Biological control of fungal plant pathogens by tomato endosphere bacteria

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Abstract

Yield losses can be the result of abiotic or biotic stressors during the development of crop plants. A large share of these yield losses in a multitude of crop species are caused by fungal plant pathogens. Fungicide-resistance and their endophytic life-style within the host makes traditional chemical control difficult and unsustainable. Using biological control agents, like endophytic bacteria, as a means to combat these pathogens could be viable method to secure future food production on a safe and sustainable way. In this experiment we test the biological control potential of several tomato endosphere derived bacteria. Infection assays with Botrytis cinerea or Verticillium dahliae on gnotophoric Arabidopsis thaliana show that several Bacillus amyloliquefaciens strains and a Bacillus subtilis strain are capable of reducing infection incidence and disease symptoms. Genomic analysis of Bacillus amyloliquefaciens AC35 revealed the presence of several genes clusters related to the production of antibiotic metabolites. Bacillus amyloliquefaciens AC35 is closely related to subspecies plantarum and has several genes for the production of auxins. Making it also a plant growth promoting bacteria, next to being a biological control agent of plant pathogenic fungi.
Introduction

Endophytic bacteria

Bacteria which complete their entire life cycle or part of it inside the plant without causing any disadvantageous side-effects to its host is referred to as endophyte (Hardoim et al., 2008). The symbiosis between plants and beneficial microbes has been a continuous field of research in the last decades. The positive effects which endophytic and rhizospheric bacteria can have on their host plants become more apparent every year. Whilst microbes are often associated with plant diseases, only a small fraction of all plant-related bacteria and fungi are pathogenic (Mei & Flinn, 2010). In general, endophytic bacteria tend to have a favourable relationship for both itself and its host-plant, through plant growth promotion, plant defence induction or the production of antimicrobial metabolites. These microbes reside within plant tissue; both intracellular and intercellular spaces are colonized (Bacon & Hinton, 2006) (Figure 1). Endophytes are of great importance to plants, until this moment they are found in all examined plants, in high abundance and great diversity (Ryan et al., 2008).

Where the term endophyte was first coined for solely endophytic fungi; it nowadays also encompasses endospheric bacteria (Hardoim et al., 2008). Whilst endophytic bacteria are studied less than their fungal counterpart, their importance to plant health and growth is described in the last fifty years. A multitude of endophytic bacterial strains has been uncovered e.g.: Pseudomonas, Enterobacter, Bacillus, Staphylococcus, among others (Ryan et al., 2008).

Bacterial endophytes could potentially be utilised in many fields of fundamental and applied science. These endophytic bacteria can have a varied and highly diverse function inside the plant. They produce a vast array of metabolites, which can positively influence the growth of plants. Growth induction is either directly or indirectly. Direct plant growth can be achieved by the synthesis of growth promoting metabolites, like auxin or ethylene, or by fixation of nitrogen or minerals, or the production of mineral-fixating agents like siderophores (Hardoim et al., 2008), (Shah et al., 1992). A plant can also indirectly be stimulated in growth by the pathogen-suppressing ability of endophytic bacteria, by means of antibiotic production or niche competition with pathogens within the plant (Lodewyckx et al., 2002).

Endophytic bacteria make a multitude of beneficial and possibly useful products. Nearly all studies are executed to increase the knowledge about useful products with the means for sustainable agricultural improvements or phytoremediation of soils (Bacon & Hinton, 2006), (Doty, 2008). Although many mechanisms behind pathogen suppression are unveiled, not all are uncovered and many still lack complete comprehension. Endophytic bacteria are known producers of a multitude of bio-active compounds. Many compounds are described, yet a vast amount are unexplored. These metabolites could have the potential to be harnessed for biological control of fungal and bacterial plant pathogens or as bio-fertilizer for crops.

