were observed in both experiments. This indicates that, contrary to our initial hypothesis, nutrient levels did not have a strong effect on multispecies interactions and on the ability of *Pseudomonas fluorescens* Pfo-1 to respond to different bacteria. It is well known that under different nutrient conditions bacteria often produce different secondary metabolites (Sanchez et al., 2010;van Wezel and McDowall, 2011;Garbeva et al., 2014b) and hence influence microbial interactions in different ways.

This work demonstrates that interspecific interactions can play an important role in soil and may influence microbial performance and consequently shape the composition of microbial communities.

Acknowledgment

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Supplementary material

Figure S5.1: Example of a sand microcosm used in this study.

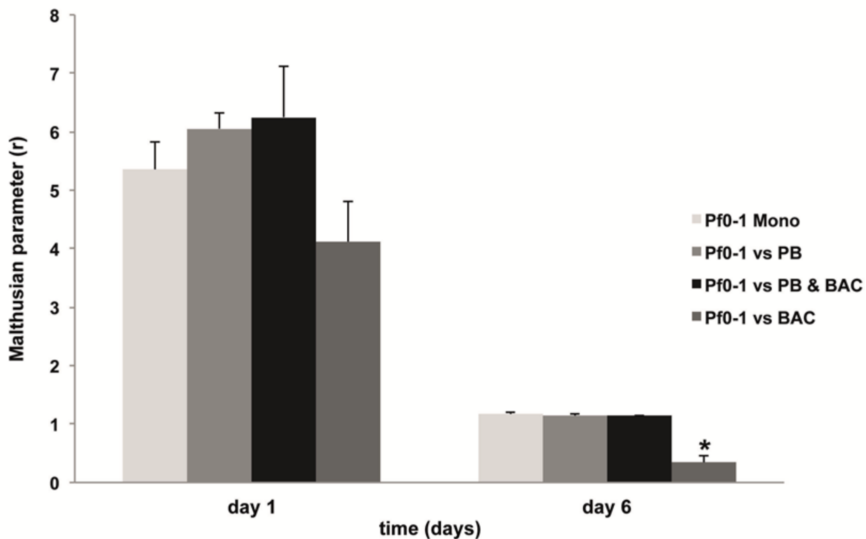

Figure S5.2: Malthusian parameter calculated for the time interval from day 0 to day 1 and for the time interval from day 0 to day 6 (nutrient rich media) representing the fitness *of Pseudomonas fluorescens* Pfo-1 in the four different microcosm treatments. Error bars are indicating standard deviation (SD) between the triplicates. Significant differences are indicated by an asterisk ($p \leq 0.05$).

Chapter 6

No apparent costs for facultative antibiotic production by the soil bacterium *Pseudomonas fluorescens* Pfo-1

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No apparent costs for facultative antibiotic production by the soil bacterium *Pseudomonas fluorescens* Pfo-1

Abstract

Background: Many soil-inhabiting bacteria are known to produce secondary metabolites that can suppress microorganisms competing for the same resources. The production of antimicrobial compounds is expected to incur fitness costs for the producing bacteria. Such costs form the basis for models on the co-existence of antibiotic-producing and non-antibiotic producing strains. However, so far studies quantifying the costs of antibiotic production by bacteria are scarce. The current study reports on possible costs, for antibiotic production by *Pseudomonas fluorescens* Pfo-1, a soil bacterium that is induced to produce a broad-spectrum antibiotic when it is confronted with non-related bacterial competitors or supernatants of their cultures.

Methodology and Principal Findings: We measured the possible cost of antibiotic production for *Pseudomonas fluorescens* Pfo-1 by monitoring changes in growth rate with and without induction of antibiotic production by supernatant of a bacterial competitor, namely *Pedobacter* sp.. Experiments were performed in liquid as well as on semi-solid media under nutrient-limited conditions that are expected to most clearly reveal fitness costs. Our results did not reveal any significant costs for production of antibiotics by *Pseudomonas fluorescens* Pfo-1. Comparison of growth rates of the antibiotic-producing wild-type cells with those of non-antibiotic producing mutants did not reveal costs of antibiotic production either.

Significance: Based on our findings we propose that the facultative production of antibiotics might not be selected to mitigate metabolic costs, but instead might be advantageous because it limits the risk of competitors evolving resistance, or even the risk of competitors feeding on the compounds produced.

Introduction

Interference competition is an important strategy of bacterial strains to establish and maintain themselves within microbial communities (Hibbing et al., 2010). A well-known mechanism of bacterial interference competition is the production of antibiotics (Raaijmakers et al., 2002).These secondary metabolites can be targeted against more or less closely related strains and species (e.g. bacteriocins) or against a wide range of competitors (e.g. many polyketides) (Riley and Wertz, 2002a;Challis and Hopwood, 2003;Riley et al., 2003). Theoretical models have demonstrated that, instead of decreasing diversity by leaving only the most aggressive strains, microbial warfare could actually promote diversity, with dynamic coexistence of many strains differing in their antibiotic production and sensitivity profiles (Czaran and Hoekstra, 2003;Gardner and West, 2004;Czaran and Hoekstra, 2007;Hibbing et al., 2010). These results are obtained when it is assumed that both resistance to- and production of antibiotics come at a fitness cost, resulting in a reduced growth rate. The ecological trade-offs involved in investment in killing, resisting or outgrowing competing strains is thus predicted to maintain diversity.

Because of its profound relevance to human health, the fitness cost of bacterial resistance to antibiotics has received far more attention than the fitness cost of bacterial antibiotic production. Whereas it has emerged that the majority of bacterial antibiotic resistance mechanisms comes at a fitness cost (Andersson and Hughes, 2010), as predicted by theory (Coustau et al., 2000), few studies have examined the cost of antibiotic production. Indications for biological costs of antibiotic production or antibiotic resistance are generally obtained by comparison of relative fitness of wild-type strains with that of antibiotic-negative mutant strains (e.g. (Ender et al., 2004;Binet and Maurelli, 2005)). However, mutations causing loss of antibiotic production may cause additional changes in the bacterial phenotype (Bjorkman et al., 1998;Bjorkman and Andersson, 2000).

Previously, we have reported on competitor-induced triggering of broad-spectrum antibiotic production in fluorescent pseudomonads (Garbeva and de Boer, 2009;Garbeva et al., 2011b). The soil isolate *Pseudomonas fluorescens* Pfo-1 exhibits antibiotic activity only when it is confronted with specific phylogenetically unrelated competitors (e.g. *Pedobacter* sp.) or their supernatant indicating that it can distinguish interspecific competition from intraspecific competition (Garbeva et al., 2011b). Although the structure of the antimicrobial compound has not yet been elucidated, upregulated genes

during confrontation with competitors point at the synthesis of a polyketide-like compound (Garbeva et al., 2011b). In addition, we have shown that it has broad-spectrum activity, acting against both Gram-positive and Gram-negative bacteria as well as against fungi (Garbeva et al., 2011b).

Competitor-dependent induction of antibiotic production allows for another possibility to examine costs of antibiotic production namely by comparing growth rates of wild-type bacteria with and without induction of antibiotic production. In the current study we used both approaches to quantify the possible fitness cost of antibiotic production in *P. fluorescens* Pf0-1: 1) comparison of the growth rate of the wild-type with and without induction of antibiotic production and 2) comparison of the growth rate of wild-type and non-antibiotic producing mutants under conditions that induce antibiotic production. All experiments were performed using nutrient-poor media, as soil-dwelling bacteria typically experience a scarcity of easily degradable carbon resources (Alden et al., 2001;Demoling et al., 2007). Moreover, growth limiting conditions represent a situation in which the costs of antibiotic production should be most pronounced as there is no surplus of energy resources (Anderl et al., 2003). Costs were measured in liquid culture as well as on semi-solid medium in an incubation chamber, allowing quantification of micro-colony growth.

Materials and methods

Bacterial and fungal cultures used

The bacterial strains used in this study are *Pseudomonas fluorescens* Pfo-1 (Gamma-proteobacteria) which was isolated from an agricultural loam soil in Sherborn, Massachusetts, USA (Compeau et al., 1988) and *Pedobacter* sp. V48 (Sphingobacteria), which was isolated from a coastal dune site in The Netherlands (de Boer et al., 2007). The strains were pre-cultured from frozen glycerol stocks on 1/10 strength Tryptic Soy Broth agar (TSBA) (Garbeva and de Boer, 2009). In addition to the parental strain, deletion mutant ΔPfl01_3463-66 (with deletion of Pfl01_3463-3464: two branched-chain alpha-keto acid dehydrogenase E1 components; Pfl01_3465: branched-chain alpha-keto acid dehydrogenase subunit E2 and Pfl01_3466: dihydrolipoamide dehydrogenase) which is unable to produce the broad-spectrum antibiotic was used as well (Garbeva et al., 2011b). The fungal isolate *Rhizoctonia solani* anastomosis group 2.2IIIB, a plant-pathogenic basidiomycete, was used as bioindicator for production of broad-spectrum antimicrobial compounds (Garbeva et al., 2011b).

Preparation of cell-free *Pedobacter* supernatant

Cell-free supernatant was prepared by centrifugation (16,000 x g for 5 min) followed by filtration (Spin-X 0.22 μm filters; Corning Costar, Cat# 8160) of over-night cultures of *Pedobacter* strain V48 grown in 1/10th strength Tryptic Soy Broth at 20 °C. An aliquot of the cell-free supernatant was boiled for 10 minutes and was used as a control. Boiling the cell-free supernatant for 10 minutes was sufficient to destroy the signalling compound(s) that trigger antibiotic production in *P. fluorescens* Pfo-1.

