Development of high-throughput methods for the detection of hydrogen cyanide-producing bacteria for the application in biocontrol

Master's thesis

Submitted by

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List of Abbreviations

AIT	Austrian Institute of Technology GmbH
ATP	Adenosine triphosphate
BCA	biocontrol agent
bp	base pairs
cDNA	complementary DNA
cfu	colony forming unit
°C	degree(s) Celsius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate or deoxyribonucleotide
dsDNA	double-stranded DNA
DRB	deleterious rhizosphere bacteria
EDTA	ethylenediaminetetraacetic acid
g	gramm
Gb	gigabases
H_2O	water
HCN	hydrogen cyanide
kb	kilobases
L	liter
M	molar
Mb	megabases
$MgCl_2$	magnesium chloride
Milli-Q	Millipore Corporation
mL	milliliter
mM	millimolar
mm	millimeter
µg	microgramm

µM	micromolar
NCBI	National Center for Biotechnology Information
N	normal
ng	nanogramm
nM	nanomolar
%	percent
PCR	polymerase chain reaction
PGPB	plant growth-promoting bacteria
PGPR	plant growth-promoting rhizobacteria
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TBE	Tris-borate-EDTA
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UV	ultraviolet
x g	multiples of earth's gravitational acceleration
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

Hydrogen cyanide-producing bacteria have been tested for their potential use as biocontrol agents for several decades now. Nevertheless, there is still a lack of fast and reliable high-throughput tests for qualitative detection of cyanogenic bacteria. Standard methods described in the literature are either too cumbersome or imprecise and error-prone. In the master's thesis at hand, we present several tests for rapid large-scale screening of hydrogen cyanide-producing bacteria. Distinct functional and molecular methods have been developed to investigate bacterial isolates as well as metagenomic libraries. We expect these assays to accelerate and facilitate research of bacterial cyanogenesis. Seven cyanogenic *Pseudomonas* isolates detected with our new tests were found to significantly improve the germination of the model plant lettuce. These plant growth-promoting bacteria could be of interest for biocontrol. Additionally, an unknown volatile compound was discovered with a function-based screening for hydrogen cyanide-producing clones of a rhizosphere cosmid metagenomic library. So far, activity-driven tests and Next-Generation Sequencing allowed to identify some of the functions and genes possibly involved in the production of this volatile.

Key words: hydrogen cyanide, biocontrol, bacteria, high-throughput, metagenomic library

Zusammenfassung

Bereits seit Jahrzehnten wurden Bakterien, die Cyanwasserstoff (Blausäure) produzieren, auf ihre Anwendbarkeit in der Biologischen Kontrolle getestet. Nichtsdestotrotz liegt immer noch ein Mangel an schnellen und zuverlässigen Tests, die für eine qualitative Erfassung cyanogener Bakterien in großem Maßstab geeignet sind, vor. Die in der Literatur beschriebenen Standardverfahren stellen sich als zu arbeitsaufwendig, unpräzise oder fehleranfällig dar. In der vorliegenden Masterarbeit werden mehrere Hochdurchsatz-Tests für die Bestimmung von Cyanwasserstoff produzierenden Bakterien vorgestellt. Unterschiedliche funktionelle und molekulare Methoden wurden für die Untersuchung von bakteriellen Isolaten sowie Metagenombibliotheken entwickelt. Diese Tests könnten zukünftig die Erforschung der Produktion von Cyanwasserstoff durch Bakterien wesentlich erleichtern und beschleunigen. Sieben cyanogene Isolate aus der Gattung *Pseudomonas*, die mit Hilfe der neu entwickelten Methoden detektiert wurden, führten zu einer signifikanten Verbesserung der Keimung der Modellpflanze Salat. Diese wachstumsfördernden Isolate könnten von Interesse für die Biokontrolle sein. Außerdem wurde in funktionellen Tests für die Bestimmung Cyanwasserstoff produzierender Klone einer Rhizosphären-Cosmid-Metagenombibliothek eine bisher unbekannte flüchtige Substanz entdeckt. Bisher konnten durch Aktivitäts-basierte Tests und Next-Generation Sequencing einige der Funktionen und potentieller Gene, die in die Produktion dieser Substanz involviert sein könnten, identifiziert werden.

Schlagwörter: Cyanwasserstoff, Biokontrolle, Bakterien, Hochdurchsatz, Metagenombibliothek

1.) Introduction

In the master's thesis at hand, new methods will be presented for the detection of hydrogen cyanide-producing bacteria. These cyanogenic bacteria could be useful agents for the biocontrol of agricultural pests. First, the theoretical background of biocontrol and bacterial hydrogen cyanide production will be illustrated, followed by a section of material and methods utilized for the practical work of this master's thesis. Next, results of all tests conducted will be demonstrated and discussed. In a final conclusion, the most relevant outcome will be summarized and a future outlook will be given.

1.1.) Global challenges in agriculture and need for sustainable cultivation techniques

Modern agriculture has to face a series of important socio-economic and environmental issues, all connected on a global scale.

First of all and most important, it should assure the provision of high-quality food for an increasing world population. The United Nations project that about 9,2 billion people will inhabit our planet by 2050, most of them living in urban areas (UNPD, 2010). In order to guarantee enough food for the growing population, agriculture will have to double its food production worldwide, with an annual increase of productivity of 1,8 %. In addition, (agricultural) policies should enable better distribution of food to minimize hunger and poverty, especially in developing countries (Singh, 2012). On the other hand, there is a growing demand for renewable energies from biomass and a change in the meat consumption behavior worldwide, both competing with primary food production from arable land to a large extent (Beddington, 2010).

Agriculture has to deal with all these socio-economic challenges while natural resources (e.g. water, land, fossil fuels, phosphorus, biodiversity) diminish and an anthropogenic global climate change alters production conditions on a broad range (Hulme, 2004; Singh, 2012). Already now, yield losses of annual crops due to abiotic stresses (e.g. drought, flooding, cold, hail) might reach 51 – 82 %, depending on the crop (Bray et al., 2000). Limitations of crop yield caused by biotic stressors (e.g. bacteria, fungi, viruses, insect pests, weeds) are usually lower (Oerke, 2006). Nevertheless, also total losses have been recorded in some cases (Borém and

Fritsche-Neto, 2012). Increasing weather extremes and a higher temperature in general might further intensify these stresses (Dhillon et al., 2012).

Up to the present, synthetic pesticides, artificial fertilizers, and genetically modified plants (GM crops), helped to mitigate negative environmental influences and thereby improving productivity. Yet, their extensive use came at a risk and led to environmental damage (e.g. water eutrophication, resistant pests, loss of biodiversity). The same is true for mismanaged cultivation techniques (e.g. tillage in high frequency, soil cultivation along a gradient, excessive irrigation), which caused (and still cause) erosion and salinization of wide areas of arable land (Robinson and Harris, 2004).

Improved sustainability of the whole agricultural system can only be achieved with a holistic approach. An increase in productivity has to be accompanied by an enhancement in the protection of the environment. A consistent application of integrated crop management systems, including integrated nutrient, water, and pest (i.e. plant pathogens, herbivory arthropods and other animals, weeds) management, is therefore necessary (Singh, 2012). Balancing agronomic, environmental, economic, and social optima has to be the central goal of sustainable agriculture in the future (Robinson and Harris, 2004).

1.2.) Biocontrol as an environmentally compatible mechanism to improve productivity of agricultural ecosystems

A high agricultural productivity is largely dependent on effective plant protection measurements. Regarding previous considerations, an integrated pest management (IPM), as described by Kogan (1998), should be established wherever possible: ‘IPM is a decision support system for the selection and use of pest control tactics, singly or harmoniously co-ordinated into a management strategy, based on cost/benefit analyses that take into account the interest of and impact on producers, society and the environment’ (Kogan, 1998).

The integrated pest management system combines several aspects, which altogether allow a case-specific decision for an appropriate plant protection measurement, as illustrated in figure 1.

One such aspect of integrated pest management is biological control or biocontrol. Eilenberg et al. (2001) define biocontrol as ‘the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be’. This definition has to be adapted in that the term ‘living organisms’ also includes viruses. Up to now, useful biological control agents (BCAs) have been found in the groups of viruses, bacteria, fungi, nematodes, insects, and mites (Eilenberg et al., 2001). Although Eilenberg et al. (2001) definitely exclude the use of genes or gene products from biological control, other authors consider their implementation either to improve biocontrol agents themselves or the plants to be protected (Charudattan et al., 2002; Gnanamanickam et al., 2002; Khetan, 2001).

According to Eilenberg et al. (2001), biocontrol is comprised of four distinct strategies, namely conservation biological control, classical biological control, inoculation biological control, and inundation biological control (see also figure 1). Conservation biological control is achieved via ‘modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests’ (Eilenberg et al., 2001). Classical biological control is based on ‘the intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control’ (Eilenberg et al., 2001). Inoculation biological control in turn, is described as ‘the intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently’ (Eilenberg et al., 2001). Finally, inundation biological control is defined as ‘the use of living organisms to control pests when control is achieved exclusively by the released organisms themselves’ (and not their progeny) (Eilenberg et al., 2001).

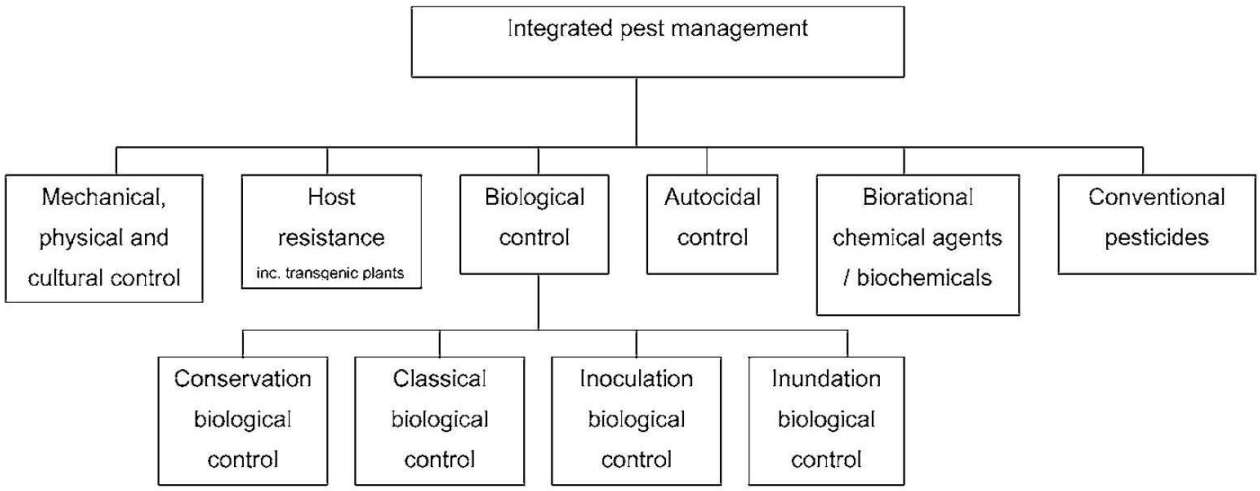

Figure 1: Biological control in the context of an integrated pest management. Biological control comprises 4 distinct strategies (Eilenberg et al., 2001).

Three major uses were identified by Eilenberg et al. (2001) for biocontrol agents in agriculture: First, the biocontrol of invertebrate pests (mainly insects and mites) with predators, parasitoids, and pathogens, second, the biocontrol of weeds with herbivorous insects and pathogens, and third, the biological control of plant pathogens using antagonistic microorganisms and induced plant resistance. The biological control of invertebrate pests will not be further discussed here. More information on the topic can be found in Bellows (1999) and Rechcigl and Rechcigl (2000). As for the biological control of weeds, another approach would be the use of non-pathogenic, however, deleterious bacteria from the rhizosphere (deleterious rhizobacteria, DRB). These bacteria do not cause disease, but produce harmful metabolites, which might inhibit plant growth (Alström and Burns, 1989; Flores-Vargas and O'Hara, 2006; Kremer and Kennedy, 1996; Kremer and Souissi, 2001). In general, biological control of weeds could be interesting for parasitic weeds, for weeds that are closely related to crop species, for weeds that are resistant against synthetic herbicides, or in case of locally occurring weeds where the development of chemical pesticides would be too expensive (Khetan, 2001). For the biological control of plant pathogens on the other hand, there are three distinct areas of application, namely, to control diseases of above-ground parts of plants, soil-borne diseases, and post-harvest diseases (Khetan, 2001). Pathogens attacking the above-ground parts of plants might be controlled by antagonistic microorganisms via competition for nutrients, hyperparasitism or antibiosis (Khetan, 2001). Research in the field of soil-borne pathogens has revealed several mechanisms of control. Important information could be gained by investigating naturally suppressive soils harboring antagonistic

microbes (Haas and Défago, 2005). Promising biocontrol agents from rhizosphere or from within plants (so-called endophytes) were found to use one or more of the following modes of action to promote plant growth and/or inhibit pathogen growth and development: competition for space and nutrients (especially for iron with the help of siderophores), production of lipopolysaccharides or antibiotics (e.g. phenazines, pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, hydrogen cyanide), hyperparasitism and excretion of lytic enzymes, detoxification of pathogen virulence factors, ice nucleation, and finally, induction of systemic resistance in plants (ISR – induced systemic resistance) (Compant et al., 2005; Gnanamanickam et al., 2002; Haas and Défago, 2005; Handelsman and Stabb, 1996). Similar mechanisms could be identified for the biological control of pathogens leading to postharvest diseases. Yet, microorganisms producing toxic metabolites or causing allergenicity have to be excluded from biocontrol if the harvested product will eventually be used for human or animal nutrition (Charudattan et al., 2002).

Biological control offers a lot of advantages, making it a central strategy of integrated pest management and plant protection. In comparison to synthetic pesticides, biocontrol agents exhibit benefits like a higher degree of safety for humans and the environment in general, a high or even higher level of host-specificity (hardly any or no non-target effects), lower costs in development, and a lower risk for the formation of resistant hosts (Charudattan et al., 2002). Although vast research has been carried out on biological control, only very few products became market-ready (Warrior et al., 2002). As a consequence, the market for biological control agents is still very small. In 2008, sales were estimated at approximately 200 Mio € in Europe, while the turnover for chemical pesticides reached 7.000 Mio € in the same period (Blum et al., 2011b). Various reasons for the relatively low amount of biocontrol plant protection products on the market have been described: First, the success of biocontrol is still less predictable than for chemical pesticides as the performance of living organisms is influenced by a large number of environmental factors. In addition, many biological control agents may only provide a short shelf life, meaning that their viability and effectiveness decline rapidly when stored. Finally, in comparison to the market potential, the registration of biocontrol agents is less cost-effective than for synthetic plant protection products (Blum et al., 2011a, b; Charudattan et al., 2002; Warrior et al., 2002).

All these findings suggest that there is still a large need for research to increase the amount of active biological control agents on the market. The development of mixtures of biocontrol agents, a better incorporation of biological control agents into the system of integrated pest management (e.g. simultaneous use of biocontrol agents and chemical pesticides), and the optimization of timing and placement of biocontrol could further increase the significance of biological control for plant protection (Charudattan et al., 2002; Gnanamanickam et al., 2002; Yang and del Rio, 2002).

1.3.) Bacteria used as biocontrol agents

Among the group of biocontrol agents, bacteria have played an important role right from the beginning (Khetan, 2001). Characteristics of bacteria, which made them attractive for biological control, are their various mechanisms of interaction with and effects on other organisms (e.g. fungi, plants, nematodes, insects), their quick generation time, and natural establishment in many environments (e.g. within soil, outside or within plants). In addition, bacteria are in general easy-to-handle and most bacteria can be used for mass-production via fermentation (Warrior et al., 2002). All modes of action for biocontrol listed in the previous chapter can also be found in bacteria (e.g. antibiosis, competition, plant growth-promotion, parasitism). 41 bacterial isolates are listed as registered biocontrol agents in the fourth edition of The Manual of Biocontrol Agents (Copping et al., 2009). The respective plant protection products or so-called biopesticides comprise insecticides, fungicides, biological plant growth regulators, bactericides, nematicides, and herbicides. Bacterial genera used as biocontrol agents involve *Agrobacterium*, *Azospirillum*, *Bacillus*, *Brevibacillus*, *Burkholderia*, *Erwinia*, *Pantoea*, *Pasteuria*, *Pseudomonas*, *Serratia*, *Streptomyces*, and *Xanthomonas* (Copping et al., 2009). Bacteria of the genera *Rhizobium* and *Bradyrhizobium* are also useful as potential biocontrol agents (Antoun et al., 1998), but are usually treated and termed differently (‘biofertilizers’) due to their symbiotic relationship with legume plants (*Fabaceae*) and capability to fixing nitrogen (Torres, 2000). The bacteria most widely used in biocontrol belong to the three genera *Bacillus*, *Pseudomonas*, and *Agrobacterium (Charudattan et al., 2002; Khetan, 2001; Warrior et al., 2002)*.

Bacillus species are usually gram-positive, rod-shaped, spore-forming, motile, aerobic or facultatively anaerobic, ubiquitous soil-inhabiting bacteria. These bacteria produce endospores within cells, allowing them to withstand unfavorable environmental conditions (Charudattan et al., 2002). Biopesticides containing isolates of several subspecies of *Bacillus thuringiensis* (*Bt*) are the commercially most successful biocontrol products (Warrior et al., 2002). During sporulation, *Bacillus thuringiensis* forms a parasporal crystal containing δ-endotoxin proteins. These toxins disrupt the midgut epithelial membranes of specific insects after ingestion, thereby killing the insect (Khetan, 2001). Susceptible hosts have been found within the Lepidoptera, Coleoptera, and Diptera (Warrior et al., 2002). Biocontrol with *Bt* isolates or their toxins alone has been adapted for use in agriculture, forestry, and against mosquitos. Certain strains of *Bacillus thuringiensis* have also been genetically modified and the genes encoding for the δ-endotoxin proteins have been successfully transferred into plants to create insect-tolerant transgenic crop plants. Vast use of the *Bt* toxins has already led to resistance development in certain insect species, calling for resistance management actions (Khetan, 2001). Isolates of other species of the genus *Bacillus* have been developed as biofungicides (*B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. mycoides*), biological plant growth regulators (*B. cereus*), bionematicides (*B. firmus*), and biobactericides (*B. subtilis*) (Copping et al., 2009). *Bacillus* species enhancing plant growth and/or controlling plant pathogens are classified as plant growth-promoting bacteria (PGPB). In general, these PGPB can be found in various niches within (endophytic) and around plants (especially in the rhizosphere – plant growth-promoting rhizobacteria, PGPR) (Compant et al., 2005; Haas and Défago, 2005). The modes of action for PGPB are the same as already described for biocontrol of plant pathogens in the previous chapter. Another large group of PGPB is comprised of *Pseudomonas* species (Haas and Défago, 2005).

Pseudomonas species are usually gram-negative, rod-shaped, non-spore-forming, motile, aerobic or facultatively anaerobic bacteria, versatile in metabolite production, and inhabiting several environments like water, soil, and (as opportunistic pathogens) plants, animals and humans (Singleton, 1992). Registered plant protection products containing isolates of *Pseudomonas* species include biofungicides (*P. aureofaciens*, *P. cepacia*, *P. chlororaphis*, *P. fluorescens*, *P. syringae*), biobactericides (*P. fluorescens*), and bioherbicides (*P. gladioli* pv. *gladioli*) (Copping et al., 2009). Further

species that could be of interest for biological control but have not yet been commercialized, are *Pseudomonas putida* (Khetan, 2001; van Loon and Pieterse, 2002) and *Pseudomonas aeruginosa* (Gallagher and Manoil, 2001; Rudrappa et al., 2008). Due to its role as opportunistic pathogen (for plants, animals, and humans), the latter has not been formulated as biopesticide because of safety issues (Handelsman, 2002; Rudrappa et al., 2008).

Several isolates of *Agrobacterium radiobacter* represent another important group of biocontrol agents on the market (Copping et al., 2009). All isolates are used for biological control of crown gall disease caused by *Agrobacterium tumefaciens*. The mechanisms of control are aggressive colonization of plant roots and production of an antibiotic called agrocin (Khetan, 2001; Yang and del Rio, 2002).

1.4.) Hydrogen cyanide-producing bacteria

The production and release of hydrogen cyanide by bacteria has been identified as one useful mechanism for biological control via antibiosis. Examples for the inhibitory effect of bacterial hydrogen cyanide have been shown for fungi (Voisard et al., 1989), plants (Alström and Burns, 1989), nematodes (Gallagher and Manoil, 2001), insects (Devi et al., 2007) and other bacteria (Rudrappa and Bais, 2008).

Hydrogen cyanide (synonyms hydrocyanic acid or prussic acid) is a linear inorganic compound with the chemical formula HCN (triple-bond between carbon and nitrogen) (Budavari et al., 1996). It is either a colorless gas or a bluish-white liquid (boiling point 25,6°C) with a characteristic odor like bitter almonds. Hydrogen cyanide is miscible with water and alcohol, and slightly soluble in ether (Paquette, 1995). The compound is highly toxic as already micromolar amounts of it inhibit several metalloenzymes (e.g. cytochrome oxidase, catalase, superoxide dismutase, nitrite and nitrate reductases, nitrogenase, and several others) as well as enzymes without involvement of a metal (e.g. ribulose diphosphate carboxylase), as reviewed in Solomonson (1981). The inhibition of the cytochrome oxidase (the terminal component of the respiratory chain) eventually leads to the death of the organism, meaning that all aerobic organisms might suffer from lethal cyanide poisoning (Solomonson, 1981). 0,5 - 3,5 mg/kg body weight are generally considered as fatal dose in humans (Ermans et al. (1972) in Solomonson (1981)).

The abilities to produce, assimilate or detoxify hydrogen cyanide can be found in a wide range of organisms (Castric, 1981). In general, the production of hydrogen cyanide can be termed as ‘cyanogenesis’, producers as ‘cyanogenic’, non-producers as ‘acyanogenic’ (Conn, 1981). Natural producers of HCN include more than 3000 plant species (among them important crop plants such as cassava, sorghum, maize, wheat, and sugarcane; reviewed in Kassim and Rumbold (2013)), various fungi (Bunch and Knowles, 1981; Knowles, 1976), some algae (Vennesland et al., 1981), several arthropods (classes Chilopoda, Diplopoda, and Insecta) (Duffey, 1981), and certain bacteria (Bunch and Knowles, 1981; Castric, 1981; Knowles, 1976; Vennesland et al., 1981).

Cyanogenesis in bacteria seems to be restricted to a relatively small number of species. Most hydrogen cyanide-producing bacteria have been detected within the genus *Pseudomonas* (a number of strains of *P. aeruginosa* and *P. fluorescens*, some strains of *P. aureofaciens* and *P. chlororaphis,* as well as of *P. entomophila* and *P. putida*), belonging to the Gammaproteobacteria (Castric, 1981; Knowles, 1976; Knowles and Bunch, 1986; Owen and Zdor, 2001; Ryall et al., 2009). Further cyanogenic strains can be found for the Betaproteobacteria *Chromobacterium violaceum* (Michaels and Corpe, 1965) and *Acidovirax delafieldii* (Owen and Zdor, 2001), for *Rhizobium leguminosarum* (Alphaproteobacteria) (Antoun et al., 1998), for all bacteria of the Burkholderia cepacia complex (Betaproteobacteria; HCN production reported under specific conditions) (Ryall et al., 2008), and certain species of the genus *Bacillus* (division Firmicutes, class Bacilli) (Ahmad et al., 2008; Grover et al., 2009; Mateescu et al., 2007; Patty, 1921). Production of hydrogen cyanide has also been found in some cyanobacteria (*Anacystis nidulans*, *Nostoc muscorum*, and *Plectonema boryanum*), as reviewed by Vennesland et al. (1981).

Lorck (1948) showed that an optimum production of hydrogen cyanide in bacteria could be achieved if glycine was added to the growth medium. Later studies revealed that glycine is the immediate metabolic precursor of cyanide in bacteria. Hydrogen cyanide and carbon dioxide (CO_2) are stochiometrically formed by oxidative dexarboxylatoin of glycine (Castric, 1977; Wissing, 1974). In *Pseudomonas* species (Askeland and Morrison, 1983) and *Chromobacterium violaceum* (Bunch and Knowles, 1982), the two components HCN and CO_2 were shown to originate from the methylene carbon and the carboxyl group of glycine, respectively. The highest level

of cyanogenesis was reported during the transition from the exponential to the stationary phase, with a sharp decline in productivity afterwards (Askeland and Morrison, 1983; Castric, 1981). The presence of oxygen was found to be necessary for the production of hydrogen cyanide, but only in restricted amounts (i.e. microaerophilic conditions) (Castric, 1981; Laville et al., 1998). Another essential compound for cyanogenesis is iron, which has to be present in sufficient amounts (Blumer and Haas, 2000a). The enzyme responsible for the conversion of glycine into HCN and CO_2 was termed HCN synthase (Castric, 1981). The enzyme was characterized as a membrane-bound flavoprotein (Wissing, 1974; Wissing and Anderson, 1981). Due to instability and sensitivity to oxygen, the enzyme and the biosynthetic pathway could not be defined first (Blumer and Haas, 2000b; Gross and Loper, 2009). Only after Laville et al. (1998) identified the genes encoding the HCN synthase in *Pseudomonas fluorescens* strain CHA0, the mechanism for bacterial cyanogenesis could be unraveled. Three contiguous genes, *hcnABC*, were found to be part of the responsible operon (total size 3286 bp) (see also figure 2). When compared to other nucleotide sequences, all HCN synthase subunits showed similarities with enzymes known to be involved in hydrogen transfer (HcnA similar to formate dehydrogenase, HcnB and HcnC similar to amino acid oxidases) (Laville et al., 1998). In accordance with Wissing (1974) and Knowles and Bunch (1986), Laville et al. (1998) postulated a dehydrogenase (or amino acid oxidase) model for HCN synthase. In a first dehydrogenase reaction, glycine is oxidized to iminoacetic acid, as can be seen in figure 2. In a subsequent second dehydrogenase reaction, iminoacetic acid is converted into an unstable nitrile derivative, of which the C-C bond is rapidly cleaved, leading to production of HCN and CO_2 (Gross and Loper, 2009; Laville et al., 1998).

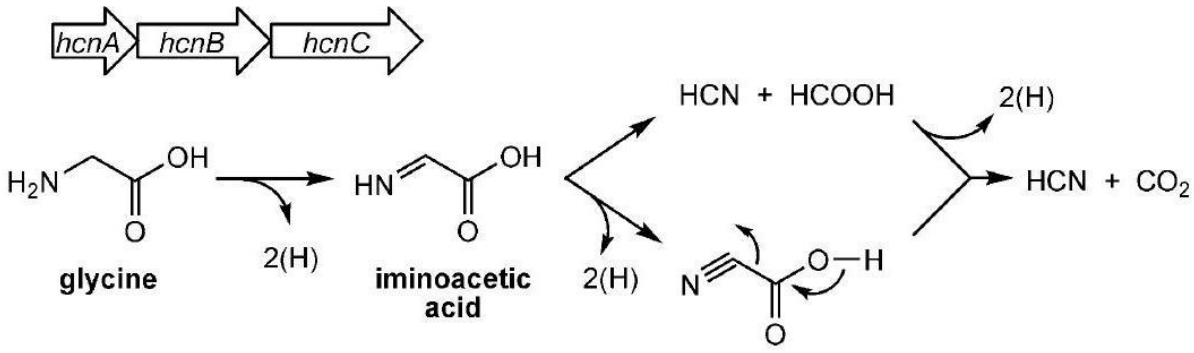

Figure 2: The HCN synthase gene cluster (*hcnABC*) and the dehydrogenase model for HCN production from glycine in *Pseudomonas* species.

Yet, the functions and interactions of the three HCN synthase subunits have not been clarified in detail (Gross and Loper, 2009). So far, the *hcnABC* operon seems to be highly conserved among Pseudomonas species, at least in sequence and organization (Loper and Gross, 2007; Ramette et al., 2003; Ryall et al., 2009). Differences might occur on a genomic context (i.e. where on the genome the HCN synthase genes will be located) (Gross and Loper, 2009). The HCN synthase gene cluster has also been found in *Chromobacterium violaceum* (Gross and Loper, 2009) and certain species of *Burkholderia* (Ryall et al., 2008).

Two components were identified as regulatory proteins for cyanogenesis in *Pseudomonas* species (Blumer and Haas, 2000a, b). The first, the anaerobic regulator ANR, activates cyanogenesis on the level of transcription upon oxygen limitation and depending on iron availability. The second, the global activator GacA, controls cyanogenesis on a transcriptional as well as on a posttranscriptional level (Blumer and Haas, 2000a, b; Pessi and Haas, 2001). In *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, the production of hydrogen cyanide was found to be further influenced by the quorum-sensing regulators LasR and RhlR (Pessi and Haas, 2000) as well as the transcriptional regulator AlgR involved in alginate production (Carterson et al., 2004).

Up to the present, no physiological function has been discovered for the highly toxic hydrogen cyanide. The production would lead to autointoxication if the producing bacteria would not possess the ability to tolerate the presence of HCN (Blumer and Haas, 2000b; Knowles, 1976). In general, hydrogen cyanide is seen as secondary metabolite due to its missing role in primary metabolism and the fact that it might provide the producing organism with an ecological advantage (Blumer and Haas, 2000b; Castric, 1975). The production and release of hydrogen cyanide might be used to outcompete other organisms, to allow parasitism of other organisms, or for protection against antagonists (Knowles, 1976), all valuable biocontrol characteristics. Nevertheless, hydrogen cyanide might also function as virulence factor for opportunistic pathogens like *Pseudomonas aeruginosa* (Blumer and Haas, 2000b; Gallagher and Manoil, 2001; Ryall et al., 2008). Castric (1975) hypothesized that cyanogenesis might also be performed to remove excessive glycine that would otherwise harm the cell, a theory which could not be proven so far.

Several mechanisms of tolerance to and detoxification of hydrogen cyanide have been illuminated in cyanogenic as well as acyanogenic organisms. Cyanide degradation to less toxic or non-toxic compounds (e.g. CO_2, ammonia, nitrite, nitrate, thiocyanate) could be achieved via enzymatic (e.g. β-cyanoalanine synthase, γ-cyano-α-aminobutyric acid synthase, rhodanese) or non-enzymatic (e.g. α-ketoglutarate) pathways (Blumer and Haas, 2000b; Knowles, 1976; Lang, 1933; Rodgers, 1981). Especially the enzyme rhodanese, first described by Lang (1933), seems to be of high importance for cyanide detoxification in a wide variety of organisms (Cipollone et al., 2007; Knowles, 1976). Rhodanese (thiosulfate:cyanide sulfurtransferase) catalyzes the reaction of cyanide with thiosulfate to form the less toxic thiocyanate and sulfite, an irreversible reaction (Lang, 1933). A very potent adaptation to allow respiration and growth in presence of HCN, is the production of alternate cyanide-insensitive terminal oxidases (or branched respiratory chains) as found both in prokaryotes and eukaryotes (Cunningham et al., 1997; Henry, 1981; Knowles, 1976; Quesada et al., 2007). Another process to tolerate exogenous hydrogen cyanide to certain concentrations is the anaerobic metabolism via fermentation, demonstrated exemplarily for *Escherichia coli* or *Pseudomonas aeruginosa* (Knowles, 1976; Zlosnik et al., 2006). Cyanogenesis might also be regulated under unfavorable conditions: For instance, *Pseudomonas aeruginosa* does not induce cyanide production during presence of nitrate and simultaneous absence of oxygen (i.e. under denitrifying conditions), in order to protect nitrate and nitrite reductases (Castric, 1975). Several bacteria (and other microbes) were found to assimilate hydrogen cyanide and use this compound as source of carbon and/or nitrogen, somehow representing the strongest resistance to hydrogen cyanide (Knowles, 1976).

1.5.) Metagenomic libraries – their use, construction, and screening

Already in early microbial research, it has been observed that most of the cells found to be active and viable under a microscope, did not grow and form colonies on media plates. Two major causes were identified for this large difference, called ‘great plate count anomaly’. First, known cultivable species might enter an unculturable state or might not grow on a certain medium due to differing nutrient requirements. Second, many of the cells detected via microscopy might belong to yet uncultivable species (Amann et al., 1995; Handelsman, 2004). Later on, DNA analyses (mainly based on

genes coding for phylogenetic markers like ribosomal RNA) revealed that the majority of microorganisms inhabiting natural environments belong to the group of still uncultivable species (Amann et al., 1995; Torsvik et al., 1990; Torsvik et al., 1996). It is now commonly accepted among microbiologists, that less than 1 % of all microorganisms can be cultured with current cultivation techniques (Amann et al., 1995; Daniel, 2004; Handelsman, 2005; Handelsman et al., 1998; The National Academy of Sciences, 2007). Up to the present, numerous natural products have been isolated from cultivable microorganisms for agricultural, pharmaceutical, and industrial use (e.g. antibiotics, phytotoxic compounds, enzymes, etc.). The existence of such large numbers of yet unculturable species indicates that natural habitats may provide many more of these useful products (Daniel, 2004; Handelsman et al., 1998).

The fact that most of the microorganisms are not amenable to culturing may be circumvented by isolating DNA or RNA directly from an environmental sample (Handelsman et al., 1998; Kao-Kniffin et al., 2013). With the help of molecular techniques termed metagenomics, it is possible to investigate a complete set of genomes present in a specific environment (Handelsman et al., 1998). Besides the above mentioned product-driven approach, metagenomics are also a useful tool in ecology, enlightening how microorganisms might interact in a specific environment and contribute to an ecosystem (e.g. their role in biogeochemical cycles or their mechanisms of nutrient acquisition and energy production) (Handelsman, 2004; Steele and Streit, 2005).

The isolated environmental or metagenomic DNA (comprised of prokaryotic and eukaryotic DNA) can be analyzed via direct sequencing or sequencing following PCR (polymerase chain reaction) or used to construct so-called metagenomic libraries for further research. Direct sequencing of metagenomic DNA is nowadays widely performed with modern sequencing technologies, termed Next Generation Sequencing (NGS) (e.g. Roche 454 Pyrosequencing, Illumina Sequencing by Synthesis, etc.). Sequencing following PCR is often used for taxonomy studies using phylogenetic markers (e.g. the genes encoding for 16S rRNA) (Kao-Kniffin et al., 2013).