Figure 1 Visualization of endophyte Pseudomonas sp. Ph6-gfp within plant tissues. Bacterial strain Pseudomonas sp. Ph6 was validated as an endophytic bacteria, via green fluorescence protein. Rye grass (Lolium multiflorum Lam.) is inoculated via root soaking. The bacteria is visually observed by fluorescence microscopy in the roots (D), stems (E) and leaves (F) of the plant; indicating a vertical transfer from roots to shoots (Sun et al., 2014).
**Botrytis cinerea and Verticillium dahliae**

*Botrytis cinerea* is a fungal pathogen with a broad host range of over 200 plant species and known to cause grey mould. This necrotrophic foliar ascomycete is an important causative agent of yield reduction and fruit spoilage throughout the world (Williamson et al., 2007). It causes disease in numerous wild species and cultivated crops. Some estimates even show that *B. cinerea* related crop damage mounts up to over two billion euros per year (Elad et al., 2007). *B. cinerea* strains possess a vast array of secondary metabolites, which enables it to be a potent plant pathogen. Metabolites such as fungal toxins, enzymes that destroy and macerate plant tissue, and hypersensitive response inducing effectors (Williamson et al., 2007). Grey mould is heavily controlled with fungicides, with fungicidal resistance as major drawback. The proportion of multi-fungicide-resistant *B. cinerea* isolates increases. Changes in agricultural practices can reduce the risk of spreading and infections. An alternative solution to chemical control of *B. cinerea* would be the use of biological control agents. Known biological control agents of *B. cinerea* are several filamentous fungi; *Trichoderma*, *Gliocladium* and *Ulocladium*, and a number of bacterial strains; *Bacillus* and *Pseudomonas* (Elad et al., 2007).

The soilborne fungal pathogen *Verticillium dahliae* is one of the causative agents responsible for the Verticillium wilting disease in a broad spectrum of plants. Over 250 species of herbaceous and woody, predominantly dicot, plant species are susceptible to this vascular pathogen. This includes a large amount of cash crops. The pathogen penetrates its host root system and migrates systemically through the xylem vessels. *V. dahliae* is therefore difficult to combat; residing in the vascular system makes it insensitive to traditional means of chemical control. After systemic colonisation of the host, the fungus creates microsclerotia; resting structures which are able to survive over 10 years in the soil. Crop-rotation and the usage of resistant plant varieties could reduce disease severity and incidence (Xiao et al., 1998), (López-Escudero et al., 2004).

Both *B. cinerea* and *V. dahliae* severely damage a broad spectrum of crop plants, reducing yields and production. Using biological control agents as a means to combat these pathogens could be viable method to secure future food production on a safe and sustainable way.

**Biological control by endophytic bacteria**

Nature-based bio pesticides and the use of biological control agents is a growing market worldwide. In 2000, the market share of bio pesticides was 0.2% of all pesticides used. This share increased to 2.5% in just six years. Bio pesticides are often part of an integrated pest management system (IPM), instead of used individually (Thakore, 2006). Endophytes are a relative young addition to the arsenal of bio pesticides, but these new additions show promising and exciting novel methods of plant protection (Glare et al., 2012).

There is an active uptake of beneficial endophytic bacteria by plants and they survive in niches similar to the pathogenic microbes they combat (Quadt-Hallmann et al., 1997). Endophytic bacteria can also be easily and cheaply added to seeds, seedlings and plantlets; making it a cheap systemic biocontrol agent for the duration of the growth season (Glare et al., 2012). These are just two reasons why endophytic bacteria are suitable as biological control agents. Interest in plant-related bacteria is increasing as a novel and ecologically sustainable alternative to chemical pesticides and traditional antibiotics.

Numerous cases of biological control of plant pathogens with endophytic bacteria are proven to be effective and efficient in cash crops (Ramesh et al., 2008), (Nejad & Johnson, 2000). Biocontrol by endophytes is predominantly the result of induced resistance, yet very little is known about direct antagonism. This direct antagonism can be characterised by antibiosis, competition and lysis of fungal pathogens (Berg & Hallmann, 2006). Some endophytic bacteria are even useful biocontrol agent against pest like nematodes and insects (Compant et al., 2010), (Kerry, 2000).
Aims of this research