Determination of growth of Pfo-1 in nutrient-poor liquid media

The effect of cell-free supernatant from *Pedobacter* sp V48 on the growth rate of Pfo-1 was determined in a nutrient-poor liquid medium (0.5 gL^{-1} KH_2PO_4; 0.1 gL^{-1} BD Bacto™ Yeast extract (Cat# 210934) and 0.1 $gL^{-1}(NH_4)_2SO_4$; adjusted to pH 6.5). This medium was supplemented with 10 % (v/v) boiled (control) or unboiled *Pedobacter* supernatant, respectively. *P. fluorescens* Pfo-1 was inoculated to an optical density (OD) (600 nm) of 0.02 which corresponds to 2.6×10^6 cells/ml^{-1}.

The 50 ml cultures were incubated for 24 hours at 20 °C shaken at 200 rpm and 1ml samples were taken every hour for OD measurements and viable counts. Additionally, the growth rate of *P. fluorescens* Pfo-1 wild-type and

mutants ΔPfl01_3463 (both supplemented with 10 % (v/v) boiled (control) or unboiled *Pedobacter* supernatant) were measured in 96-well plates (Greiner bio-one, Cat# 655180) using Synergy Microplate Reader. The OD measurements were performed every 30 minutes for total period of 8.5 hours.

Agar-incubation chambers for observation of bacterial growth on semi-solid medium

1 ml of *P. fluorescens* Pfo-1 overnight culture was centrifuged for 3 min at 16,000 g. The cells were resuspended in 10mM phosphate buffer (pH 6.5) containing 10 % non-boiled or boiled cell-free supernatant of *Pedobacter* sp V48 to an optical density of 0.002 at 600 nm; so, the initial number of cells per ml was 10 times lower than in the liquid growth experiments. The lower number was used as it allows distinguishing individual bacteria on the agar slices (see below). An aliquot of 280 µl of *P. fluorescens* Pfo-1 bacterial suspension was equally spread on a thin layer (2 mm) of water agar containing 5g L^{-1} NaCl; 1g L-1 KH_2PO_4 and 0.1g L-1 $(NH_4)_2SO_4$ adjusted to pH 6.5 as previously described (Garbeva and de Boer, 2009). Since bacterial growth was observed on this water-agar medium no extra substrate (yeast extract) was added (Garbeva et al., 2011b). Apparently, the substrates that were added with the 10 % *Pedobacter* supernatant and the substrates that became available after autoclaving of agar were sufficient for growth. After inoculation the plates were dried for about 5 minutes in a flow cabinet and 3 slices of 1 cm^2 were cut and carefully transferred to incubation chambers. The incubation chamber described in **Figure 6. 1** is adapted from Reinhard and Van der Meer (Reinhard and Van der Meer, 2011) and Robin Tecon (unpublished data).

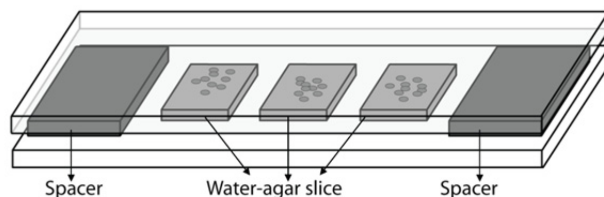

Figure 6.1: Incubation chamber for determination of bacterial growth made of a glass slide with cardboard spacers on both sides. Between the spacers 3 pre-inoculated water-agar (WA) slices of 1 cm^2 are placed. A glass coverslip is slightly pressed on top of the agar slices. The sides of the incubation chamber are sealed with parafilm.

Microscopical counting and data analysis

Twenty randomized pictures at 400-fold magnification representing an area of 1 mm^2 were taken of each slice with an Axio Imager M1 microscope (Carl Zeiss, Oberkochen, Germany) under phase-contrast illumination and an AxioCam MRm camera. Microscopic enumerations were performed every hour for a period of 9 hours at room temperature. During this period microcolonies remained two-dimensional, i.e. no stacking took place. Digital images were analyzed using the AXIO VISION v4.7 Software (Carl Zeiss Imaging Solutions GmbH, Germany) for enumeration and area determination of bacterial colonies.

Extraction of antimicrobial compound and test for inhibition

P. fluorescens Pf0-1 overnight liquid cultures exposed to 10 % boiled or unboiled supernatant of *Pedobacter* sp V48 (initial volume 35 ml) were acidified with trifluoroacetic acid (0.1 % (v/v)), mixed with 2 volumes of ethylacetate and shaken vigorously for 5 min. as described by Raaijmakers et al. (Raaijmakers et al., 1999). After overnight incubation at -20 °C the unfrozen liquid (ethylacetate) fraction containing the active compounds was carefully transferred to a new flask and dried under constant air-flow. The dried extract was dissolved in 150 µl 50 % (v/v) methanol and tested for inhibition of the bacterial isolate *Pedobacter* sp V48 and the soil borne plant-pathogenic fungus *Rhizoctonia solani* as described by Garbeva et al. (Garbeva et al., 2011b).

RNA extraction, cDNA synthesis and real-time PCR

RNA was extracted from *P. fluorescens* Pf0-1 grown in nutrient-poor liquid medium that had been exposed to 10 % boiled or non-boiled supernatant of *Pedobacter* sp V48 as described above. Cells for RNA extraction were collected at 4 time points (3h, 6h, 8h and 24h) and diluted with sterile phosphate buffer to the same optical density (600nm) to obtain equal amounts of cells. The RNA extraction and cDNA synthesis were performed as described previously by Garbeva et al (Garbeva et al., 2011b). For real-time PCR assessment of expression of genes involved in antibiotic production two different primer combinations were used: (1) primer combination 3463F835 (GAT TTT TAC GCG GTC TAC GC) and 3463R1036 (TGA TCA GGT TGC TGT TTC AGG) amplifying 206bp from gene Pf01_3463 encoding the two branched-chain alpha-keto acid dehydrogenase E1 component and (2) primer combination 3465T1F (CAG GGC CCG ATG GTT GC) and 3465T1R (TTG CTT TTT GTG CCG CGC TCG) amplifying 348bp from gene Pf01_3465 encoding branched-

chain alpha-keto acid dehydrogenase subunit E2. As a control, a 210bp fragment of the house keeping 16S rDNA gene was amplified using primer combination 16SPf01F (TTG GGA GCC TTG AGC TCT TA) and 16SPf01R (AAG GCA CCA ATC CAT CTC TG). Real-time PCR was performed using a Corbett Research Rotor-Gene 3000 thermal cycler (Westburg, Leusden, The Netherlands) with the following conditions: an initial cycle of 95 °C for 15 min followed by 40 cycles of: 95 °C for 15 sec; 56 °C for 50sec and 72 °C for 50 sec. Standard curves were established for each primer combination.

Statistical analysis

All experiments were performed in triplicate. Differences in optical densities of liquid cultures between treatments were tested for significance for each time interval by one-way analysis of variance. Bacterial viable count data that were used to calculate the maximum yield (maximum number of colony forming units per ml) were also log-transformed prior to one-way analysis of variance. For the analysis of microcolony development the data were log transformed and the slopes of the regression lines were compared in GraphPad Prism 5 (GraphPad Software, Inc., CA, USA) using a two-tailed t-test. The statistical analyses of quantitative real-time PCR data were carried out with XLStat 2010 (Addinsoft, New York, USA) using a two-tailed t-test. Data were considered to be statistically different at $p \leq 0.05$.

Results

P. fluorescens Pfo-1 antibiotic production and growth rate

The OD measurements indicated that Pfo-1 grew only a short period (1 – 2 hr) exponentially in nutrient-poor liquid media followed by linear increase (**Figure 6.2**, **Figure 6.3, Figure S6.1**). This rapid decline in growth rates occurred for all treatments and stationary phase was reached after 8 hours. At none of the time intervals (performed on 96-well plates every 30 min) were there significant differences in OD between cultures exposed to boiled and unboiled *Pedobacter* supernatant (**Figure 6. 2**). The growth rate of Pfo-1 mutant Δ3463, which is deficient in the production of the broad-spectrum antimicrobial compound triggered by *Pedobacter* supernatant, was also compared with that of the wild-type strain in the presence of *Pedobacter* supernatant. Again, no differences in OD were observed (**Figure 6.2**).

Similar observations were made for the 50 ml cultures where OD measurements were performed every hour (**Figure S6.1**). For the latter cultures, the maximum yield (highest number of viable cells per ml of growth medium) was calculated and again no differences were found between the treatments (**Table 6.1.**).

Table 6.1: Maximum yield of *P. fluorescens* Pfo-1 wild-type and mutant Δ3463 strains in nutrient-poor liquid medium.

Treatment	Maximum yield * (CFU per ml)
Pfo-1 wild-type + 10% cell-free *Pedobacter* supernatant	**7.99 ±0.013**
Pfo-1 wild-type + 10% boiled cell-free *Pedobacter* supernatant	**7.96 ±0.022**
Δ3463 + 10% cell-free *Pedobacter* supernatant	**8.00 ±0.021**
Δ3463 + 10% boiled cell-free *Pedobacter* supernatant	**7.99 ±0.016**

* The maximum yield calculated at time point =7.5 h is the mean of tree replicates per treatment. The variation between the replicates is indicated as ± s.d.