The construction of metagenomic libraries (living DNA collections) on the other hand, allows to study metagenomic DNA on a sequence-based as well as on a functional level (Handelsman, 2004). For library preparation, the extracted environmental DNA

is purified, fragmented, and then inserted into a vector. The vector is then used for transformation of a suitable bacterial host (e.g. *Escherichia coli, Streptomyces lividians, Agrobacterium tumefaciens, Burkholderia graminis, Pseudomonas putida, Saccharomyces cerevisiae*). Under optimum conditions, each of the resulting cells (clones) contains a different piece of metagenomic DNA (Daniel, 2005; Handelsman et al., 1998; Kao-Kniffin et al., 2013). This process allows to stably conserve, to multiply, and to study DNA of otherwise uncultivable organisms. Different microorganisms can thereby be analyzed in parallel on a genetic or functional level (Mühlhardt, 2009; Neufeld et al., 2011).

The choice of vector determines the size of the insert DNA. For small inserts of up to 15 kb, plasmids are used. Whereas for larger DNA fragments, cosmids, fosmids (both up to 40 kb) or bacterial artificial chromosomes (BAC) (even more than 100 kb) are utilized (Kao-Kniffin et al., 2013). Small-insert libraries as well as large-insert libraries each provide certain advantages and disadvantages. Small-insert libraries usually can be prepared more easily and vectors can be found in higher abundance within a host cell (high-copy vectors). This enables a detection of even weakly-expressed insert genes. Due to the small insert size, the expression of foreign genes might be enhanced and manipulated by a promoter on the vector more easily. The construction of large-insert libraries on the other hand, is much more difficult. Nevertheless, for metagenomics, large-insert vectors are more commonly used as they allow the analysis of large gene clusters or operons coding for whole biosynthetic pathways even in a single vector. With large-insert vectors, a lower number of bacterial cells have to be transformed to cover the same amount of metagenomic DNA. Therefore, the genes or compounds of interest might be found by screening a smaller number of clones than for small-insert libraries (Daniel, 2005).

As mentioned earlier, metagenomic libraries can be screened using either a sequence-based or a function-based approach. Sequence-based screenings using PCR or hybridization can be applied for phylogenetic analyses, for targeted gene search or as random sequencing of clones (Handelsman, 2004; Kao-Kniffin et al., 2013). For the sequencing process itself, standard Sanger sequencing or modern NGS technologies can be used, as already described above. Sequencing with phylogenetic markers (e.g. 16S rRNA genes) is used to gain information about the distribution and abundance pattern of the organisms found in a certain environment

(same as the sequencing of metagenomic DNA described above). As a result, otherwise still uncultivable species might be identified and a first insight in the ecological role of certain organisms and in the composition of a certain community might be gained. In addition, phylogenetic markers can be utilized to sequence surrounding genes. With this approach, it might be possible to connect taxonomy and function if the functions of the surrounding genes have already been described (combination of sequence-based and function-based approaches). Metagenomic libraries can also be screened for the presence of specific genes or genes showing homology to known genes. Using conserved gene regions, different members of the same gene family might be detected. Yet, this excludes the possibility of finding completely new genes. Finally, random sequencing and sequencing of complete inserts are well suited for linking of traits, for unraveling the genomic organization and for analyzing the significance of horizontal gene transfer for specific genes and communities (Daniel, 2005; Handelsman, 2004, 2005; Schmeisser et al., 2007; Simon and Daniel, 2009). Unlike functional analyses, a sequence-based approach does not depend on the expression of the foreign genes (Daniel, 2005).

Successful transformation of a bacterial host cell alone does not necessarily mean that all proteins and products encoded by the foreign genes are finally produced, excreted and detectable. Efficient transcription and translation of metagenomic DNA is often restricted to genes of closely related species of the host (Gabor et al., 2004; Kao-Kniffin et al., 2013; Streit and Schmitz, 2004). Fortunately, several bacterial species have already been employed as potential hosts (see above). Furthermore, shuttle-vectors and broad-host vectors have been developed for transferring metagenomic DNA from one host species into another and for expression in several hosts, respectively. Taken together, these improvements will reduce incompatibility problems and further increase screening efficiency of functional tests in the future (Craig et al., 2010; Kao-Kniffin et al., 2013; Streit and Schmitz, 2004). In general, the frequency of metagenomic clones expressing a certain function is very low (one in several ten or even hundred thousand clones), so that large numbers of clones have to be tested (Daniel, 2005; Handelsman, 2004; Simon and Daniel, 2009; Uchiyama and Miyazaki, 2009). Nevertheless, function-based screenings are widely used in metagenomics because in contrast to sequence-based analyses, they allow to identify compounds with new modes of action, structure or genetic sequence (Daniel, 2005; Kao-Kniffin et al., 2013). So far, novel antibiotics, antibiotic resistance genes,

enzymes, and ion transporters have been detected with functional tests (Daniel, 2005; Handelsman, 2004; Schmeisser et al., 2007).

Many different methods for function-based analyses of metagenomic libraries have been established. Each functional test depends on one of three distinct types of detection, namely direct detection, indirect detection or induced detection. Direct detection systems use phenotypic traits to screen for clones of interest. Examples for such phenotypic traits are atypical colony morphology or pigmentation, reaction with added substances, antibiosis, and substrate use (the latter two mostly indicated by zones of clearing around the clone of interest). Colorimetric or fluorometric compounds might be added to the substrate to facilitate detection. Indirect detection of a certain function can be achieved with reporter genes, heterologous complementation or reporter trap assays. The expression of reporter genes (e.g. *lacZ* gene for blue-white screening or the gene for the green fluorescent protein (GFP)), which are located on a reporter microbe, might be influenced by the activity of clones with certain inserts. As a consequence, the reporter microbe might change in color or fluoresce. The reporter gene-based approach has especially been used to identify genes involved in quorum sensing (a cell-density dependent gene regulation system in bacteria essential for coordination of a number of biological functions). In a heterologous complementation system, the host cells are lacking an essential function and will only grow if the foreign DNA insert provides the genes for full functionality. For the reporter trap assay, a specific vector is used. This vector contains the *gfp* gene, but not the according promoter. Only if a promoter is located on the insert, the host cells will produce the green fluorescent protein which can be detected via fluorescence microscopy (Ekkers et al., 2012; Guan et al., 2007; Kao-Kniffin et al., 2013; Simon and Daniel, 2009). Three individual systems have been employed for induced detection analyses, all of which use *gfp* as reporter gene. Other than the above described screenings, all systems based on induced detection do not necessarily call for plating of clones. Clones of interest can be detected via fluorescence microscopy or fluorescence-activated cell sorting (FACS) (Kao-Kniffin et al., 2013). The observations that the expression of many catabolic genes is induced by the corresponding substrate and that genes responsible for a certain catabolic pathway are often organized as operon including a substrate-sensitive promoter were the basis for the development of the so-called substrate-induced gene expression screening (SIGEX). By placing the inserted metagenomic DNA upstream of the *gfp*

gene (‘operon trap’), the production of the green fluorescent protein is regulated by a promoter within the insert. If the substrate induces gene expression of the metagenomic DNA, GFP will also be produced. This method allows to identify clones which contain metagenomic DNA that encodes for enzymes converting a certain substrate (Uchiyama et al., 2005). Metabolite regulated expression (METREX) is a similar screening approach. Production of green fluorescent protein is regulated by a promoter that is induced intracellularly by quorum sensing signals at a certain threshold. The metagenomic DNA, which encodes for the compounds responsible for quorum sensing, is present within the same cell as the reporter gene (Guan et al., 2007; Williamson et al., 2005). The third induced detection strategy connects product formation with *gfp* gene expression and is termed product-induced gene expression (PIGEX). The *gfp* expression is under regulation of genes which are induced by a specific product. Upon addition of a substrate, metagenomic DNA encoding for enzymes involved in the product synthesis can be identified (Uchiyama and Miyazaki, 2010).

The development of new screening strategies, the improvement of vector-host systems, and advances in sequencing technologies will further enhance the efficiency of metagenomics in microbiological research (Daniel, 2005; Kao-Kniffin et al., 2013).

1.6.) Next-Generation Sequencing – Illumina Sequencing by Synthesis (SBS)

In the last few years, a lot of new sequencing technologies have been invented. All these technologies provide a higher throughput (longer DNA fragments in total per run), speed (massively parallel sequencing of different fragments), and resolution (each base covered by sequencing multiple times) than the standard methods of sequencing, and are therefore called Next-Generation Sequencing (NGS) (Illumina Inc., 2013; Mühlhardt, 2009).

One of these NGS technologies is the Illumina Sequencing by Synthesis (SBS) system. Similar to the modern Sanger sequencing, the SBS technology uses fluorescent labels and laser detection for the sequencing of relatively short DNA fragments. Various sources of DNA can be analyzed (e.g. genomic DNA, cDNA). In addition, it is also possible to sequence RNA (Illumina Inc., 2013).

Illumina provides several systems for sequencing (e.g. MiSeq, HiSeq). The various systems differ in their capacity (i.e. the size of the genome to be analyzed) and the according data output, while the general sequencing principle (SBS) is the same for all systems. The SBS workflow is comprised of 4 separate processes, namely library preparation, cluster generation, sequencing, and data analysis. The library preparation is largely dependent on the nucleic acid sample used (e.g. genomic DNA, cDNA, small RNA, rRNA metagenomics, etc.). Yet, there are some common steps involved in the procedure. In general, the nucleic acid molecule is fragmented into shorter pieces and all small fragments are tagged with two so-called adapters. These oligonucleotides allow the binding of the DNA fragments to the flow cell, on which the sequencing reactions occur. In addition, they contain primer binding sites needed for the actual sequencing process. While modern Sanger sequencing uses fluorescence and gel electrophoresis to determine the base sequence, the SBS system relies on a combination of fluorescence and fixed positions of nucleic acid fragments attached to the surface of the flow cell. To enable the sequencing of nucleic acids of different origin (i.e. different genomes) in parallel (multiplexing), further oligonucleotides called indices can be attached to the fragmented nucleic acids. These indices serve as recognition sequences for the different genomes. As soon as the library has been prepared, all necessary ingredients (i.e. flow cell, buffers, reaction cartridge) are filled into the sequencing system and the library is added. The short tagged single-stranded fragments randomly hybridize with the flow cell, which also carries the two adapters in large quantity (complementary binding between the two adapters). In a subsequent process, each small fragment is amplified many times in a small area of the flow cell. Millions of these clusters are formed by so-called bridge amplification with the help of a polymerase and dNTPs (deoxynucleotides). Each generated cluster contains multiple clonal copies of the same nucleic acid fragment. Next, the sequencing process itself starts. The sequencing procedure is comprised of a number of cycles (currently, maximal 300 cycles, depending on the system), where each cycle represents the addition and characterization of one base of the nucleic acid fragment. The number of cycles hence determines the ‘read length’ for each fragment. At the beginning of each cycle, a polymerase, a universal primer (binding site in adapters), and all four newly developed ddNTPs (dideoxynucleotides) labelled with distinct fluorescent dyes, are added (but no dNTPs). Same as for the modern Sanger sequencing, these ddNTPs inhibit a further elongation of the synthesized

strand. A laser excites the fluorescent dyes and the fluorescence emitted by each cluster is measured, leading to base identification. Both the fluorescent dye and the compound blocking the 3' end of the newly synthesized strand can be chemically removed afterwards ('reversible terminator chemistry'), allowing further strand elongation. Before the next cycle is initiated, all undesirable components are washed away. As the position of each cluster containing copies of one small fragment remains the same for all cycles and because all clusters are analyzed in parallel, many bases of the initial large fragment can be identified at the same time. Finally, one sequence will be obtained for each cluster with a base length resembling the number of cycles. Fragments of different origin will be sorted according to their index recognition sequences using Illumina software. Afterwards, fragments of the same origin can be aligned to form larger contigs using specific algorithms, either with or without a reference sequence. With the SBS technology, it is currently possible to sequence up to 1,5 terabases (10^{12} bases) of DNA in one run (Illumina Inc., 2014; Inc., 2010).

1.7.) Research questions

The practical work for the master's thesis at hand was carried out as part of a series of projects dealing with bacterial biocontrol. The aims of this thesis were to develop new methods for the detection of hydrogen cyanide-producing bacteria and to test the influence of these bacteria on the germination of the model plant lettuce (*Lactuca sativa* L.).

The following research questions should be answered in the course of this master's thesis:

1.) Which method is best suited to screen for hydrogen cyanide-producing bacterial isolates?
2.) Which method is best suited to screen for hydrogen cyanide-producing clones in a metagenomic library?
3.) How do hydrogen cyanide-producing bacteria (isolates or metagenomic clones) influence the germination of lettuce (*Lactuca sativa* L. var. *capitata*)?

2.) Material and Methods

In the following chapters, the tests conducted and the material used for the master's thesis at hand will be described in detail. For a better understanding, tests were separated into two sections, one for testing bacterial isolates and the other for testing a metagenomic library.

Tests for bacterial isolates included the process of isolation of bacterial strains from plant material itself, the optimization of a standard functional assay for the detection of cyanogenic bacteria, and the development of a new high-throughput functional screening method for hydrogen cyanide-producing strains. Besides, a PCR based molecular assay to screen for strains harboring the *hcnC* gene will be introduced. A description of the phylogenetic analysis of bacterial isolates via PCR and first tests for the biocontrol ability of hydrogen cyanide-producing strains (using lettuce as a model plant) will also be part of this first section of material and methods.

In the subsequent chapter, a newly developed high-throughput functional screening method for cyanogenic clones of a metagenomic library will be presented.

2.1.) Testing bacterial isolates

For all tests of this section, isolated bacterial strains were used. All of those strains could be cultivated on growth media.

2.1.1.) Isolation of bacteria from plant material

To allow a screening of endophytic bacterial communities of weeds for hydrogen cyanide-producing strains, isolation of bacteria from those plants was necessary first.

In this work at hand, isolation was carried out only once due to time limitations. Roots and shoots of common ragweed (*Ambrosia artemisiifolia* L.) plants collected in a field in Schützen am Gebirge, on July 14th, 2013, were chosen for the isolation process.

In a first step, the plant material was cleansed of larger particles of soil by rinsing the plants under Milli-Q water (Milli-Q Integral Water Purification System; EMD Millipore Corporation, Billerica, MA, USA). The plants were then cut into smaller pieces with a sterile scalpel and approximately 1 g of material (either roots or stems) was weighed in for further processing. Next, the plant material was surface-sterilized to get rid of superficially living microorganisms. The sterilization of the surface was carried out in

a laminar flow bench under sterile conditions. The plant material was first immersed in 70 % ethanol, followed by 2,5 % sodium hypochlorite (for the preparation of all media and solutions see appendix). The surface sterilization was concluded by three subsequent washing steps in sterile Milli-Q water. All single treatments were conducted for 5 minutes (25 minutes in total for all steps) in sterile plastic boxes (Magenta^{TM} vessel GA-7, Magenta Corporation, Chicago, IL, USA). The plant material was transferred in between boxes with sterile forceps only. After the third washing step in sterile Milli-Q water, the plant material was grinded with sterile mortar and pestle under addition of 10 mL of 0,85 % sodium chloride solution. By way of grinding the surface-sterilized plant material, the endophytic bacteria were set free. The obtained solution was mixed on a Vortex Lab dancer (VWR International, Radnor, PA, USA) and tenfold diluted in a series from 1/10 up to 1/10^{9}. 100 µL of the undiluted sample and each dilution were plated on 94 mm Petri dishes containing approximately 25 mL of R2A Agar medium (10 Petri dishes per dilution series). 100 µL of the Milli-Q water of the third washing step were plated on R2A Agar medium as a control for the sterilization process. The Petri dishes were then put at 28°C for a maximum of 5 days. Plates with bacterial colonies were stored at 4°C until further used. The whole procedure described above was carried out three times for roots and stems, respectively.

From plates with bacterial colonies, morphologically different (isolated) colonies were picked with sterile inoculation loops (1 µL, rigid plastic; VWR International, Radnor, PA, USA) and streaked on 60 mm Petri dishes containing approximately 5 mL of R2A Agar medium (see also figure 3). Plates were again incubated at 28°C for a maximum of 5 days. Plates with grown bacteria were stored at 4°C.

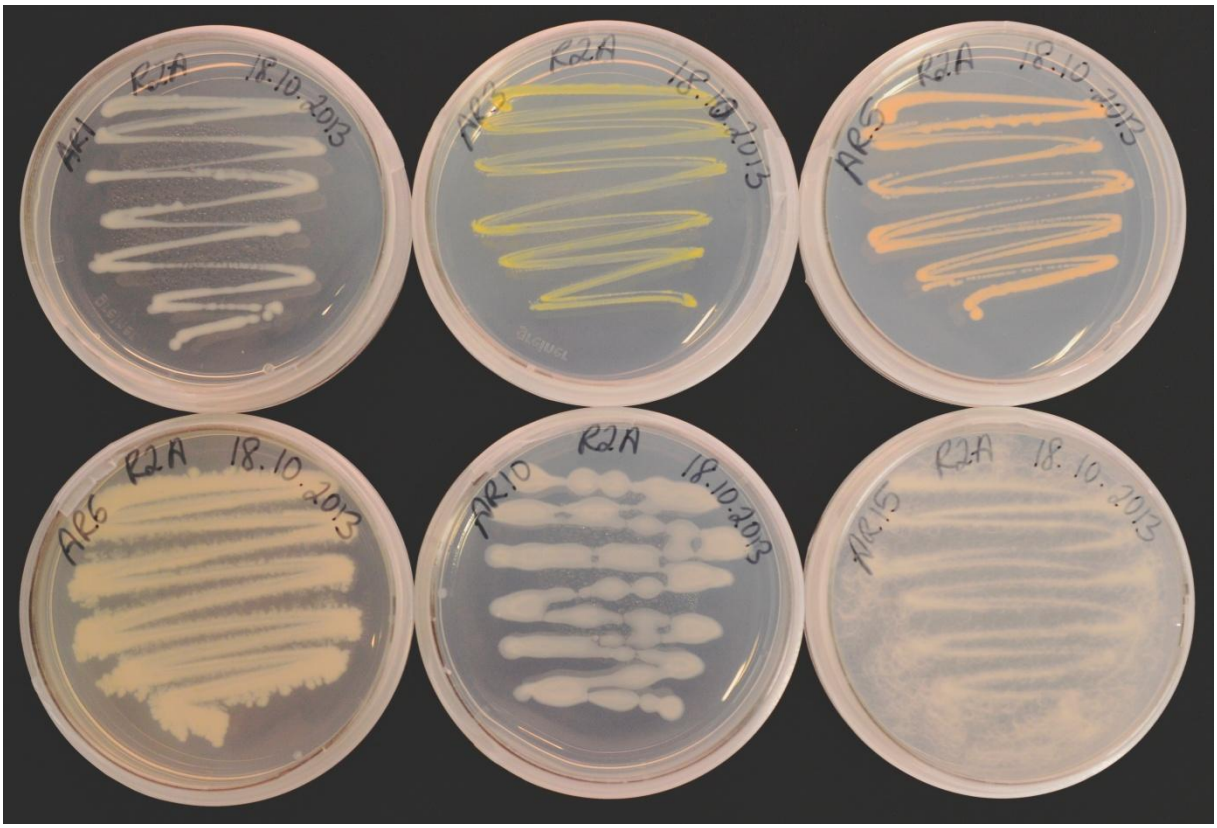

Figure 3: Examples of morphologically different bacteria isolated from ragweed plants (July, 2013).

Long-term storage of isolates in glycerol stocks

For long-term storage, 300 µL of a 50 % glycerol solution were added to 700 µL of a bacterial overnight culture in cryogenic vials (Nunc® CryoTubes®, 1,8 mL; Thermo Scientific, Waltham, MA, USA) to gain a final concentration of 15 % glycerol. This solution was then mixed on a Vortex Lab dancer and put at -80°C. Two cryogenic vials were filled for each bacterial isolate.

The bacterial overnight culture itself was prepared by transferring an isolated bacterial colony into a sterile 24 mL glass test tube containing 5 mL of R2A Broth. The test tube was then put on a shaker (KS-15, Edmund Bühler GmbH, Tübingen, DE) at moderate speed at 28°C for approximately 15 hours.

Phylogenetic analysis of isolates

The phylogenetic analysis and discrimination of all isolates was based on 16S rRNA PCR to amplify the *16S rRNA* gene. For this purpose, DNA was extracted from all isolates with the help of InstaGene™ Matrix (Bio-Rad, Hercules, CA, USA) (Bio-Rad Laboratories, 2014).

The 16S rRNA PCR itself was performed using FIREPol® DNA Polymerase (Solis BioDyne, Tartu, EE) and primers 8F (5' AGAGTTTGATCCTGGCTCAG 3') and 1520R (5' AAGGGAGGTGATCCAGCCGCA 3') (both synthesized by Microsynth AG, Balgach, CH), leading to an amplicon size of 1512 bp. The exact mastermix composition for one reaction can be seen in table 1. For all PCR set-ups, sterile water was used as negative control. The PCR was run on a Thermocycler T1 (Biometra GmbH, Göttingen, DE), starting with an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 1 minute, and elongation at 72°C for 2 minutes. The PCR was completed by a final elongation step at 72°C for 10 minutes. PCR products were then kept at 4°C until further used.

Table 1: Mastermix composition for 16S rRNA PCR for one reaction.

Component	Stock	Final	Amount per 20 µl
H_20 *(Aqua ad iniectabilia, sterile, for PCR; B. Braun Austria GmbH, Maria Enzersdorf, AT)*			2,8 µL
$MgCl_2$ *(Solis BioDyne, Tartu, EE)*	25 mM	2,25 mM	1,8 µL
Reaction buffer BD *(Mg^{2+} and detergent free; Solis BioDyne, Tartu, EE)*	10 x	1 x	2 µL
Primer 8F *(synthesized by Microsynth AG, Balgach, CH)*	1,5 µM	0,045 µM	0,6 µL
Primer 1520R *(synthesized by Microsynth AG, Balgach, CH)*	1,5 µM	0,045 µM	0,6 µL
dNTP Mix *(Thermo Scientific, Waltham, MA, USA)*	2 mM	0,2 mM	2 µL
FIREPol® DNA Polymerase *(Solis BioDyne, Tartu, EE)*	5 U/µL	1 U	0,2 µL
DNA template *(extracted with InstaGene™ Matrix)*			10 µL
TOTAL			***20 µL***

The quality of all PCR products was analyzed by gel electrophoresis. For this, a 1 % agarose gel was prepared by dissolving 1 g of agarose per 100 mL of 1 x TBE buffer (both Sigma-Aldrich, St. Louis, MO, USA). This mix was then brought to the boil in a microwave and let cool down again for several minutes after complete dissolving of the agarose. Next, 1 drop of an ethidium bromide solution (0,125 mg/mL) was added per 50 mL of the agarose gel solution to gain a final concentration of 0,125 µg of ethidium bromide per mL. The solution was then poured into a gel tray carrying one or more combs to mark positions of wells. The tray with the solidified gel was put into a gel chamber (Sub-Cell® GT System, Bio-Rad, Hercules, CA, USA), covered with 1 x TBE buffer, and all combs were removed. 1,5 µL of loading dye (6 x, ready-to-

use; Thermo Scientific, Waltham, MA, USA) were added to 5 µL of PCR product before pipetting into the gel wells. For each gel electrophoresis run, an appropriate marker was used to detect the size of tested DNA (for a list of all size markers used for agarose gel electrophoresis see appendix). For 16S rRNA PCR, the GeneRuler 1 kb DNA Ladder (ready-to-use, Thermo Scientific, Waltham, MA, USA) was used as size marker (3 µL per well). As soon as all samples were pipetted into the according wells, the gel chamber was connected to PowerPac™ Basic (Bio-Rad, Hercules, CA, USA) for power supply. The gel was then run at 120 V for 60 minutes. Afterwards, the gel was checked with the help of a UV Imaging System (BioSpectrum® Imaging System with software VisionWorks LS 5.5.4 Image Acquisition and Analysis System, both UVP Ultra-Violet Products Ltd, Upland, CA, USA) at a wavelength of 280 nm.

PCR products, which lead to positive results in gel electrophoresis, were sent to LGC Genomics GmbH (Berlin, DE) for Sanger sequencing. Obtained sequences were edited with the software BioEdit 7.0.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and compared to the NCBI database in BLASTN 2.2.29 (search nucleotide databases using a nucleotide query; http://blast.ncbi.nlm.nih.gov/Blast.cgi; NCBI, Bethesda, MD, USA) (Zhang et al., 2000). The 16S rRNA sequences were also submitted to analysis with the RDP Naïve Bayesian rRNA Classifier 2.6 (http://rdp.cme.msu.edu/classifier/classifier.jsp; Michigan State University, MI, USA) (Wang et al., 2007) for a reliability check of sequence data, and the given phylogenetic information. Discrimination of tested strains was then based on the phylogenetic level confirmed by the RDP Classifier.

2.1.2.) Optimization of a standard functional screening method for the detection of hydrogen cyanide-producing bacteria

A standard functional screening method for the detection of hydrogen cyanide-producing bacteria (Lorck, 1948) was optimized to verify results of all newly developed methods under ideal conditions.

Gaseous hydrogen cyanide released by bacteria was detected via a color change of a filter paper fixed to the lid of a Petri dish containing culture medium on which the tested bacteria grew on. A color change of the filter paper from yellow to orange, dark orange or reddish brown could be observed for HCN-producing isolates, as shown in

figure 4. Isolates, which did not produce hydrogen cyanide, did not cause a color change of the filter paper. One bacterial isolate could be tested per 94 mm Petri dish.

Figure 4: Colour reaction of the filter paper for a hydrogen cyanide-producing strain (pictures taken after 4, 24, 48, and 72 hours of incubation).

The Petri dishes used for this test contained approximately 25 mL of growth medium. A list of tested media can be seen in table 2 (for the preparation of all media and solutions see appendix). Every medium was prepared in a standard type as given in the list below and in a second type with supplemented glycine, which is the natural precursor of the hydrogen cyanide synthesis in bacteria (see section introduction for further details). For this, 0,5 g of glycine (for molecular biology, Sigma-Aldrich, St. Louis, MO, USA) were added to 1 liter of medium before autoclaving. The filter papers (90 mm Whatman® qualitative filter paper for technical use, Grade Shark Skin®, creped; GE Healthcare, Chalfont St Giles, UK) were autoclaved, saturated with 1 % picric acid solution, let dry in the laminar flow bench overnight and wetted with 10 % sodium carbonate solution prior to final use. Different amounts of chemicals for the preparation of the filter papers were tested, as given in table 2. Isolated bacterial colonies were streaked onto the test plates with a sterile inoculation loop (1 µL, rigid plastic; VWR International, Radnor, PA, USA). The two *Pseudomonas* strains KS15 and KS28 were tested with the standard test first and then used to optimize this test. All other strains were tested later under improved testing conditions. A list of all bacterial isolates tested with this standard functional method for HCN detection can be seen in tables 10 and 11 (chapter 3.1.4.). To guarantee good growth of bacteria, two of the 10 tested media were used in parallel for all isolates (see results for optimum media). By utilizing R2A Agar, which is low in nutrient content, as well as LB Agar medium, which is a richer and more complex medium, different growth requirements of isolates could be considered. Both media contained additional glycine. After the bacteria had been streaked onto the media, the filter paper was fixed to the inner surface of the plate lid. Table 2 also shows the

different techniques of fixation that were examined. Finally, Petri dishes were sealed with Parafilm® M (BRAND GMBH + CO KG, Wertheim, DE) to prevent a gas exchange and put at 28°C. The optimum duration of the testing period was also evaluated. For this, plates were checked after 4, 24, 48, and 72 hours of incubation. A negative control without bacteria was included in each test run.

Table 2: Parameters tested for the optimization of the standard test for HCN detection.

Parameter	Parameter level				
Growth medium *(standard type and type with supplemented glycine)*	King's B Agar medium, modified				
	Quarter-strength King's B Agar medium, modified				
	R2A Agar medium				
	LB Agar medium				
	10% TSA medium				
Amount of chemicals for filter papers *(90mm)*	Amount of 1% picric acid solution (saturating autoclaved filter papers)				
	600 µL	800 µL	1000 µL		
	Amount of drops of 10% sodium carbonate pipetted with a sterile 153 MM Pasteur pipette (greiner bio-one, Frickenhausen, DE) (wetting dried filter papers)				
	3	5	7	10	12
Fixation of filter paper to Petri dish lid	Fixation with UHU Patafix (UHU GmbH & Co KG, Bühl / Baden, DE)				
	Fixation with Scotch Magic 3M tape (3M, Maplewood, MN, USA)				
	Without specific fixation (fixation only by moisture of filter paper and moisture caused by transpiration of bacteria)				
Duration of testing period *(time of incubation)*	4 hours	24 hours	48 hours	72 hours	

2.1.3.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacteria

A high-throughput functional screening method for the detection of hydrogen cyanide-producing bacteria was developed based on the same principle as the standard test described above. Again, gaseous HCN released by bacteria growing on a culture medium led to a color change of a filter paper.

As other chemicals were used to impregnate the filter papers, the color change differed from the standard test. Instead of a yellow filter paper turning brown after

reaction with hydrogen cyanide, a white filter paper was turning blue (Feigl and Anger, 1966). To allow a screening of large numbers of isolates, we developed a high-throughput screening assay for 96 well microtiter plates, as can be seen in figure 5.

Figure 5: High-throughput functional screening method for the detection of hydrogen cyanide-producing bacteria in 96 well microtiter plates. HCN-producing strains lead to a blue color change of the filter paper. Pictures of the upper and lower site of plates taken after 24 hours of incubation.

Filter papers and microtiter plates had to be prepared in advance. Whatman® 3MM filter paper (GE Healthcare, Chalfont St Giles, UK) was cut into pieces of 8 x 12 cm size and autoclaved. The autoclaved filter papers were saturated with a mix of equal amounts of 1 % copper(II)ethylacetotacetate solution and 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution (for the preparation of all media and solutions see appendix) and let dry under the fume hood. Filter papers, which were not used immediately, were stored at 4°C. The wells of the microtiter plates were filled with 200 µL of medium, respectively. All isolates were tested on LB Agar medium as well as on R2A agar medium (for reasons, see above). Both media contained additional glycine (0,5 g per liter). All wells of a microtiter plate were filled with either one of the media. Isolated bacterial colonies were streaked onto the medium with a sterile inoculation loop (1 µL, rigid plastic; VWR International, Radnor, PA, USA). Each isolate was tested in 3 separate wells per microtiter plate and medium. For a detailed list of all bacterial isolates tested with this high-throughput functional screening method, see tables 10 and 11 (chapter 3.1.4.). A negative control without bacteria was included in 3 wells of every test plate. With this set-up, 31 bacterial strains could be tested per 96 well microtiter plate. After the bacteria had been streaked into the wells, a filter paper was laid above the microtiter plate and the plate was closed with a completely dry lid. The microtiter plates were then sealed with Parafilm® M (BRAND GMBH + CO KG, Wertheim, DE) and put at 28°C. The lids of the plates were

weighed down with a glass bottle filled with water to assure a close contact of the filter paper and the well borders and to prevent a gas exchange between neighboring wells. Any color change was recorded after 4, 24, 48, and 72 hours of incubation. Test runs were stopped as soon as the blue coloring spread across well borders or latest after 72 hours of incubation.

2.1.4.) Detection of hydrogen cyanide-producing bacteria with a PCR-based molecular assay

In addition to the functional screening methods for cyanogenic bacterial isolates, a PCR based molecular assay was established. For this purpose, degenerated primers were designed for the *hcnC* gene, which is part of the *hcnABC* gene cluster that encodes for the HCN synthase. All isolates tested with the high-throughput functional screening method for isolates were also tested with the HCNC PCR (for a complete list, see tables 10 and 11, chapter 3.1.4.). Primer design was carried out by Gajender Aleti (AIT) by ClustalW aligning (Thompson et al., 1994) of *Pseudomonas aeruginosa* protein sequences of the HcnC subunit (*P. aeruginosa* strain PAO1 with NCBI accession number NP_250885.1 as starting sequence for initial protein blast). Using wobble codes, degenerate primers were designed for the most conserved regions.

The HCNC PCR was then performed using FIREPol® DNA Polymerase (Solis BioDyne, Tartu, EE) and the degenerated primers HCNC-F (5' GARTTYYTNTGYGAYCAYCA 3') and HCNC-R (5' ATYTCNCCRTTRTCYTTYTG 3') (both synthesized by Microsynth AG, Balgach, CH), leading to an amplicon size of 360 bp. The exact mastermix composition for one reaction can be seen in table 3. Again, 10 µL of DNA extracted with InstaGene™ Matrix was used as template. The PCR was run on a Thermocycler T1 (Biometra GmbH, Göttingen, DE), starting with an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 1 minute. The PCR was completed by a final elongation step at 72°C for 10 minutes. PCR products were then kept at 4°C until further used.

Table 3: Mastermix composition for HCNC PCR.

Component	Stock	Final	Amount per 20 µl
$H_2 0$ *(Aqua ad iniectabilia, sterile, for PCR; B. Braun Austria GmbH, Maria Enzersdorf, AT)*			1,8 µL
$MgCl_2$ *(Solis BioDyne, Tartu, EE)*	25 mM	2,5 mM	2 µL
Reaction buffer BD *(Mg^{2+} and detergent free; Solis BioDyne, Tartu, EE)*	10 x	1 x	2 µL
Primer HCNC-F *(synthesized by Microsynth AG, Balgach, CH)*	100 µM	4 µM	0,8 µL
Primer HCNC-R *(synthesized by Microsynth AG, Balgach, CH)*	100 µM	4 µM	0,8 µL
dNTP Mix *(Thermo Scientific, Waltham, MA, USA)*	2 mM	0,2 mM	2 µL
BSA *(Thermo Scientific, Waltham, MA, USA)*	1 ng/µL	0,4 ng	0,4 µL
FIREPol® DNA Polymerase *(Solis BioDyne, Tartu, EE)*	5 U/µL	1 U	0,2 µL
DNA template *(extracted with InstaGene™ Matrix)*			10 µL
TOTAL			***20 µL***

The quality of all PCR products was analyzed by gel electrophoresis as already described above. For HCNC PCR, the GeneRuler 100 bp DNA Ladder (ready-to-use, Thermo Scientific, Waltham, MA, USA) was used as size marker (3 µL per well) (for a list of all size markers used for agarose gel electrophoresis see appendix). The gel was run at 120 V for 60 minutes.