A collection of 122 bacterial endophytes were extracted from the endosphere of *Solanum lycopersicum*. With nearly 82% of the identified species being Gram-positive. The majority, over 44%, showed high levels of similarity with the genera of *Bacillus* based on 16S ribosomal RNA-gene sequencing. *Pseudomonas* are with 10% the most numerous Gram-negative plant-related bacterial species isolated (Figure 2). Some of these isolated bacteria are known to be endophytic, others are described in literature as plant growth promoting (PGPB), and several are unknown in their plant-related function. The antimicrobial effect of these endophytes is tested *in vitro* against several common and aggressive plant pathogens: *Verticillium dahliae, Cladosporium fulvum* and *Phytophthora infestans*. Bacterial strains that showed to contain antimicrobial properties to *Verticillium dahliae* were tested on *Lepidium sativum* in order to examine plant growth promotion and pathogen suppression. Several of these tested bacteria revealed to be both a stimulant of plant growth in *L. sativum* and a suppressor of plant pathogens *in vitro*.

Numerous studies are conducted with endophytic bacteria and their application as biocontrol agents of soilborn and foliar pathogenic fungi (Weller, 1988), (Paulitz & Bélanger, 2001). Nonetheless, knowledge gaps concerning specifically their role and mechanisms in plant defence are still present. This study will further increase the understanding of endophytic bacterial strains and the characteristics of their pathogen suppressing metabolites. We show that plant related bacteria are a valuable source of novel antimicrobial and plant growth promoting metabolites.

These metabolites are screened on biophysical properties. *In planta* tests will be conducted in gnotophoric Arabidopsis thaliana to screen the individual biological control potential of endophytically derived bacterial strains. Gnotophoric *Arabidopsis thaliana* will be tested against foliar fungal pathogen *Botrytis cinerea* and the soilborn pathogen *Verticillium dahliae*. Comparing the genome of two de novo sequenced *Bacillus amyloliquefaciens* strains will enable to us examine genes of interest. Differences between inhibition level or colonisation success can be analysed on a genotypic level.

Several bacteria show a reduction in pathogenic symptoms and induce plant growth, making these bacteria potentially interesting for further research. Although *B. amyloliquefaciens* AC35 and AC116 are similar species, they reveal to be different in metabolic secretion; either in abundance or alteration in metabolic structure. Genomic analysis reveals that *B. amyloliquefaciens* AC35 has several antibiotic gene clusters, with antifungal properties. Results of a gene annotation programme showed that AC35 has genes to produce auxin. Next to being a suppressor of fungal plant pathogens it is a plant growth promoting bacteria.

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**Figure 2.** *Solanum lycopersicum* endosphere derived bacteria communities. The outer ring reveals the percentage of individual bacterial species found, ranked on genus level taxonomy. Where labelling is absent, the percentage is 1%. The inner ring reveals the percentage of gram-positive and gram-negative bacterial species.

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Materials and Methods

Screening and characterisation of antifungal bacteria

**In vitro screening of antifungal properties.**
The 122 *Solanum lycopersicum* derived bacterial strains are tested against the plant pathogens *Cladosporium fulvum*, *Verticillium dahliae* JR2, *Phytophthora infestans* and in this case *B. cinerea* B05.10. Both the inhibition of hyphae growth and the germination of spores of *B. cinerea* by these bacterial strains is determined. Bacterial strains are inoculated with a toothpick on a PDA + 0.5%YE plate, unless stated otherwise. A circular disc of *B. cinerea* mycelia was acquired with a sterilized cork borer (diameter: 7.5 mm) from advancing zone of fresh culture (cultured on a PDA + 0.5%YE plate) and is placed in the centre of the PDA plate. The mycelial plugs do not show any sign of sporulation and are placed mycelial side down, the plates are cultured in the dark at 20°C, for a period of three to four days.

Isolated bacteria were screened for *B. cinerea* inhibiting effect in monoculture (one strain per plate) and multiculture (six strains per plate). Inhibition is shown either through a zone of inhibition were no hyphal growth is present, or the discolouration and stress growth of the fungus. Inhibition is scored as either absent or present and all *B. cinerea* inhibition plates are cultured in the dark at 20°C, for a period of three to four days, unless stated otherwise.