One-way ANOVA did not reveal difference between the treatments p>0.05.

The liquid cultures used for growth rate determinations were extracted at the end of the incubation to confirm that the wild-type Pfo-1 was induced by *Pedobacter* supernatant to produce antibiotics. The presence of antibiotic activity in the extracts was tested by determining the effect of the extracts on growth of *Pedobacter* sp V48 and the soil-borne pathogenic fungus *Rhizoctonia solani.* Inhibition of *Rhizoctonia* and *Pedobacter* was observed only with the extracts from wild type Pfo-1 cultures exposed to 10 % cell-free *Pedobacter* supernatant (**Figure S6.2A, B**). There was no such inhibition by the extracts obtained from Pfo-1 cultures exposed to 10 % boiled cell-free *Pedobacter* supernatant or from Pfo-1 mutant Δ3463 cultures exposed to 10 % cell-free *Pedobacter* supernatant.

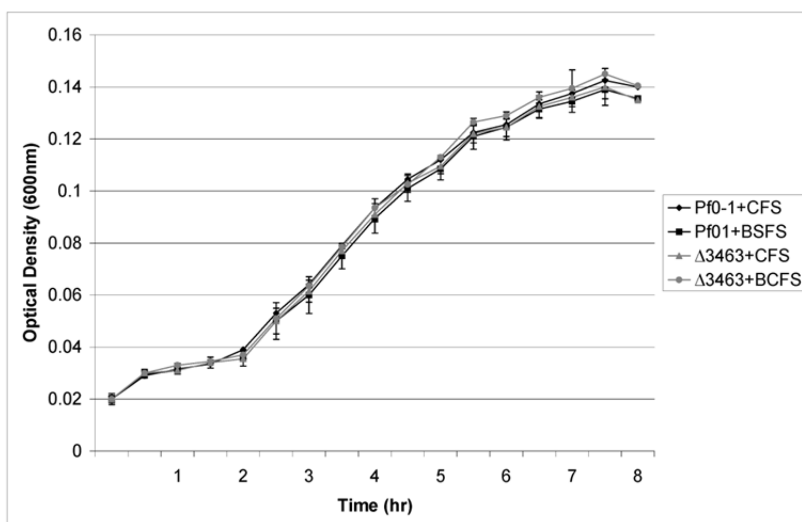

Figure 6.2: Bacterial growth (Optical Density at 600 nm) in nutrient-poor liquid medium in microplates: Pfo1wt+CFS - *P. fluorescens* Pfo-1 wild type with 10 % cell-free supernatant from *Pedobacter* sp. V48; Pfo1wt+BCFS - *P. fluorescens* Pfo-1 wild type with 10 % boiled cell-free supernatant from *Pedobacter* sp. V48; Δ3463+CFS – *P. fluorescens* Pfo-1 mutant Δ3463 with 10 % cell-free supernatant from *Pedobacter* sp. V48 and Δ3463+BCFS – *P. fluorescens* Pfo-1 mutant Δ3463 with 10 % boiled cell-free supernatant from *Pedobacter* sp. V48. The measurements were performed every 30 min for total period of 8.5 h. Symbols represent means of 3 replicate measurements; error bars represent standard deviations.

Expression of genes involved in antimicrobial compound production

At four time points (t = 3h, 6h, 8h and 24h) during growth in liquid medium, *P. fluorescens* Pfo-1 cells were collected for RNA isolation, cDNA synthesis and quantitative RT-PCR. Primers targeting genes Pfl01_3463 and Pfl01_3465 were used for quantitative RT-PCR. Genes Pfl01_3463 and Pfl01_3465 encode branch-chain alpha-keto acid dehydrogenase E1 components and branched-chain alpha-keto acid dehydrogenase subunit E2 that were previously demonstrated to be essential for the production of broad-spectrum antimicrobial activity by *P. fluorescens* Pfo-1 (Garbeva et al., 2011b). Quantitative RT-PCR revealed that triggering of Pfl01_3463 and Pfl01_3465 genes expression by *Pedobacter* supernatant was already apparent after three hours, as there was a significant (3.16 and 2.57 fold respectively) increase in comparison with the expression of these genes in cultures of Pfo-1 exposed to 10 % boiled *Pedobacter* supernatant (**Table 6.2**). The expression of genes Pfl01_3463 and Pfl01_3465 was always at least two-fold higher in the Pfo-1 cultures exposed to 10 % cell-free *Pedobacter* supernatant than in the controls (Pfo-1 cultures exposed to 10 % boiled cell-free *Pedobacter* supernatant).

Table 6.2: Quantitative real-time PCR comparison of gene expression in *P. fluorescence* Pfo-1 with triggered antibiotic production (treatment) and non-triggered antibiotic production (control).

Time points hours	**Fold change treatment/ to control gene Pf01_3463**	**Fold change treatment/ to control *gene Pf01_3465***
3	**3.16 ±0.03**	**2.57 ±0.11**
6	**2.39 ±0.06**	**2.68 ±0.03**
8	**2.11 ±0.11**	**3.21 ±0.14**
24	**2.50 ±0.10**	**2.6 ± 0.10**

Differential expression is given as fold-changes treatment relative to the control.

The numbers are the means of tree replicates per treatment. The variation between the replicates is given as ± s.d. All fold changes of gene expression in the treatment were significantly different ($p < 0.05$) from the control.

Effect of antibiotic production on *P. fluorescens* Pfo-1 growth rate on agar and microcolony morphology

Using water-agar incubation chambers we determined growth rate and size of Pfo-1 colonies with and without induction of antibiotic production by *Pedobacter* supernatant. Growth (increase of surface areas micro-colonies) was exponential during the period of examination (**Figure 6. 3**). There was no significant difference in growth rates between the two treatments (**Figure 6. 3A**). No significant differences in growth rates were observed between the colonies of wild type and mutant Δ3463 strains either (**Figure 6. 3B**). In fact, the average colony size of Pfo-1 supplied with 10 % cell-free supernatant from *Pedobacter* sp V48 was slightly (but not significantly) bigger than that of Pfo-1 supplied with boiled cell-free supernatant. However, there was a clear difference in colony morphology. After 7 hours of incubation, wild-type Pfo-1 exposed to 10 % *Pedobacter* supernatant started to form spherical colonies, a phenomenon not observed for Pfo-1 exposed to boiled *Pedobacter* supernatant or for Pfo-1 mutant Δ3463 exposed to unboiled Pedobacter supernatant (**Figure 6. 4**).

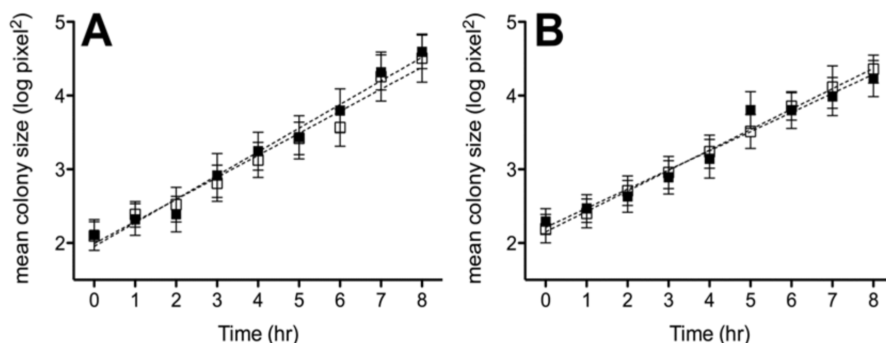

Figure 6.3: Bacterial growth in micro-colonies measured microscopically using Water-Agar incubation chambers (**A**) white squares -wild type *P. fluorescens* Pfo-1 exposed to 10 % cell-free supernatant from *Pedobacter* sp. V48 and black squares- wild type *P. fluorescens* Pfo-1 exposed to 10 % boiled cell-free supernatant from *Pedobacter* sp. V48; (**B**) white squares -wild type *P. fluorescens* Pfo-1 exposed to 10 % cell-free supernatant from *Pedobacter* sp. V48 and black squares- mutant Δ3463 exposed to 10 % cell-free supernatant from *Pedobacter* sp. V48. Squares represent the means of micro-colony sizes and the error bars represent the standard deviations. Statistical analysis revealed no significant differences ($p > 0.05$) between the growth rates (slopes) of the treatments.

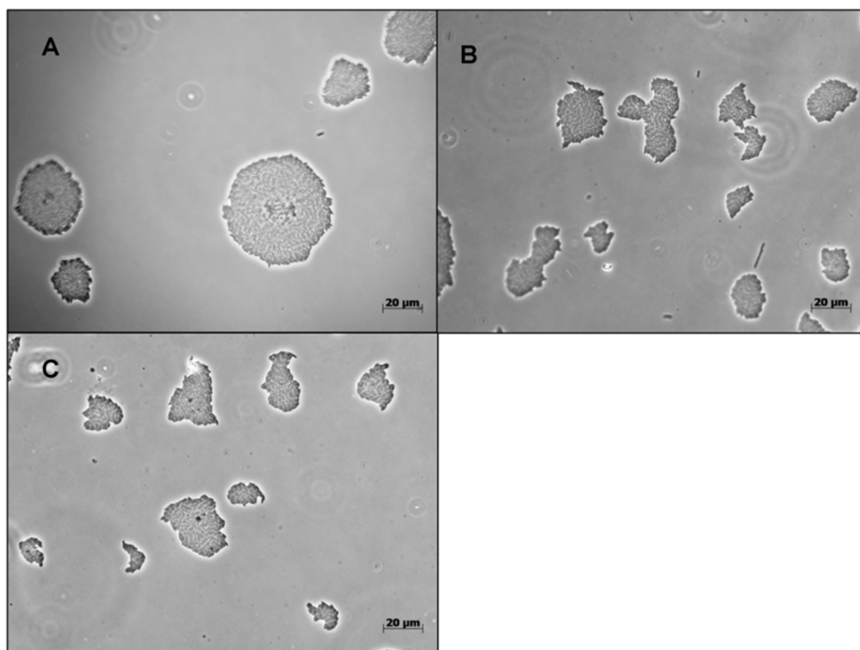

Figure 6.4: Morphology of bacterial micro-colonies after 7h of incubation on Water-Agar of (**A**) wild type *P. fluorescens* Pfo-1 exposed to 10 % cell-free supernatant from *Pedobacter* sp. V48 (**B**) wild type *P. fluorescens* Pfo-1 exposed to 10 % boiled cell-free supernatant from *Pedobacter* sp. V48 and (**C**) - Mutant Δ3463 exposed to 10 % cell-free supernatant from *Pedobacter* sp. V48.