Due to the degeneracy of the HCNC primers, amplicons could not be sequenced directly. Therefore, PCR products had to be cloned into *Escherichia coli* with the help of the StrataClone PCR Cloning Kit (Stratagene, Agilent Technologies, La Jolla, CA, USA): The StrataClone PCR Cloning Kit Instruction Manual (Agilent Technologies Inc., 2008) was used and adapted accordingly. For the initial ligation process, 1 µL of StrataClone Cloning buffer was mixed with 0,66 µL of PCR product, and 0,33 µL of StrataClone Vector Mix amp/kan. All other steps were conducted as described in the manual. For the selection of cells containing the cloning vector, LB-Ampicillin Agar medium was utilized (final concentration of 100 µg ampicillin per mL of medium). After the outgrowth period, 50 and 100 µL of the transformation mixture were plated on LB-Ampicillin-X-gal plates, respectively. Plates with grown bacteria were sealed with Parafilm® M (BRAND GMBH + CO KG, Wertheim, DE) and stored at 4°C. Blue-white screening based on *lacZ'* α-complementation was used to detect clones of interest (which appear white).

To obtain PCR products that could be used for Sanger sequencing, an M13 Colony PCR was performed next. Binding sites for both M13 primers used (see below) are located on the StrataClone PCR cloning vector pSC-A-amp/kan close to the insert position. White colonies of transformed *E. coli* clones (blue-white screening, as explained above) were picked with a sterile wooden toothpick (PAPSTAR GmbH, Kall, DE) and transferred into the previously prepared M13 Colony PCR mastermix. For the M13 Colony PCR, FIREPol® DNA Polymerase (Solis BioDyne, Tartu, EE) and primers M13F (5' GTAAAACGACGGCCAG 3') and M13R (5' CAGGAAACAGCTATGAC 3') (both synthesized by Microsynth AG, Balgach, CH) were used, leading to an amplicon size of 620 bp (260 bp of vector and 360 bp of insert). The exact mastermix composition for one reaction can be seen in table 4. The PCR was run on a Thermocycler T1 (Biometra GmbH, Göttingen, DE), starting with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 58°C for 1 minute, and elongation at 72°C for 2 minutes. The PCR was completed by a final elongation step at 72°C for 10 minutes. PCR products were then kept at 4°C until further used.

Table 4: Mastermix composition for M13 Colony PCR for one reaction.

Component	Stock	Final	Amount per 25 µl
H_20 *(Aqua ad iniectabilia, sterile, for PCR; B. Braun Austria GmbH, Maria Enzersdorf, AT)*			16,3 µL
$MgCl_2$ *(Solis BioDyne, Tartu, EE)*	25 mM	3 mM	3 µL
Reaction buffer BD *(Mg^{2+} and detergent free; Solis BioDyne, Tartu, EE)*	10 x	1 x	2,5 µL
Primer M13F *(synthesized by Microsynth AG, Balgach, CH)*	5 µM	0,05 µM	0,25 µL
Primer M13R *(synthesized by Microsynth AG, Balgach, CH)*	5 µM	0,05 µM	0,25 µL
dNTP Mix *(Thermo Scientific, Waltham, MA, USA)*	2 mM	0,2 mM	2,5 µL
FIREPol® DNA Polymerase *(Solis BioDyne, Tartu, EE)*	5 U/µL	1 U	0,2 µL
DNA template *(colony transferred with sterile toothpick)*			1 colony
TOTAL			***25 µL***

The quality of all PCR products was analyzed by gel electrophoresis as already described above. For M13 Colony PCR, the GeneRuler 100 bp DNA Ladder (ready-to-use, Thermo Scientific, Waltham, MA, USA) was used as size marker (3 µL per

well) (for a list of all size markers used for agarose gel electrophoresis see appendix). The gel was run at 120 V for 60 minutes.

PCR products, which lead to positive results in gel electrophoresis, were sent to LGC Genomics GmbH (Berlin, DE) for Sanger sequencing. Obtained sequences were edited with the software BioEdit 7.0.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and compared to the NCBI database in BLASTX 2.2.29 (search protein databases using a translated nucleotide query; http://blast.ncbi.nlm.nih.gov/Blast.cgi; NCBI, Bethesda, MD, USA) (Altschul et al., 1997).

An illustration of the whole workflow for the PCR-based molecular assay for the detection of hydrogen cyanide-producing bacteria can be seen in figure 6.

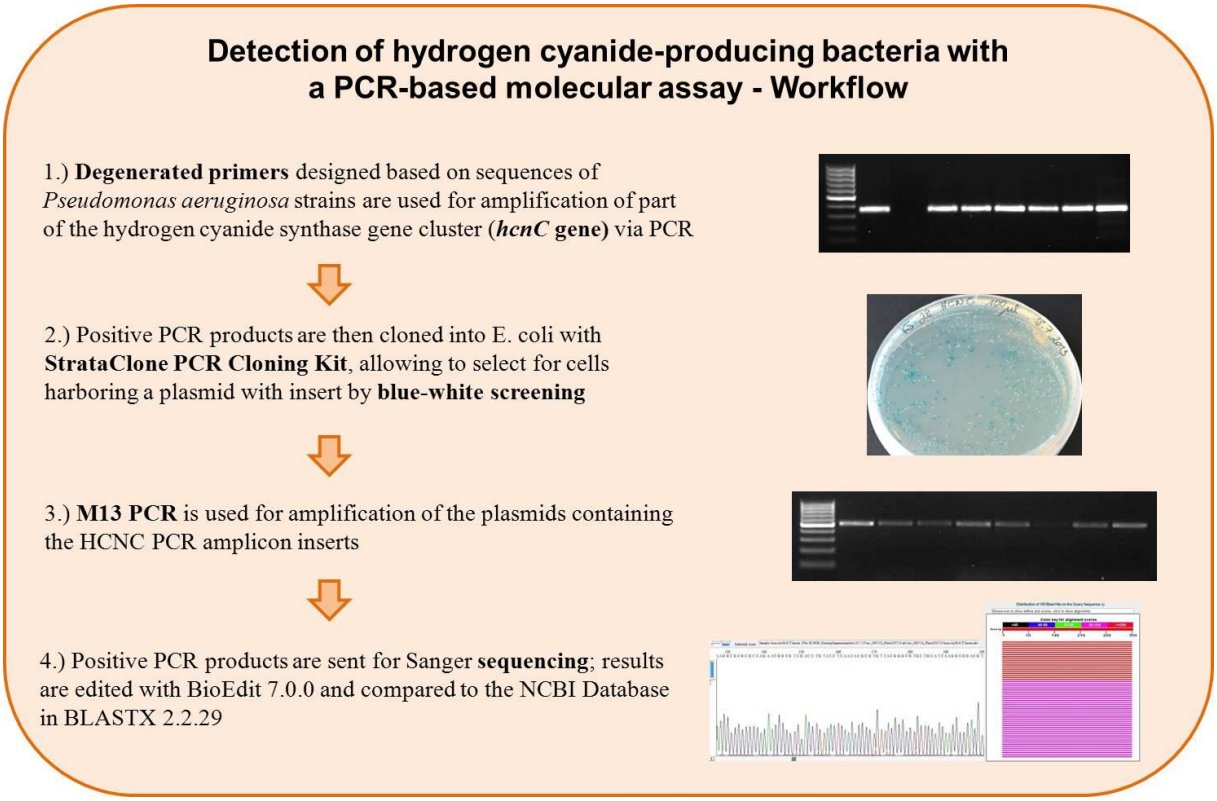

Figure 6: Workflow for the PCR-based molecular assay for the detection of hydrogen cyanide-producing bacteria.

2.1.5.) Phylogenetic analysis of hydrogen cyanide-producing bacteria with PCR-based molecular assays

The standard phylogenetic discrimination was carried out with 16S rRNA PCR, as described above (see chapter 2.1.1. for further information).

In addition, PCR amplification and characterization of the 16S-23S rDNA intergenic spacer region (IGS) was applied to allow a distinct separation of different strains for the same phylogenetic information (same species) gained with 16S rRNA PCR sequence analysis (Idris et al., 2004). The 16S-23S IGS PCR was performed using FIREPol® DNA Polymerase (Solis BioDyne, Tartu, EE) and primers pHr (5' TGCGGCTGGATCACCTCCTT 3') and P23SR01 (5' GGCTGCTTCTAAGCCAAC 3') (both synthesized by Microsynth AG, Balgach, CH). Each of the 2 primers binds to a highly conserved region of either the 16S rDNA (pHr; position 1518 – 1541 in *E. coli*) or the 23S rDNA (P23SR01; position 1069 – 1052 in *E. coli*). Due to variability in sequence length of the 16S-23S rDNA intergenic spacer region, the amplicon size might differ even for strains of the same species (approximate size 1600 bp) (Massol-Deya et al., 1995). 8 µL of DNA extracted with InstaGene™ Matrix (Bio-Rad, Hercules, CA, USA) (Bio-Rad Laboratories, 2014) were used as template. The exact mastermix composition for one reaction can be seen in table 5. The PCR was run on a Thermocycler T1 (Biometra GmbH, Göttingen, DE), starting with an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 90 seconds, and elongation at 72°C for 2 minutes. The PCR was completed by a final elongation step at 72°C for 10 minutes. PCR products were then kept at 4°C until further used.

Table 5: Mastermix composition for 16S-23S IGS PCR for one reaction.

Component	Stock	Final	Amount per 20 µl
$H_2 0$ *(Aqua ad iniectabilia, sterile, for PCR; B. Braun Austria GmbH, Maria Enzersdorf, AT)*			5,46 µL
$MgCl_2$ *(Solis BioDyne, Tartu, EE)*	25 mM	2,25 mM	1,8 µL
Reaction buffer BD *(Mg^{2+} and detergent free; Solis BioDyne, Tartu, EE)*	10 x	1 x	2 µL
Primer pHr *(synthesized by Microsynth AG, Balgach, CH)*	15 µM	0,2025 µM	0,27 µL
Primer P23SR01 *(synthesized by Microsynth AG, Balgach, CH)*	15 µM	0,2025 µM	0,27 µL
dNTP Mix *(Thermo Scientific, Waltham, MA, USA)*	2 mM	0,2 mM	2 µL
FIREPol® DNA Polymerase *(Solis BioDyne, Tartu, EE)*	5 U/µL	1 U	0,2 µL
DNA template *(extracted with InstaGene™ Matrix)*			8 µL
TOTAL			***20 µL***

The quality of all PCR products was analyzed by gel electrophoresis as already described above. For 16S-23S IGS PCR, the GeneRuler 1 kb DNA Ladder (ready-to-use, Thermo Scientific, Waltham, MA, USA) was used as size marker (3 µL per well) (for a list of all size markers used for agarose gel electrophoresis see appendix). The gel was run at 120 V for 60 minutes.

PCR products of both PCR set-ups (16S rRNA PCR and 16S-23S IGS PCR), which lead to positive results in gel electrophoresis, were sent to LGC Genomics GmbH (Berlin, DE) for Sanger sequencing. Obtained sequences were edited with the software BioEdit 7.0.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and compared to the NCBI database in BLASTN 2.2.29 (search nucleotide databases using a nucleotide query; http://blast.ncbi.nlm.nih.gov/Blast.cgi; NCBI, Bethesda, MD, USA) (Zhang et al., 2000). 16S rRNA sequences were also submitted to analysis with the RDP Naïve Bayesian rRNA Classifier 2.6 (http://rdp.cme.msu.edu/classifier/classifier.jsp; Michigan State University, MI, USA) (Wang et al., 2007) for a reliability check of sequence data, and the given phylogenetic information. Discrimination of tested strains was then based on the phylogenetic level confirmed by the RDP Classifier. 16S-23S IGS PCR sequences, which yielded identical species hits when compared to the NCBI database, were further analyzed by ClustalW alignment with the software BioEdit 7.0.0. It was supposed that similar sequences with varying nucleotide composition belong to different strains.

2.1.6.) Testing the influence of hydrogen cyanide-producing bacteria on the germination of lettuce (*Lactuca satica* L. var. *capitata*)

As a first test of the biocontrol ability of the hydrogen cyanide-producing isolates found, we performed a germination assay with lettuce seedlings inoculated with a bacterial suspension of cyanogenic bacteria. This test was carried out to check whether cyanogenic bacteria are able to inhibit the germination of the model plant lettuce. Isolates causing inhibition would be of interest for further germination assays with other plants, especially weeds.

Sixteen isolates were chosen for this test, one of them an isolate unable to produce HCN (*Pseudomonas* isolate KS15). A list of all bacterial isolates utilized in the lettuce germination assay can be seen in table 15 (chapter 3.1.6.). Experiments described below were replicated three times for all isolates.

The preparation of the bacterial suspension for every single isolate was performed as follows: First, an overnight culture was prepared. For this purpose, an isolated bacterial colony was transferred into a sterile 24 mL glass test tube containing 5 mL of LB medium (for the preparation of all media and solutions see appendix) with a sterile inoculation loop (1 µL, rigid plastic; VWR International, Radnor, PA, USA). The test tube was then put on a shaker (KS-15, Edmund Bühler GmbH, Tübingen, DE) at moderate speed at 28°C for approximately 15 hours. From this overnight culture, 100 µL were pipetted into a new test tube containing LB medium to generate a fresh culture (see also figure 7). The test tube for the fresh culture was also put on the shaker, for approximately 4 hours.

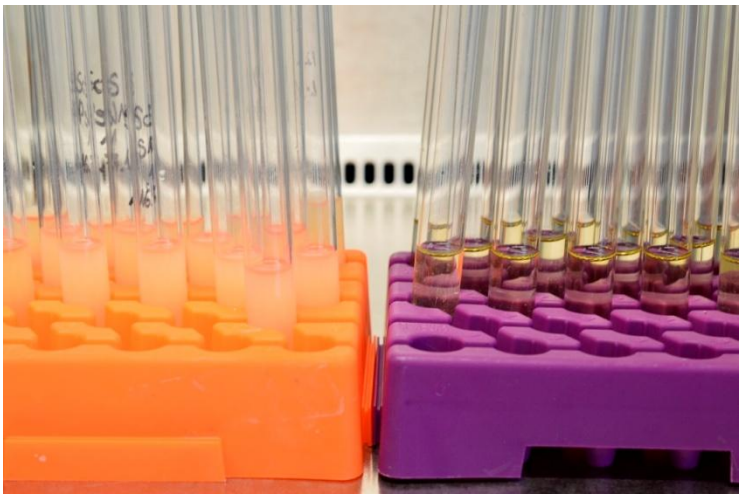

Figure 7: Bacterial overnight cultures (left) and fresh cultures before incubation (right).

During the second growth phase, seeds of lettuce (*Lactuca satica* L. var. *capitata*, variety 'Winterkönig'; Austrosaat AG, Wien, AT) were surface-sterilized. For this, seeds were immersed in 70 % ethanol for 1 minute, next, in 3,5 % sodium hypochlorite with additional 0,05 % Tween-20 for 15 minutes, and finally, 6 times in sterile Milli-Q water for 1 minute, respectively. All single treatments were conducted in sterile plastic boxes (Magenta^{TM} vessel GA-7, Magenta Corporation, Chicago, IL, USA). The plant material was transferred in between boxes with sterile forceps and sterile metal wires only. After the surface-sterilization process, 25 to 30 seeds were put into an empty sterile 94 mm Petri dish for each of the tested isolates, respectively. An additional Petri dish was filled with seeds to allow a mock treatment without bacteria as a control (see below). While the seeds were let dry in the Petri dishes, the optical density of the bacterial fresh cultures (proportional to bacterial growth or propagation in a certain range) was determined on the NanoDrop® ND-1000 Spectrophotometer (software version 3.7.1; Thermo Scientific, Waltham, MA, USA). Thereto, 1 µL of a fresh culture was pipetted onto the test pedestals and the absorbance was read at 600 nm (optical density at 600 nm = NanoDrop®-value for absorbance at 600 nm * 10). The fresh cultures of all isolates were then diluted to an optical density of 0,2 (range 0,17 – 0,23) with LB medium. 100 µL of these dilutions were then again diluted in 10 mL of a 20 mM $MgCl_2$ solution (to a final optical density of 0,002), for each isolate separately. For the mock treatment, 100 µL of LB medium were diluted in 10 mL of 20 mM $MgCl_2$. These final dilutions were then filled into the 17 Petri dishes containing the surface-sterilized lettuce seeds (16 isolates and 1 mock treatment). The lettuce seeds were incubated in the bacterial suspensions under sterile conditions for 25 minutes at room temperature. Afterwards, for each treatment, 20 seeds were plated on a 145 mm Petri dish containing approximately 60 mL of 1,5 % Water Agar medium. Finally, the Petri dishes were closed and randomly distributed in the plant culturing room (20°C, 50 % air humidity) under conditions of 16/8 hours of day/night. Different germination parameters were then evaluated over a testing period of 6 days, as can be seen in table 6. Parameters were defined in accordance with the International Rules for Seed Testing 2011 (ISTA, 2011). Representative images of all parameter levels of the parameter 'seedling morphology' can be seen in figures 8 to 10. Tests were stopped after 6 days of incubation before plant behavior and morphology were rather influenced by abiotic stresses (e.g. lacking of nutrients) than by bacteria.

Statistical analyses for all parameters were performed using one-way ANOVA or Kruskal-Wallis test (software SAS®, version 9.1; SAS Institute Inc., Cary, NC, USA). For this purpose, data were first analyzed for normal distribution (Kolmogorov-Smirnoff test) and homogeneity of variance (Brown-Forsythe test). Data, which fulfilled these two requirements, were tested via ANOVA, all other data sets with the non-parametric Kruskal-Wallis test. The null hypothesis for all tests was expressed as 'there are no significant differences among tested groups', meaning that the bacterial isolates did not have a significant influence on the germination of lettuce if compared to a control treatment without bacteria. The alternative hypothesis was 'there are significant differences among tested groups', meaning that one or several bacterial isolates have a significant influence on the germination of lettuce if compared to a control treatment without bacteria. The null hypothesis was rejected if the p-value for the performed test was lower than the significance level of $\alpha = 0,05$. A post-hoc Tukey's test or a Wilcoxon signed-rank test was performed for data showing significant differences for ANOVA or Kruskal-Wallis test, respectively. Results will be depicted using means, standard error for means and p-values.

Table 6: Parameters evaluated for the germination assay with lettuce.

Parameter	Parameter level(s) or unit	Parameter evaluated on day(s) after incubation	Parameter evaluated for
Emergence of radicle	Radicle clearly visible	1 to 6	All 20 seedlings of a Petri dish
	Radicle not visible		
Emergence of cotyledons	Cotyledons clearly visible	1 to 6	All 20 seedlings of a Petri dish
	Cotyledons not visible		
Expansion of cotyledons	Cotyledons fully expanded	1 to 6	All 20 seedlings of a Petri dish
	Cotyledons not fully expanded		
Morphology of seedling	Seedling vital, strong	6	All 20 seedlings of a Petri dish
	Seedling weak or small, but otherwise healthy		
	Seedling abnormal		
	Seed failed to germinate		
Length of radicle	mm	6	10 randomly chosen seedlings per Petri dish
Weight of seedling	mg	6	Same 10 randomly chosen seedlings as for parameter 'length of radicle'

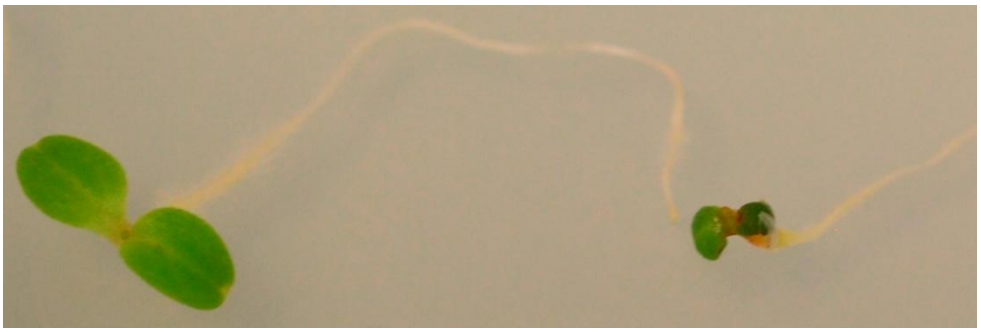

Figure 8: A vital, strong seedling (left) next to a weak, but otherwise healthy seedling (right).

Figure 9: An abnormal seedling (left) next to a vital, strong seedling (right).

Figure 10: A vital, strong seedling (left) next to a seed that did not germinate at all (right) (left root belongs to a third seedling, right root to vital, strong seedling).

2.2.) Testing a metagenomic library

In this second section of material and methods, a test will be presented to detect hydrogen cyanide-producing clones in a metagenomic library. We developed this high-throughput functional screening method for a metagenomic library in addition to the previously described tests for bacterial isolates especially because we were hoping to find novel mechanisms for HCN production in bacteria. For our studies, we were using the Peru Rhizosphere Library (14PR) created by a research team of the University of Waterloo, ON, Canada, in 2012 (http://www.cm2bl.org/; see also Neufeld et al. (2011)). This *Escherichia coli* library contains 540.500 clones harboring the Gateway® entry cosmid pJC8 (Gateway® Life Technologies Corporation, Carlsbad, CA, USA; Cheng et al. (2014)) with an average insert of 28,1 kb. The DNA of the inserts was extracted from potato rhizosphere samples from Peru.

2.2.1.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacterial clones

The high-throughput functional screening method for the detection of hydrogen cyanide-producing clones in a metagenomic library was based on the same principle as the high-throughput screening method for isolates in 96 well microtiter plates. The test set-up was modified slightly to guarantee easy and optimum testing conditions. Instead of 96 well microtiter plates, 145 mm Petri dishes were used. The Petri dishes were filled with a bilayer of approximately 130 mL of 1,5 % Water Agar medium (lower layer), and 70 mL of LB-Tetracycline Agar medium (final concentration of 20 µg tetracycline per mL of medium) with supplemented glycine (0,5 g per liter medium) (upper layer) (for the preparation of all media and solutions see appendix). Such high amounts of media per Petri dish were chosen to minimize the air volume above the growing clones and thereby prevent undesirable dilution of HCN to an undetectable concentration. Whatman® 3MM filter paper (GE Healthcare, Chalfont St Giles, UK) was cut into pieces of 145 mm diameter and autoclaved. The autoclaved filter papers were saturated with a mix of equal amounts of 1 % copper(II)ethylacetotacetate solution and 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution and let dry under the fume hood (Feigl and Anger, 1966), as already described above. Filter papers, which were not used immediately, were stored at 4°C. A solution containing approximately 1000 colony forming units (clones)

per 250 µL was prepared as follows: A small amount of the glycerol stock of a metagenomic library (Peru Rhizosphere Library with cosmid pJC8 in our case) was transferred into a sterile 1,5 mL safe-lock tube (Eppendorf AG, Hamburg, DE) containing 100 µL of 0,85 % sodium chloride solution. The optical density of this solution was measured with the NanoDrop® ND-1000 Spectrophotometer (software version 3.7.1; Thermo Scientific, Waltham, MA, USA), as described above. The final density was set to a value of approximately 1000 colony forming units per µL (range 0,027 – 0,030 for the value of absorbance at 600 nm in NanoDrop® for the Peru Rhizosphere Library with cosmid pJC8) by diluting with 0,85 % sodium chloride solution. 1 µL of this solution was then diluted in 249 µL of 0,85 % sodium chloride solution for proper plating of 250 µL per 145 mm Petri dish using a sterile glass rod. As a negative control, 250 µL of 0,85 % sodium chloride solution were plated on one Petri dish per test run. The hydrogen cyanide-producing, but not tetracycline resistant, strain KS28 was included as a positive control. For this, a small piece of LB-Tetracycline Agar medium with supplemented glycine was cut out from the Petri dish (after plating the metagenomic library) with the wide end of a sterile 1000 µL pipette tip (VWR International, Radnor, PA, USA). The arising gap was refilled with a piece of LB Agar medium with supplemented glycine cut out from a separate Petri dish with a sterile 200 µL pipette tip (VWR International, Radnor, PA, USA). On this piece of medium, a small amount of an isolated colony of strain KS28 was streaked with a sterile inoculation loop (1 µL, rigid plastic; VWR International, Radnor, PA, USA), as can be seen in figure 11. This procedure was carried out for every fifth Petri dish of a test run. As soon as the bacterial dilution had been streaked onto every dish, the filter papers were fixed to the Petri dish lids with photo-tapes (KLS Alben + Rahmen GmbH, Kaarst, DE). Petri dishes were closed and sealed with Parafilm® M (BRAND GMBH + CO KG, Wertheim, DE) to prevent a gas exchange. Finally, plates were put at 37°C for a testing period of 7 days.

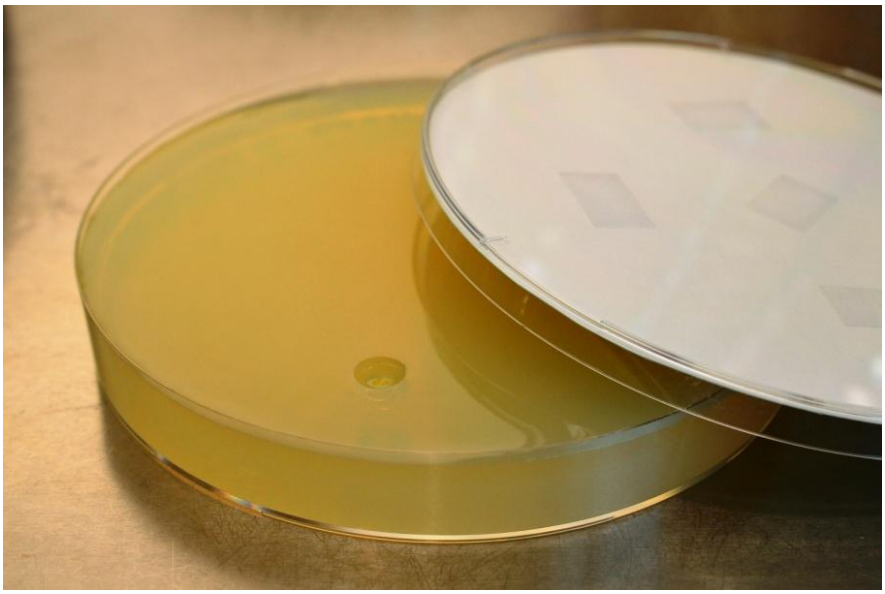

Figure 11: Test plate for the high-throughput functional screening method for the detection of hydrogen cyanide-producing clones in a metagenomic library. Plate contains positive control strain KS28 on a replaced piece of culture medium.

Clones, which led to a color change of the filter paper, were isolated and streaked on 94 mm Petri dishes containing approximately 25 mL of LB-Tetracycline Agar medium with supplemented glycine. A functional screening in 96 well microtiter plates (as described above) containing the same medium was carried out for a quality check of the isolation process.

3.) Results

Results for all the tests performed for this Master's thesis will be presented in the upcoming chapters. First, findings for tests with bacterial isolates will be demonstrated, followed by a section of results for testing a metagenomic library.

3.1.) Testing bacterial isolates

3.1.1.) Isolation of bacteria from plant material

Roots and shoots of common ragweed (*Ambrosia artemisiifolia* L.) plants collected in a field in Schützen am Gebirge, on July 14th, 2013, were chosen for the isolation process, as described above.

Morphologically different bacteria were isolated from plates of dilution steps 1/10^4 to 1/10^6. Control plates (with water from last washing step) contained either no or very few colonies (less than 5).

Thirty-two bacterial isolates were isolated from ragweed roots (isolates AR1 to AR32), and eight from ragweed stems (isolates AS1 to AS8), leading to a total amount of 40 endophytic bacterial isolates isolated from ragweed plants. All isolates grew well on R2A agar medium. Suitable growth was achieved on average after 2 or 3 days of incubation at 28°C.

The phylogenetic analysis and discrimination of all isolates was based on the 16S rRNA gene amplified via PCR. A list of all 40 isolates and their phylogenetic identification can be seen in tables 7 and 8.

The 40 isolates were subsequently tested for production of hydrogen cyanide (see chapters 3.1.3. and 3.1.4.). The according results can be seen in table 11 (chapter 3.1.4.).

Table 7: Bacterial isolates isolated from ragweed plants and their phylogenetic classification. Part I. Isolates AR1 to AR20.

Bacterial isolate	Closest match with NCBI database, accession number and percentage of identity	Phylogenetic classification	Isolate designation
AR1	*Rhizobium* sp. O-MR28 16S ribosomal RNA gene, partial sequence; JN613487.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR1
AR2	*Sphingomonas* sp. DC2a-27 gene for 16S rRNA, partial sequence; AB552856.1; 99 % identity	*Sphingomonas* sp.	*Sphingomonas* sp. AR2
AR3	*Sphingomonas* sp. DC2a-27 gene for 16S rRNA, partial sequence; AB552856.1; 99 % identity	*Sphingomonas* sp.	*Sphingomonas* sp. AR3
AR4	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR4
AR5	*Clavibacter* sp. Aws13 16S ribosomal RNA gene, partial sequence; JQ977405.1; 100 % identity	*Clavibacter* sp.	*Clavibacter* sp. AR5
AR6	*Bacillus anthracis* partial 16S rRNA gene, isolate KJS20; HG424433.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR6
AR7	*Curtobacterium* sp. W2.10-183 16S ribosomal RNA (16S rRNA) gene, complete sequence; JX458460.1; 100 % identity	*Curtobacterium* sp.	*Curtobacterium* sp. AR7
AR8	*Bacillus anthracis* strain WBD8 16S ribosomal RNA gene, partial sequence; KF550440.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR8
AR9	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR9
AR10	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR10
AR11	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR11
AR12	*Bacillus idriensis* strain MOSEL-NES3 16S ribosomal RNA gene, partial sequence; KF307644.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR12
AR13	*Nocardioides* sp. V6B23 16S ribosomal RNA gene, partial sequence; GQ921951.1; 99 % identity	*Nocardioides* sp.	*Nocardioides* sp. AR13
AR14	*Rhizobium leguminosarum* bv. *viciae* strain SWD43-1 16S ribosomal RNA gene, partial sequence; KF749034.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR14
AR15	*Bacillus pseudomycoides* strain IHB B 1512 16S ribosomal RNA gene, partial sequence; KF475882.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR15
AR16	*Stenotrophomonas rhizophila* strain MOSEL-tnc3 16S ribosomal RNA gene, partial sequence; KF307697.1; 100 % identity	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. AR16
AR17	*Rhizobium* sp. G3Ec4 16S ribosomal RNA gene, partial sequence; KF465965.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR17
AR18	*Stenotrophomonas* sp. HH10 16S ribosomal RNA gene, partial sequence; KC857480.1; 100 % identity	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. AR18
AR19	*Rhizobium* sp. A2Ec4 16S ribosomal RNA gene, partial sequence; KF465964.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR19
AR20	*Stenotrophomonas* sp. HH10 16S ribosomal RNA gene, partial sequence; KC857480.1; 100 % identity	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. AR20

Table 8: Bacterial isolates isolated from ragweed plants and their phylogenetic classification. Part II. Isolates AR21 to AS8.

Bacterial isolate	Closest match with NCBI database, accession number and percentage of identity	Phylogenetic classification	Isolate designation
AR21	*Rhizobium* sp. A2Ec4 16S ribosomal RNA gene, partial sequence; KF465964.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR21
AR22	*Rhizobium* sp. A2Ec4 16S ribosomal RNA gene, partial sequence; KF465964.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR22
AR23	*Rhizobium* sp. A2Ec4 16S ribosomal RNA gene, partial sequence; KF465964.1; 100 % identity	*Rhizobium* sp.	*Rhizobium* sp. AR23
AR24	*Stenotrophomonas maltophilia* strain ex9 culture-collection CGMCC:7.110 16S ribosomal RNA gene, partial sequence; KF317879.1; 100 % identity	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. AR24
AR25	*Curtobacterium* sp. W2.10-183 16S ribosomal RNA (16S rRNA) gene, complete sequence; JX458460.1; 100 % identity	*Curtobacterium* sp.	*Curtobacterium* sp. AR25
AR26	*Bacillus* sp. MBEP23 gene for 16S rRNA, partial sequence; AB733567.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR26
AR27	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR27
AR28	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR28
AR29	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR29
AR30	*Bacillus anthracis* partial 16S rRNA gene, isolate KJS20; HG424433.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR30
AR31	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AR31
AR32	*Stenotrophomonas rhizophila* strain MOSEL-tnc3 16S ribosomal RNA gene, partial sequence; KF307697.1; 100 % identity	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. AR32
AS1	*Clavibacter* sp. Aws13 16S ribosomal RNA gene, partial sequence; JQ977405.1; 100 % identity	*Clavibacter* sp.	*Clavibacter* sp. AS1
AS2	*Bacillus aryabhattai* partial 16S rRNA gene, isolate KJS 17; HG424432.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AS2
AS3	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AS3
AS4	*Bacillus aryabhattai* partial 16S rRNA gene, isolate KJS 17; HG424432.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AS4
AS5	*Arthrobacter nitroguajacolicus* strain d 16S ribosomal RNA gene, partial sequence; KF555632.1; 100 % identity	*Arthrobacter* sp.	*Arthrobacter* sp. AS5
AS6	*Bacillus simplex* partial 16S rRNA gene, isolate KJS 4; HG424431.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AS6
AS7	Bacillus anthracis partial 16S rRNA gene, isolate KJS20; HG424433.1; 100 % identity	*Bacillus* sp.	*Bacillus* sp. AS7
AS8	*Pantoea agglomerans* strain ACW 55802 16S ribosomal RNA gene, partial sequence; FJ611807.1; 99 % identity	*Pantoea* sp.	*Pantoea* sp. AS8

A phylogenetic composition of this small sub-population of the whole bacterial community present in ragweed plants is shown in figure 12. The genus *Bacillus* represents the largest group of bacterial isolates, for all isolates in total as well as for isolates of roots or shoots, respectively. *Rhizobium* spp. and *Stenotrophomonas* spp., comprising the 2nd and 3rd largest groups in total, could only be found in roots. The same is true for *Sphingomonas* spp., *Curtobacterium* spp., and *Nocardioides* spp. On

the other hand, *Arthrobacter* spp. and *Pantoea* spp. were only isolated from shoots. Finally, *Clavibacter* spp. were isolated from both roots and shoots.