Plates containing PDA(1.2% Agar) + 0.5%YE are imbedded with \(10^4\) spores \(\times\) mL\(^{-1}\) within the agar. These plates are created by distributing 10ml agar in sterile tubes (*Starstedt* Tube 13ml, round base) and cooling the medium down in a 42°C water bath. When cooled properly the spore suspension is added; the plates are air-dried and the bacterial strains are inoculated unto the PDA. The bacterial strains were tested in both multiculture and monoculture.

**Spectrophotometric germination and fungal growth assay.**
The metabolic properties of bacterial strains which showed a positive result in inhibiting one or multiple fungal pathogens were further examined. Bacterial supernatant was obtained by growing bacteria in 50ml tubes (*Starstedt* Tube 50ml, flat/conical base) in 10mL PDB + 0.5%YE medium. The tubes were positioned at an angle of 40° in increase the air/medium interface area and stimulate pellicle formation. The bacteria were grown at 25°C for a minimum of 5 days. The supernatant was extracted by centrifugation of the bacterial colonies at 5520 RCF for a duration of 20 minutes. The supernatant was further sterilised by filtration through a Whatman FP30/0.2 CA-S 0.2µM filter. The supernatant was stored at 4°C till further processing.

Sterile 96-well microplates are used for this antifungal bacterial supernatant assay (0.5 mL volume, *Cellstar* by *Greiner bio-one*). Each well contains a spore suspension of 100µL PDB + 0.5%YE to which \(10^5\) spores \(\times\) mL\(^{-1}\) are added (Broekaert et al., 1990). Each well is augmented with 75µL of the bacterial supernatant, unless stated otherwise. The germination and growth of the fungal hyphae is determined by absorbance level of the well, at a wavelength of 600nm. This optical density is measured at several time points during the experiment with a *Bio-Rad Model 680 series Microplate Reader*. 96-wells plates were stored in the dark at 20°C between measurements, unless stated otherwise.

**Thin layer chromatography (TLC) bioautographic assay.**
The bacterial supernatant of some inhibiting bacterial strains is acquired as described in the spectrophotometric germination assay. This supernatant is drawn over a thin layer chromatography (TLC Silica gel 60 *Merck KGaA*) plate. Drops of several µLs are applied upon TLC plates (20cm length \(\times\) 3cm width) using a sterile Pasteur’s pipette. The liquid phase of the TLC was an ethanol:methanol (1:1) mixture. After chromatography the plates are removed from the liquid phase and air dried. Experiments with TLC plates were executed in quadruplicate at the same time. Two will be used for the bioautography assay, whilst the other two will purpose as donor plates for sample analysis for GC-MS.
Arabidopsis thaliana infection assay.

Growth conditions of Arabidopsis thaliana plantlets.
A. thaliana seeds were surface sterilized by means of vapour-phase sterilization, which uses chlorine gas to sterilize the seeds for up to 4 hours. Columbia-0 seeds were grown in vitro on MS medium (Sarstedt 100×100×20mm Petri dish), 2.5 cm from the top of a square plate, spaced 0.8 from each other and 1.0 cm from the sides of the dish. The Petri dish was sealed with three layers of clear cling-foil. Prior to germination, the seeds are stratified in the dark at a temperature of 4°C for the duration of 5 days. After stratification, the seeded plates were placed at an angle in growth chambers with 12-hour light and 12-hour dark period, 70% humidity, and fluctuating temperature (21°C at day, 19°C during night-time). After 8 days in the growth chambers the roots of the seedlings were inoculated with 2.0 µL OD₆₀₀=0.02 bacterial suspension and air dried. The control was mock inoculated with 2.0 µL PDB + 0.5% YE. The seedlings were transferred to pots (7×7×8cm) of sterile potting soil (Unifarm) 14 days post bacterial inoculation. Growth conditions remained unchanged.