Discussion

Many, if not most, bacteria produce a range of secondary metabolites that can target competing microorganisms (Raaijmakers et al., 2002;Challis and Hopwood, 2003). Natural bacterial populations have been found to consist of a wide variety of genotypes that differ in their ability to both suppress and withstand conspecifics (Ott et al., 2001;Riley et al., 2003;Davelos et al., 2004;Vos and Velicer, 2009). A trade-off between an advantage in growth (resource or scramble competition) and an advantage in 'killing capacity' or 'resistance capacity' (interference competition) lies at the basis of theoretical models attempting to explain the coexistence of strains differing in antibiotic production and sensitivity (Hsu and Waltman, 1997;Czaran and Hoekstra, 2003;Gardner and West, 2004;Czaran and Hoekstra, 2007;Brown et al., 2009;Hibbing et al., 2010). The reason that not all strains evolve to produce antibiotics thus is explained by the fact that the production of these compounds (and their corresponding immunity factors) incurs a metabolic cost. In competition in a structured environment, antibiotic producing cells will displace sensitive (non-antibiotic producing) cells, whereas sensitive (non-antibiotic producing) cells have a growth rate advantage over resistant (non-antibiotic producing) cells that in turn displace antibiotic producing cells because they do not carry the cost of antibiotic production (Kerr et al., 2002).

Previously, it was hypothesized that the facultative- rather than the constitutive production of antibiotics represents a cost-effective strategy, as the antibiotic compound is only produced in situations where it is needed (Garbeva et al., 2011b). Our finding that the cost of antibiotic production in the *P. fluorescens* Pfo-1 system is not significant is not in line with the cost-based assumption on basis of which theoretical models aim to explain how microbial warfare can promote microbial diversity. If these costs are truly insignificant then why do not all strains produce antimicrobial compounds constitutively? Two alternative ecological trade-offs could be envisaged to be at work. First, facultative antibiotic production could prevent competitors evolving resistance to the antibiotic by reducing exposure (Garbeva et al., 2011b). It is evident from clinical studies that increased exposure to antibiotics (through patient consumption) can result in higher resistance levels in a pathogen population (e.g. (Bergman et al., 2004)). Second, it has recently emerged that many bacteria can actually subsist on antibiotic compounds (D'Costa et al., 2006;Dantas et al., 2008). Although antibiotic production

could inhibit the growth of some strains competing for resources, it could promote the growth of others. It is presently unknown how important both mechanisms are in bacterial populations in soil but they certainly seem worthy of future attention.

Whilst we did not observe significant costs of antibiotic production here, biological costs associated with antibiotic resistance have been reported to vary from significant (e.g. (Andersson, 2003; 2006;Andersson and Hughes, 2010), to no-cost (e.g. (Bjorkman and Andersson, 2000;Kanai et al., 2004;Kugelberg et al., 2005;Zhang et al., 2006)) to even enhanced fitness (e.g. (Zhang et al., 2006)). This variation in costs might be explained by the fact that the genetic systems underlying antibiotic resistance are diverse and furthermore might not be readily comparable to those that underlie antibiotic production (including the production of a molecule conferring autoimmunity). Mutations conferring resistance arising in sensitive cells often modify the molecule targeted by the antibiotic in such a way that, although the cell is protected from the antibiotic, its function is severely compromised (Andersson and Hughes, 2010). Such non-additive, pleiotropic fitness costs often are severe.

With antibiotic production on the other hand, it could be hypothesized that the cells mainly bear the additive, metabolic cost of the production of the antibiotic and the immunity molecules (which must be small compared to the sum of all other molecules produced by the cell) and suffer less from pleiotropic costs. In addition, compensatory mutations that mitigate pleiotropic fitness costs have been identified in resistant bacteria (Andersson and Hughes, 2010). It could be that compensatory mutations are more readily accessible for genetic systems mediating antibiotic production than they are for resistance mutations thus lowering the fitness cost in the former.

Although we did not observe costs for antibiotic production, clear changes in *P. fluorescens* Pfo-1 colony morphology during antibiotic production were apparent from the agar chamber experiments. Antibiotic producing *P. fluorescens* Pfo-1 cells formed spherical colonies whereas the non-producing mutant as well as wild-type strain in the control situation did form irregular shaped colonies. Recently it was reported that different *P. fluorescens* colony morphology variants have distinct metabolic profiles (Workentine et al., 2010).

It seems plausible that the spherical colony-shape of Pfo-1 here is a response to the produced antibiotic and not to a signal of *Pedobacter*, as the mutant deficient in the production of the antibiotics did not produce spherical colonies in the presence of *Pedobacter* supernatant. Formation of such spherical colonies may coincide with antibiotic production to obtain the highest protection against antagonizing organisms. Further studies are needed to understand the mechanism and the biological relevance of changes in colony morphology of *P. fluorescens* Pfo-1 during antibiotic production.

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Supplementary material

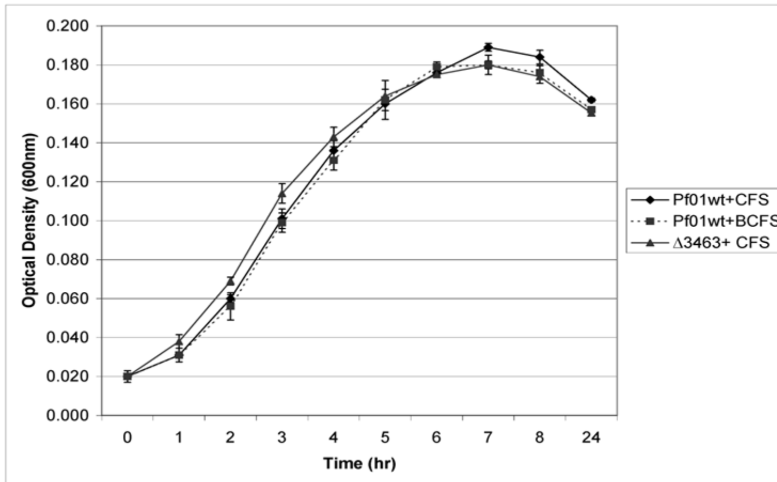

Figure S6.1: Bacterial growth (Optical Density 600 nm) in 50ml nutrient-poor liquid cultures: Pf01wt+CFS - wild type *P. fluorescens* Pf0-1 with 10 % cell-free supernatant from *Pedobacter* sp. V48; Pf01wt+BCFS - wild type *P. fluorescens* Pf0-1 with 10 % boiled cell-free supernatant from *Pedobacter* sp. V48; Δ3463+CFS – *P. fluorescens* Pf0-1 mutant Δ3463 with 10 % cell-free supernatant from *Pedobacter* sp. V48. The measurements are presented as mean ± SD (n= 3 replicates).

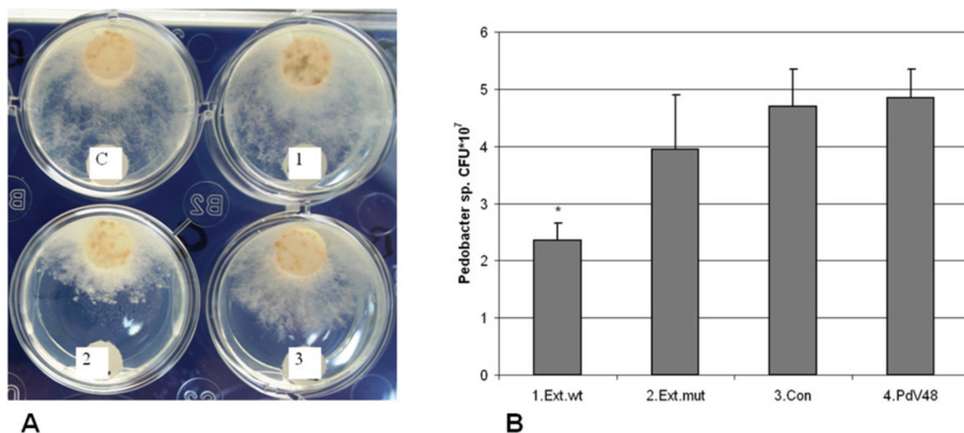

Figure S6.2: (**A**) Antagonist assay against the fungus *Rhizoctonia solani* with extracts from liquid cultures (Fig 1 SI) 1- Mutant Δ3463 with 10 % cell-free supernatant from *Pedobacter* sp. V48; 2- wild type *P. fluorescens* Pfo-1 with 10 % cell-free supernatant from *Pedobacter* sp. V48; 3- wild type *P. fluorescens* Pfo-1 with 10 % boiled cell-free supernatant from *Pedobacter* sp. V48 and C- control 50 % methanol. (**B**) Effect of different extracts from liquid cultures (**Figure S6.1**) on growth of *Pedobacter* sp. V48 (growing on 1/10 TSBA for 24h) 1.Ext.wt- extract from wild type *P. fluorescens* Pfo-1 with 10 % cell-free supernatant from *Pedobacter* sp. V48; 2. Ext.mut- extract from mutant Δ3463 with 10 % cell-free supernatant from *Pedobacter* sp. V48 and 3.Con- extract from wild type *P. fluorescens* Pfo-1 with 10 % boiled cell-free supernatant from *Pedobacter* sp. V48. 4. PdV48- *Pedobacter* sp. V48 without any extract. Data are presented as mean ± SD (n= 3 replicates).