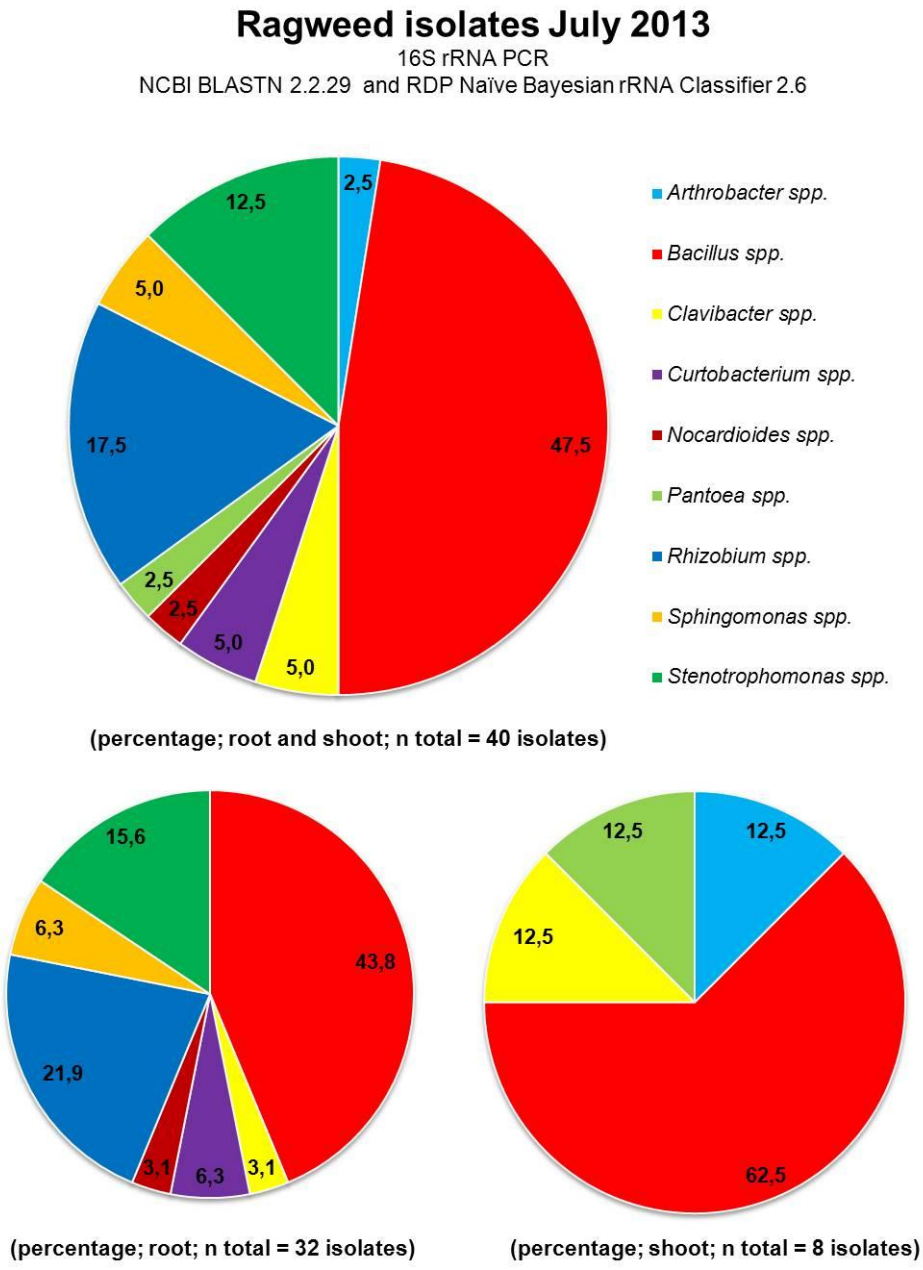

Figure 12: Pro-rata phylogenetic distribution of the 40 bacterial strains isolated from ragweed plants.

3.1.2.) Optimization of a standard functional screening method for the detection of hydrogen cyanide-producing bacteria

In a first test run, the two *Pseudomonas* isolates KS15 and KS28 (isolated by Aleti, 2011, unpublished) were tested with the standard test (Lorck, 1948). A color change of the filter paper from yellow to orange, dark orange or reddish brown was interpreted as ability to produce and release hydrogen cyanide. Isolate KS15 was found to be a non-producer of hydrogen cyanide, whereas isolate KS28 proofed to be positive for HCN production, as shown in figure 13.

Figure 13: Results for the standard functional screening method for the detection of hydrogen cyanide-producing bacteria for *Pseudomonas strains* KS15 and KS28.

Unsatisfying testing conditions (e.g. chemicals used for the filter paper impregnation running on the agar surface where bacteria were growing) were eliminated in a subsequent optimization process using the two strains previously mentioned. In addition, 10 different media were tested to enable ideal growth as well as production of HCN. Ideal settings for all parameters tested can be seen in table 9. As for the culture medium, it has to be said that in general, HCN production seemed to be higher (darker coloring of the filter paper) if isolates grew better. Therefore, it was not possible to find an optimum medium for all isolates due to differing growth requirements. For isolate KS28, HCN production was always highest on LB Agar medium with supplemented glycine. Hence, this medium was chosen for all further

tests. To compensate for divergent nutrient requirements, a second medium (R2A Agar medium with supplemented glycine) was chosen, as already described above. 48 hours of incubation at 28°C were found to be ideal for the detection of hydrogen cyanide-producing isolates with this test. In most cases, detection was already possible after 24 hours, but increasing release of HCN over another 24 hours guaranteed a safe and clear detection after 48 hours. An extension of the testing period to 72 hours did not change or improve test results for any of the tested strains. None of the tested isolates led to a color change of the filter paper after 4 hours of incubation.

Table 9: Ideal testing conditions for the standard functional screening method for the detection of hydrogen cyanide-producing bacterial isolates.

Parameter	Optimum parameter level
Growth medium	***Any medium possible****;* the better the strains grow, the more intense the color change of the filter paper (For strain KS28 LB Agar medium with supplemented glycine)
Amount of chemicals for filter papers (90mm)	***800µl of 1% picric acid solution and 10 drops of 10% sodium carbonate*** *(*the second pipetted with a sterile 153 MM Pasteur pipette (greiner bio-one, Frickenhausen, DE))
Fixation of filter paper to Petri dish lid	***Without specific fixation*** (fixation only by moisture of filter paper and transpiration of bacteria)
Duration of testing period	***48h***

A list of all bacterial isolates tested with the optimized standard test can be seen in tables 10 and 11 (chapter 3.1.4.). The list also includes the 15 bacterial isolates, which were found to produce hydrogen cyanide. The isolates, which had been isolated from rhizosphere of either common grapevine (*Vitis vinifera* L.) or hoary cress (*Cardaria draba* (L.) Desv.), had been initially tested with the high-throughput test for isolates and then results were verified with the standard test (see table 11). Each positive isolate caused a darker coloring of the filter paper if grown on LB Agar medium with supplemented glycine instead of R2A Agar medium with supplemented glycine. None of the *Pseudomonas* strains from the AIT strain collection was identified as a producer of hydrogen cyanide.

3.1.3.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacteria

The proof of principle for the newly developed high-throughput functional screening method for hydrogen cyanide-producing bacteria could be provided with the *Pseudomonas* isolates KS15 and KS28, which had been tested with the standard test before. Again, isolate KS15 did not cause a color change of the filter paper, whereas isolate KS28 always induced a blue color reaction.

In general, optimum detection could be achieved after 24 hours of incubation. Some positive strains already showed a blue color reaction on the filter paper after 4 hours of incubation, especially on the lower side of the filter paper facing the well. In some cases, an extension of the testing period to 48 hours already led to a spreading of the color across well borders.

A complete list of all bacterial isolates tested with this high-throughput functional screening method is given in tables 10 and 11 (chapter 3.1.4.). Out of the 117 bacterial isolates tested, 15 were detected as HCN-producers. One of them, of course, was isolate KS28 while further eight cyanogenic isolates originated from the rhizosphere of grapevine, of which 62 bacterial isolates were tested in total (yet, the 62 isolates did not represent the whole community isolated from grapevine; the remaining isolates were tested by Abdul Samad (AIT); results unpublished). The additional six cyanogenic isolates, which had been isolated from the rhizosphere of hoary cress (*Cardaria draba (L.) Desv.*; 'CdR'-strains), were tested together with other bacteria from the same isolation process by Abdul Samad (2013, unpublished). They were included in the analysis of this thesis and used for the germination assay with lettuce seedlings (see also chapter 3.1.6.). None of the *Pseudomonas* strains of the AIT strain collection produced hydrogen cyanide. The same was true for all bacterial isolates derived from ragweed plants. As isolating bacteria from environmental samples (plant material, rhizosphere, or soil) was not a central goal of this work at hand, no further isolation was carried out. Instead isolates from other co-workers were used for the detection of hydrogen cyanide-producing bacteria.

The production of HCN could be confirmed with the standard test for all isolates, which showed a positive reaction with the high-throughput test.

3.1.4.) Detection of hydrogen cyanide-producing bacteria with a PCR-based molecular assay

With the HCNC PCR set-up described above, amplification was recorded for all isolates, which were detected as producers of hydrogen cyanide with both functional screenings methods. A detailed list of all bacterial isolates tested with the functional and the PCR-based molecular screening methods (117 isolates in total) is depicted in tables 10 and 11. All 15 cyanogenic isolates are highlighted in yellow.

The PCR products could always be detected with a sequence length of approximately 360 bp when analyzed with agarose gel electrophoresis. Moreover, no amplification occurred for any of the acyanogenic isolates.

After cloning, M13 PCR amplification of the insert, and Sanger sequencing of the M13 PCR amplicons, obtained sequences were edited with the software BioEdit 7.0.0 and compared to the NCBI database in BLASTX 2.2.29, as explained above.

The presence of the *hcnC* gene could be confirmed for all isolates causing a positive reaction in the functional assays. The results for the BLASTX analyses are shown for 1 clone per isolate in table 12.

Table 10: Bacterial isolates tested for production of hydrogen cyanide with two functional screening methods and a PCR-based molecular assay. Part I. HCN+ = production of hydrogen cyanide and presence of *hcnC* gene. HCN- = no production of hydrogen cyanide and *hcnC* gene not present. Cyanogenic strains are highlighted in yellow.

Bacterial isolate/strain	Origin	Optimized standard functional assay	High-throughput functional assay	PCR-based molecular assay
KS15	Isolated from Ja! Natürlich Grüner Daumen compost tea bag B (without comfrey (*Symphytum* sp.)) (Aleti, 2011, unpublished)	HCN-	HCN-	HCN-
KS28	Isolated from Ja! Natürlich Grüner Daumen compost tea bag E (with comfrey (*Symphytum* sp.)) (Aleti, 2011, unpublished)	HCN+	HCN+	HCN+
***Pseudomonas syringae* pv. *tomato* DC3000**	Strain collection AIT	HCN-	HCN-	HCN-
***Pseudomonas alcaligenes* LMG1224**	Strain collection AIT	HCN-	HCN-	HCN-
Pseudomonas fluorescens WCS 365	Strain collection AIT	HCN-	HCN-	HCN-
***Pseudomonas luteola* LMG7041**	Strain collection AIT	HCN-	HCN-	HCN-
***Pseudomonas stutzeri* ATCC11607**	Strain collection AIT	HCN-	HCN-	HCN-
***Pseudomonas stutzeri* DSM 5190**	Strain collection AIT	HCN-	HCN-	HCN-
***Pseudomonas stutzeri* LMG 5838**	Strain collection AIT	HCN-	HCN-	HCN-

Table 11: Bacterial isolates tested for production of hydrogen cyanide with two functional screening methods and a PCR-based molecular assay. Part II. HCN+ = production of hydrogen cyanide and presence of *hcnC* gene. HCN- = no production of hydrogen cyanide and *hcnC* gene not present. Cyanogenic strains are highlighted in yellow.

Bacterial isolate	Origin	Optimized standard functional assay	High-throughput functional assay	PCR-based molecular assay
AR1 – AR32 (32 isolates)	Isolated from roots of common ragweed (*Ambrosia artemisiifolia* L.) collected in a field in Schützen am Gebirge, Burgenland, Austria, on July 14th, 2013	Not tested	All HCN-	All HCN-
AS1 – AS8 (8 isolates)	Isolated from shoots of common ragweed (*Ambrosia artemisiifolia* L.) collected in a field in Schützen am Gebirge, Burgenland, Austria, on July 14th, 2013	Not tested	All HCN-	All HCN-
2BSD1 – 2BSD29 (29 isolates)	Isolated from rhizosphere of common grapevine (*Vitis vinifera* L.) collected in a vineyard at Illmitz on June 21st, 2013 (Samad, 2013, unpublished)	All HCN+ confirmed, rest not tested	5 HCN+: 2BSD2, 2BSD5, 2BSD8, 2BSD14, 2BSD15	5 HCN+: 2BSD2, 2BSD5, 2BSD8, 2BSD14, 2BSD15
2BSE1 – 2BSE33 (33 isolates)	Isolated from rhizosphere of common grapevine (*Vitis vinifera* L.) collected in a vineyard at Illmitz on June 21st, 2013 (Samad, 2013, unpublished)	All HCN+ confirmed, rest not tested	3 HCN+: 2BSE4, 2BSE5, 2BSE9	3 HCN+: 2BSE4, 2BSE5, 2BSE9
CdR-d1	Isolated from rhizosphere of hoary cress (*Cardaria draba* (L.) Desv.) collected in a vineyard at Illmitz on April 15th, 2013 (Samad, 2013, unpublished); *High-throughput test carried out by Samad (2013, unpublished)*	HCN+	HCN+	HCN+
CdR-d3		HCN+	HCN+	HCN+
CdR-d6		HCN+	HCN+	HCN+
CdR-d8		HCN+	HCN+	HCN+
CdR-d15		HCN+	HCN+	HCN+
CdR-e6		HCN+	HCN+	HCN+

Table 12: Confirmation of the presence of the *hcnC* gene in hydrogen cyanide-producing isolates via HCNC PCR, cloning, M13 PCR, and Sanger sequencing.

Clone	Length of sequence (expected length)	Result for BLASTX 2.2.29 and Accession Number	Identity (%)	Query cover (%)	Putative phylum and class
KS28-7	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas mosselii]; WP_023629672.1	87	99	Proteobacteria; Gammaproteobacteria
2BSD2-10	335 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	95	99	Proteobacteria; Gammaproteobacteria
2BSD5-12	350 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	95	99	Proteobacteria; Gammaproteobacteria
2BSD8-14	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	98	99	Proteobacteria; Gammaproteobacteria
2BSD14-10	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	92	99	Proteobacteria; Gammaproteobacteria
2BSD15-10	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	95	99	Proteobacteria; Gammaproteobacteria
2BSE4-9	352 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas moraviensis]; WP_024013371.1	99	99	Proteobacteria; Gammaproteobacteria
2BSE5-16	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	97	99	Proteobacteria; Gammaproteobacteria
2BSE9-16	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas sp. CFII68]; WP_018612789.1	97	99	Proteobacteria; Gammaproteobacteria
CdR-d1-12	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas fluorescens]; WP_016986231.1	97	99	Proteobacteria; Gammaproteobacteria
CdR-d3-5	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas fluorescens]; WP_016986231.1	97	99	Proteobacteria; Gammaproteobacteria
CdR-d6-4	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas fluorescens]; WP_016986231.1	97	99	Proteobacteria; Gammaproteobacteria
CdR-d8-4	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas fluorescens]; WP_016986231.1	97	99	Proteobacteria; Gammaproteobacteria
CdR-d15-5	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas fluorescens]; WP_016986231.1	96	99	Proteobacteria; Gammaproteobacteria
CdR-e6-11	353 bp (360 bp)	hydrogen cyanide synthase HcnC [Pseudomonas]; WP_018928981.1	96	99	Proteobacteria; Gammaproteobacteria

3.1.5.) Phylogenetic analysis of hydrogen cyanide-producing bacteria with PCR-based molecular assays

The phylogenetic classification for hydrogen cyanide-producing bacteria was based on 16S rRNA PCR and 16S-23S IGS PCR, as described above. Amplicons for the 16S rRNA gene PCR were analyzed by Sanger sequencing and subsequent comparison with the NCBI (BLASTN 2.2.29) and RDP databases (RDP Classifier) (sequence lengths 905-1429 bp). The sequence analysis for the 16S rRNA gene revealed that all cyanogenic isolates and the acyanogenic (control) isolate KS15 belong to the genus *Pseudomonas*, as shown in table 13.

Table 13: Phylogenetic analysis for hydrogen cyanide-producing isolates of this study based on 16S rRNA gene sequences.

Bacterial isolate	Phylogenetic classification	Isolate designation
KS15 (HCN-)	*Pseudomonas* sp.	*Pseudomonas* sp. KS15
KS28	*Pseudomonas* sp.	*Pseudomonas* sp. KS28
2BSD2	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSD2
2BSD5	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSD5
2BSD8	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSD8
2BSD14	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSD14
2BSD15	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSD15
2BSE4	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSE4
2BSE5	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSE5
2BSE9	*Pseudomonas* sp.	*Pseudomonas* sp. 2BSE9
CdR-d1	*Pseudomonas* sp.	*Pseudomonas* sp. CdR-d1
CdR-d3	*Pseudomonas* sp.	*Pseudomonas* sp. CdR-d3
CdR-d6	*Pseudomonas* sp.	*Pseudomonas* sp. CdR-d6
CdR-d8	*Pseudomonas* sp.	*Pseudomonas* sp. CdR-d8
CdR-d15	*Pseudomonas* sp.	*Pseudomonas* sp. CdR-d15
CdR-e6	*Pseudomonas* sp.	*Pseudomonas* sp. CdR-e6

Discrimination of bacterial isolates with 16S rRNA sequences was only possible on the genus level. Therefore, 16S-23S IGS PCR sequences (sequence lengths 985-1477 bp) could not be used to exactly identify the listed bacteria on a strain level. Nevertheless, database comparison and subsequent ClustalW alignment were performed. Cyanogenic isolates with similar 16S-23S IGS PCR sequences, which yielded identical species hits when compared to the NCBI database but varied on a nucleotide level, were supposed to belong to different strains. Phylogenetic analysis based on 16S-23S IGS PCR sequences indicated that 9 different strains had been tested (one of them acyanogenic). The according results are depicted in table 14.

Table 14: Phylogenetic analysis based on 16S-23S IGS PCR sequences (NCBI database comparison in BLASTN 2.2.29 and clustalW alignment in BioEdit 7.0.0). 9 differing strains could be identified.

Bacterial isolate(s)	**Phylogenetic classification of bacterial isolates based on 16S-23S IGS PCR sequences (database comparison and ClustalW alignment)** *Closest matches with NCBI database (BLASTN2.2.29) and accession number*
KS15 (acyanogenic)	*Pseudomonas putida* HB3267, complete genome; CP003738.1 (100 % identity, 100 % query cover)
KS28	*Pseudomonas putida* H8234, complete genome; CP005976.1 (99 % identity, 100 % query cover)
2BSD8	*Pseudomonas fluorescens* F113, complete genome; CP003150.1 (100 % identity, 100 % query cover)
2BSE4	*Pseudomonas* sp. UW4, complete genome; CP003880.1 (100 % identity, 100 % query cover)
CdR-d6	*Pseudomonas* sp. UW4, complete genome; CP003880.1 (98 % identity, 100 % query cover)
CdR-e6	*Pseudomonas fluorescens* SBW25 complete genome; AM181176.4 (99 % identity, 100 % query cover)
2BSD2, 2BSD5, 2BSD14, 2BSD15	*Pseudomonas brassicacearum* subsp. *brassicacearum* NFM421, complete genome; CP002585.1 (97 % identity, 99 % query cover)
2BSE5, 2BSE9	*Pseudomonas fluorescens* Pf29Arp contig024, whole genome shotgun sequence; ANOR01000024.1 (99 % identity, 100 % query cover)
CdR-d1, CdR-d3, CdR-d8, CdR-d15	*Pseudomonas* sp. UW4, complete genome; CP003880.1 (99 % identity, 100 % query cover)

3.1.6.) Testing the influence of hydrogen cyanide-producing bacteria on the germination of lettuce (*Lactuca satica* L. var. *capitata*)

The influence of 16 bacterial isolates (15 cyanogenic isolates and the acyanogenic isolate KS15) on the germination of lettuce was evaluated, as described above. A mock treatment without bacteria was incorporated in the test as a negative control. A list of all bacterial isolates utilized for the lettuce germination assay can be seen in table 15.

Table 15: Bacterial isolates used for the lettuce germination assay. The list includes 15 cyanogenic and 1 acyanogenic strain.

Bacterial isolate	Origin
KS15 (not producing HCN)	Isolated from Ja! Natürlich Grüner Daumen compost tea bag B (without comfrey (*Symphytum* sp.)) (Aleti, 2011, unpublished)
KS28	Isolated from Ja! Natürlich Grüner Daumen compost tea bag E (with comfrey (*Symphytum* sp.)) (Aleti, 2011, unpublished)
2BSD2	Isolated from rhizosphere of common grapevine (*Vitis vinifera* L.) collected in a vineyard at Illmitz on June 21st, 2013 (Samad, 2013, unpublished)
2BSD5	
2BSD8	
2BSD14	
2BSD15	
2BSE4	
2BSE5	
2BSE9	
CdR-d1	Isolated from rhizosphere of hoary cress (*Cardaria draba* (L.) Desv.) collected in a vineyard at Illmitz on April 15th, 2013 (Samad, 2013, unpublished)
CdR-d3	
CdR-d6	
CdR-d8	
CdR-d15	
CdR-e6	

Results for the statistical analyses for all germination parameters tested are shown in tables 16 to 19. Data for the two parameters 'length of the radicle on day 6 of incubation' and 'weight of the seedling on day 6 of incubation' were analyzed using one-way ANOVA. For all other sets of data, Kruskal-Wallis tests were performed.

No significant differences among treatments were found for the parameters 'emergence of the radicle', 'emergence of the cotyledons', and 'expansion of the cotyledons'. The same is true for all parameter levels of the parameter 'morphology

of seedlings on day 6 of incubation'. Graphical illustrations for these results can be seen in figures 14 to 17.

Means for the two parameters 'length of the radicle on day 6 of incubation' and 'weight of the seedling on day 6 of incubation' differed highly significantly among treatments ($p < 0,001$). Treatment with six bacterial isolates (i.e. CdR-d8, CdR-e6, 2BSE4, 2BSD14, 2BSE9, and 2BSE5) led to significantly longer radicles in comparison to the control treatment, as can be seen in figure 18. Finally, inoculation with bacterial isolate CdR-d6 caused a significant increase of seedling weight if compared to the control treatment, as depicted in figure 19.

To sum up, all bacterial isolates tested failed to inhibit the germination of lettuce.

Table 16: Results for the statistical analysis for the germination parameter 'emergence of the radicle'. Depicted are means for each treatment as well as p-value of Kruskal-Wallis test. n = 60.

	Bacterial isolate (mean)																
Germination parameter	*Control*	*KS15*	*KS28*	*2BSD2*	*2BSD5*	*2BSD8*	*2BSD14*	*2BSD15*	*2BSE4*	*2BSE5*	*2BSE9*	*CdR-d1*	*CdR-d3*	*CdR-d6*	*CdR-d8*	*CdR-d15*	*CdR-e6*	**p-value**
Percentage of radicles emerged on day 1	81,67	93,33	88,33	91,67	85,00	80,00	91,67	86,67	90,00	86,67	90,00	90,00	76,67	96,67	83,33	93,33	85,00	**0,3261**
Percentage of radicles emerged on day 2	95,00	96,67	98,33	96,67	96,67	88,33	100,00	95,00	95,00	98,33	95,00	95,00	93,33	100,00	95,00	98,33	93,33	**0,3432**
Percentage of radicles emerged on day 3	95,00	98,33	98,33	96,67	96,67	93,33	100,00	95,00	95,00	100,00	95,00	96,67	95,00	100,00	96,67	98,33	95,00	**0,4876**
Percentage of radicles emerged on day 4	96,67	98,33	98,33	96,67	96,67	95,00	100,00	95,00	96,67	100,00	95,00	96,67	95,00	100,00	96,67	98,33	96,67	**0,6556**
Percentage of radicles emerged on day 5	98,33	98,33	98,33	96,67	96,67	95,00	100,00	95,00	96,67	100,00	96,67	96,67	96,67	100,00	98,33	98,33	98,33	**0,8451**
Percentage of radicles emerged on day 6	98,33	98,33	98,33	96,67	96,67	95,00	100,00	96,67	96,67	100,00	96,67	96,67	96,67	100,00	98,33	98,33	98,33	**0,8690**

Table 17: Results for the statistical analysis for the germination parameter 'emergence of the cotyledons'. Depicted are means for each treatment as well as p-value of Kruskal-Wallis test. n = 60.

	Bacterial isolate (mean)																
Germination parameter	*Control*	*KS15*	*KS28*	*2BSD2*	*2BSD5*	*2BSD8*	*2BSD14*	*2BSD15*	*2BSE4*	*2BSE5*	*2BSE9*	*CdR-d1*	*CdR-d3*	*CdR-d6*	*CdR-d8*	*CdR-d15*	*CdR-e6*	**p-value**
Percentage of cotyledons emerged on day 1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**1,0000**
Percentage of cotyledons emerged on day 2	73,33	73,33	73,33	65,00	73,33	68,33	70,00	70,00	58,33	68,33	75,00	70,00	73,33	85,00	65,00	70,00	70,00	**0,9834**
Percentage of cotyledons emerged on day 3	93,33	95,00	93,33	98,33	96,67	91,67	96,67	90,00	95,00	95,00	96,67	95,00	90,00	98,33	95,00	96,67	90,00	**0,8121**
Percentage of cotyledons emerged on day 4	96,67	98,33	95,00	98,33	96,67	96,67	100,00	95,00	95,00	95,00	96,67	95,00	95,00	98,33	96,67	98,33	96,67	**0,9142**
Percentage of cotyledons emerged on day 5	98,33	100,00	98,33	98,33	100,00	96,67	100,00	96,67	98,33	100,00	96,67	98,33	95,00	100,00	100,00	98,33	96,67	**0,4741**
Percentage of cotyledons emerged on day 6	100,00	100,00	98,33	98,33	100,00	96,67	100,00	98,33	98,33	100,00	96,67	98,33	96,67	100,00	100,00	98,33	96,67	**0,6616**

Table 18: Results for the statistical analysis for the germination parameter 'expansion of the cotyledons'. Depicted are means for each treatment as well as p-value of Kruskal-Wallis test. n = 60.

	Bacterial isolate (mean)																
Germination parameter	*Control*	*KS15*	*KS28*	*2BSD2*	*2BSD5*	*2BSD8*	*2BSD14*	*2BSD15*	*2BSE4*	*2BSE5*	*2BSE9*	*CdR-d1*	*CdR-d3*	*CdR-d6*	*CdR-d8*	*CdR-d15*	*CdR-e6*	**p-value**
Percentage of cotyledons fully expanded on day 1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**1,0000**
Percentage of cotyledons fully expanded on day 2	5,00	8,33	3,33	11,67	11,67	1,67	5,00	8,33	8,33	8,33	10,00	6,67	5,00	6,67	0,00	8,33	3,33	**0,6540**
Percentage of cotyledons fully expanded on day 3	51,67	58,33	56,67	51,67	56,67	50,00	55,00	45,00	46,67	55,00	58,33	48,33	38,33	48,33	50,00	53,33	51,67	**0,9856**
Percentage of cotyledons fully expanded on day 4	71,67	68,33	63,33	68,33	65,00	66,67	66,67	58,33	55,00	63,33	78,33	63,33	60,00	61,67	61,67	60,00	63,33	**0,9769**
Percentage of cotyledons fully expanded on day 5	80,00	73,33	65,00	75,00	65,00	68,33	70,00	63,33	56,67	70,00	81,67	71,67	65,00	68,33	65,00	66,67	68,33	**0,9178**
Percentage of cotyledons fully expanded on day 6	70,00	68,33	53,33	75,00	56,67	63,33	68,33	61,67	53,33	66,67	78,33	60,00	58,33	61,67	60,00	55,00	68,33	**0,5390**

Table 19: Results for the statistical analysis for the germination parameters 'morphology of seedlings on day 6', 'length of radicle on day 6', and 'weight of seedling on 6'. Depicted are means for each treatment as well as p-values of Kruskal-Wallis test and ANOVA. n = 60 ('morphology of seedlings on day 6'), n = 30 ('length of radicle on day 6', 'weight of seedling on 6').

	Bacterial isolate (mean)																
Germination parameter	*Control*	*KS15*	*KS28*	*2BSD2*	*2BSD5*	*2BSD8*	*2BSD14*	*2BSD15*	*2BSE4*	*2BSE5*	*2BSE9*	*CdR-d1*	*CdR-d3*	*CdR-d6*	*CdR-d8*	*CdR-d15*	*CdR-e6*	**p-value**
Percentage of strong seedlings on day 6	90,00	90,00	71,67	90,00	76,67	76,67	88,33	75,00	81,67	86,67	91,67	83,33	80,00	85,00	83,33	86,67	83,33	**0,6715**
Percentage of weak but otherwise healthy seedlings on day 6	8,33	8,33	18,33	6,67	18,33	11,67	6,67	13,33	11,67	10,00	5,00	10,00	10,00	10,00	8,33	10,00	10,00	**0,9506**
Percentage of abnormal seedlings on day 6	1,67	1,67	10,00	1,67	5,00	8,33	5,00	11,67	5,00	3,33	1,67	5,00	6,67	5,00	8,33	1,67	5,00	**0,2094**
Percentage of seeds which failed to germinate till day 6	0,00	0,00	0,00	1,67	0,00	3,33	0,00	0,00	1,67	0,00	1,67	1,67	3,33	0,00	0,00	1,67	1,67	**0,3967**
Length of radicle on day 6 [mm]	35,47	41,03	39,27	42,50	41,37	38,67	45,00	41,73	44,00	49,83	45,43	41,87	41,17	42,37	42,87	41,90	43,40	**< 0,001**
Weight of seedling on day 6 [mg]	10,87	12,83	10,50	12,53	12,17	12,23	12,40	11,10	13,00	12,83	12,97	12,80	12,00	13,53	11,73	12,40	12,87	**< 0,001**

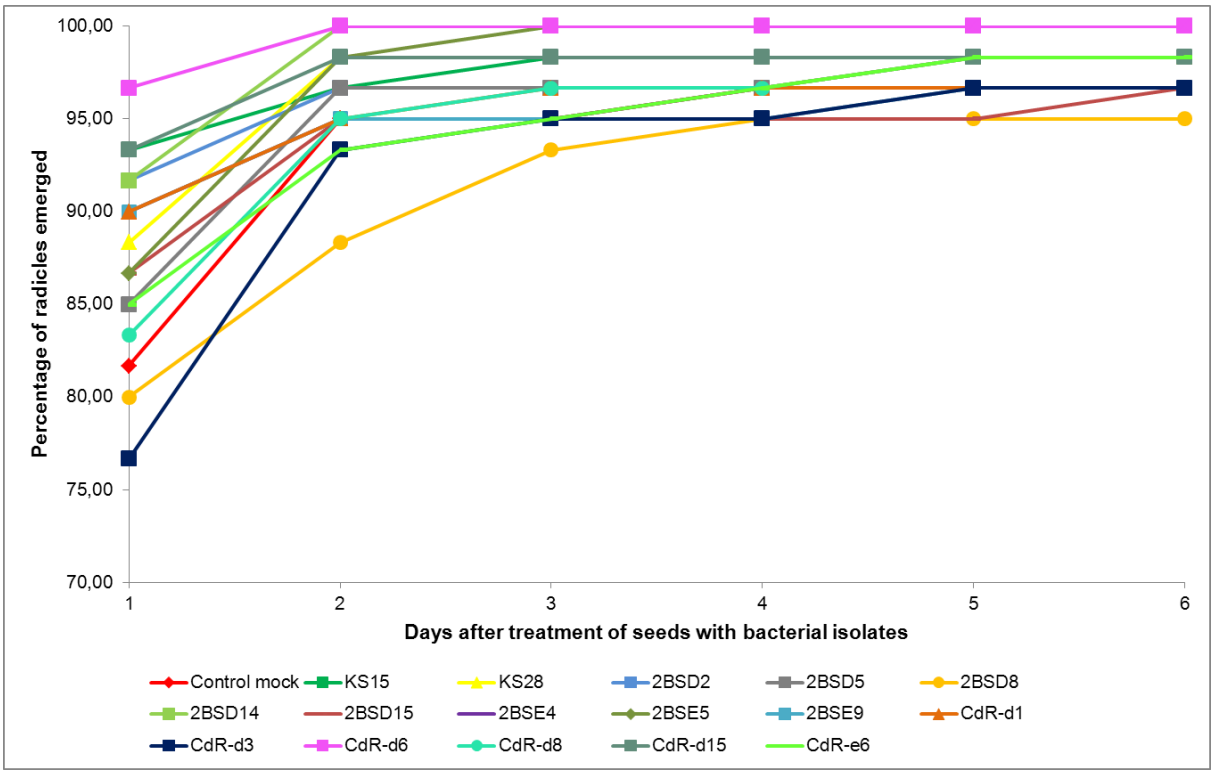

Figure 14: Percentage of radicles emerged between days 1 and 6 after treatment with bacterial isolates. Treatments do not differ significantly (Kruskal-Wallis test). n = 60.