Bacterial strains.
Isolates and bacterial strains used in this experiment are shown in Table 1. Bacterial colonies are incubated for a minimum of 5 days in 13ml round base tubes (Starstedt) with PDB + 0.5% YE at 25°C, the tubes were shaken at an angle. After incubation, the OD was defined and calibrated to OD₆₀₀=0.02 (10⁷ CFUxmL⁻¹). Bacterial strains were isolated from Solanum lycopersicum which originated from Unifarm and an organic farm near Wageningen and characterised by Albane Ruaud and Christina Papazlatani.

Table 1 Bacterial strains used to inoculate Arabidopsis thaliana seedlings

<table>
<thead>
<tr>
<th>Strain¹</th>
<th>Isolation Source²</th>
<th>Pathogen inhibition³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus altitudinis AC09</td>
<td>Stems</td>
<td>V. dahliae JR2</td>
</tr>
<tr>
<td>Bacillus altitudinis AC39</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2</td>
</tr>
<tr>
<td>Bacillus altitudinis AC86</td>
<td>Leaves' vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens AC116</td>
<td>-80°C Fusarium stock</td>
<td>B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens AC35</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens AC59</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens AC60</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus cereus AC01</td>
<td>Stems, leaves</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus flexus AC30</td>
<td>Roots</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus megaterium AC72</td>
<td>Leaves</td>
<td>V. dahliae JR2</td>
</tr>
<tr>
<td>Bacillus sp. AC113</td>
<td>Leaves</td>
<td>V. dahliae JR2</td>
</tr>
<tr>
<td>Bacillus sp. AC71</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus subtilis AC47</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Bacillus subtilis AC95</td>
<td>Leaves, vessels</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
<tr>
<td>Micrococcus luteus AC06</td>
<td>Stems, Leaves</td>
<td>None⁴</td>
</tr>
<tr>
<td>Pseudomonas alcaligenes AC17</td>
<td>Roots</td>
<td>V. dahliae JR2 &amp; B. cinerea B05.10</td>
</tr>
</tbody>
</table>

¹ All strains were identified in a previous study, carried out by Albane Ruaud and Christina Papazlatani.
² Strains were isolated from Solanum lycopersicum from Unifarm and an organic farm near Wageningen.
³ Bacterial strains revealed a inhibiting effect in vitro on fungal pathogens, when co-cultured.
⁴ Micrococcus luteus AC09 shows no inhibition of V. dahliae JR2 or B. cinerea B05.10, but is used as negative control.
Fungal strains.
The two fungal strains used in these experiment are *B. cinerea* B05.10 and *V. dahliae* JR2. Colonies were grown on PDA + 0.5% YE medium, spores were harvested by adding autoclaved MilliQ onto the plate and scraping the spores with a sterile scraper. The MilliQ/spore suspension is sieved through a glass fibres, centrifuged at 1000 rpm for 8 minutes after which the liquid phase is discarded. Spore concentrations were ascertained with a Bürker Türk haemocytometer, and stored at 4°C in autoclaved MilliQ at a concentration of $10.0 \times 10^7$ spores × mL$^{-1}$. Harvested spores are grown and tested for growth and viability, prior to further experiments.

Inoculation and infection of plants with *Verticillium dahliae*.
When *A. thaliana* seedlings are reallocated from the MS-plate to the potting soil, the roots of the plantlets are *in vitro* drenched with a *V. dahliae* JR2 conidial suspension. The roots were infected with 10.0 mL PDB+ 0.5% YE / spore suspension ($10^6$ spores × mL$^{-1}$) for duration of 30 minutes on a horizontal shaking plate. The control seedlings were mock inoculated with 10.0 mL PDB+0.5% YE. The seedlings were potted in potting soil and monitored for signs of infection for 30 days, the plant fresh weight is determined at the end of the experiment.

Inoculation and infection of plants with *Botrytis cinerea*.
*A. thaliana* seedlings are relocated from the MS-plate to the potting soil, 27 days after relocation the plants were inoculated with a *B. cinerea* B05.10 spore suspension. Three leaves of the same size per plant were chosen at random and inoculated with a droplet of 2.0 µL spore suspension ($1.0 \times 10^6$ spores × mL$^{-1}$) suspended in a PDB+0.5% YE medium. Four days after inoculation the lesion size was determined using a digital pair of calipers, the percentage of lesion outgrowth was calculated and the plant freshweight is examined.