* - Indicates significant reduction of *Pedobacter* sp. V48 CFU as compared to other treatments ($p < 0.05$) as analyzed by one-way ANOVA followed by Tukey`s HSD test.

Chapter 7

General discussion

Impact of interspecific interactions on antimicrobial activity

What did we know about the influence of interspecific interactions on antimicrobial activity in soil bacteria?

When I started my PhD project in January 2012 several studies indicated already the importance of interspecific bacterial interactions for triggering of antibiotic production in bacteria (Slattery et al., 2001;de Boer et al., 2007;Garbeva et al., 2011b). Such interaction-mediated induction of antibiotic production was proposed as a promising approach for the discovery of novel antibiotics. For example, co-culturing of two bacteria namely *Streptomyces endus* S-522 and *Tsukamurella pulmonis* lead to the discovery of the novel antibiotic compound alchievemycin A (Onaka et al., 2011). However, the frequency of interaction-mediated antibiotic triggering in bacterial communities was unknown and to date most studies on interactions altering antibiotic production are focused mainly on *Streptomyces* spp. (Ueda et al., 2000;Traxler et al., 2013;Kinkel et al., 2014;Abrudan et al., 2015).

What did my research reveal about the influence of interspecific interactions on antimicrobial activity in soil bacteria?

In ***chapter two*** I screened for the first time the influence of interspecific interactions on antimicrobial activity of phylogenetically different bacteria. The results obtained in chapter two indicate clearly that interspecific mediated induction of antibiotic activity is commonly occurring among bacterial species. From all tested bacterial isolates 42 % showed antimicrobial activity only during interactions and 33 % showed antimicrobial activity in monoculture. This frequency is in line with previous studies on frequencies of antimicrobial activity during intra-specific interactions within the genus *Streptomyces* (Davelos et al., 2004;Kinkel et al., 2014). Yet, the strains that were involved in interaction-mediated triggering of antibiotic production did not produce or induce antibiotic production in all of the tested interactions. This indicates that the identity of the interacting partner is an important factor in the induction of antibiotic production. This is in line with previous observations on a *P. fluorescens* strain for which antibiotic production was found to be dependent on the identity of the interacting species (Garbeva et al., 2011b). The strong influence of the identity of the interacting bacteria could be related to specific signals from neighbouring microorganisms which has been referred to as competitor sensing (Cornforth and Foster, 2013).

In this thesis bacterial interactions that induced antibiotic production often involved combinations of phylogenetically different bacteria or interactions among beta-Proteobacteria and among Actinobacteria. For the latter phyla several antibiotics have been already identified and described (Pantanella et al., 2007;Berdy, 2012;Cornforth and Foster, 2013;Debois et al., 2013;Zhu, 2014). Hence, it is possible that the screening method revealed bacteria that produce known antibiotics but only during co-cultivation. This would indicate that the regulation is different from known antibiotic-producing bacteria that produce these antibiotics in monoculture.

Some isolates from the phylogenetic classes of Flavobacteria and alpha-Proteobacteria showed antimicrobial activity only in monocultures, however the majority of these isolates exhibited antimicrobial activity only during interactions. This observation indicates that for these phylogenetic groups there is a clear potential to discover novel antibiotics.

Besides induction of antibiotic production, suppression of antibiotic production was also observed. In total 22 % of all tested interactions suppressed antibiotic production and only 13 % of all tested isolates revealed antimicrobial activity in both monocultures and mixed cultures. Interestingly, this suppressing effect on antibiotic production was more abundant than the induction of antibiotic production. The suppression of antimicrobial activity can be due to several mechanisms e.g. interference with signal transduction systems like the quorum sensing system or other regulation mechanisms involved in antibiotic production (Gonzalez and Keshavan, 2006;Venturi and Subramoni, 2009;Christensen, 2013). Direct growth inhibition of the antibiotic producing strain is also a possible explanation for this observation (Straight et al., 2007;Hibbing et al., 2010;Schneider et al., 2012). Another mechanism for the observed silencing of antibiotic production during interaction could be also the lower nutrient availability, as nutrient supply and nutrient composition play an important role in the production of antimicrobial compounds (van Wezel and McDowall, 2011).

So far it was proposed that the main driving force for antibiotic production is defensive rather than offensive, serving to protect the resources (Chater and Merrick, 1979). Indeed, the results of ***chapter two*** show that more bacteria produce antimicrobials during interactions when sensing the presence of a competitor. This is also in line with other studies suggesting that competition

rather than cooperation dominates interactions of cultivable microbial species (D'Onofrio et al., 2010;Foster and Bell, 2012).

In conclusion, the results obtained in ***chapter two*** show that interspecific interactions can significantly affect antimicrobial activity in soil bacteria in both directions: induction and silencing. The high-throughput screening described in chapter two allows for a quick detection of antibiotics produced by bacteria as result of interaction. Such screening of interacting bacteria for antimicrobial activity might be a useful strategy for "waking up" cryptic genes and revealing novel antibiotics and other useful bioactive metabolites.

Impact of interspecific interactions on volatile compound production in bacteria

What did we know about the impact of bacterial interspecific interactions on volatile production in soil bacteria?

Most studies on microbial volatiles to date are focused on volatiles produced by bacteria cultivated in monocultures (Schulz and Dickschat, 2007;Kai et al., 2009;Wenke et al., 2010;Garbeva et al., 2014a) without considering the effect of bacterial interspecific interactions on volatile compound production in bacteria.

Influence of interspecific interactions on volatile production and volatile blend composition

In ***chapter three*** and ***four*** I describe the results of studies on the influence of interspecific interactions on the production of volatile organic compounds in bacteria. I compared the volatile blends emitted by six phylogenetically different soil-bacteria grown either in monoculture or in pair wise cultures.

The results obtained in ***chapter three*** and ***four*** showed that the volatile blends emitted during interaction differed from the volatile blends of the respective monocultures. In ***chapter three*** the volatile blend produced by interacting strains consisted mostly of the volatile compounds produced by the monocultures of those strains. However some compounds that were produced by monocultures were not detected in the mixtures. For example indole was detected in monocultures of *Chryseobacterium* sp. AD48 but was not detected during interaction with *Tsukamurella* sp. AD106. Indole and its derivatives are known to play an important role in microbial interactions and

to be involved in intercellular and multispecies signalling controlling diverse bacterial physiological processes (Wang et al., 2001;Di Martino et al., 2003;Diggle et al., 2006;Nikaido et al., 2008;Lee et al., 2009;Lee and Lee, 2010). In addition to the features as a signal molecule indole has been shown to have inhibitory effects on fungi and stimulating effects of plant growth (Kamath and Vaidyanathan, 1990;Blom et al., 2011). A similar observation was made in ***chapter five*** where two volatile compounds that were produced by monocultures of *Burkholderia* sp. AD24 (S-Methyl methanethiosulfonate and an unknown compound) were not detected during the interaction with *Paenibacillus* sp. AD87. However, in ***chapter four*** induction of volatile compound production during interaction of strains is also reported. One compound identified as 2,5-bis(1-methylethyl)-pyrazine, was produced in higher abundance when *Burkholderia* sp. AD24 was interacting with *Paenibacillus* sp. AD87.

Influence of bacterial volatiles on fungal and oomycetal organisms

Many studies have shown that bacterial volatiles play a major role in the suppression of soil-borne fungi (Zou et al., 2007;Garbeva et al., 2011a;van Agtmaal et al., 2015). In ***chapter four*** I also detected strong anti-fungal and anti-oomycetal activity caused by the volatile blends produced by the four tested bacteria cultivated in monocultures or mixed cultures. In line with previous studies I observed strong fungal sensitivity to bacterial volatiles. One possible explanation for the observed strong fungal and oomycetal growth inhibition is the production of sulfur containing volatiles such as dimethyl disulfide, dimethyl trisulfide, which were detected in high abundance. Sulfur containing volatiles like dimethyl di- and trisulfide have been shown to inhibit the growth of different plant pathogenic fungi (Li et al., 2010;Huang et al., 2012;Wang et al., 2013;Kanchiswamy et al., 2015).

Influence of bacterial volatiles on other bacteria

In ***chapter three*** I describe the growth inhibition of *E. coli* WA321 by volatiles emitted by *Chryseobacterium* sp. AD48 and by volatiles emitted by interacting *Chryseobacterium* and *Tsukamurella* strains. Besides growth inhibition I also observed growth promotion caused by volatiles, namely the stimulation of growth of *S. aureus* 533R4 by monocultures of *Dyella* sp. AD56. However, this growth promotion of *S. aureus* was not observed when *Dyella* sp. AD56 was interacting with *Janthinobacterium* sp. AD80. This coincided with an observed shift in volatile blend composition between monocultures and mixed cultures.