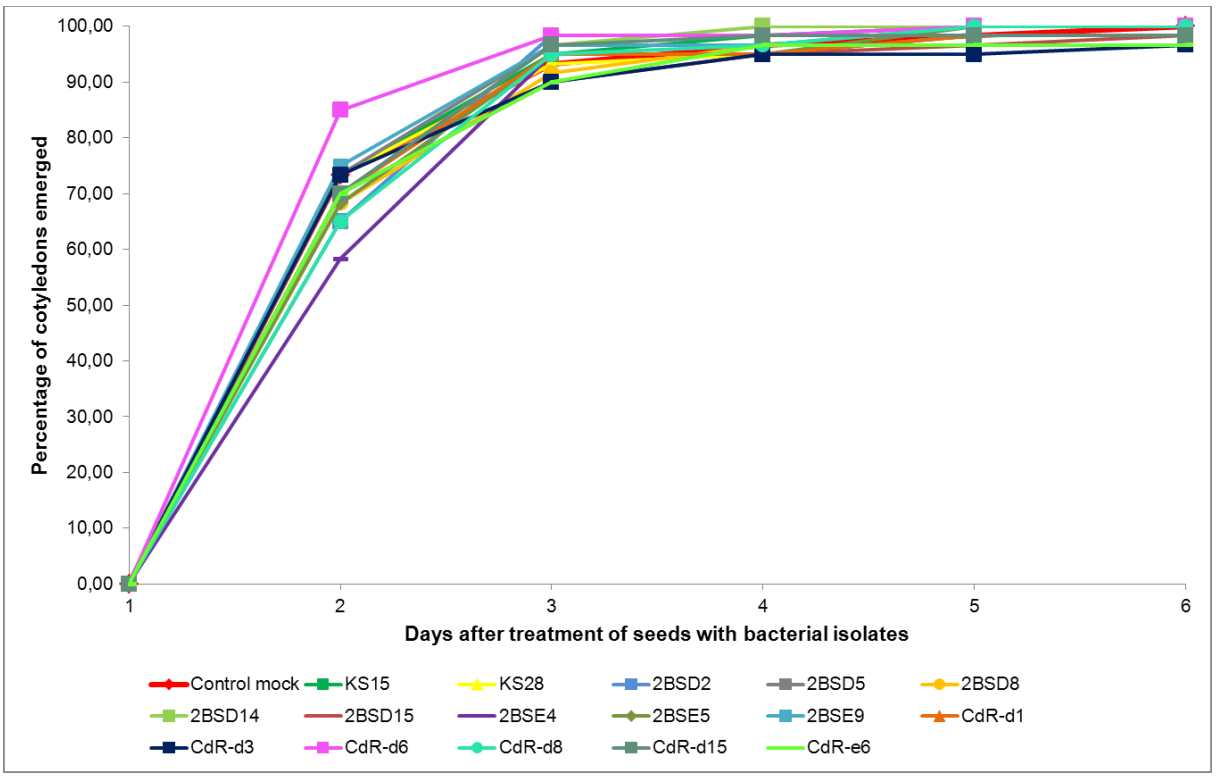

Figure 15: Percentage of cotyledons emerged between days 1 and 6 after treatment with bacterial isolates. Treatments do not differ significantly (Kruskal-Wallis test). n = 60.

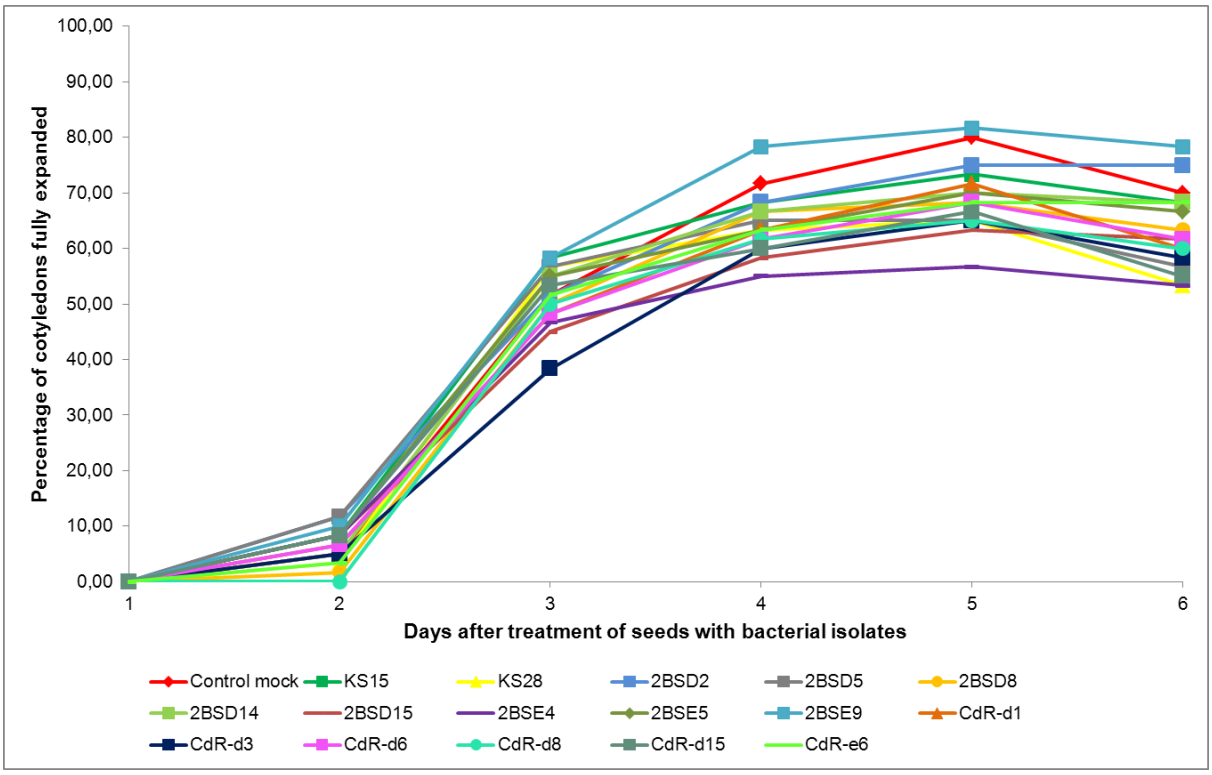

Figure 16: Percentage of cotyledons fully expanded between days 1 and 6 after treatment with bacterial isolates. Treatments do not differ significantly (Kruskal-Wallis test). n = 60.

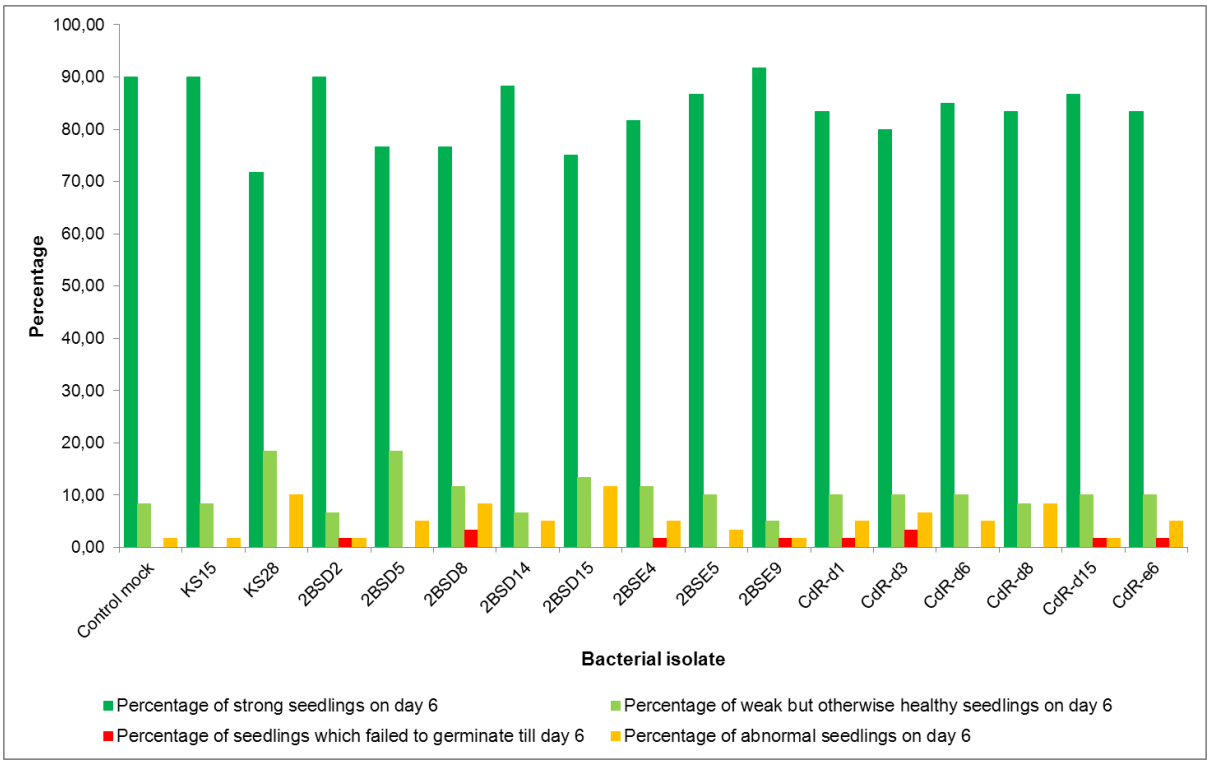

Figure 17: Morphology of seedlings on day 6 after treatment with bacterial isolates. Treatments do not differ significantly (Kruskal-Wallis test). n = 60.

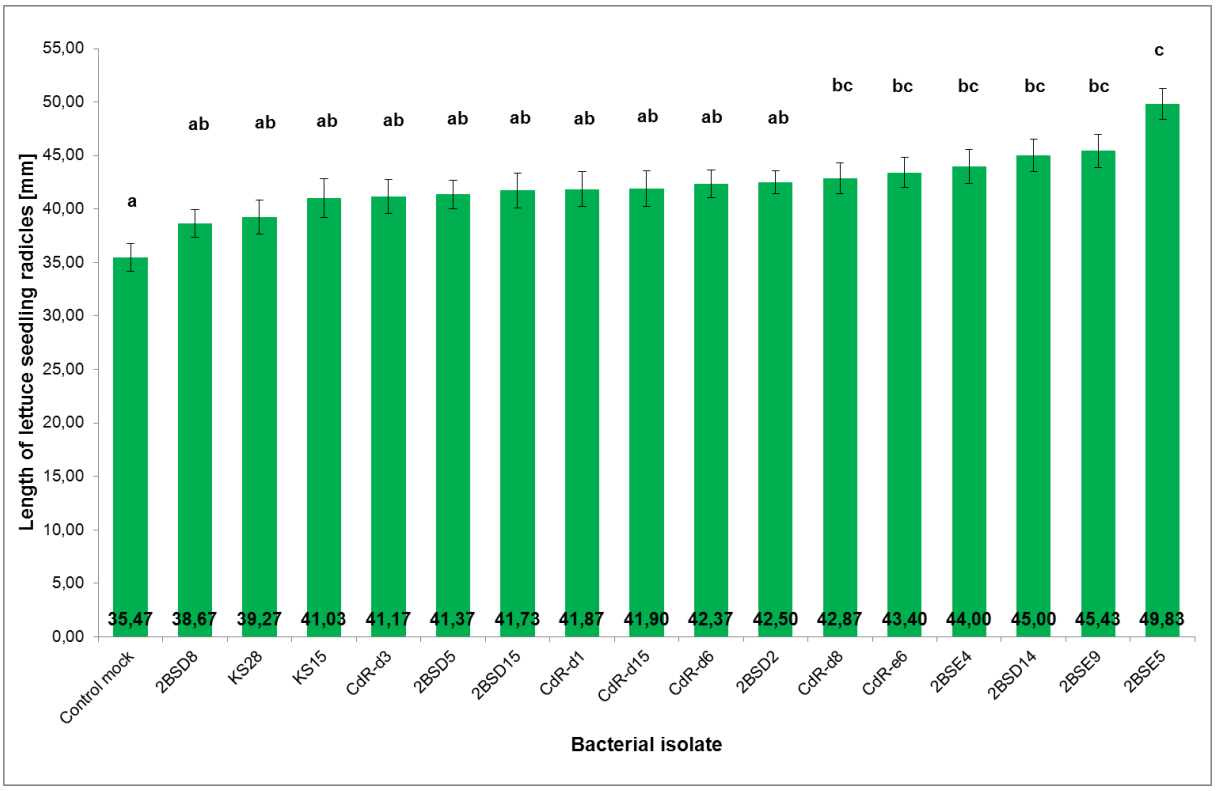

Figure 18: Length of lettuce seedling radicles [mm] 6 days after treatment with bacterial isolates. Means and standard error are depicted. Labeling with different letters refers to significant differences evaluated by ANOVA and post-hoc Tukey test. n = 30.

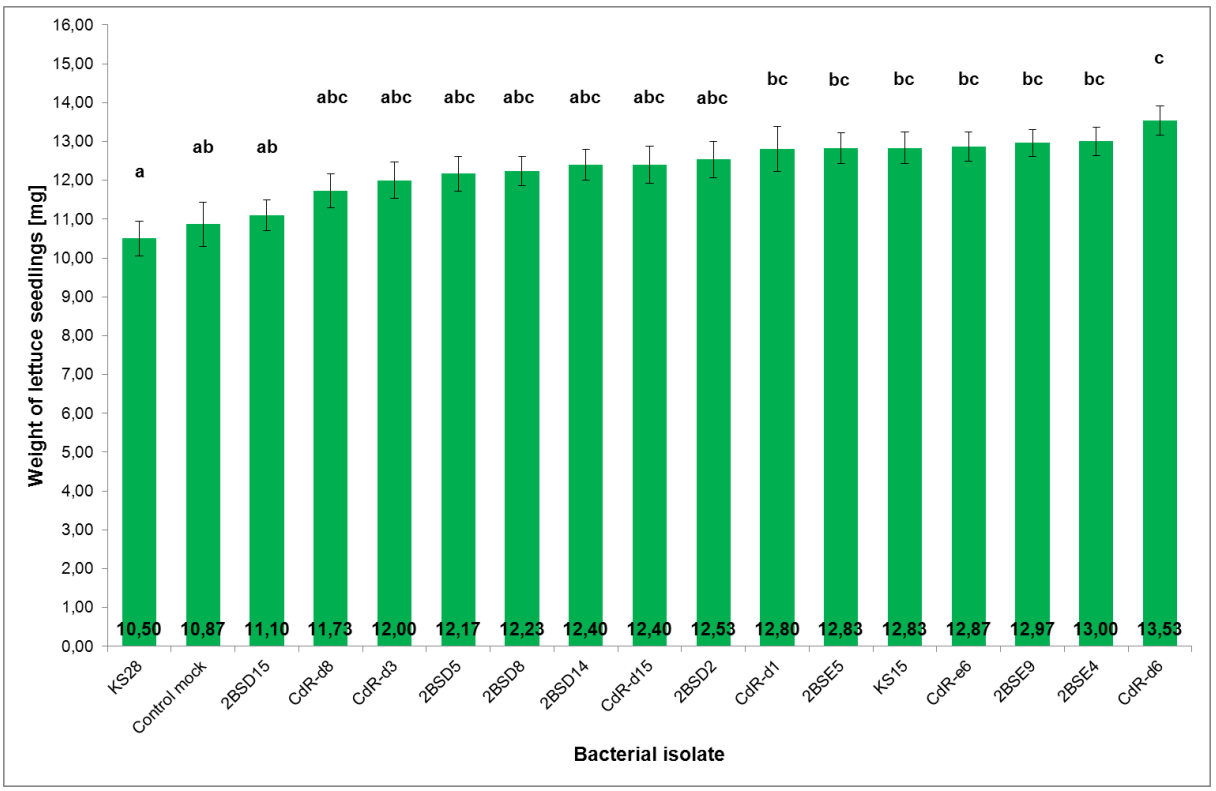

Figure 19: Weight of lettuce seedlings [mg] 6 days after treatment with bacterial isolates. Means and standard error are depicted. Labeling with different letters refers to significant differences evaluated by ANOVA and post-hoc Tukey test. n = 30.

3.2.) Testing a metagenomic library

3.2.1.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacterial clones

The proof of principle for the newly developed high-throughput functional screening method for hydrogen cyanide-producing clones could also be provided with the 2 *Pseudomonas* isolates KS15 and KS28, which had been tested with the standard test before. Due to different morphology of these isolates (colonies of KS15 appear white, those of KS28 yellow), single colonies could be easily identified if grown on the same Petri dish. Dishes were prepared with up to approximately 1.300 cfu of KS15 and less than 10 cfu of KS28. All colonies of KS28 caused a color change of the filter paper (blue reaction). Colonies of KS15 never led to a color change.

About 110.000 clones (or rather colony forming units) of the Peru Rhizosphere Library with cosmid pJC8 were tested with this high-throughput functional screening method in four separate test runs. Taking the average insert size into account (28,1 kb), we screened approximately 3,09 Gb of metagenomic DNA.

All clones failed to induce a blue color reaction on the filter paper. Therefore, it can be assumed that none of the 110.000 clones tested was able to produce hydrogen cyanide. The positive control isolate KS28 always released HCN, leading to a color change of the filter paper in all cases. One of these positive reactions of KS28 can be seen in figure 20.

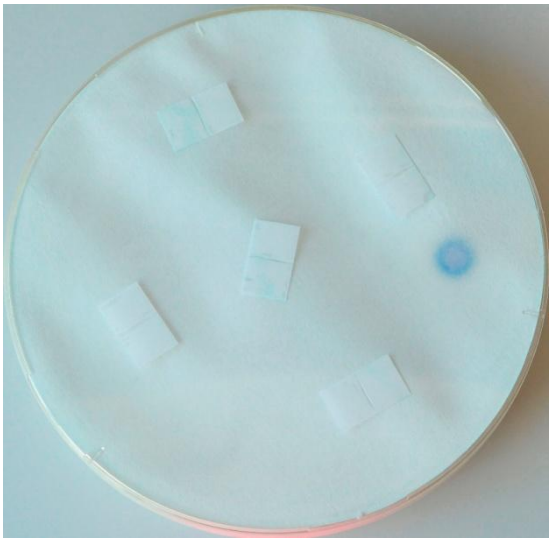

Figure 20: Test plate for the high-throughput functional screening method for the detection of hydrogen cyanide-producing bacterial clones including positive control strain KS28 (blue reaction on filter paper).

Instead of a blue color change, we observed a brown color change of the filter paper above clone colonies on seven different test plates, as shown exemplarily in figure 21.

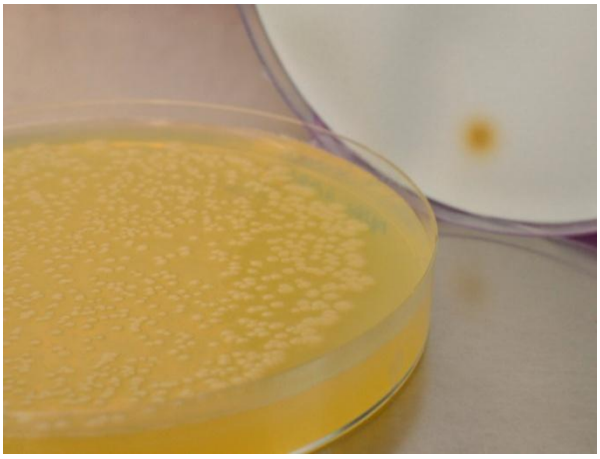

Figure 21: High-throughput functional screening plate for hydrogen cyanide-producing clones with brown color change of the filter paper above clone colonies.

50 clones were isolated in total from colonies below brown spots on the filter paper. They were re-grown on LB-Tetracycline Agar medium with supplemented glycine, as described above. High-throughput tests in microtiter plates (as described for isolates) containing the same medium were carried out to identify the seven clones causing the brown reaction, as can be seen in figure 22. For each of the seven original test plates, one clone, which led to a brown color change of the filter paper, could be detected. The seven clones were simply labelled according to their number during isolation (i.e. clones 1, 5, 11, 25, 27, 31, and 36).

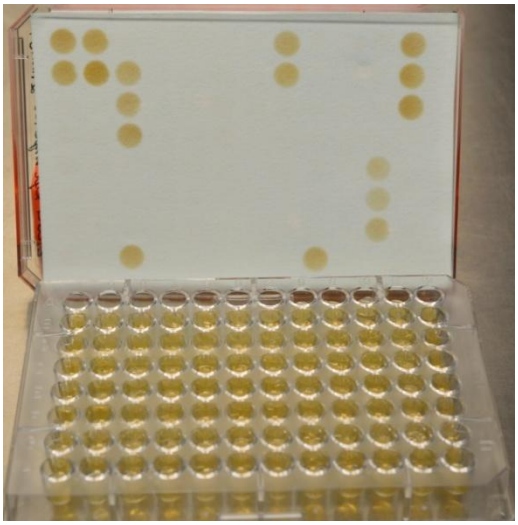

Figure 22: High-throughput test in a microtiter plate to identify the 7 clones, which led to a brown color change of the filter paper of the high-throughput functional screening for hydrogen cyanide-producing clones.

For the detection of the brown reaction on the filter paper, a slightly longer time period was necessary as for the detection of the blue reaction caused by hydrogen cyanide-producing bacteria. Testing periods were adapted accordingly. Nevertheless, large areas of the filter paper were turning brown if clones were streaked densely on the medium, as depicted in figure 23. In addition to the brown coloring on the filter paper, we also observed a strange, fusty smell arising from colonies of all seven clones.

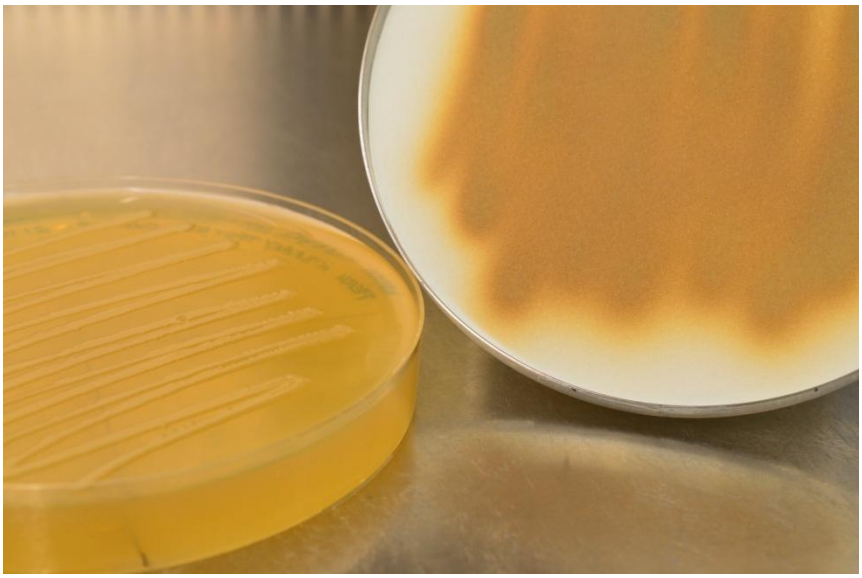

Figure 23: Filter paper turning brown in large areas over densely growing colonies of clone 1.

Taking the test set-up and the reaction behavior into account, we came to the conclusions that all seven clones produce at least one volatile compound that causes the brown color change of the filter paper and that this volatile compound is most probably the same for all seven clones. As the detection of the unknown volatile compound involved in the brown reaction on the filter paper had not been described before, we carried out further functional and molecular assays to identify the chemical structure of the unknown compound as well as the genes involved in its biosynthesis. Results for these tests will be described in the upcoming chapter.

3.2.2.) Identification of the unknown volatile compound discovered with the high-throughput functional screening method for hydrogen cyanide-producing clones

Several functional and molecular screenings were performed in order to identify the unknown volatile compound and its genetic background. A short description of each test and the respective results will be given below. The tests comprise 5 functional assays (tests 1 to 5) as well as 3 molecular assays (tests 6 to 8).

Functional screenings:

Test 1 – Testing the clones with the standard functional screening method for the detection of hydrogen cyanide-producing bacteria

All seven clones were tested with the standard functional screening method for the detection of hydrogen cyanide-producing bacteria to check if volatile HCN is involved in the reaction process. The assay was carried out as described above with LB-Tetracycline Agar medium with supplemented glycine as culture medium (for the preparation of all media and solutions see appendix) (see also chapter 2.1.2.). Test plates were put at 37°C for 72 hours. A color change of the filter papers from yellow to orange, brown or reddish brown was interpreted as presence of HCN (or CN^-) molecules. No reaction on the filter papers indicated that there was no hydrogen cyanide present.

All seven clones of interest failed to induce a color change of the filter papers used for the standard test to detect hydrogen cyanide, as shown for one clone in figure 24. These seven clones do definitely not produce hydrogen cyanide.

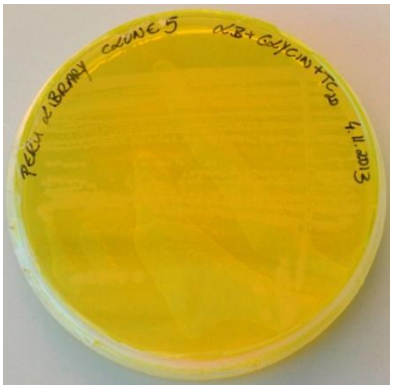

Figure 24: Standard functional screening for the production of hydrogen cyanide for the 7 clones releasing an unknown volatile compound (only clone 5 depicted).

Test 2 – Testing the influence of the antibiotic tetracycline on the production of the unknown volatile compound

Test 2 was carried out to check whether the inserts of the seven clones of interest contain additional genes, which code for proteins involved in a degradation process of tetracycline different to that encoded by genes of the cosmid. Thereto, all seven clones were streaked on LB Agar medium with supplemented glycine (without tetracycline) in 145 mm Petri dishes (for the preparation of all media and solutions see appendix). The same filter papers as for the high-throughput test were utilized (see chapter 2.2.1.). The Petri dishes were closed, sealed with Parafilm® M (BRAND GMBH + CO KG, Wertheim, DE) and put at 37°C for a maximum of seven days. The plates were removed earlier, if there was an obvious reaction on the filter paper. No reaction on the filter paper after seven days or a color change other than brown was interpreted as tetracycline having an influence on the production of the unknown volatile compound. A brown reaction on the filter paper indicated that tetracycline did not influence the production of the unknown volatile compound. To assure that all clones were still harboring the cosmid after this test on medium without selective compound, the clones were streaked from these testing plates onto 94 mm Petri dishes containing LB-Tetracycline Agar medium.

All seven clones still produced the unknown volatile compound if grown on medium without the antibiotic tetracycline. The same brown color change of the filter paper could be observed as for the tests with antibiotic, as shown for one clone in figure 25. In addition, all clones tested on plates without antibiotic were still able to grow on medium containing tetracycline afterwards.

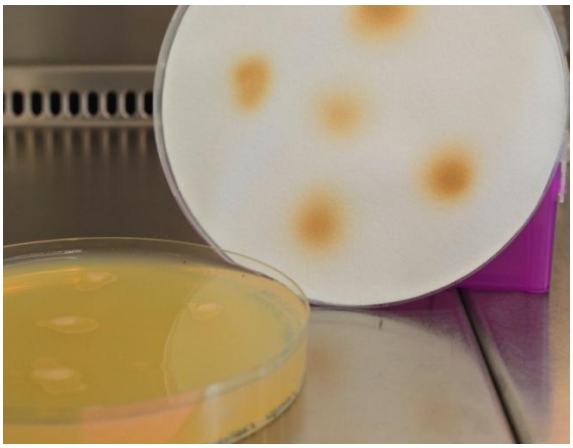

Figure 25: Testing the influence of the antibiotic tetracycline on the production of the unknown volatile compound.

Test 3 – Co-detection of hydrogen cyanide and the unknown volatile compound

Every fifth Petri dish of the initial high-throughput screening contained the cyanogenic *Pseudomonas* isolate KS28 as positive control. None of the seven clones, which caused a brown reaction on the filter papers, had been discovered on any of these dishes. Therefore, test 3 was conducted to check whether hydrogen cyanide and the unknown volatile compound can be detected together on the same filter paper. The seven clones and KS28 were streaked on several 145 mm Petri dishes containing LB Agar medium with supplemented glycine (always one or two clones and isolate KS28 on one dish) (for the preparation of all media and solutions see appendix). The same filter papers as for the high-throughput test were utilized (see chapter 2.2.1.). The Petri dishes were closed, sealed with Parafilm® M (BRAND GMBH + CO KG, Wertheim, DE) and put at 37°C for several days. The plates were controlled daily for color reactions on the filter papers.

Streaking the seven clones of interest on the same plate as the hydrogen cyanide-producing isolate KS28 revealed that the unknown volatile compound and hydrogen cyanide can both be detected on the same filter paper, as shown for two clones and isolate KS28 in figure 26. In areas where both volatile compounds react with the filter paper, the blue and the brown reaction could be observed in parallel.

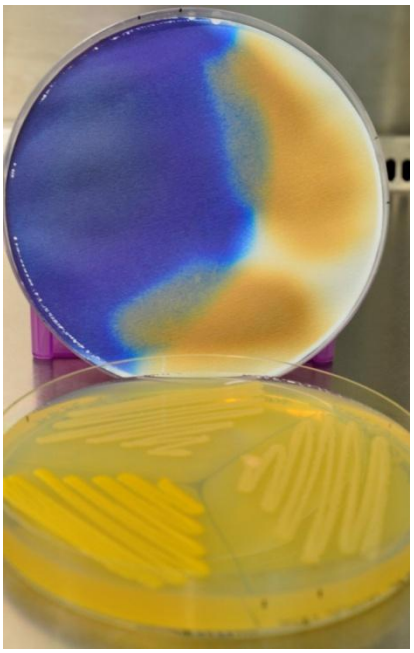

Figure 26: Co-detection of hydrogen cyanide (blue reaction) and the unknown volatile compound (brown reaction). Colonies of the cyanogenic isolate KS28 appear yellow, those of the *E. coli* clones beige.

Test 4 – Testing the chemicals used for filter paper impregnation for their influence on the color reaction on the filter papers

Hydrogen cyanide reacts with 4,4'-dimethylenebis-(N,N-dimethylaniline) to form a blue complex that is visually detectable on the filter paper. Nevertheless, this color reaction is only possible if also copper(II)ethylacetotacetate is present (Feigl and Anger, 1966). The brown coloring of the filter paper instead, might originate from a precipitation of the unknown volatile compound without influence of the other two chemicals (copper(II)ethylacetotacetate and 4,4'-dimethylenebis-(N,N-dimethylaniline), from a reaction of the unknown volatile compound with either one of the two chemicals used for filter impregnation or, finally, from a reaction involving both chemicals. Filter papers without chemicals and such impregnated with only one of the two chemicals were prepared and utilized to detect the reaction partner(s) of the unknown compound (for the preparation of all media and solutions see appendix). Test 4 was carried out on either 145 mm (no chemicals) or 94 mm Petri dishes (single chemicals) containing LB-Tetracycline Agar medium with supplemented glycine.

In test 4 with filter papers without chemicals or with filter papers impregnated with either one of the two chemicals (copper(II)ethylacetotacetate or 4,4'-dimethylenebis-(N,N-dimethylaniline)), all seven clones only caused a color change on filter papers with copper(II)ethylacetotacetate as reaction partner, as can be seen in figure 27.

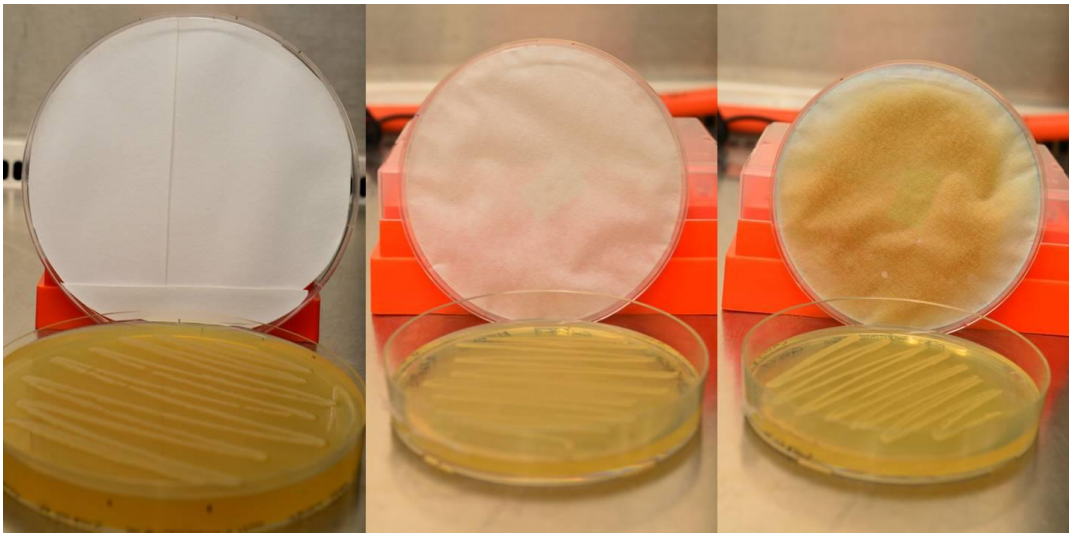

Figure 27: Testing the chemicals used for filter paper impregnation for their influence on the color reaction on the filter papers. Filter paper without chemicals (left), filter paper impregnated with 4,4'-dimethylenebis-(N,N-dimethylaniline) (middle), and filter paper impregnated with copper(II)ethylacetotacetate (right).

Test 5 – Gas chromatography measurements

Gas chromatography measurements were conducted with the help of the Division for Organic Chemistry of the University of Natural Resources and Life Sciences, Vienna, to elucidate the chemical composition of the unknown volatile compound.

So far, the unknown volatile compound could not be identified in several test runs.

Molecular screenings:

All functional screenings (tests 1 to 5) led to equal results for all seven clones. Therefore, we assumed that the unknown chemical compound and the genes involved in its biosynthesis would be the same for each of the seven clones. As a consequence, we conducted three experiments to find out if all seven clones are in fact the copy of only one clone or if there are several different clones harboring either the same or also different genes.

Before any of the molecular assays could be performed, the cosmid DNA had to be extracted from the clones first. For cosmid DNA isolation, the Alkaline Lysis Plasmid Mini-Prep Protocol (Sambrook and Russell, 2001) was used and adapted accordingly. Overnight cultures were prepared for all clones in separate 50 mL Erlenmeyer flasks containing 10 mL of LB-Tetracycline medium (for the preparation of all media and solutions see appendix). For the process of alkaline lysis, the threefold amounts for each of the solutions were used (i.e. 300 µL of ice-cold Alkaline Lysis solution 1, 600 µL of freshly prepared Alkaline Lysis solution 2, and 450 µL of ice-cold Alkaline Lysis solution 3). Purification steps were carried out using in succession phenol : chloroform-isoamyl alcohol mixture (for molecular biology, 25 : 24 : 1; Sigma-Aldrich, St. Louis, MO, USA) and chloroform-isoamyl alcohol mixture (BioUltra, for molecular biology, 24 : 1; Fluka Analytical, St. Gallen, CH). Following precipitation (with ethanol), the nucleic acids were resuspended in 100 µL of sterile Milli-Q water. In order to get rid of potential RNA contaminations, an RNA digest with RNase A (Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA) was performed. Thereto, 1 µL of a dilution of RNase A (20 mg/mL stock diluted 1:4 in sterile Milli-Q water) was added to the solution containing nucleic acids. The tubes were then put in a Thermomixer comfort heating block (Eppendorf AG,

Hamburg, DE) at 37°C for 1 hour. After that, the DNA was again precipitated with 2,5 volumes of ice-cold ethanol (absolute, for analysis; Merck KGaA, Darmstadt, DE) and 1/10 of volume of 3 M sodium acetate solution (pH 5,2) at -20°C for 2 hours. After the precipitation procedure, the tubes of all clones were again filled with 100 µL of sterile Milli-Q water.

The quality and concentration of the cosmid DNA extracted was analyzed with the NanoDrop® ND-1000 Spectrophotometer (software version 3.7.1; Thermo Scientific, Waltham, MA, USA) and gel electrophoresis, as described above. For the agarose gel electrophoresis, the Lambda DNA/Hind III Marker (2, ready-to-use, Thermo Scientific, Waltham, MA, USA) was used as size standard (3 µL per well) (for a list of all size markers used for agarose gel electrophoresis see appendix). 1 µL of the isolated cosmid DNA was diluted with 4 µL of sterile Milli-Q water and 1,5 µL of loading dye (6 x, ready-to-use; Thermo Scientific, Waltham, MA, USA) were added before pipetting into the wells of the gel.

Results for the quality check of the cosmid DNA isolation process with the NanoDrop® ND-1000 Spectrophotometer and agarose gel electrophoresis can be seen in table 20 and figure 28, respectively.

The different bands of each sample on the agarose gel were interpreted as distinct topological forms of the cosmid (i.e. relaxed circular, full-length linear, supercoiled) (Nikolic, 2013, personal communication).

The isolated cosmid DNA of all clones was of good quality according to measurements with the NanoDrop® ND-1000 Spectrophotometer and agarose gel electrophoresis.

Table 20: Results for the quality check of the cosmid DNA isolation process with the NanoDrop® ND-1000 Spectrophotometer.

Cosmid DNA of Clone	DNA content [ng/µL]	Ratio of absorbance at 260 and 280 nm [260/280]	Ratio of absorbance at 260 and 230 nm [260/230]
1	956,5	1,91	2,73
5	1015,0	1,93	2,71
11	677,5	1,95	2,75
25	987,4	1,93	2,71
27	1003,2	1,96	2,75
31	726,7	1,93	2,69
36	755,9	2,00	2,71

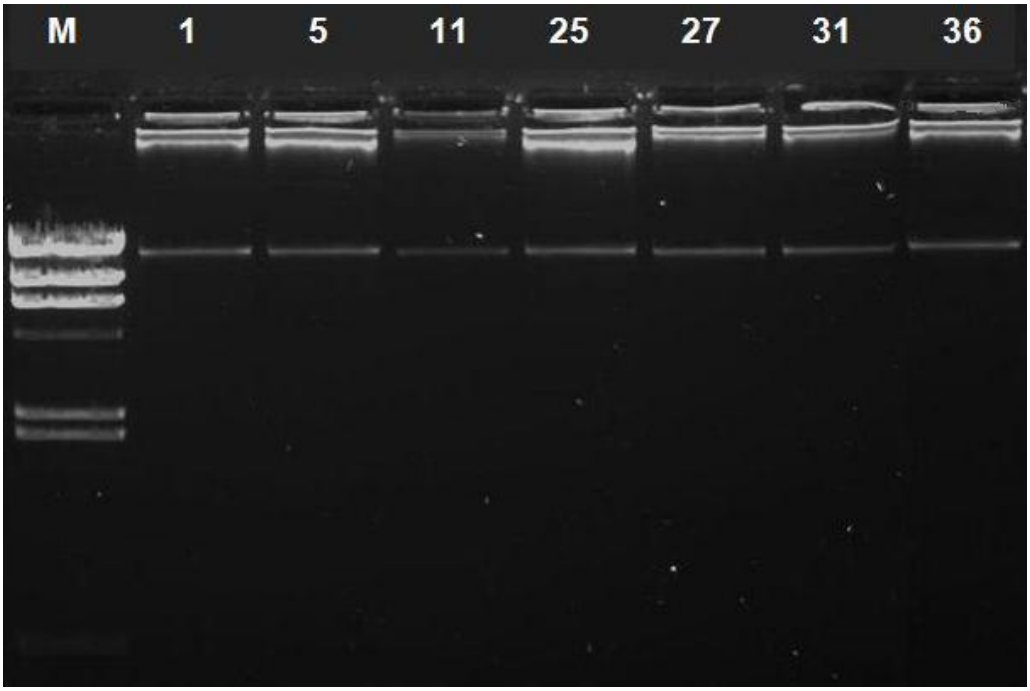

Figure 28: Results for the quality check of the cosmid DNA isolation process with agarose gel electrophoresis. M = Lambda DNA/Hind III Marker (largest band at 23.130 bp, second largest band at 9.416 bp). Cosmid DNA of clones is represented with respective clone numbers.

Test 6 – Restriction digest for cosmid DNA

Separate restriction digests with four different restriction endonucleases were carried out as a first indicator of variability of insert sequences. Four restriction enzymes were chosen to facilitate analysis and resolution. For a complete list of restriction enzymes and their recognition sites, see table 21. The reaction mixture for one enzyme-cosmid combination was comprised as follows (10 µL in total):

5,5 µL of sterile Milli-Q water
1 µL of reaction buffer
0,5 µL of restriction enzyme
3 µL of isolated cosmid DNA (concentration as given in table 20)

All enzymes and buffers were purchased from Fermentas (Thermo Scientific, Waltham, MA, USA).

Reaction mixtures were prepared in sterile 1,5 mL safe-lock tubes (Eppendorf AG, Hamburg, DE) and tubes were incubated for an appropriate amount of time and at optimum temperature (Thermomixer comfort heating block; Eppendorf AG, Hamburg, DE), as can be seen in table 22.

Restriction digests with enzymes *Bam*HI and *Eco*RI were performed for cosmids of all seven clones, whereas digests with enzymes *Not*I and *Sma*I were only conducted for cosmids of clones 1, 31, and 36.

Results were analyzed with agarose gel electrophoresis, as described above. Always, the whole amount of reaction mixture was pipetted into the wells of the gel.

Table 21: Restriction enzymes used for the restriction digest of cosmid DNA. Restriction site information based on nucleotide sequence of cosmid vector pJC8 (Cheng and Charles, 2012).

Restriction enzyme (reaction buffer)	Restriction site and cutting point	Number and position of restriction sites in the cosmid vector pJC8
***Bam*HI FastDigest** (FastDigest Green buffer)	5' - G↓G A T C C - 3' 3' - C C T A G↑G - 5'	**2** (382; 1.444)
***Eco*RI FastDigest** (FastDigest Green buffer)	5' - G↓A A T T C - 3' 3' - C T T A A↑G - 5'	**1** (269)
***Not*I** (buffer O (orange))	5' - G C↓G G C C G C - 3' 3' - C G C C G G↑C G - 5'	**3** (10.493; 10.906; 12.681)
***Sma*I** (buffer Tango (yellow))	5' - C C C↓G G G - 3' 3' - G G G↑C C C - 5'	**1** (4.487)

Table 22: Time and temperature conditions used for the restriction digest of cosmid DNA.

Restriction enzyme	Time period of digest	Temperature for digest
***Bam*HI FastDigest**	1 hour	37°C
***Eco*RI FastDigest**	1 hour	37°C
***Not*I**	4 hours	37°C
***Sma*I**	4 hours	30°C

Agarose gel electrophoresis for restriction digests with the two enzymes *Bam*HI and *Eco*RI showed three different banding patterns for the cosmid DNA of the seven clones, as presented in figure 29. The first pattern was shared by clones 1 and 27, the second by clones 5, 11, 25, and 36, and the third pattern was only found for clone 31.

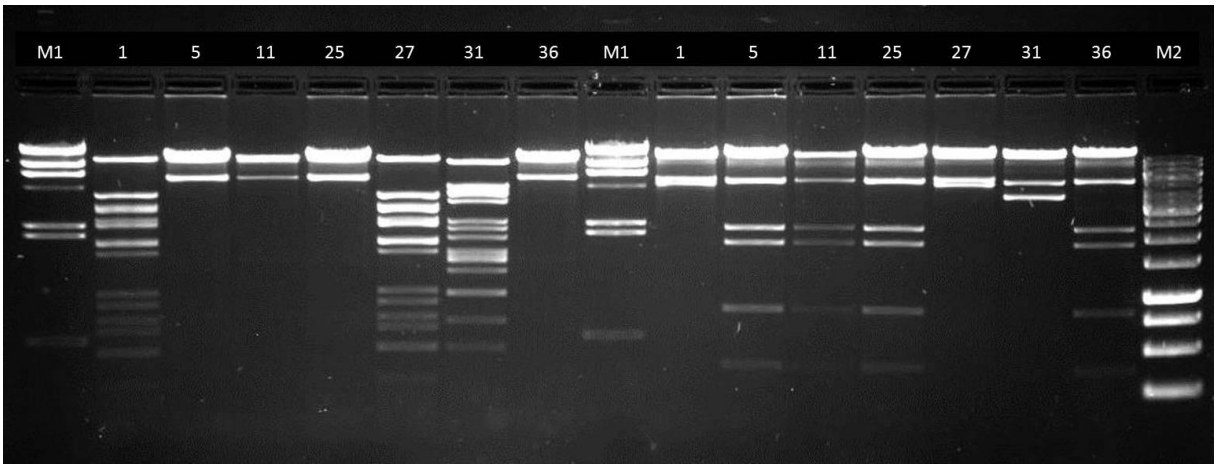

Figure 29: Restriction digest for cosmid DNA with enzymes *Bam*HI and *Eco*RI. M1 = Lambda DNA/Hind III Marker, M2 = GeneRuler 1 kb DNA Ladder. Cosmid DNA of clones is represented with respective clone numbers. Left series of clones represent digest with *Bam*HI, right series of clones represent digest with *Eco*RI.

Two more restriction digests with enzymes *Not*I and *Sma*I were carried out for clones 1, 31, and 36, which were all showing diverse banding patterns after digest with *Bam*HI and *Eco*RI. Again, 3 different patterns could be observed after gel electrophoresis of the digests, as can be seen in figure 30.

An exact size estimation of the insert sequences was not possible due to too many double bands on the agarose gel.

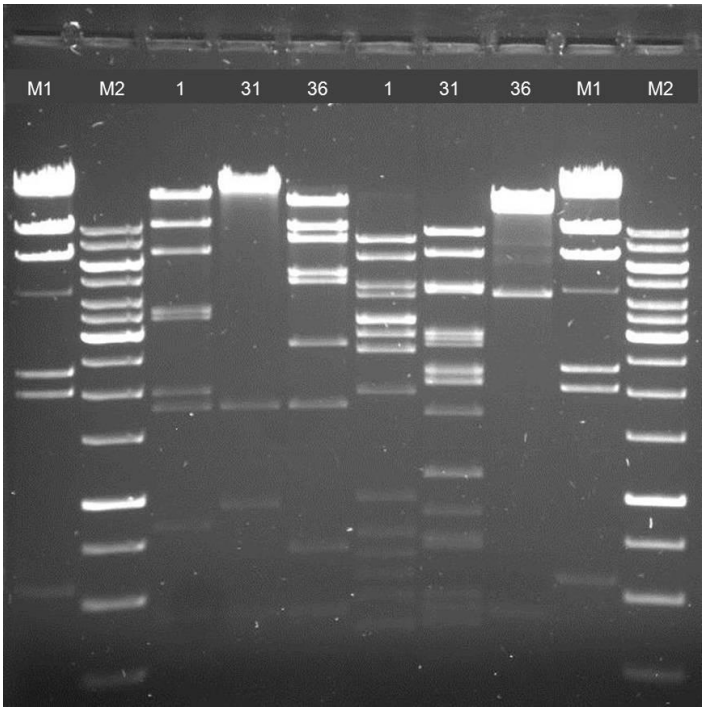

Figure 30: Restriction digest for cosmid DNA with enzymes *Not*I and *Sma*I. M1 = Lambda DNA/Hind III Marker, M2 = GeneRuler 1 kb DNA Ladder. Cosmid DNA of clones is represented with respective clone numbers. Left series of clones represent digest with *Not*I, right series of clones represent digest with *Sma*I.

Test 7 – Sanger sequencing from the border of the insert

Due to the large size of the inserts, sequencing of the whole construct for all seven clones would have been quite expensive and unnecessary if only copies of one clone (or cosmid) would have been analyzed. Therefore, marginal insert sequences were checked first by Sanger sequencing to give additional information and support restriction digest findings if possible. For that purpose, 5 µL of cosmid DNA (diluted to 300 ng/µL with sterile Milli-Q water according to NanoDrop® measurements) were mixed with 5 µL of either primer M13F or M13R (both 5 µM; for more information on primers, see chapter 2.1.4.) and sent to GATC Biotech AG (Konstanz, DE) for sequencing. Binding sites for both primers are located close to the insert position on cosmid vector pJC8 and, hence, allow Sanger sequencing from the border of the insert (M13F binding site 114 – 129 bp upstream of insert position, M13R binding site 134 – 118 bp downstream of insert position (Cheng and Charles, 2012)). Obtained sequences were edited with the software BioEdit 7.0.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and compared to the NCBI database in BLASTN 2.2.29 (http://blast.ncbi.nlm.nih.gov/Blast.cgi; NCBI, Bethesda, MD, USA) (Zhang et al., 2000).

Results for the database comparison of these sequences are shown in tables 23 and 24.

Closest matches with database entries were consistent for both ends of the insert for each clone (slight change for clone 1). Homology was found at percentages from 71 to 93 %. For qualitatively poor sequences (i.e. M13 forward for clone 11 and M13 reverse for clone 27), query coverage was very low. For all other sequences, coverage was detected at 83 to 99 %. Sequence lengths reached from 339 bp to 1012 bp.

Based on sequence comparisons, the same diversity of clones (inserts) could be found as for the restriction digest: clones 1 and 27 share the same insert, so do 5, 11, 25, and 36, but clone 31 carries an insert differing from all others (at least in length). Best hits showed highest similarity with database entries of *Aeromonas hydrophyla* strains (clones 1, 27, and 31) or *Comamonas testosteroni* strain CNB-2 (clones 5, 11, 25, and 36).

Table 23: Results for the Sanger sequencing for marginal insert sequences with primer M13F.

Cosmid of clone	Length of sequence	Primer M13forward Closest match with NCBI BLASTN 2.2.29 and accession number	Range on closest match and feature (protein)	Identity (%)	Query cover (%)	Putative phylum and class
1	775 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	146810 to 147577; LysR family transcriptional regulator	89	99	Proteobacteria; Gammaproteobacteria
5	879 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2566328 to 2567186; 2-oxo-acid dehydrogenase E1 subunit	75	97	Proteobacteria; Betaproteobacteria
11	436 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2566580 to 2566764; 2-oxo-acid dehydrogenase E1 subunit	71	42	Proteobacteria; Betaproteobacteria
25	936 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2566314 to 2567241; 2-oxo-acid dehydrogenase E1 subunit	83	98	Proteobacteria; Betaproteobacteria
27	887 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	146810 to 147666; LysR family transcriptional regulator	80	85	Proteobacteria; Gammaproteobacteria
31	606 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	150038 to 150554; putative transport system permease ABC transporter protein, ABC-type sugar transporter permease component	81	85	Proteobacteria; Gammaproteobacteria
36	945 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2566315 to 2567230; 2-oxo-acid dehydrogenase E1 subunit	84	98	Proteobacteria; Betaproteobacteria

Table 24: Results for the Sanger sequencing for marginal insert sequences with primer M13R.

Cosmid of Clone	Length of sequence	Primer M13reverse Closest match with NCBI BLASTN 2.2.29 and accession number	Range on closest match and feature (protein)	Identity (%)	Query cover (%)	Putative phylum and class
1	785 bp	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966, complete genome; CP000462.1	280911 to 281568; ribosomal RNA small subunit methyltransferase B	89	83	Proteobacteria; Gammaproteobacteria
		3rd best hit: *Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	192632 to 193381; trkA product, rsmB product	85	96	
5	791 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2599286 to 2600084; hypothetical conserved protein, hypothetical protein	91	99	Proteobacteria; Betaproteobacteria
11	531 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2599552 to 2600034; hypothetical conserved protein, hypothetical protein	79	90	Proteobacteria; Betaproteobacteria
25	886 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2599263 to 2600084; hypothetical conserved protein, hypothetical protein	91	95	Proteobacteria; Betaproteobacteria
27	339 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	193210 to 193312; rsmB product	74	30	Proteobacteria; Gammaproteobacteria
31	822 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	187634 to 188246; NapF protein, hypothetical protein; 188281 to 188536; DNA recombination protein	93	98	Proteobacteria; Gammaproteobacteria
36	1012 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.1	2599186 to 2600084; hypothetical conserved protein, hypothetical protein	91	91	Proteobacteria; Betaproteobacteria

Test 8 – Illumina® MiSeq Sequencing by Synthesis for total insert sequences

The Illumina® MiSeq Sequencing technology was used to identify the whole DNA sequences of inserts of 3 cosmids, which showed diverse results for the restriction digest and Sanger sequencing (i.e. cosmids of clones 1, 31, and 36).

An exact estimation of the concentration of double-stranded DNA in the samples to be tested was necessary as only 5 µL of a 0,2 ng/µL DNA solution (1 ng of dsDNA in total) were needed for the preparation of the MiSeq procedure. For this, the quantity of cosmid DNA was evaluated by PicoGreen measurements using the Quant-iT™ PicoGreen® dsDNA Kit (Molecular Probes™, Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA). PicoGreen® is a fluorochrome that selectively binds double-stranded DNA and therefore, can be used for quantitation of such DNA in a fluorescence detection system (Ahn et al., 1996). All steps of the procedure were based on the Molecular Probes™ manual (Spence and Johnson, 2010). The cosmid DNA of all 7 clones was diluted to several degrees (1/10, 1/50, 1/100, and 1/200) to ensure that the DNA concentration of at least one dilution level would be covered by the values of a standard calibration curve based on known DNA content. For this calibration curve, a dilution series of λ DNA was prepared (final concentrations of 500, 250, 125, 62,5, 31,25, 15,625, and 7,8125 ng/mL). All solutions were mixed appropriately in a white 96 well hard shell PCR plate (Bio-Rad, Hercules, CA, USA). Always, the fluorochrome was added last. Two columns of the 96 well plate were filled with λ DNA standards for a better validation of the calibration curve. After all samples were pipetted into the wells, the plate was incubated in the dark at room temperature for 5 minutes. Subsequently, fluorescence was measured in a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (with the software Gen5™ Data Analysis Software; both BioTek, Winooski, VT, USA) with excitation at 485 nm and emission measurement at 538 nm. The concentration of double-stranded DNA present in the cosmid samples was then calculated based on the calibration curve (depicted in figure 31), taking into account all dilution steps.

Results for the DNA quantification can be seen in table 25.

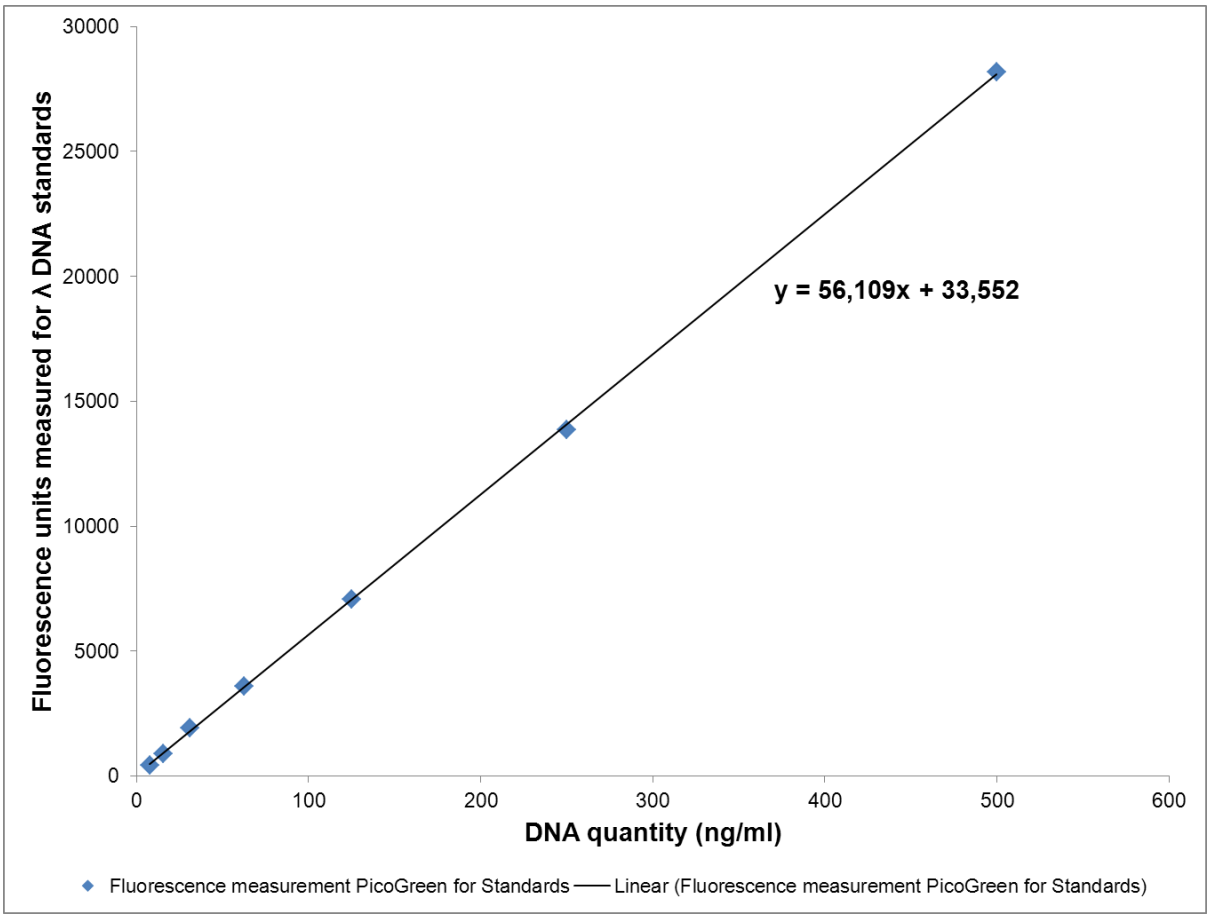

Figure 31: Calibration curve for the cosmid DNA quantification with PicoGreen®. Dilutions of λ DNA were used as standards. Equation for the calibration curve given as y = 56,109x + 33,552.

Table 25: Results for the DNA quantification with PicoGreen® for cosmid DNA of all 7 clones of interest.

Quantity of undiluted cosmid DNA (ng/µL) isolated with the Alkaline Lysis Plasmid Mini-Prep Protocol according to PicoGreen® measurements							
Calculated via dilution	***Clone 1***	***Clone 5***	***Clone 11***	***Clone 25***	***Clone 27***	***Clone 31***	***Clone 36***
1/10	105,65	149,87	51,06	164,69	108,69	156,75	151,57
1/10	106,64	148,79	47,70	168,93	109,76	147,99	150,84
1/100	104,21	142,35	47,98	171,14	98,24	157,86	146,81
1/200	92,58	142,30	43,74 (outlier)	162,80	169,75	137,49	141,95
Mean	**102,27**	**145,83**	**48,91 (without outlier)**	**166,89**	**121,61**	**150,02**	**147,79**

Preparation of the DNA library for the Illumina® MiSeq Sequencing run was based on the PicoGreen® measurements. The cosmid DNA of clones 1, 31, and 36 was diluted to 0,2 ng/µL (with sterile Milli-Q water), respectively, and then pooled in one sample for the Illumina® MiSeq sequencing (leading to a final concentration of 0,066 ng/µL for each clone).

The preparation for the Illumina® MiSeq Sequencing by Synthesis process was then exactly performed as described in the Nextera® XT DNA Sample Preparation Guide (Illumina Inc., 2012) using the Nextera® XT DNA Sample Preparation Kit (for 24 samples). In a last step of preparation for sequencing, the pooled sequencing library was diluted in hybridization buffer and heat denatured prior to Illumina® MiSeq sequencing. The flow cell, on which the sequencing reaction occurs, was integrated into the MiSeq apparatus. 6 µL were removed from the 600 µL of pooled library sample and replaced by 6 µL of Phix library DNA (12,5 pM; Phix Control v3; Illumina, Inc., San Diego, CA, USA) for quality control of the sequencing process. The pooled library sample was then pipetted into the reagent cartridge. The reagent cartridge, containing all necessary clustering and sequencing reagents, and the according buffer were also placed in the MiSeq apparatus according to the manufacture protocol. Finally, the Illumina® MiSeq Sequencing by Synthesis process was started by following instructions on the MiSeq screen.

The sequence data obtained from the MiSeq run were used for contig formation (assembly) (all steps performed by Livio Antonielli). For this purpose, the sequence data were first quality checked with the software FastQC (Babraham Bioinformatics, Cambridge, UK; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). In a second step, the single reads were edited with the software Trimmomatic (Lohse et al. (2012); http://www.usadellab.org/cms/?page=trimmomatic). Then, all reference indices were created in Bowtie 2 (Johns Hopkins University, Baltimore, MD, USA; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). During the subsequent mapping process (comparison to the indexed references), some contigs were found to contain sequences from *Escherichia coli*, indicating that the initial DNA extraction was not selective enough for cosmid and insert alone. These impurities in the contigs were removed. The final sequence assembly was then performed using the SPAdes Genome Assembler (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences, St. Petersburg, RU). For clone 36, which yielded

matches for *Comamonas testosteroni* for both Sanger sequencing runs, contig formation could easily be achieved. On the contrary, contig assembly was more difficult for clones 1 and 31, which both showed similar results for sequences obtained from Sanger sequencing (*Aeromonas* spp.). To obtain the complete insert sequences for these two clones, smaller contigs were assembled based on the restriction pattern of digestion with enzymes *Eco*RI and *Bam*HI and ClustalW alignment with the software BioEdit 7.0.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) (Thompson et al., 1994) (performed by Günter Brader). The resulting final contigs were compared to the NCBI database in BLASTN 2.2.29 (search nucleotide databases using a nucleotide query; http://blast.ncbi.nlm.nih.gov/Blast.cgi; NCBI, Bethesda, MD, USA) (Altschul et al., 1997).

Data assembly yielded five contigs, one for clone 36 and two with similar size for each of the clones 1 and 31, respectively. Two contigs had to be assembled for each of the two clones 1 and 31 as one small fragment could not be assigned exactly. In both cases, the two different contigs showed similarity on the nucleotide level of 99,94 %. The slight differences did neither change the results for the database comparisons nor the genome annotation (see below). The contigs are 39.474, 39.035, and 38.281 bp in size for inserts of clones 1, 31, and 36, respectively. The closest matches and the respective features (proteins) for comparisons with the NCBI database in BLASTN 2.2.29 are listed in tables 26 to 28. Contigs derived from Illumina® MiSeq Sequencing confirmed Sanger sequencing results. For inserts of clones 1 and 31, closest hits were found for *Aeromonas hydrophila* strain 4AK4 (91 and 96 % identity, respectively), while for clone 36, the best match was again *Comamonas testosteroni* strain CNB-2 (90 % identity). For clones 1 and 31, similar protein hits were also identified in related *Aeromonas* species (data not shown). The two (four) contigs for these clones showed 59,31 % identity when compared to each other after clustalW alignment (and 26,47 % before). Based on comparison to *Aeromonas hydrophila* strain 4AK4 (at accession number CP006579.1), 30 identical proteins could be found for the two clones 1 and 31. Contigs of both clones showed similarity mainly to two genomic regions of *Aeromonas hydrophila* strain 4AK4 (i.e. to approximately 150.000 to 156.000 and to 171.000 to 188.000). Similarities to the contig of clone 36 are described below. Overall, the genomic organization of our insert contigs and of database entries varied widely. Besides, even for the closest matches query coverage did not exceed 82 % and large fragments of the insert

sequences could not be connected to database entries (approximately 6000 bp for each contig). These findings indicated that the inserts actually originated from species closely related to those with entries in the NCBI database. Therefore, a genome annotation using RAST (Rapid Annotation using Subsystem Technology) (Aziz et al., 2008) was finally performed for all three inserts (performed by Günter Brader). Results for the genome annotation process are depicted in tables 29 to 34. 40, 38, and 35 coding sequences (CDS) could be identified for clones 1, 31, and 36, respectively. Neither direct database comparison nor genome annotation led to the discovery of identical genes of both *Aeromonas hydrophila* and *Comamonas testosteroni* on a nucleotide level. Hence, the exact gene(s) responsible for the brown coloring on the Feigl-Anger filter papers could not yet be identified. Still, genes with related functions could be found, including transcriptional regulators from the LysR family, several dehydrogenases (oxidoreductases), and genes involved in reactions with aldehydes (as highlighted in tables).

Table 26: Results from BLASTN 2.2.29 NCBI database comparison for contig 1 derived from Illumina® MiSeq Sequencing. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Length of sequence	Closest match with NCBI BLASTN 2.2.29 and accession number	Putative phylum and class	Identity (%)	Query cover (%)
1 (355)	39.474 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	Proteobacteria; Gamma-proteobacteria	**91**	**82** (more than 6.000 bp gap/no match)
Range on closest match and features (proteins) (36 in total)					
146810 to 147668 (859 bp): LysR family transcriptional regulator; **149286 to 151485 (2202 bp):** putative transport system permease ABC transporter protein, ABC-type sugar transporter permease component; **151543 to 156764 (5236 bp):** ABC transporter, nucleotide binding/ATPase protein, basic membrane lipoprotein, hypothetical protein, DNA helicase II; **167429 to 169328 (1901 bp):** 3-oxoacyl-ACP synthase II, HxlR family transcriptional regulator; **170357 to 171040 (694 bp):** hypothetical protein; **171638 to 176159 (4600 bp):** hypothetical protein, LysR family transcriptional regulator, amino acid transporter LysE, YeeE/YedE family protein, hypothetical protein, amino acid transporter LysE; **176181 to 179962 (3794 bp):** AraC/XylS family transcriptional regulator, rarD-1 product, hypothetical protein, hypothetical protein, putative aldehyde dehydrogenase; **180056 to 188246 (8224 bp):** DNA-binding protein, Omega-amino acid--pyruvate aminotransferase, mmsA-1 product, glutamine amidotransferase, class I, putative cys regulon transcriptional activator, carbohydrate kinase, Rrf2 family protein, NapF protein, hypothetical protein; **188281 to 192669 (4391 bp):** DNA recombination protein, potassium uptake protein TrkH, trkA product; **192632 to 193379 (757 bp):** trkA product, rsmB product; **2006186 to 2006590 (408 bp):** methylmalonate-semialdehyde dehydrogenase;					

Table 27: Results from BLASTN 2.2.29 NCBI database comparison for contig 31 derived from Illumina® MiSeq Sequencing. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Length of sequence	Closest match with NCBI BLASTN 2.2.29 and accession number	Putative phylum and class	Identity (%)	Query cover (%)
31 (358)	39.035 bp	*Aeromonas hydrophila* 4AK4, complete genome; CP006579.1	Proteobacteria; Gamma-proteobacteria	**96**	**82** (more than 6.000 bp gap/no match)
Range on closest match and features (proteins) (39 in total)					
149958 to 151485 (1528 bp): putative transport system permease ABC transporter protein, ABC-type sugar transporter permease component; **151543 to 154482 (2955 bp):** ABC transporter, nucleotide binding/ATPase protein, basic membrane lipoprotein, hypothetical protein; **154534 to 156764 (2231 bp):** hypothetical protein, DNA helicase II; **156894 to 157268 (388 bp):** 130 bp at 5' side: DNA helicase II, 21 bp at 3' side: DNA helicase II; **160581 to 161600 (1020 bp):** transcriptional regulator; **161670 to 162572 (903 bp):** hypothetical protein; **164613 to 169326 (4727 bp):** hypothetical protein, transport associated protein, hypothetical protein, hypothetical protein, 3-oxoacyl-ACP synthase II, HxlR family transcriptional regulator; **169933 to 170983 (1061 bp):** Selenium-binding protein YdfZ, hypothetical protein; **170996 to 171586 (591 bp):** hypothetical protein; **171626 to 188246 (16684 bp):** hypothetical protein, LysR family transcriptional regulator, amino acid transporter LysE, YeeE/YedE family protein, hypothetical protein, amino acid transporter LysE, AraC/XylS family transcriptional regulator, rarD-1 product, hypothetical protein, hypothetical protein, putative aldehyde dehydrogenase, DNA-binding protein, Omega-amino acid--pyruvate aminotransferase, mmsA-1 product, glutamine amidotransferase, class I, putative cys regulon transcriptional activator, carbohydrate kinase, Rrf2 family protein, NapF protein, hypothetical protein; **188281 to 188535 (255 bp):** DNA recombination protein; **2006186 to 2006590 (413 bp):** methylmalonate-semialdehyde dehydrogenase; **2994267 to 2994646 (388 bp):** 184 bp at 5' side: tryptophan synthase subunit alpha, 528 bp at 3' side: hypothetical protein;					

Table 28: Results from BLASTN 2.2.29 NCBI database comparison for contig 36 derived from Illumina® MiSeq Sequencing. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Length of sequence	Closest match with NCBI BLASTN 2.2.29 and accession number	Putative phylum and class	Identity (%)	Query cover (%)
36	38.281 bp	*Comamonas testosteroni* CNB-2, complete genome; CP001220.2	Proteobacteria; Beta-proteobacteria	**90**	**81** (more than 6.000 bp gap/no match)
Range on closest match and features (proteins) (26 in total)					
2255330 to 2255955 (626 bp): fatty acid desaturase; **2566316 to 2572687 (6414 bp):** 2-oxo-acid dehydrogenase E1 subunit, pyruvate dehydrogenase complex dihydrolipoamide, conserved hypothetical protein, dihydrolipoamide dehydrogenase; **2574694 to 2575348 (668 bp):** glutathione-dependent formaldehyde-activating; **2575486 to 2577401 (1920 bp):** major facilitator superfamily MFS_1; **2577454 to 2578078 (635 bp):** alkylphosphonate utilization operon protein; **2578153 to 2581096 (2946 bp):** conserved hypothetical protein, ATPase associated with various cellular; **2581170 to 2592172 (11033 bp):** transcriptional regulator, LysR family, conserved hypothetical protein, amidohydrolase, sodium/hydrogen exchanger, hypothetical conserved protein, diguanylate cyclase, hypothetical protein, putative transmembrane protein, aldo/keto reductase; **2593006 to 2600080 (7087 bp):** major facilitator superfamily MFS_1, Amidohydrolase 3, membrane protein-like protein, putrescine importer, hypothetical conserved protein, hypothetical protein; **4882336 to 4882628 (297 bp):** amidohydrolase					