Interestingly the volatiles emitted by the monoculture of *Chryseobacterium* sp. AD48 and the mixture of *Dyella* sp. AD56 with *Janthinobacterium* sp. AD80 induced clear changes in colony morphology of *S. marcescens* P87. The exposed colonies of *S. marcescens* P87 appeared to be more spherical as compared to the control. A similar observation was made for *P. fluorescens* Pf0-1 in ***chapter six***, where the micro-colonies also formed round colonies during the exposure to supernatant of a competing bacterium. Hence, it is plausible that this change in bacterial colony morphology is an adaptive response to the produced antimicrobial compounds.

Influence of dimethyl disulfide and dimethyl trisulfide on bacteria

Most of the volatile compounds detected in the experiments performed in ***chapter three*** and ***four*** were sulfur-containing volatiles like dimethyl disulfide, dimethyl trisulfide and methyl thiocyanate. Experiments were performed with pure dimethyl di- and trisulfide to test their effect on a variety of bacterial model organisms. The assays revealed strong growth inhibitory capacities on all tested bacterial model organisms, when applied in a concentration of 50 µM. Such bacterial growth suppression by dimethyl disulfide was already reported for *P. fluorescens* strains against crown-gall diseases causing *Agrobacterium* sp. (Dandurishvili et al., 2011;Popova et al., 2014). In addition to growth inhibition I also observed influence on colony morphology in *S. marcesens P87*. Colonies of *S. marcesens* P87 exposed to dimethyl trisulfide (50 µM) showed reduced growth and were lacking the production of prodigiosin. It is possible that this is related to the inhibition of quorum-sensing as previously reported by Chernin in 2011 (Chernin et al., 2011).

Influence of interspecific interactions on the induction of novel volatiles

In ***chapter four*** I detected the induction of volatile compound production, namely the production of 2,5-bis(1-methylethyl)-pyrazine with a strong antimicrobial activity. This compound was produced in higher concentration during the interaction of *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD87. Hence, the interspecific interactions may lead to the discovery of novel volatile compounds with valuable antimicrobial activities.

In conclusion the results obtained in ***chapter three*** and ***four*** revealed that interspecific bacterial interactions alter volatile blend composition in both directions induction and suppression.

Impact of interspecific interactions on bacterial fitness
What did we know about the impact of bacterial interspecific competitive interactions on bacterial fitness?

Several studies have been done on competitive interactions between bacteria (Hibbing et al., 2010;Foster and Bell, 2012). However there is still little knowledge on competitive interactions between Gram-positive and Gram-negative bacteria.

Effect of interspecific interactions on bacterial fitness
In two chapters of this thesis the influence of interspecific interactions on bacterial fitness was studied. In ***chapter four*** I describe the influence of interspecific interactions on bacterial fitness of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 and in ***chapter five*** I describe the influence of interspecific interactions on *P. fluorescens* Pfo-1 fitness under two nutrient conditions in sand microcosms.

The results of ***chapter four*** showed that the interaction between *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 had a negative effect on *Burkholderia* sp. AD24 cell counts whereas the cell counts of *Paenibacillus* sp. AD87 were not affected. Hence it seems that *Paenibacillus* sp. A87 is a better competitor than *Burkholderia* sp. AD24. A similar observation was made in ***chapter five*** where the growth of *P. fluorescens* Pfo-1 was negatively affected when confronted with *Bacillus* sp. V102 resulting in significantly lower cell counts under both nutrient conditions. Although *P. fluorescens* Pfo-1 cell counts were lower in nutrient poor sand microcosms than in nutrient rich sand microcosms, similar growth patterns were seen under both nutritional conditions. This result indicates that nutrient supply did not have a significant effect on the outcome of the competitive interactions. Yet, it is well known that nutrient conditions can affect the production of secondary metabolites (Sanchez et al., 2010;van Wezel and McDowall, 2011;Garbeva et al., 2014b) and hence can influence the outcome of microbial interactions. Such growth reduction of *P. fluorescens* Pfo-1 in confrontation with *Bacillus* sp. V102 was already observed on nutrient-poor agar even without direct cell-cell contact (Garbeva and de Boer, 2009). Overall, the results obtained in ***chapter four*** and *five* suggests that the fitness of soil bacteria depends greatly on the presence and identity of the neighboring microorganisms.

Effect of interspecific interactions on gene expression

To understand the mechanism of interactions between Gram-positive and Gram-negative bacteria in ***chapter four*** I performed transcriptome analysis. The outcome of the transcriptome analysis for *Burkholderia* sp. AD24 revealed differential expression of ribosomal proteins pointing to a general stress response (Ishige et al., 2003;Silberbach and Burkovski, 2006;Picard et al., 2013). Furthermore several genes related to signal transduction, secondary metabolite production and to cell motility were up-regulated *Burkholderia* sp. AD24 during the interaction with *Paenibacillus* sp. AD87. The elevated expression of genes associated with cellular motility (Hu et al., 2009) indicates that motility is important during bacterial interspecific interactions. This observation could imply that *Burkholderia* sp. AD24 is trying to escape from *Paenibacillus* sp. AD87. Such elevated expression of genes related to cell motility during interspecific interaction between Gram-negative and Gram-positive bacteria was previously observed for *P. fluorescens* Pfo-1 (Garbeva et al., 2011b).

For *Paenibacillus* sp. AD87, genes encoding antibiotic resistance were highly up regulated and in particular genes related to the Vancomycin B-type resistance, suggesting protection against antimicrobial compounds produced by *Burkholderia* sp. AD24. Recently, Letoffe and co-workers (Letoffe et al., 2014) reported the increase of antibiotic resistance due to possible synergistic effects between volatile compounds, thus it is possible that in this particular case, the produced volatile compounds such as 2,5-bis(1-methylethyl)-pyrazine induced increased expression of genes related to antibiotic resistance in *Paenibacillus* sp. AD87.

Effect of interspecific interactions on secondary metabolite production

The performed metabolomic analysis in ***chapter four*** revealed that the produced metabolites by monocultures and interactions of Burkholderia sp. AD24 and Paenibacillus sp. AD87 differed. The interaction increased the production of specific antimicrobial compounds such as 2,5-bis(1-methylethyl)-pyrazine and as well of a unknown soluble pederin like compound ($C25H45NO9$, m/z= 504.316) [M^+H^+]. The production of 2,5-bis(1-methylethyl)-pyrazine was so far only reported for a few bacteria (Beck et al., 2003;Dickschat et al., 2005;Rajini et al., 2011).

In ***chapter four*** strong synergistic effects between 2,5-bis(1-methylethyl)-pyrazine and the soluble secondary metabolites produced during interaction of *Burkholderia* sp. AD24 with *Paenibacillus* sp. AD87 was observed. This stronger antimicrobial activity might be related to synergistic effects between non-volatile and volatile compounds enhancing the overall antimicrobial activity (Schmidt et al., 2015). Such synergistic effects between volatile and non-volatile compounds were already reported for other antibiotics like e.g. beta-lactam antibiotics (Hemaiswarya and Doble, 2010).

In conclusion from the results described in ***chapter four*** and ***five*** it is obvious that interspecific bacterial interactions are important and influence microbial fitness, gene expression and the production of secondary (volatile and soluble) metabolites and consequently affect the structure of microbial communities.

Biological costs for the production of antimicrobial compounds

What did we knew about the possible costs of antimicrobial compound production in bacteria?

The knowledge about the possible costs for the production of antimicrobial compound by bacteria is scarce. While there are several studies showing the possible biological costs of antibiotic resistance (Gagneux et al., 2006;Andersson and Hughes, 2010;Melnyk et al., 2015) so far there were no studies showing the costs for antimicrobial compound production in bacteria. Yet, it is generally assumed that the production of antimicrobial compounds and their corresponding immunity factors incurs metabolic costs for the producing organism.

In ***chapter six*** I studied the possible costs for facultative antimicrobial compound production in *P. fluorescens* Pfo-1 by monitoring microscopically the growth rate of micro-colonies with and without induction of antibiotic production. The results obtained in this chapter showed that the production of antimicrobial compounds does not incur significant costs for *P. fluorescens* Pfo-1. An alternative explanation for reduced costs of antibiotic production is that such competition-mediated induction of antimicrobial compound production lowers the risk of increasing resistance of competitors against the produced antibiotic compound as well it lowers the risk of competing organisms to feed on the produced antimicrobial compound.

In conclusion the results revealed in ***chapter six*** showed that production of antimicrobial compounds is not necessarily costly for the antibiotic compound produced by *P. fluorescens* Pfo-1 as no reduction of growth was observed. A similar observation was made in ***chapter five*** were *P. fluorescens Pfo-1* growing in microcosms together with *Pedobacter* sp. V48 was triggered for the productions of antibiotics but didn't showed reduced growth. If this observation is generally valid for antibiotic production by soil bacteria then it will have consequences for the prediction of producer /non-producer (cheaters) dynamics in bacterial populations.

Outlook on the discovery of novel antibiotics and general conclusion

There is a need for new antimicrobial drugs with activity against both pathogenic bacteria and fungi. In the last four decades antibiotic resistance in pathogenic bacteria has become a global rising health problem (Crisóstomo et al., 2001;Tenover et al., 2001;Al-Gheethi et al., 2013;Economou et al., 2013). However, so far, only two classes of new antibiotics have reached the clinical practice, so there is a clear discrepancy between the number of newly discovered antibiotics and the number of novel compounds which would be needed to fight the problem of antimicrobial resistance (Barbachyn and Ford, 2003;Kern, 2006;ECDC/EMEA, 2009). Antimicrobial resistance is a complex problem that requires efforts of microbiologists, ecologists, health care specialists, educationalists, the industry, policy makers and the public in order to be solved.

This PhD project was financed by the BE- Basic consortium with a practical background aiming for the discovery of novel antimicrobial compounds by using a so called "intelligent mining" approach by screening interacting bacteria for novel antimicrobial activity. The high-throughput screening method developed in this work allows a fast screening of interacting bacteria for antimicrobial activity.

Mining into bacterial interspecific interactions is one way of "waking up" cryptic gene clusters in order to reveal novel and potentially useful secondary metabolites. Furthermore studying bacterial interspecific interactions that affect antimicrobial activity may be very beneficial to better understand the composition and the dynamics of soil microbial communities (Velicer, 2003;Mitri and Foster, 2013;Abrudan et al., 2015;Aziz et al., 2015). Further understanding of such bacterial interspecific interactions can be additionally beneficial for selecting the right players in synthetic communities that fulfil

specific ecosystem services like disease suppression in agricultural crop systems (Weller et al., 2002;Garbeva et al., 2004;De Roy et al., 2013;Grosskopf and Soyer, 2014).
So far most of the studies on secondary metabolites produced by bacteria are focused either on soluble or on volatile organic compounds alone but do not consider the importance and the possible synergistic effects of both compound classes together as shown in this thesis.

In this thesis we show that volatile compounds can exert synergistic effects with soluble compounds and enhance the overall antimicrobial activity. However, very often the application of bacteria to control soil-borne diseases fails, as the diseases suppression is not consistent or minimal. Thus, selection of a mixture of interacting strains with different properties may lead to more consistent disease control in agricultural production systems.

Overall this work contributes to better understanding of microbial interactions and in particular the interaction between soil bacteria. However, interactions in nature are much more complex and can vary depending on the occurring environmental conditions. Thus laboratory experiments are only a snapshot of the “real” situation and will not give a complete picture of the complex interactions occurring in nature.

To finalize, the results described in this thesis show that the screening of interspecific bacterial interactions can be important (1) to understand competitive mechanisms occurring in soil bacterial communities and (2) for the discovery of novel volatile and soluble antimicrobial compounds important for agriculture as well for medical purposes.

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Summary

Summary

The soil habitat is one of the most important resources of microbial natural products of human interest such as antibiotics, enzymes and other secondary metabolites. Soil is a very complex environment where many microorganisms are constantly competing for limiting nutrients and space. So far, only a small fraction of the terrestrial bacterial species has been explored for novel pharmaceutical compounds. Facing the worldwide problem of increasing antibiotic resistance in pathogenic bacteria, novel antimicrobials are urgently needed. New methods and screening strategies are needed to access the full range of antimicrobials produced by terrestrial microbes. Current screening methods for discovering bioactive compounds do often target only well-examined genera that are known to produce antibiotics in monocultures. Such screening methods do not consider the importance of interspecific interactions, which represent the natural situation in which microorganisms produce and use antibiotics. This thesis is focused on competitive bacterial interactions with the aim to explore soil bacteria for novel antimicrobial compounds produced resulting from interspecific interactions.

A major objective of this thesis was to study the frequency of interspecific interaction induced antibiotic production in soil bacteria. For this purpose a high-through-put screening method was developed (*chapter two*) to screen soil bacteria for their antibiotic activity in monocultures and pairwise cultures. The results indicate clearly that interspecific interaction mediated induction of antibiotic activity is commonly occurring among soil bacteria. From all tested bacterial isolates 42 % showed antimicrobial activity only during interactions and 33 % of all tested isolates showed antimicrobial activity only in monoculture. Furthermore, the results indicated that the identity of the interacting partner is an important factor in the induction of antibiotic production. Besides induction of antibiotic production also suppression of antibiotic production was observed for 22 % of all tested interactions and only 13 % of all tested isolates showed antimicrobial activity in both monocultures and mixed cultures.

More detailed studies of the effect of interspecific interactions on the production of secondary metabolites by soil bacteria are presented in *chapter three* and *four*. Particular attention was given to the production of volatile organic compounds.

Volatile blends emitted by phylogenetically different soil-bacteria grown either in monoculture or mixed cultures were compared. The results revealed that the volatile blends emitted during interactions differed from the volatile blends emitted by the respective monocultures. Interestingly some compounds that were produced by monocultures were not detected in the mixtures. Furthermore, the effect of bacterial volatiles on performance of target microbes (bacteria, fungi and oomycetes) was investigated. The results revealed that volatiles produced by bacteria can inhibit the growth of other bacteria, influence their colony morphology and can inhibit the growth of fungi and oomycetes. Interestingly, also the induction of volatile compound production (2,5-bis(1-methylethyl)-pyrazine) was observed (*chapter four*). This volatile compound was found to be produced in higher abundance during co-cultivation of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87. The effect of interspecific interactions on the fitness, gene expression and on the production of soluble metabolites in soil bacteria was also studied. The results of *chapter four* revealed that the interaction between *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 had a negative effect on numbers of *Burkholderia* sp. AD24 whereas those of *Paenibacillus* sp. AD87 were not affected.

A similar observation was described in *chapter five*. Here the growth of *P. fluorescens* Pfo-1 was negatively affected when confronted with *Bacillus* sp. V102. The additional performed transcriptome analysis in *chapter four* revealed differential expression of several genes related to ribosomal proteins, signal transduction, secondary metabolite production, antibiotic resistance, defense mechanisms and cell motility. The metabolomic analysis performed on soluble secondary metabolites revealed that the produced metabolites of monocultures of *Burkholderia* sp. AD24 and *Paenibacillus* sp. AD87 differed from the metabolites produced during interactions. Interaction-mediated triggering of antibiotic production provides a new opportunity to estimate the biological costs for the production of antimicrobial compounds. I investigated the possible costs for interaction-mediated antimicrobial compound production in *P. fluorescens* Pfo-1 by monitoring the growth rate with and without induction of antibiotic production. The results of this study are reported in *chapter six* and revealed that the production of an antimicrobial compound by *P. fluorescens* Pfo-1 did not incur detectable metabolic costs.

In summary this thesis extends our knowledge on the impact of interspecific interactions on secondary metabolite production in soil bacteria thereby providing better insight in the competitive mechanisms occurring in soil microbial communities. This thesis further highlights the influence of interactions between Gram-negative and Gram-positive bacteria on bacterial fitness and on the production of volatile and soluble secondary metabolites. Furthermore, the thesis research strengthens the importance of interspecific microbial interactions for the discovery of novel antibiotics.

Samenvatting

Samenvatting

De bodem is een van de belangrijkste bronnen van microbiële natuurlijke producten die toegepast worden voor industriële of therapeutische doeleinden. Voorbeelden zijn antibiotica en enzymen. De bodem is een zeer complexe omgeving waar micro-organismen voortdurend met elkaar concurreren om beperkt beschikbare voedingsstoffen en ruimte. Hierbij worden secundaire metabolieten ingezet om concurrenten te onderdrukken. Slechts een klein deel van de secundaire metabolieten die door bodembacteriën geproduceerd kunnen worden is onderzocht voor toepassingsdoeleinden. Om het wereldwijde probleem van toenemende resistentie in pathogene bacteriën tegen de algemeen gebruikte antibiotica te kunnen oplossen zijn nieuwe antibiotica dringend nodig. Ontwikkeling van nieuwe screeningsmethoden- is dan ook essentieel om het volledige potentieel aan antibiotische stoffen in bodembacteriën te ontdekken. De huidige screeningsmethoden voor het ontdekken van bioactieve stoffen richten zich alleen op bacteriële soorten waarvan bekend is dat ze antibiotica produceren in een monocultuur. Dergelijke screeningsmethoden houden geen rekening met competitieve interacties tussen microbiële soorten, terwijl dit de natuurlijke situatie is waarin micro-organismen antibiotica produceren en gebruiken. Met het in dit proefschrift beschreven onderzoek heb ik geprobeerd om vanuit een ecologische invalshoek strategieën te ontwikkelen die kunnen leiden tot ontdekking van nieuwe antibiotica. Daarvoor zijn interspecifieke interacties tussen bodembacteriën als uitgangspunt genomen.

Als eerste is de frequentie van geïnduceerde productie van antibiotica onderzocht voor een groep van bodembacteriën. Voor dit doel werd een high-through-put screening methode ontwikkeld (*hoofdstuk twee*) om bodembacteriën te screenen op hun antibiotische activiteit in zowel monocultuur als gemengde (paarsgewijze) kweken. De resultaten gaven duidelijk aan dat inductie van antibiotica productie door interspecifieke interactie vaak optreedt bij bodembacteriën. Van alle geteste bacteriële isolaten toonde 42 % alleen antimicrobiële activiteit tijdens interacties. Het onderzoek gaf echter ook aan dat de identiteit van de interacterende partner een belangrijke factor is bij de inductie van antibioticaproductie. Naast de inductie van antibioticaproductie heb ik ook onderdrukking van antibioticaproductie in gemengde kweken waargenomen.