Table 29: Genome annotation for the contig of clone 1 based on RAST technology (Rapid Annotation using Subsystem Technology). Part I. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Feature ID	Start-position of coding sequence (nt)	Stop-position of coding sequence (nt)	Length of coding sequence (bp)	Function (protein)
1 (355) (*Aeromonas* sp.) **PART I**	fig|642.82.peg.1	41	859	819	Transcriptional regulator, LysR family, in formaldehyde detoxification operon
	fig|642.82.peg.2	1902	937	966	Predicted nucleoside ABC transporter, permease 2 component
	fig|642.82.peg.3	3023	1905	1119	Predicted nucleoside ABC transporter, permease 1 component
	fig|642.82.peg.4	4588	3020	1569	Predicted nucleoside ABC transporter, ATP-binding component
	fig|642.82.peg.5	5673	4675	999	Predicted nucleoside ABC transporter, substrate-binding component
	fig|642.82.peg.6	6078	8255	2178	ATP-dependent DNA helicase UvrD/PcrA
	fig|642.82.peg.7	9094	8372	723	Homolog of eukaryotic DNA ligase III
	fig|642.82.peg.8	9584	9045	540	hypothetical protein
	fig|642.82.peg.9	10977	9571	1407	hypothetical protein
	fig|642.82.peg.10	11593	11228	366	hypothetical protein
	fig|642.82.peg.11	11873	12370	498	hypothetical protein
	fig|642.82.peg.12	13928	12681	1248	3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.41)
	fig|642.82.peg.13	14482	13955	528	Transcriptional regulator, HxlR family
	fig|642.82.peg.14	14745	15245	501	hypothetical protein
	fig|642.82.peg.15	15879	15433	447	Uncharacterized conserved protein
	fig|642.82.peg.16	16669	16013	657	Miscellaneous; Unknown
	fig|642.82.peg.17	16966	17466	501	Acetyltransferases
	fig|642.82.peg.18	17611	18279	669	hypothetical protein
	fig|642.82.peg.19	19221	18292	930	Transcriptional regulator, LysR family
	fig|642.82.peg.20	19290	19910	621	Putative threonine efflux protein

Table 30: Genome annotation for the contig of clone 1 based on RAST technology (Rapid Annotation using Subsystem Technology). Part II. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Feature ID	Start-position of coding sequence (nt)	Stop-position of coding sequence (nt)	Length of coding sequence (bp)	Function (protein)
1 (355) (*Aeromonas* sp.) **PART II**	fig|642.82.peg.21	21115	20006	1110	hypothetical protein
	fig|642.82.peg.22	21359	21219	141	Threonine efflux protein
	fig|642.82.peg.23	22004	21363	642	Threonine efflux protein
	fig|642.82.peg.24	23116	22298	819	Transcriptional regulator, AraC family
	fig|642.82.peg.25	24084	23188	897	Protein rarD
	fig|642.82.peg.26	24378	24205	174	hypothetical protein
	fig|642.82.peg.27	26000	24504	1497	Aldehyde dehydrogenase (EC 1.2.1.3)
	fig|642.82.peg.28	26723	26181	543	Transcriptional regulator, MerR family
	fig|642.82.peg.29	26891	28228	1338	Omega-amino acid--pyruvate aminotransferase (EC 2.6.1.18)
	fig|642.82.peg.30	28336	29832	1497	Methylmalonate-semialdehyde dehydrogenase [inositol] (EC 1.2.1.27)
	fig|642.82.peg.31	29916	30644	729	GMP synthase (EC 6.3.5.2)
	fig|642.82.peg.32	30814	31770	957	Cys regulon transcriptional activator CysB
	fig|642.82.peg.33	31833	32672	840	Carbohydrate kinase, PfkB family
	fig|642.82.peg.34	32961	33413	453	Nitrite-sensitive transcriptional repressor NsrR
	fig|642.82.peg.35	33933	33481	453	Ferredoxin-type protein NapF (periplasmic nitrate reductase)
	fig|642.82.peg.36	34132	34347	216	FIG00361587: hypothetical protein
	fig|642.82.peg.37	34344	35675	1332	DNA recombination protein RmuC
	fig|642.82.peg.38	37190	35748	1443	Potassium uptake protein TrkH
	fig|642.82.peg.39	38660	37284	1377	Trk system potassium uptake protein TrkA
	fig|642.82.peg.40	39474	38821	654	Ribosomal RNA small subunit methyltransferase B (EC 2.1.1.-)

Table 31: Genome annotation for the contig of clone 31 based on RAST technology (Rapid Annotation using Subsystem Technology). Part I. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Feature ID	Start-position of coding sequence (nt)	Stop-position of coding sequence (nt)	Length of coding sequence (bp)	Function (protein)
31 (358) (*Aeromonas* sp.) **PART I**	fig|642.85.peg.1	1467	349	1119	Predicted nucleoside ABC transporter, permease 1 component
	fig|642.85.peg.2	3053	1467	1587	Predicted nucleoside ABC transporter, ATP-binding component
	fig|642.85.peg.3	4248	3145	1104	Predicted nucleoside ABC transporter, substrate-binding component
	fig|642.85.peg.4	4713	6653	1941	hypothetical protein
	fig|642.85.peg.5	6694	7119	426	hypothetical protein
	fig|642.85.peg.6	7182	9359	2178	ATP-dependent DNA helicase UvrD/PcrA
	fig|642.85.peg.7	10494	10111	384	hypothetical protein
	fig|642.85.peg.8	12401	10971	1431	hypothetical protein
	fig|642.85.peg.9	13595	12702	894	Transcriptional regulator, LysR family
	fig|642.85.peg.10	14670	13885	786	FIG01217123: hypothetical protein
	fig|642.85.peg.11	14928	15086	159	hypothetical protein
	fig|642.85.peg.12	15114	15533	420	Osmotically inducible protein Y
	fig|642.85.peg.13	15540	15845	306	hypothetical protein
	fig|642.85.peg.14	16128	17516	1389	hypothetical protein
	fig|642.85.peg.15	18904	17627	1278	3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.41)
	fig|642.85.peg.16	19428	18901	528	Transcriptional regulator, HxlR family
	fig|642.85.peg.17	19815	19618	198	FIG00362285: hypothetical protein
	fig|642.85.peg.18	20106	20606	501	hypothetical protein
	fig|642.85.peg.19	21273	20839	435	Uncharacterized conserved protein
	fig|642.85.peg.20	22020	21364	657	Miscellaneous; Unknown

Table 32: Genome annotation for the contig of clone 31 based on RAST technology (Rapid Annotation using Subsystem Technology). Part II. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Feature ID	Start-position of coding sequence (nt)	Stop-position of coding sequence (nt)	Length of coding sequence (bp)	Function (protein)
31 (358) (*Aeromonas* sp.) **PART II**	fig|642.85.peg.21	22295	22942	648	hypothetical protein
	fig|642.85.peg.22	23888	22959	930	Transcriptional regulator, LysR family
	fig|642.85.peg.23	23957	24577	621	Putative threonine efflux protein
	fig|642.85.peg.24	25735	24653	1083	hypothetical protein
	fig|642.85.peg.25	26652	26011	642	Threonine efflux protein
	fig|642.85.peg.26	27550	26732	819	Transcriptional regulator, AraC family
	fig|642.85.peg.27	28515	27619	897	Protein rarD
	fig|642.85.peg.28	28931	28641	291	hypothetical protein
	fig|642.85.peg.29	30442	28940	1503	Aldehyde dehydrogenase (EC 1.2.1.3)
	fig|642.85.peg.30	31177	30635	543	Transcriptional regulator, MerR family
	fig|642.85.peg.31	31345	32682	1338	Omega-amino acid--pyruvate aminotransferase (EC 2.6.1.18)
	fig|642.85.peg.32	32786	34282	1497	Methylmalonate-semialdehyde dehydrogenase [inositol] (EC 1.2.1.27)
	fig|642.85.peg.33	34441	35094	654	GMP synthase (EC 6.3.5.2)
	fig|642.85.peg.34	35265	36221	957	Cys regulon transcriptional activator CysB
	fig|642.85.peg.35	36287	37126	840	Carbohydrate kinase, PfkB family
	fig|642.85.peg.36	37413	37865	453	Nitrite-sensitive transcriptional repressor NsrR
	fig|642.85.peg.37	38476	37958	519	Ferredoxin-type protein NapF (periplasmic nitrate reductase)
	fig|642.85.peg.38	38608	38823	216	FIG00361587: hypothetical protein

Table 33: Genome annotation for the contig of clone 36 based on RAST technology (Rapid Annotation using Subsystem Technology). Part I. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Feature ID	Start-position of coding sequence (nt)	Stop-position of coding sequence (nt)	Length of coding sequence (bp)	Function (protein)
36 (*Comamonas* sp.) **PART I**	fig|6666666.61607.peg.1	3	395	393	hypothetical protein
	fig|6666666.61607.peg.2	426	914	489	Protein of unknown function DUF55
	fig|6666666.61607.peg.3	1204	2616	1413	Putrescine importer
	fig|6666666.61607.peg.4	2732	3802	1071	hypothetical protein
	fig|6666666.61607.peg.5	5608	3851	1758	Predicted metal-dependent hydrolase with the TIM-barrel fold
	fig|6666666.61607.peg.6	6995	5763	1233	putative integral membrane protein
	fig|6666666.61607.peg.7	7845	7336	510	Oxidoreductase, aldo/keto reductase family
	fig|6666666.61607.peg.8	8174	7803	372	Oxidoreductase, aldo/keto reductase family
	fig|6666666.61607.peg.9	8389	9240	852	FIG00349370: hypothetical protein
	fig|6666666.61607.peg.10	9328	10080	753	hypothetical protein
	fig|6666666.61607.peg.11	10143	10457	315	Uncharacterized protein conserved in bacteria
	fig|6666666.61607.peg.12	11705	10464	1242	diguanylate cyclase
	fig|6666666.61607.peg.13	12096	11869	228	Putative cytoplasmic protein
	fig|6666666.61607.peg.14	13028	12051	978	Putative cytoplasmic protein
	fig|6666666.61607.peg.15	14301	13018	1284	hypothetical protein
	fig|6666666.61607.peg.16	15874	14699	1176	Catalyzes the cleavage of p-aminobenzoyl-glutamate to p-aminobenzoate and glutamate, subunit A
	fig|6666666.61607.peg.17	17233	15890	1344	membrane protein, putative
	fig|6666666.61607.peg.18	17411	18361	951	regulatory protein, LysR:LysR, substrate-binding
	fig|6666666.61607.peg.19	18559	19200	642	hypothetical protein
	fig|6666666.61607.peg.20	19308	20375	1068	ATPase associated with various cellular activities, AAA_5

Table 34: Table 32: Genome annotation for the contig of clone 36 based on RAST technology (Rapid Annotation using Subsystem Technology). Part II. Proteins with related functions to those detected in other contigs are highlighted in yellow.

Contig	Feature ID	Start-position of coding sequence (nt)	Stop-position of coding sequence (nt)	Length of coding sequence (bp)	Function (protein)
36 (*Comamonas* sp.) **PART II**	fig|6666666.61607.peg.21	20372	22231	1860	Sll7028 protein
	fig|6666666.61607.peg.22	22642	22241	402	Putative cytoplasmic protein
	fig|6666666.61607.peg.23	23997	22639	1359	Multidrug resistance protein B
	fig|6666666.61607.peg.24	24086	24997	912	LysR-family transcriptional regulator
	fig|6666666.61607.peg.25	25310	25549	240	Alkylphosphonate utilization operon protein PhnA
	fig|6666666.61607.peg.26	26313	27683	1371	Major facilitator family transporter
	fig|6666666.61607.peg.27	29231	27825	1407	hypothetical protein
	fig|6666666.61607.peg.28	29742	29617	126	hypothetical protein
	fig|6666666.61607.peg.29	30330	29914	417	Gfa-like protein
	fig|6666666.61607.peg.30	30671	31603	933	Mobile element protein
	fig|6666666.61607.peg.31	31613	31891	279	Mobile element protein
	fig|6666666.61607.peg.32	33821	31986	1836	Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex (EC 1.8.1.4)
	fig|6666666.61607.peg.33	34800	33916	885	Enolase (EC 4.2.1.11)
	fig|6666666.61607.peg.34	36592	34907	1686	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)
	fig|6666666.61607.peg.35	38242	36611	1632	Pyruvate dehydrogenase E1 component (EC 1.2.4.1)

4.) Discussion

In the following chapters, all results of the practical work for this master's thesis will be discussed. Again, tests for bacterial isolates and such for metagenomic libraries will be considered separately.

4.1.) Testing bacterial isolates

4.1.1.) Isolation of bacteria from plant material

In total, 40 endophytic bacterial isolates were isolated from common ragweed plants. Thirty-two of those isolates originated from roots, the other eight were isolated from stems. The isolates represent a rather low diversity, with *Bacillus* spp. making up for nearly half of all bacterial isolates. With the 16S rRNA PCR and Sanger sequencing alone, the isolates could only be identified on a genus level, but a more detailed classification was not yet necessary. The culture plates, from which the bacteria were isolated, contained relatively few bacterial colonies, when compared to other isolation procedures carried out in our working group. The reduced number of colonies cannot be attributed to a too harsh isolation process as some colonies were also found on the control plates (water from last washing step). A reason for the lacking diversity and the small number of isolates could be that the plant material was used for isolation more than a week after its collection. Although the ragweed plants were stored at 4°C, many bacteria might have died due to desiccation. The remaining strains might be adapted to a rather dry (plant) environment. Unfortunately, none of the isolated bacteria proofed to be positive for hydrogen cyanide production. Nevertheless, some of those isolates could be of interest for biocontrol if they possess features like production of other antibiotics or capability to induce systemic resistance in plants. The according screenings have not been carried out so far. Even though these 40 isolates did not produce hydrogen cyanide, cyanogenic isolates originating from common ragweed plants have been detected in our working group (Widhalm, unpublished results). Due to time limitations, no further isolation process was carried out and other strains from the AIT strain collection or from co-workers were used for the development of the HCN detection tests.

4.1.2.) Optimization of a standard functional screening method for the detection of hydrogen cyanide-producing bacteria

The standard test for HCN detection as first described by Lorck (1948) allows a safe and easy identification of hydrogen cyanide-producing bacterial strains. Yet, this functional screening method is quite cumbersome as only one isolate can be tested per Petri dish and filter paper. Nevertheless, we optimized this test to verify the results of all newly developed tests under ideal conditions. Optimum parameter levels could be found for the growth medium, the amount of chemicals for the filter papers, the fixation of the filter paper to the Petri dish lid, and the duration of the testing period (see section results for further details). Production of hydrogen cyanide (indicated by a color change of the filter paper) was always found to be higher if the isolates grew on LB medium instead of R2A medium. Supplemented glycine led to an increase of HCN production in all cases. The same has been reported earlier (Alström and Burns, 1989; Castric, 1977; Kremer and Souissi, 2001; Wissing, 1974). Apparently, cyanogenesis is largely dependent on the growth medium as the different media provide unequal amounts of the natural precursor glycine. Similar findings were also documented by Michelsen and Stougaard (2012). LB medium, which is a more complex medium than R2A, provides higher amounts of glycine for the metabolic conversion into hydrogen cyanide. In many other studies (Alström and Burns, 1989; Kremer and Souissi, 2001; Ramette et al., 2003), King's B agar was used as single culture medium. This medium is generally used for the nonselective isolation, cultivation and (fluorescent) pigment production of *Pseudomonas* species (Atlas and Parks, 1993). In our tests, we did not utilize King's B agar for two reasons. First, we did not have all the necessary compounds. We substituted the lacking proteose peptone with tryptone (modified King's B medium), but still cultivation on LB medium yielded darker coloring of the filter papers. Second, we did not want to discriminate potential hydrogen cyanide-producers from outside the genus *Pseudomonas*. As for the culture medium, we suggest to rely on the medium on which the bacteria have been isolated. In addition, we have to point out that this standard test only allows to screen for cyanogenic bacteria, but not to analyze the amount of HCN produced. Differences in the coloring of the initially yellow filter paper (from light orange to dark reddish-brown) are the result of the hydrogen cyanide production ability as well as of the cell density and the time of incubation.

4.1.3.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacteria

All isolates leading to a positive reaction with the newly developed high-throughput test could also be confirmed to produce hydrogen cyanide with the above mentioned standard test. On the other hand, acyanogenic isolates did not induce a positive reaction with either of the two tests. Both functional screening methods seem to be equally valuable in terms of reliability. The high-throughput test offers several advantages, including of course the higher number of isolates to be tested with one plate and filter paper (up to 96 isolates depending on the degree of replication and the integration of a negative control), a faster optimum detection (after 24 instead of 48 hours) and a higher sensitivity due to the smaller air volume above the bacterial colonies, and the use of a dry instead of a wetted filter paper. In screenings for hydrogen cyanide-producing bacteria, we therefore recommend to replace the standard test with our new test. A very similar high-throughput test using the same filter papers and microtiter plates has been described by Takos et al. (2010) to detect cyanogenic plants. Our newly developed test can be seen as modification of this method. A test presented by Castric and Castric (1983) already allowed the screening of multiple isolates on one Petri dish. HCN detection was also based on the same filter papers, as described by (Feigl and Anger, 1966). To allow the parallel testing, Castric and Castric (1983) suggested a bi-layer agar system with pits in the upper layer. These depressions were created with a sterile cork borer barrel to guarantee equal dimensions (up to 50 per plate). The bacterial isolates to be tested were then streaked into the pits and production of hydrogen cyanide was determined on the filter paper fixed to the Petri dish lid. This system appears to have several disadvantages in comparison to our newly developed high-throughput test. First, the preparation of the culture plate is much more cumbersome. Second, the pits are not strongly separated like the wells of a microtiter plate, allowing motile strains to move between pits. This could lead to misinterpretations. Third, reactions on the filter paper are not as accurate as all tested strains share a common air volume and the spots on the filter paper might not always be restricted only to the area above the according pit. Fourth, on a round Petri dish it is much harder to link the spot on the filter paper to the pit containing the responsible HCN producer (we observed similar problems with our high-throughput test for metagenomic libraries). Other than on the

rectangular microtiter plate, one or several position signs are obligatory to guarantee correct linkage. Taking all these considerations into account, we also suggest to use our newly developed test in microtiter plates instead of the test described by Castric and Castric (1983).

Again, it has to be mentioned that both of the tests used for the practical work of this master's thesis are qualitative assays. This means that these tests allow the detection of HCN-producing bacteria, but not a quantification of the hydrogen cyanide released. Different intensities of color change cannot be interpreted as varying potential to produce and release HCN, because the cell density streaked onto the culture medium was not fixed to a certain value for any of the bacterial strains tested. Nevertheless, we could observe a darker coloring of the filter papers in both tests if LB Agar medium with supplemented glycine was used instead of R2A Agar medium with supplemented glycine. This was true for all isolates, as already mentioned above. We conclude that the growth medium has a large influence on the HCN production of bacteria. Therefore, we have to point out that results of any quantitative measurement of hydrogen cyanide produced by bacteria can only be true for a specific growth medium, a certain (initial) cell density, and an exact duration and temperature of the growing period. Such a quantitative measurement of the hydrogen cyanide produced by bacteria could be assessed as described by Alström and Burns (1989), Askeland and Morrison (1983), Kremer and Souissi (2001) or Zlosnik and Williams (2004). Unfortunately, these studies were using different references for the production of HCN (e.g. mg HCN/L medium, µmoles HCN/number of viable cells, nmoles HCN/mg cellular protein, etc.). This discrepancy makes a comparison of the production ability of cyanogenic bacteria rather difficult.

4.1.4.) Detection of hydrogen cyanide-producing bacteria with a PCR-based molecular assay

In pre-tests for our PCR-based molecular assay for the detection of hydrogen cyanide-producing bacteria, four pairs of primers had been tested with the cyanogenic isolate KS28 and the acyanogenic isolate KS15 (results not shown). A temperature-gradient PCR set-up was used to identify the optimum annealing temperature. For the HCNC primers, this temperature was found to be 58°C, as already mentioned above. All other three primer pairs failed to distinguish clearly between the two differing isolates with our PCR set-up. Hence, they were not used

for the PCR assay. Two of these three primer pairs were specific primers which had previously been published by Ramette et al. (2003) (primer pairs ACa and ACb for *hcnBC*, and H19a and H9b for *hcnAB*). The third set of primers (degenerated primers) had been designed by Gajender Aleti, in the same fashion as the HCNC primers, but for the *hcnB* gene. With the HCNC primers, we always obtained a single product for HCN-producing isolates, whereas non-producers did not yield an amplicon in any of the cases. Ramette et al. (2003) reported the same for the primers ACa and ACb, while the primers H19a and H9b were identified as being either nonspecific or specific for only a few strains. It might be that the slightly differing PCR mastermix composition and PCR conditions are the reason why especially the ACa and ACb primers did not work well in our case. For the future it might be of interest to use these primers instead of the HCNC primers, because of the additional amount of work with our degenerated primers (cloning, M13 PCR). Another set of specific primers for the HCN synthase was published by Svercel et al. (2007) (primers PM2 and PM7-26R for *hcnAB*). Together with the ACa and ACb primers, these primers could be used to check strains for the presence of all three genes of the HCN synthase (*hcnABC*). Although the amplification process with degenerated primers is much more time-consuming and labor-intensive, it can also be of advantage. Due to the degeneracy of the primers, it might be possible to detect a wider range of cyanogenic bacteria with slight modifications on the nucleotide level.

For the characterization of hydrogen cyanide-producing bacteria, it is advisable to perform a combination of functional and PCR-based molecular assays. Even though the PCR set-up described in the master's thesis at hand works well, it cannot be excluded that misinterpretations occur without confirmation via functional methods. On the one hand, it is possible to detect strains which harbor (part of) the HCN synthase genes (in our case only the *hcnC* gene), but not a complete or functioning operon. This might be helpful to gain insight into the bacterial hydrogen cyanide production, but such strains are not useful for biocontrol. On the other hand, a PCR-based molecular assay only allows to detect bacterial strains which contain the genes for which the primers have been designed. This constraint limits the possibility to find novel genes responsible for bacterial HCN synthesis. In our tests, all isolates leading to a positive reaction with the two functional screening methods (standard test and high-thoughput test) also yielded a similar amplicon. All (HCNC) PCR products showed highest similarity to database entries of the hydrogen cyanide synthase

subunit HcnC of *Pseudomonas* species. These findings suggest that at least the *hcnC* gene is highly conserved among our isolates and cyanogenic *Pseudomonas* species.

4.1.5.) Phylogenetic analysis of hydrogen cyanide-producing bacteria with PCR-based molecular assays

The prokaryotic 16S and 23S ribosomal RNA (rRNA) genes have been used for phylogenetic classification for more than 25 years now (Olsen et al., 1986; Woese, 1987). Ribosomal RNAs are among the most highly conserved cellular molecules due to their essential role in protein translation. Nevertheless, their sequences provide enough variability to allow distinction of more or less related organisms or organism groups (Devereux and Willis, 1995). Together with the 16S and 23S rRNA genes, also the intergenic spacer region (16S-23S IGS) between these two genes has been widely used for phylogenetic characterization. Other than the rRNA genes themselves, the IGS region shows a high degree of sequence, length and frequency variation. Hence, the 16S-23S IGS region is especially used to distinguish closely related organisms (e.g. different strains of the same species) (Massol-Deya et al., 1995). In our case, database comparisons for the 16S PCR sequences only yielded phylogenetic information on a genus level (*Pseudomonas* spp.). Apparently, the genes encoding for 16S rRNA are too much conserved among *Pseudomonas* species to allow reliable distinction on a species level. Further analysis based on 16S-23S IGS PCR indicated that the cyanogenic isolates described in the master's thesis at hand most likely belong to eight different strains. Unfortunately, an exact phylogenetic characterization on a species or even strain level has to remain incomplete.

Finally, we have to point out again that all cyanogenic isolates were found to be most likely *Pseudomonas* strains. Taken together with the fact, that the presence of the *hcnC* gene could be confirmed for the same isolates, these results support previous studies (Loper and Gross, 2007; Ramette et al., 2003; Ryall et al., 2009) in that the hydrogen cyanide synthase genes (or at least the *hcnC* gene) are highly conserved among *Pseudomonas* species.

4.1.6.) Testing the influence of hydrogen cyanide-producing bacteria on the germination of lettuce (*Lactuca sativa* L. var. *capitata*)

Hydrogen cyanide-producing bacteria were shown to be potential biocontrol agents active against plants, fungi, other bacteria, nematodes, and arthropods (see section introduction for further details). In our preliminary studies, we were only focusing on the influence of cyanogenic bacteria on the germination of the model plant lettuce (*Lactuca sativa* L. var. *capitata*). Further research about the effect on other trophic levels still has to be carried out for the isolates presented in the master's thesis at hand. In a bioassay on Petri dishes, all of the cyanogenic isolates tested failed to inhibit the germination of lettuce. On the contrary, treatments with six different isolates (i.e. CdR-d8, CdR-e6, 2BSE4, 2BSD14, 2BSE9, and 2BSE5; isolated from either rhizosphere of *Cardaria draba* (L.) Desv. or *Vitis vinifera* L.) led to a significant increase of the length of the lettuce seedling radicles while treatment with isolate CdR-d6 (isolated from rhizosphere of *Cardaria draba* (L.) Desv.) improved lettuce seedling weight. Again, it has to be mentioned that most probably nine different strains had been tested according to phylogenetic analysis (instead of sixteen strains, as assumed). Out of the seven isolates, which had a significant influence on the germination of lettuce, only two (i.e. 2BSE5 and 2BSE9) most probably are the same strain (based on comparisons for 16S-23S IGS PCR sequence data).

So far, very contrasting results for the influence of cyanogenic bacteria on plant growth and development have been reported. Bakker and Schippers (1987) hypothesized that certain hydrogen cyanide-producing *Pseudomonas* strains might be responsible for the reduction of potato (*Solanum tuberosum* L.) yield in short rotations via depressing root cell energy metabolism (blocked ATP formation by inhibiting the cellular respiration). Kremer and Souissi (2001) showed that cyanogenic *Pseudomonas* strains are able to significantly inhibit the seedling growth of lettuce (*Lactuca sativa* L.) and barnyardgrass (*Echinochloa crus-galli* L.). This inhibition could be observed with bacteria directly in contact with the roots and even more so if the volatile metabolites of these bacteria were tested. The authors concluded that hydrogen cyanide produced by bacteria directly in contact with the plant roots might be more easily and rapidly degraded or deactivated than volatile HCN. In seedling bioassays using standard concentrations of potassium cyanide (KCN), Kremer and Souissi (2001) observed varying degrees of root growth retardation for different plant

species. While root growth of lettuce (*Lactuca sativa* L.), barnyardgrass (*Echinochloa crus-galli* L.), and green foxtail (*Setaria viridis* (L.) P. Beauv.) was already significantly reduced at concentrations of 12,5 to 100 µmoles, the same was true for field bindweed (*Convolvulus arvensis* L.) only at concentrations of 60 µmoles or higher. Apparently, susceptibility for hydrogen cyanide poisoning varies with the plant species. The same has been demonstrated by Zeller et al. (2007) for barnyardgrass (*Echinochloa crus-galli* L.), hedge bedstraw (*Galium mollugo* L.), and carrot (*Daucus carota* L. cv. Nantaiser II) (high susceptibility) in contrast to wheat (*Triticum aestivum* L. cv. Arina), wall barley (*Hordeum murinum* L.), and brown knapweed (*Centaurea jacea* L.) (significantly lower susceptibility). To our knowledge, it is not yet known whether different varieties of the same species respond to hydrogen cyanide unequally. Anyhow, bacterial biocontrol of weeds seems to be more profitable for susceptible plant species. In experiments with the above mentioned six plant species and eight rhizobacterial strains (four of which were cyanogenic), Zeller et al. (2007) could not detect a significant influence of the bacteria on the shoot height or aboveground biomass of the plants on average when all combinations were analyzed together. Yet, Zeller et al. (2007) found the interaction term to be highly significant, meaning that certain plant species combined with certain rhizobacteria differed in their reaction from each other. The authors found a reduction of 95 % of the aboveground biomass in comparison to the untreated plants for one combination (*Echinochloa crus-galli* L. with the cyanogenic *Pseudomonas* strain G11 isolated from *Galium mollugo* L.). Such host-plant selectivity and host-specific interactions have already vastly been described for rhizobacteria (Åström and Gerhardson, 1988; Harris and Stahlman, 1996; Kennedy et al., 1991; Li and Kremer, 2006; Schippers et al., 1987) and also for cyanogenic bacteria (Flores-Vargas and O'Hara, 2006; Kremer and Souissi, 2001; Owen and Zdor, 2001). Flores-Vargas and O'Hara (2006) reported of a cyanogenic *Pseudomonas fluorescens* strain which impeded the growth of wild radish (*Raphanus raphanistrum* L.), but had no adverse effects on common grapevine (*Vitis vinifera* L.) or subterranean clover (*Trifolium subterraneum* L.). Similar findings were presented by Owen and Zdor (2001) for velvetleaf (*Abutilon theophrasti* Medik.) and corn (*Zea mays* subsp. *mays* L.), where only velvetleaf was found to be inhibited by hydrogen cyanide-producing bacteria. These examples of selectivity hold great promise for the development of weed-specific biocontrol with HCN-producing bacteria. In a study with *Arabidopsis thaliana* Col-0, Rudrappa et al.

(2008) could provide evidence that root growth retardation was caused by cyanogenesis of *Pseudomonas* strains due to the suppression of auxin signaling and/or perception at the root tip. Whether the negative effect on the root growth is caused by a cyanide-mediated suppression of auxin induced gene expression directly or if the altered auxin biosynthesis is a consequence of the impeded cellular respiration, still has to be unraveled. In further experiments, Rudrappa et al. (2008) confirmed the role of hydrogen cyanide as multi-host virulence factor. Besides the above mentioned root growth inhibition of *A. thaliana*, cyanogenesis was also responsible for suppressing *Arabidopsis* root colonization and biofilm formation by the plant growth-promoting bacterium *Bacillus subtilis* (Rudrappa et al., 2008). However, how these multitrophic interactions vary under non-sterile conditions in natural soil are still unclear. Suppressive effects of deleterious rhizobacteria might be compensated by concurring plant growth-promoting microorganisms or environmental influences (Li and Kremer, 2006). The contradictory influence of cyanogenic bacteria on plant growth was best demonstrated by Alström and Burns (1989). Out of two hydrogen cyanide-producing *Pseudomonas fluorescens* strains, one (strain 5241) was found to be an inhibitor while the other (strain S97) was found to be a promoter of lettuce (*Lactuca sativa* L. var. *sativa* cv. Montana or Market Favourite) and common bean (*Phaseolus vulgaris* cv. Bonita) growth. Antoun et al. (1998) also reported direct positive as well as negative influence of cyanogenic strains of *Rhizobium leguminosarum* on wild radish (*Raphanus sativus* L.). Many other researchers attributed hydrogen cyanide-producing bacteria plant growth-promoting activity, but all of these studies were focusing on pathosystems, including the host plant, a pathogen, and one or several hydrogen cyanide-producing bacteria. Plant protection and disease suppression by cyanogenic bacteria were shown exemplarily for the pathosystems tobacco-*Thielaviopsis basicola* (Laville et al., 1998; Voisard et al., 1989), wheat-*Septoria tritici* and wheat-*Puccinia recondita* f. sp. *tritici* (Flaishman et al., 1996), cucumber-*Pythium ultimum* and tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* (Ramette et al., 2003; Rezzonico et al., 2007; Sharifi-Tehrani et al., 1998). The exact interactions in these systems are still not fully understood, but direct antagonism and the induction of plant defense mechanisms might be involved (Voisard et al., 1989). For our experiments, we were not intentionally using pathogens and could not observe fungal growth or development on any of the test plates. Anyway, we have to point out that although hydrogen

cyanide-producing bacteria had been tested in all of the above mentioned studies as well as in ours, the biocontrol effect of these bacteria cannot be solely attributed to HCN, as other toxic or stimulating compounds might be produced as well. For instance, the concomitant production of hydrogen cyanide and the antibiotic 2,4-diacetylphloroglucinol (Phl) was shown to be involved in biocontrol of soil-borne fungal plant diseases (Rezzonico et al., 2007; Sharifi-Tehrani et al., 1998).

As for the set-up of our lettuce seedling bioassay with the cyanogenic *Pseudomonas* isolates, several critical points could be identified. These points should be reconsidered, leading probably to adaptations for future tests. First of all, we did not seal the test plates with Parafilm® in contrast to previous studies (Alström and Burns, 1989; Kremer and Souissi, 2001; Zeller et al., 2007). Therefore, we could not prevent a natural gas exchange and the produced HCN might have been diluted to a concentration where it was no longer inhibitory to plant growth. Furthermore, oxygen concentration might have been too high to induce *anr* expression regulating cyanogenesis (Laville et al., 1998). The use of smaller Petri dishes might also help to reduce excessive dilution. Due to the direct contact of the bacteria with the plant roots, additional degradation processes might have been involved, as already mentioned above. Hence, testing the volatile metabolites of the same isolates could lead to differing results. For this purpose, a paired-plate assembly could be performed as described by Alström and Burns (1989) or Kremer and Souissi (2001). In principle, cyanogenic bacteria are grown on a Petri dish containing culture medium while plant seeds or seedlings are placed on a second Petri dish containing water agar. The two plates are then put together and sealed with Parafilm®. Similar tests could be performed using compartment plates which allow the separate growth of different organisms on the same Petri dish with the help of a barrier in between compartments (Rudrappa et al., 2008). Another advantage of the paired-plate assemblies or of compartment plates is the fact that specific media could be used for either bacteria or plants and that bacteria could be pre-cultured accordingly. In our tests, we only used water agar without additional nutrients. Without providing exogenous glycine and iron, production of hydrogen cyanide might only have occurred on a low level if at all. We cannot tell whether lettuce root exudates contained enough of these crucial compounds to enhance cyanogenesis (Schippers et al., 1987). On the other hand, a combined test with direct contact between plants and bacteria might be useful to detect if the cyanogenic bacteria are able to colonize

or even enter the roots of the plant of interest. So far, we did not investigate the proliferation of our test isolates on lettuce roots. In the future, it would be interesting to test other plant species than lettuce due to the fact that lettuce seeds germinated rapidly (in general, about 90 % of seeds had germinated after 24 hours). Hence, bacterial establishment on plant roots and cyanogenesis might not have been fast enough to adversely affect root growth (conditions in the plant culturing room might not be ideal for bacterial proliferation and HCN synthesis). In contrast to our experiments, where we immersed sterile seeds into a bacterial suspension, other studies were based on the use of pre-germinated seedlings inoculated with a bacterial solution (Kremer and Souissi, 2001; Rudrappa et al., 2008; Zeller et al., 2007). We cannot assess to which extent this change in methods led to the contrasting results observed. Yet, bacterial establishment may be easier on roots than on seed coats and root cells may be more easily harmed by hydrogen cyanide than seed coat cells, though we cannot proof this hypothesis. For future tests for the biocontrol ability of the cyanogenic isolates used in this master's thesis, we recommend to perform quantitative assays to allow estimation of the potential of bacterial cyanogenesis. In addition, a chemical control (e.g. with potassium cyanide) could be implied in the test set-up to check at which concentrations of HCN (or KCN) inhibition of plant growth arises. We did neither include quantitative assays nor a chemical control in our experiments, as previous studies (Alström and Burns, 1989; Kremer and Souissi, 2001) clearly showed that lettuce seedling growth could be reduced by cyanogenic bacteria. Yet, taking the varying cyanogenesis potential of different bacteria into account (Gilchrist et al., 2011; Kremer and Souissi, 2001; Rudrappa et al., 2008), an implementation of the above mentioned tests (i.e. quantitative assay, chemical control) in biocontrol assays with hydrogen cyanide-producing bacteria seem to be highly favorable. Modifications of the optical density of the bacterial suspensions and of the inoculation period could also be of interest for future studies.

Before any of the herein presented cyanogenic bacteria could be utilized as biocontrol agents, further tests to characterize the isolates (e.g. production of antibiotics or phytohormones, phylogenetic classification, etc.) have to be carried out. Future research should also address other plant species or even pathosystems to determine the degree of host-specificity and the best suited field of application.

4.2.) Testing a metagenomic library

4.2.1.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacterial clones

For the master's thesis at hand, we decided in favor of a function-based approach to screen for hydrogen cyanide-producing clones of a metagenomic library. We did not rely on a sequence-based strategy, so that we could possibly detect new genes and mechanisms involved in HCN production. To our knowledge, this is the first report of an activity-based detection system for cyanogenic clones of a metagenomic library. As mentioned above, we could provide the proof of principle for our test set-up with the cyanogenic *Pseudomonas* isolate KS28. Unfortunately, we did not discover any clone producing hydrogen cyanide among the 110.000 clones tested from the Peru Rhizosphere Library with cosmid pJC8. Yet, our functional screening system seems to be sensitive enough as isolate KS28 could always be easily and rapidly detected.

Several reasons could be considered as relevant for the lacking detection of cyanogenic clones. First, there might be no fully functional HCN operon present in any of the inserts of this library. Yet, we cannot tell if this was true as we did only partially screen the Peru Rhizosphere metagenomic library in one host (*Escherichia coli*). Out of the 540.500 clones in total, we tested approximately 110.000 clones. Besides, the clones investigated were derived from a pool of the library and we performed four separate test runs. Hence, it might be very likely that several clones were screened more than once and that the total number of individual clones tested was indeed smaller. While in some screens using cosmid or fosmid vectors, a single positive clone could be identified among a few hundred or thousand clones (Meilleur et al., 2009; Voget et al., 2006), several ten or even hundred thousand clones had to be tested in many other studies (Chung et al., 2008; Heath et al., 2009; Lee et al., 2006; Pang et al., 2009). Therefore, it might be possible that we simply did not screen enough clones. In general, a high number of clones has to be tested for function-based analysis, as gene expression and protein formation in a foreign host might not always work due to incompatibility problems of the vector-host system and the insert DNA (Simon and Daniel, 2009; Uchiyama and Miyazaki, 2009). In a heterologous system, a limited function or no function at all might be found for each of the steps involved in protein biosynthesis and subsequent HCN production (i.e.

transcription, translation, protein folding, HCN synthesis and excretion) (Ekkers et al., 2012; Streit and Schmitz, 2004; Uchiyama and Miyazaki, 2009). Gabor et al. (2004) estimated on a theoretical basis that only about 40 % of randomly cloned foreign DNA might be expressed accordingly in *Escherichia coli*. For genes of the cyanogenic strain *Pseudomonas aeruginosa* PAO1 (Blumer and Haas, 2000b; Rudrappa et al., 2008), Warren et al. (2008) observed only reduced expression in *E. coli* (to which extend this also affected the genes encoding for HCN synthesis is unclear). Although Laville et al. (1998) demonstrated that *E. coli* is able to produce hydrogen cyanide upon transformation with *hcnABC* genes derived from *Pseudomonas fluorescens* CHA0, the same must not necessarily be true for *E. coli* and random metagenomic DNA encoding for HCN production. In addition, hydrogen cyanide-degrading enzymes have been reported for *E. coli* (Volini and Alexander, 1981). Therefore, any potentially produced hydrogen cyanide might be degraded intracellularly to prevent autointoxication. For future tests, it might hence be advantageous to use acyanogenic strains of otherwise cyanogenic species as host organisms. Such strains might be found for *Pseudomonas aeruginosa*, *P. putida* or *Rhizobium leguminosarum*, which all have been used for the construction of metagenomic libraries so far (Craig et al., 2010; Li et al., 2005; Martinez et al., 2004; Ono et al., 2007; Schipper et al., 2009). The broad-host range vector pJC8 has already been utilized for transformation of *Pseudomonas putida* and subsequent metagenomic library screening (Cheng et al., 2014). The same process might lead to detection of cyanogenic clones with the Peru Rhizosphere Library.

For future function-based screenings for hydrogen cyanide-producing clones of a metagenomic library, we recommend to adapt our test set-up insofar that Petri dishes with reduced height are used. This modification would allow to save material (culture medium) and, hence, presents an economically interesting alternative.

Although no cyanogenic clones could be detected with the presented functional assay, seven clones which produced an unknown volatile compound were discovered. Results for tests carried out to identify this compound will be discussed in the following chapter.

4.2.2.) Identification of the unknown volatile compound discovered with the high-throughput functional screening method for hydrogen cyanide-producing clones

Two observations led to the conclusion that the compound causing a brown spot on the filter papers above seven colonies is volatile: Firstly, the specific test design where no direct contact between clone colonies and the filter paper was established. Secondly, the occurrence of a distinct smell if the seven clones of interest were growing on culture plates. The volatile compound was characterized to be definitely not hydrogen cyanide by using the optimized standard test described above (*Test 1*). A test without antibiotic (*Test 2*) indicated that tetracycline was not involved in the reaction on the filter paper. Growth of the same clones on medium containing once again antibiotic showed that the clones would not lose the cosmid after a short growth period on medium without selective reagent (important for *Test 3*). In an assay using the cyanogenic isolate KS28 and one or two of the seven clones (*Test 3*), filter papers turned blue and brown. In overlapping zones, both colors could be detected. We therefore hypothesized that hydrogen cyanide and the unknown volatile compound might react with the chemicals on the filter paper in different ways. Further evidence for this theory was found in testing the chemicals used for filter paper impregnation (*Test 4*). In tests with filter papers without chemicals or with filter papers impregnated with either one of the two chemicals (copper(II)ethylacetotacetate or 4,4'-dimethylenebis-(N,N-dimethylaniline)), all seven clones only caused a color change on filter papers with copper(II)ethylacetotacetate as reaction partner, as described above. These results demonstrated three important characteristics of the unknown volatile compound: First, the compound could not have been detected without these specific chemicals on the filter paper. Second, the volatile compound itself is not of brown color. Third, the volatile compound definitely reacts with copper(II)ethylacetotacetate to produce a brown substance without influence of 4,4'-dimethylenebis-(N,N-dimethylaniline). This is in contrast to the reaction responsible for hydrogen cyanide detection: The blue coloring indicating the presence of HCN results from an oxidation of 4,4'-dimethylenebis-(N,N-dimethylaniline), but only if also copper(II)ethylacetotacetate is present (Feigl and Anger, 1966). The exact reaction can be seen in table 35.

Table 35: Reaction involved in hydrogen cyanide detection based on Feigl-Anger chemistry. $CuAc_2$ = Copper(II)ethylacetotacetate, $C_{17}H_{22}N_2$ = 4,4'-dimethylenebis-(N,N-dimethylaniline), HCN = hydrogen cyanide (Feigl and Anger, 1966).

$2\ CuAc_2 + C_{17}H_{22}N_2 + 2\ HCN \rightarrow 2\ CuCN + [C_{17}H_{21}N_2]Ac + 3\ HAc$

In our case, a reduction of the copper(II) to copper(I) and subsequent formation of copper(I)oxide (Cu_2O), which is of brownish red color, might be the cause for the brown color change (Budavari et al., 1996; Greenwood and Earnshaw, 1984). Hence, the volatile compound could have undergone a redox reaction itself. One of the dehydrogenases (oxidoreductases) identified by database comparison and genome annotation of the contigs derived from Illumina sequencing could be involved in this reaction (see below). Another reddish-brown copper compound would be copper(II)hexacyanoferrate ($Cu_2Fe(CN)_6$) (Budavari et al., 1996). Yet, it seems rather unlikely that a reaction with the unknown volatile led to formation of this compound as the presence of cyanide could not be observed with the optimized standard test for cyanide detection. Further brownish copper compounds can be excluded as they would decompose too easily (e.g. copper(I)hydride, copper(II)azide), change their color under moist conditions (e.g. copper(II)chloride), or the elements involved would most likely not be present in the medium or the bacteria (e.g. copper(II)chromate, copper(I)mercury iodide, copper(II)tungstate) (Perry, 2011). Gas chromatography measurements (*Test 5*) conducted with the help of the Division for Organic Chemistry of the University of Natural Resources and Life Sciences, Vienna, did not help to identify the volatile compound. These findings indicate that the clones used for testing either did not produce enough of the volatile compound or that the compound itself is too volatile (low molecular weight) to be detected with the standard equipment used. Anyway, further optimized gas chromatography measurements should be carried out.

Results from restriction digests (*Test 6*) and Sanger sequencing (*Test 7*) for all seven clones suggest that the seven clones are in fact most probably three different clones (or contain three different inserts): Clones 1 and 27 are most likely the copy of the same clone. The same is true for clones 5, 11, 25, and 36. Only clone 31 differs from all other clones. The relatively poor quality of some of the sequences obtained from Sanger sequencing might have originated from an overestimation of the content of cosmid DNA based on NanoDrop® ND-1000 Spectrophotometer measurements.

While PicoGreen® measurements only quantify dsDNA, the NanoDrop® analysis includes all nucleic acids. Therefore, the high values of nucleic acids detected with the NanoDrop® ND-1000 Spectrophotometer might result from a mixture of digested RNA, DNA from the cosmid and insert, and DNA from *E. coli*. The presence of *E. coli* DNA impurities in the cosmid DNA extracts was attested by Illumina Sequencing. These findings clearly indicate that the cosmid DNA extraction process could be improved.

Illumina® MiSeq Sequencing confirmed the presence of three distinct inserts for clones 1, 31, and 36. Unfortunately, neither direct database comparison nor genome annotation led to the discovery of identical genes for all three inserts. So far, only genes with related functions could be identified. Among those are transcriptional regulators from the LysR family, several dehydrogenases (oxidoreductases), and genes involved in reactions with aldehydes. Either different nucleotide sequences encode for a similar function or different volatile compounds caused the same reaction on the filter paper. At the present state, the exact gene(s) and the reaction(s) responsible for the brown coloring on the filter papers cannot be identified.

5.) Conclusion

In the master's thesis at hand, several methods for the detection of hydrogen cyanide-producing bacteria have been examined. On a functional basis, a standard test could be optimized and a new high-throughput test could be developed. Together with a herein presented PCR-based molecular assay, the activity-driven high-throughput test represents a powerful tool for fast and reliable detection of cyanogenic bacterial isolates.

The influence of a number of hydrogen cyanide-producing bacterial isolates of the genus *Pseudomonas* on the germination of the model plant lettuce was tested in a bioassay. Treatment with six bacterial isolates caused a significant increase in the length of radicles in comparison to the control treatment while treatment with another isolate led to higher seedling weight. All other isolates did not significantly influence the germination of lettuce. Based on our preliminary studies, further tests have to be carried out to determine the biocontrol potential of the isolates presented in this master's thesis. The seven isolates enhancing lettuce germination could be of interest for experiments with plant pathogens or weeds.

In addition to tests with bacterial isolates, a high-throughput functional screening for cyanogenic clones of a metagenomic library was established. This method might allow the discovery of yet uncharacterized genes for hydrogen cyanide production. Unfortunately, we failed to detect any cyanogenic clones. Nevertheless, for some clones, the production of an unknown volatile compound could be observed. Tests revealed that the compound definitely reacts with a copper complex used for the high-throughput screening. The complete sequences of three distinct inserts responsible for the formation of the unknown volatile could be mapped via Illumina® Sequencing by Synthesis. Up to now, no identical genes and functions could be identified for the three inserts. Encoded proteins with similar functions comprise transcriptional regulators from the LysR family, multiple dehydrogenases (oxidoreductases), and genes involved in reactions with aldehydes. Future investigations will hopefully identify the exact genes accounting for the production of the unknown volatile compound.

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7.) Appendix

Preparation of media and solutions

Ad 2.1.1.) Isolation of bacterial strains from plant material

- 70 % ethanol solution:
  For 1 liter:
  - 700 mL of ethanol absolute (for analysis, Merck KGaA, Darmstadt, DE)
  - 300 mL of Milli-Q water

- 2,5 % sodium hypochlorite solution:
  For 1 liter:
  - 250 mL of 10 % sodium hypochlorite solution (Sigma-Aldrich, St. Louis, MO, USA)
  - 750 mL of Milli-Q water

- 0,85 % sodium chloride solution:
  For 1 liter:
  - 8,5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- R2A Agar medium:
  For 1 liter:
  - 18,2 g of Difco™ R2A Agar powder (Becton, Dickinson and Company, Sparks, MD, USA)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 50 % glycerol solution:
  For 1 liter:
  - 500 mL of glycerol (for molecular biology, Sigma-Aldrich, St. Louis, MO, USA)
  - 500 mL of Milli-Q water

  Autoclave at 121°C for 20 minutes.

- R2A Broth:
  For 1 liter:
  - 3 g of R2A Broth powder (LabM Limited, Bury, UK)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 1 x TBE buffer:
  For 1 liter:
  - 100 mL of 10 x TBE buffer (Sigma-Aldrich, St. Louis, MO, USA)
  - 900 mL of Milli-Q water

- Ethidium bromide solution (0,125 mg/mL):
  For 50 mL:
  - 625 µL of 10 mg/mL ethidium bromide solution (Sigma-Aldrich, St. Louis, MO, USA)
  - 49,375 mL of Milli-Q water

Ad 2.1.2.) Optimization of a standard functional screening method for the detection of hydrogen cyanide-producing bacteria

- King's B Agar medium (Atlas and Parks, 1993), modified:
  For 1 liter:
  - 20 g of tryptone (instead of proteose peptone) (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 1,5 g of dipotassium hydrogen phosphate (K_2HPO_4), anhydrous (ultra, Sigma-Aldrich, St. Louis, MO, USA)
  - 1,5 g of heptahydrated magnesium sulfate ($MgSO_4*7H_2O$) (Fluka Analytical, St. Gallen, CH)
  - 10 mL of glycerol (for molecular biology, Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes (autoclave heptahydrated magnesium sulfate ($MgSO_4*7H_2O$) separately and add to rest of medium after cooling down to approximately 60°C).

- Quarter-strength King's B Agar medium (Atlas and Parks, 1993), modified:
  For 1 liter:
  - 5 g of tryptone (instead of proteose peptone) (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 0,375 g of dipotassium hydrogen phosphate (K_2HPO_4), anhydrous (ultra, Sigma-Aldrich, St. Louis, MO, USA)
  - 0,375 g of heptahydrated magnesium sulfate ($MgSO_4*7H_2O$) (Fluka Analytical, St. Gallen, CH)
  - 2,5 mL of glycerol (for molecular biology, Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes (autoclave heptahydrated magnesium sulfate ($MgSO_4*7H_2O$) separately and add to rest of medium after cooling down to approximately 60°C).

- R2A Agar medium:
  For 1 liter:
  - 18,2 g of Difco™ R2A Agar powder (Becton, Dickinson and Company, Sparks, MD, USA)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- LB Agar medium (Atlas and Parks, 1993):
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 10% TSA medium:
  For 1 liter:
  - 3 g of CASO broth (Casein peptone Soybean flour peptone Broth; Fluka Analytical, St. Gallen, CH)
  - 15 g of agar agar
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 1% picric acid solution:
  For 50 mL:
  - 0,5 g of picric acid powder (Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 ml with Milli-Q water

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

- 10 % sodium carbonate solution:
  For 50 mL:
  - 5 g of sodium carbonate (Na_2CO_3)
  - Bring volume to 50 ml with Milli-Q water

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

Ad 2.1.3.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing bacteria

- 1 % copper(II)ethylacetotacetate solution:
  For 50 mL:
  - 0,5 g of copper(II)-ethylacetotacetate powder (97%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

- 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution:
  For 50 mL:
  - 0,5 g of 4,4'-dimethylenebis-(N,N-dimethylaniline) (98%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

- LB Agar medium (Atlas and Parks, 1993) with supplemented glycine:
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- R2A Agar medium with supplemented glycine:
  For 1 liter:
  - 18,2 g of Difco™ R2A Agar powder (Becton, Dickinson and Company, Sparks, MD, USA)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

Ad 2.1.4.) Detection of hydrogen cyanide-producing bacteria with a PCR-based molecular assay

- LB medium (Atlas and Parks, 1993):
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- LB-Ampicillin Agar medium (Atlas and Parks, 1993):
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.
  - Let the medium cool down to 55°C, and then add 1 mL of 100 mg/mL ampicillin solution to get a final concentration of 100 µg ampicillin per mL of medium

- 100 mg/mL ampicillin solution:
  For 1 mL:
  - 100 mg of ampicillin sodium salt (Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 1000 µL with sterile Milli-Q water

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

- 2 % X-gal solution:
  For 1 ml:
  - 20 µg of X-gal powder (Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 1000 µL with dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA)

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

Ad 2.1.6.) Testing the influence of hydrogen cyanide-producing bacteria on the germination of lettuce (*Lactuca sativa *L. var.* capitata*)

- LB medium (Atlas and Parks, 1993):
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 70 % ethanol solution:
  For 1 liter:
  - 700 mL of ethanol absolute (for analysis, Merck KGaA, Darmstadt, DE)
  - 300 mL of Milli-Q water

- 3,5 % sodium hypochlorite with additional 0,05 % Tween-20:
  For 1 liter:
  - 350 mL of 10 % sodium hypochlorite solution (Sigma-Aldrich, St. Louis, MO, USA)
  - 650 mL of Milli-Q water
  - 500 µL of Tween-20 (Bio-Rad, Hercules, CA, USA)

- 20 mM $MgCl_2$ solution:
  For 1 liter:
  - 4,066 g of magnesium chloride hexahydrate (BioReagent, suitable for cell culture, suitable for insect cell culture; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 1,5 % Water Agar medium:
  For 1 liter:
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

Ad 2.2.1.) Development of a high-throughput functional screening method for the detection of hydrogen cyanide-producing clones

- 1,5 % Water Agar medium:
  For 1 liter:
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- LB-Tetracycline Agar medium (Atlas and Parks, 1993) with supplemented glycine and LB Agar medium (Atlas and Parks, 1993) with supplemented glycine:
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

Adjust pH to 7.

- Bring volume to 1000 mL with Milli-Q water

Autoclave at 121°C for 20 minutes.

- Let the medium cool down to 55°C, and then add 1 mL of 20 mg/mL tetracycline solution to get a final concentration of 20 µg tetracycline per mL of medium. For LB Agar medium with supplemented glycine, omit this final step.

- 20 mg/mL tetracycline solution:
  For 1 mL:
  - 20 mg of tetracycline (Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 1000 µL with ethanol absolute (for analysis; Merck KGaA, Darmstadt, DE)

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

- 1 % copper(II)ethylacetotacetate solution:
  For 50 mL:
  - 0,5 g of copper(II)-ethylacetotacetate powder (97%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

- 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution:
  For 50 mL:
  - 0,5 g of 4,4'-dimethylenebis-(N,N-dimethylaniline) (98%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

Ad 3.2.2.) Identification of the unknown volatile compound detected with the high-throughput functional screening method

Test 1 – Testing the clones with the standard functional screening method for the detection of hydrogen cyanide-producing bacteria

- LB-Tetracycline Agar medium (Atlas and Parks, 1993) with supplemented glycine:
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

Autoclave at 121°C for 20 minutes.

- Let the medium cool down to 55°C, and then add 1 mL of 20 mg/mL tetracycline solution to get a final concentration of 20 µg tetracycline per mL of medium.

- 1% picric acid solution:
  For 50 mL:
  - 0,5 g of picric acid powder (Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 ml with Milli-Q water

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

- 10 % sodium carbonate solution:
  For 50 mL:
  - 5 g of sodium carbonate (Na_2CO_3)
  - Bring volume to 50 ml with Milli-Q water

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

Test 2 – Testing the influence of the antibiotic tetracycline on the production of the unknown volatile compound

- 1,5 % Water Agar medium:
  For 1 liter:
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- LB-Tetracycline Agar medium (Atlas and Parks, 1993) with supplemented glycine and LB Agar medium (Atlas and Parks, 1993) with supplemented glycine:
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.
  - Let the medium cool down to 55°C, and then add 1 mL of 20 mg/mL tetracycline solution to get a final concentration of 20 µg tetracycline per mL of medium. For LB Agar medium with supplemented glycine, omit this final step.

- 1 % copper(II)ethylacetotacetate solution:
  For 50 mL:
  - 0,5 g of copper(II)-ethylacetotacetate powder (97%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

- 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution:
  For 50 mL:
  - 0,5 g of 4,4'-dimethylenebis-(N,N-dimethylaniline) (98%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

Test 3 – Co-detection of hydrogen cyanide and the unknown volatile compound

- 1,5 % Water Agar medium:
  For 1 liter:
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- LB-Tetracycline Agar medium (Atlas and Parks, 1993) with supplemented glycine and LB Agar medium (Atlas and Parks, 1993) with supplemented glycine:
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.
  - Let the medium cool down to 55°C, and then add 1 mL of 20 mg/mL tetracycline solution to get a final concentration of 20 µg tetracycline per mL of medium. For LB Agar medium with supplemented glycine, omit this final step.

- 1 % copper(II)ethylacetotacetate solution:
  For 50 mL:
  - 0,5 g of copper(II)-ethylacetotacetate powder (97%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

- 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution:
  For 50 mL:
  - 0,5 g of 4,4'-dimethylenebis-(N,N-dimethylaniline) (98%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

Test 4 – Testing the chemicals used for filter impregnation for their influence on the color reaction on the filter papers

- 1,5 % Water Agar medium:
  For 1 liter:
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- LB-Tetracycline Agar medium (Atlas and Parks, 1993) with supplemented glycine:
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 0,5 g of glycine (bioultra, for molecular biology; Sigma-Aldrich, St. Louis, MO, USA)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.
  - Let the medium cool down to 55°C, and then add 1 mL of 20 mg/mL tetracycline solution to get a final concentration of 20 µg tetracycline per mL of medium.

- 1 % copper(II)ethylacetotacetate solution:
  For 50 mL:
  - 0,5 g of copper(II)-ethylacetotacetate powder (97%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

- 1 % 4,4'-dimethylenebis-(N,N-dimethylaniline) solution:
  For 50 mL:
  - 0,5 g of 4,4'-dimethylenebis-(N,N-dimethylaniline) (98%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with chloroform (ACS reagent; Sigma-Aldrich, St. Louis, MO, USA)

Cosmid DNA isolation

- LB-Tetracycline Agar medium (Atlas and Parks, 1993):
  For 1 liter:
  - 10 g of tryptone (peptone from casein, for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of yeast extract (for microbiology; Merck KGaA, Darmstadt, DE)
  - 5 g of sodium chloride (NaCl) (suitable for use as excipient, Merck KGaA, Darmstadt, DE)
  - 15 g of agar agar (standard grade; GERBU Biotechnik GmbH, Heidelberg, DE)
  - 800 mL of Milli-Q water

  Adjust pH to 7.
  - Bring volume to 1000 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.
  - Let the medium cool down to 55°C, and then add 1 mL of 20 mg/mL tetracycline solution to get a final concentration of 20 µg tetracycline per mL of medium.

- Alkaline Lysis solution 1 (Sambrook and Russell, 2001):
  Final:
  - 50 mM glucose
  - 25 mM Tris-HCl (pH 8)
  - 10 mM EDTA (pH 8)

  For 5 mL:
  - 500 µL of 500 mM glucose solution
  - 125 µL of 1 M Tris-HCl solution (pH 8)
  - 100 µL of 500 mM EDTA solution (pH 8)
  - 4,275 mL of sterile Milli-Q water

  Mix under sterile conditions. Store at 4°C.

- 500 mM glucose solution:
  For 50 mL:
  - 4,504 g of D-(+)-glucose (BioXtra; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with sterile Milli-Q water

  Subsequently, filtersterilize solution with a 0,22 µm filter (Rotilabo®-syringe filter; Carl Roth GmbH und Co. KG, Karlsruhe, DE).

- 1 M Tris-HCl solution (pH 8):
  For 50 mL:
  - 6,057 g of Tris base (ultra-pure; Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA)
  - 40 mL of Milli-Q water

  Adjust pH to 8 with HCl (25 %, for analysis; Merck KGaA, Darmstadt, DE).
  - Bring volume to 50 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 500 mM EDTA solution (pH 8):
  For 50 mL:
  - 9,305 g of EDTA disodium salt dihydrate (ACS reagent, 99.0-101.0%; Sigma-Aldrich, St. Louis, MO, USA)
  - 40 mL of Milli-Q water

  Adjust pH to 8 with NaOH (for analysis; Merck KGaA, Darmstadt, DE) under vigorous stirring on a magnetic stirrer. EDTA disodium salt dehydrate will only dissolve at pH 8.
  - Bring volume to 50 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- Alkaline Lysis solution 2:
  Final:
  - 0,2 N NaOH (freshly diluted from a 10 N NaOH solution)
  - 1 % SDS

  For 10 mL:
  - 200 µL of 10 N NaOH solution
  - 500 µL of 20 % SDS solution
  - 9,3 mL of sterile Milli-Q water

  Mix under sterile conditions. Store at room temperature.

- 10 N NaOH solution:
  For 50 mL:
  - 20 g of NaOH pellets (for analysis; Merck KGaA, Darmstadt, DE)
  - Bring volume to 50 mL with Milli-Q water (beware, highly exothermic reaction, dissolve in plastic beaker)

- 20 % SDS solution:
  For 50 mL:
  - 10 g of SDS (molecular biology grade; Promega Corporation, Madison, WI, USA)
  - 40 mL of Milli-Q water

  Heat to 68°C and stir on a magnetic stirrer to assist dissolution.
  Adjust pH to 7,2.
  - Bring volume to 50 mL with Milli-Q water

- Alkaline Lysis solution 3:
  For 10 mL:
  - 6 mL of 5 M potassium acetate
  - 1,15 mL of glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA)
  - 2,85 mL of sterile Milli-Q water

  Mix under sterile conditions. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store at 4°C.

- 5 M potassium acetate:
  For 50 mL:
  - 24,535 g of potassium acetate (SigmaUltra, minimum 99,0%; Sigma-Aldrich, St. Louis, MO, USA)
  - Bring volume to 50 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

- 70 % ethanol solution:
  For 1 liter:
  - 700 mL of ethanol absolute (for analysis, Merck KGaA, Darmstadt, DE)
  - 300 mL of Milli-Q water

- 3 M sodium acetate solution (pH 5,2):
  For 50 mL:
  - 20,412 g of sodium acetate trihydrate (for analysis, Merck KGaA, Darmstadt, DE)
  - 40 mL of Milli-Q water

  Adjust pH to 5,2 with glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA).
  - Bring volume to 50 mL with Milli-Q water

  Autoclave at 121°C for 20 minutes.

Test 8 – Illumina® MiSeq Sequencing for total insert sequences

- 1 x TE buffer:
  For 48 reactions:
  - 5,225 mL of sterile Milli-Q water
  - 275 µL of 20 x TE buffer (provided by the Quant-iT™ PicoGreen® dsDNA Kit; Molecular Probes™, Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA)

  Keep at ice.

- 1 x Quant-iT™ PicoGreen® dsDNA reagent:
  For 48 reactions:
  - 2,4875 mL of 1 x TE buffer
  - 12,5 µL of 200 x Quant-iT™ PicoGreen® dsDNA reagent (provided by the Quant-iT™ PicoGreen® dsDNA Kit; Molecular Probes™, Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA)

  Keep at room temperature, protected from light.

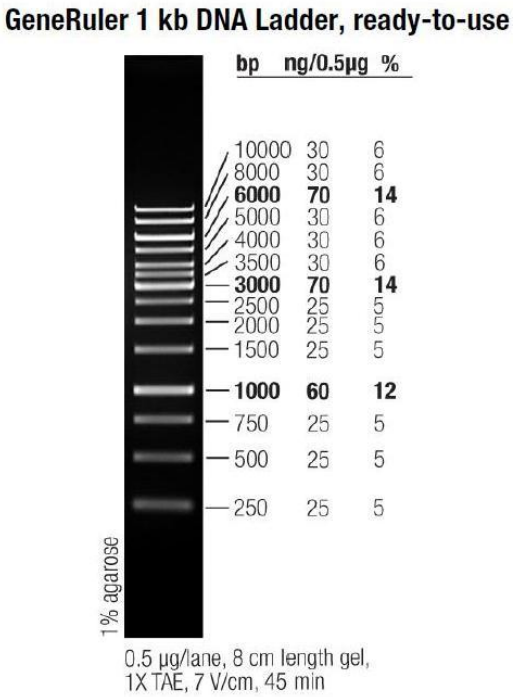
- Dilution series of λ DNA as standards for a calibration curve:
  For a double set-up of the calibration curve:
  - 196 µL of 1 x TE buffer
  - 4 µL of 100 µg/mL λ DNA (provided by the Quant-iT™ PicoGreen® dsDNA Kit; Molecular Probes™, Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA)

  From this 2000 ng/mL stock, prepare a series of 7 1 : 2 dilutions by mixing with 1 x TE buffer (1000, 500, 250, 125, 62,5, 31,25, and 15,625 ng/mL).

Size markers used for agarose gel electrophoresis

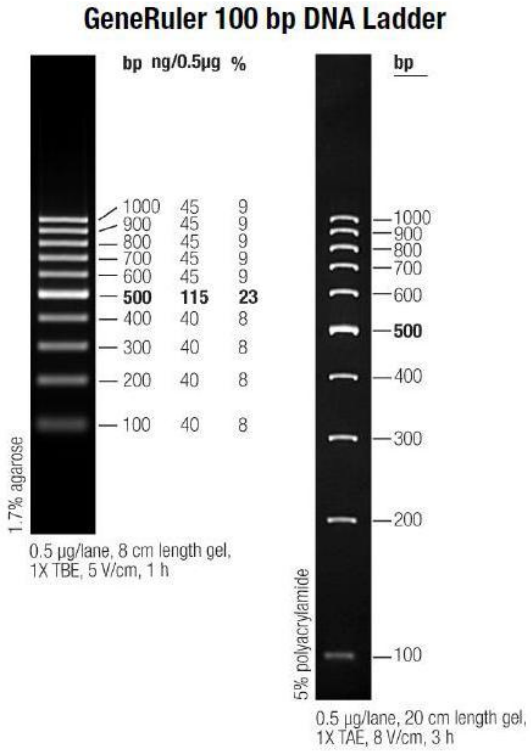
- *GeneRuler 1 kb DNA Ladder, ready-to-use*

  Designed by Thermo Scientific, Waltham, MA, USA



- *GeneRuler 100 bp DNA Ladder*

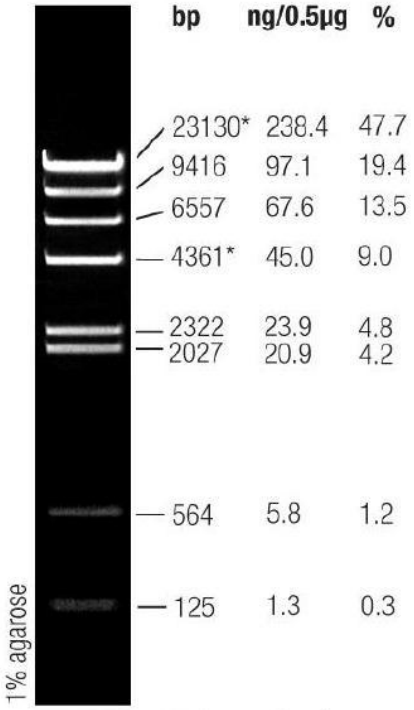
  Designed by Thermo Scientific, Waltham, MA, USA



- Lambda DNA/HindIII Marker, 2, ready-to-use

  Designed by Thermo Scientific, Waltham, MA, USA

Lambda DNA/HindIII Marker, 2, ready-to-use



0.5 µg/lane, 8 cm length gel,
1X TAE, 7 V/cm, 45 min

* The cohesive ends (the 12 nt *cos* site of bacteriophage lambda) of fragments 23130 bp and 4361 bp may anneal and form an additional band at 27491 bp. These fragments can be separated by heating at 65°C for 5 min and then cooling on ice for 3 min.