In *hoofdstuk drie en vier* worden meer gedetailleerde studies van het effect van interspecifieke interacties op de productie van secundaire metabolieten door bodembacteriën beschreven. Bijzondere aandacht werd hierbij besteed aan de productie van vluchtige organische stoffen door verschillende soorten bodem-bacteriën in monocultuur en gemengde kweken. Uit de resultaten bleek dat de samenstelling van vluchtige stoffen die vrijkomt in mengkweken verschilde van die in monoculturen Een aantal verbindingen die werden geproduceerd door monoculturen werden niet meer gedetecteerd in mengculturen. Daarnaast is het effect van bacteriële vluchtige stoffen op de groei van doelmicroben (bacteriën, schimmels en oömyceten) onderzocht. Uit de resultaten bleek dat vluchtige stoffen afkomstig van bacteriekweken de groei van andere bacteriën kunnen remmen, de kolonie morfologie kunnen beïnvloeden en de groei van schimmels en oomyceten kunnen remmen. Opvallend is ook de inductie van de productie van een vluchtige verbinding (2,5-bis(1-methylethyl)-pyrazine) (*hoofdstuk vier*). De productie van deze vluchtige verbinding nam sterk toe tijdens de interactie van *Burkholderia* sp. AD24 met *Paenibacillus* sp. AD87.

Het effect van interspecifieke bacteriële interacties op fitness, genexpressie en productie van oplosbare metabolieten werd ook bestudeerd. Uit de resultaten van *hoofdstuk vier* bleek dat de interactie tussen *Burkholderia* sp. AD24 en *Paenibacillus* sp. AD87 een negatief effect had op de *Burkholderia* aantallen, terwijl die van *Paenibacillus* niet werden beïnvloed. Een soortgelijke waarneming is beschreven in *hoofdstuk vijf*. Hier werd de groei van *Pseudomonas fluorescens* Pfo-1 negatief beïnvloed tijdens confrontatie met *Bacillus* sp. V102. Uit de aanvullende transcriptoom analyse in *hoofdstuk vier* bleek er sprake te zijn van differentiële expressie van verschillende genen betrokken bij ribosomale eiwit productie, signaaltransductie, secundaire metaboliet productie, resistentie tegen antibiotica, afweermechanismen en celmotiliteit. Uit de metaboloom analyse uitgevoerd op oplosbare secundaire metabolieten, is gebleken dat de geproduceerde metabolieten van monoculturen van *Burkholderia* sp. AD24 en *Paenibacillus* sp. AD87 verschillend zijn van de metabolieten geproduceerd in mengkweken.

De activering van antibioticaproductie tijdens interacties biedt een nieuwe kans om de biologische kosten voor de productie van antimicrobiële verbindingen te schatten. Ik onderzocht de mogelijke kosten voor interactie gemediëerde antibiotica productie in *P. fluorescens* Pfo-1 door het kwantificeren van de groei met en zonder inductie van antibiotica productie.

De resultaten van dit onderzoek zijn beschreven in *hoofdstuk zes* en gaven aandat de productie van een antimicrobiële verbinding door *P. fluorescens* Pfo-1 niet detecteerbare metabole kosten met zich meebrengen.

Kort samengevat. Dit proefschrift vergroot onze kennis over (1) de gevolgen van interspecifieke interacties op de productie van secundaire metabolieten in bodem bacteriën en (2) de concurrerende mechanismen die in bodem microbiële gemeenschappen voorkomen. Dit proefschrift onderstreept het belang van verder onderzoek naar de invloed van interacties tussen Gram-negatieve en Gram-positieve bacteriën. Uit dit proefschrift komt naar voren dat het onderzoek naar interspecifieke microbiële interacties een belangrijke bijdrage kan leveren aan de ontdekking van nieuwe antibiotica.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Competition mediated discovery of cryptic encoding for novel antimicrobials

Writing of project proposal (4.5 ECTS)

- Competition mediated discovery of cryptic encoding for novel antimicrobials

Post-graduate courses (5 ECTS)

- Introduction to R for statistical analysis; PE&RC (2011)
- Bioinformatics: an users approach; EPS (2013)
- Spring School host microbe interactions; WGS (2014)
- Microbial ecology; PE&RC (2015)

Laboratory training and working visits (3.9 ECTS)

- Use of mass spectrometric techniques for identification of secondary metabolites produced by bacteria by using LESA and mass spectrometry techniques; Chemische Ökologie , Max-Plank-Institute Jena (2013)
- Identification of a Pederin like compound using VLC technique, NMR and HPLC techniques; Chemische Pharmakologie , Universität Tübingen (2015, 2016)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Methods in Ecology and Evolution: Identification of response classes from heavy metal-tolerant soil microorganism communities by highly resolved concentration dependent screening in a microfluidic system (2014)
- Frontiers in Microbiology: Trichoderma volatiles effecting Arabidopsis: from inhibition to protection against pathogenic fungi (2015)

Competence strengthening / skills courses (2.7 ECTS)

- Reviewing a scientific paper; WGS (2013)
- Scientific integrity; WGS (2013)
- Scientific publishing; WGS (2013)
- Information literacy PhD including Endnote introduction; WUR Library (2013)
- Scientific writing; Wageningen Into Languages (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Day (2014)
- PE&RC Day (2015)
- Current themes in ecology (2015)
- NERN Days (2016)

Discussion groups / local seminars / other scientific meetings (6.5 ECTS)

- NIOO PhD Literature discussion group meeting (2012-2013)
- Microbial Ecology group meeting (2012-2016)
- NIOO Microbial Ecology PhD group meeting (2014-2016)

International symposia, workshops and conferences (16.4 ECTS)

- MiCom International student conference; Jena, Germany (2012)
- ICAR International conference on antibiotic research; Lissabon, Portugal (2012)
- FEMS; Leipzig, Germany (2013)
- BAGECO; Ljubjana, Slovenia (2013)
- SGM Society for general microbiology conference; Liverpool, UK (2014)
- SGM Society for general microbiology conference; Birmingham, UK (2015)
- Workshop volatile analysis; NIOO (2015)
- Micrope international symposium; Vienna, Austria (2015)
- KNVM Annual microbial ecology meeting; Amsterdam, the Netherlands (2016)

Lecturing / supervision of practicals / tutorials (3 ECTS)

- Practical student Course EABI; WUR (2014)

Supervision of MSc student (3 ECTS)

- Bacterial interactions and antibiotics (Sytske Drost)

Publications

Peer-reviewed and published articles:

Garbeva, P., **Tyc, O.**, Remus-Emsermann, M.N.P., Van Der Wal, A., Vos, M., Silby, M., and De Boer, W. (2011). No Apparent Costs for Facultative Antibiotic Production by the Soil Bacterium Pseudomonas fluorescens Pfo-1. **Plos One** 6. doi: ARTN e27266 doi: 10.1371/journal.pone.0027266.

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Submitted articles:

Olaf Tyc, Victor de Jager, Marlies van den Berg, Saskia Gerards, Thierry Janssens, Niels Zaagman, Marco Kai, Ales Svatos, Hans Zweers, Cornelis Hordijk, Harrie Besselink, Wietse de Boer and Paolina Garbeva
"*Exploring bacterial interspecific interactions for discovery of novel antimicrobial compounds*"

Stijn van Gils, Giovanni Tamburini, Lorenzo Marini, Arjen Biere, Maaike van Agtmaal, **Olaf Tyc**, Martine Kos, Roxina Soler, David Kleijn and Wim H. van der Putten
"*Soil pathogen-aphid interactions under differences in soil organic matter and mineral fertilizer*"

Olaf Tyc, Laura Tomás-Menor, Paolina Garbeva, Enrique Barrajón-Catalán and Vicente Micol
"*Validation of the AlamarBlue® assay as a fast screening method to determine the antimicrobial activity of botanical extracts*"

Articles in preparation:

Olaf Tyc, Chunxu Song, Michiel Vos and Paolina Garbeva
Review article
"*The ecological role of secondary metabolites produced by soil bacteria*"

Olaf Tyc was born on the 25th of June 1981 in Balingen, Germany. After he attended a technical college for commerce and business administration and finishing his education as business administrator assistant in 2001 he did his civil service as paramedic at the German Red-Cross KV Zollernalb e.V.. In 2002 Olaf attended the Kolping-Kolleg in Stuttgart, Germany were he finished his high school degree in 2005. Driven by his great interest and curiosity for nature and microorganisms he decided to start a study related to biology, he therefore started to study agricultural biology at the Hohenheim University in Stuttgart, Germany. During his study his interests in microbiology was growing and particularly in microbe-microbe interactions. This interest brought him in 2009 to the Netherlands Institute of Ecology in Heteren were he did an ERASMUS internship and his diploma thesis in 2011, supervised by Prof. Dr. Andreas Kuhn (Hohenheim University, Stuttgart, Germany), Prof. Dr. Johannes A. van Veen and Dr. Paolina Garbeva (NIOO-KNAW, Netherlands). In his diploma thesis he studied the effect of interspecific bacterial interactions on the fitness and secondary metabolite production in *Pseudomonas fluorescens* Pfo-1. In 2012 Olaf started his PhD project “Competition-mediated discovery of novel antimicrobials” at the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen, which is presented in this thesis. At present he is a postdoctoral researcher at the department of Microbial Ecology at NIOO-KNAW.

The research presented in this thesis was conducted at the Department of Microbial Ecology at the Netherlands Institute of Ecology (**NIOO-KNAW**) in Wageningen.

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