Reisolation of endophytic bacteria from inoculated *Arabidopsis thaliana*.
Bacterial colonies were isolated from the inoculated plants by surface sterilisation of leaf or seedling material. The leaf or plant material weight was determined prior to further processing, after which it was submerged in 70% ethanol for 2 minutes, during which the material was shaken. Next the material was washed twice with autoclaved MilliQ and air-dried in 1.5 mL Eppendorf-tubes. Plant material was broken down to a homogenous substrate, using a sterile plastic pestle attached as a drillbit to a mechanically driven drill. The substrate was suspended in 500µL MilliQ, of which 25µL was plated onto TSA plates using 5–8 glass beads spread the suspension over the plates. Plates were incubated at 25°C for three to four days.

Genomic DNA analysis

**Isolation and minION Nanopore sequencing**
Genomic DNA was isolated from *Bacillus* strains AC35, AC116 conform the protocol "Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria", (by Promega) with the addition that the pellet was mechanically disintegrated prior to the addition of Nuclei Lysis Buffer, and the DNA was ultimately rehydrated in T10E0.5 (Tris 10mM+EDTA 0.5mM). The gDNA was stored at 4°C, untill further processing.

The isolated gDNA is sheared in 10kbp pieces via hydrodynamic shearing by centrifuging g-TUBESTM (Covaris) at 6000 rpm for 60 seconds. The sheared gDNA was is used for de novo genome sequencing with minION Nanopore sequencing. MinIon preparation was executed conform the protocol SQK-MAP-004, as published by Oxford Nanopore MinION™. The Pre-sequencing mix and elution of the library from His-beads deviated slightly from the protocol as all steps are executed in 1.5 ml Protein LoBind Eppendorf tubes. Next to this, the elution step of the library from His-beads was executed twice to ensure the complete absence of any remaining His-beads. One Nanopore flow cell and Nanopore MinIon R7.3 chemistry datasets were used for a standard 48hour run.
De novo genome assembly was performed conform the methodology and alterations as described by Loman et al. (Loman et al., 2015). In our approach, all the reads were used and we set a read length cut-off for the seed reads of ~5.9 Kb. Moreover, only two rounds of correction were done. The assembly step was performed by Celera assembly using similar parameters as Loman et al. (Loman et al., 2015). The assembly generated by Celera was scaffolded by PBJelly2 software (English et al., 2012). The final assembly was polished using Nanopolish software (Loman et al., 2015). Quality check of the assembly was performed using QUAST software (Gurevich et al., 2013) and genome alignment to the reference genome was performed using Nucmer software (Kurtz et al., 2004).

Analysis and metabolite detection.
The created FASTA file of AC35 is analysed using several bio-informatics tools. Antibiotics and Secondary Metabolites Analysis Shell, or anti- SMASH, is used for the detection of secondary metabolite biosynthesis pathways. Biosynthesis pathways are often located in close proximity of each other in specific gene clusters. The programme cross-links all identified regions to other related known gene clusters (Medema et al., 2011). PoRtable Front End for the Command Terminal BLAST, pfefctBLAST 2.0, is usefull to do nucleotide or amino acid sequence similarity searches against public or usercreated genomic databases (Santiago-Sotelo & Ramirez-Prado, 2012). Rapid Annotation Subsystem Technology, RAST 2.0, which is designed to rapidly call and annotate the genes of a prokaryotic genome (Aziz et al., 2008). This can be used in combination with the SEED Viewer. The SEED Viewer currently curates 1511 subsystems, which represent the collection of functional roles that make up a metabolic pathway, a complex or a class of proteins (Overbeek et al., 2014).

Transformation of Bacillus sp.
Two gfp-containing plasmids were separately transformed into Bacillus sp.. Plasmids pCM29 and pAD642-25 originating from Bacillus subtilis 168 were inserted into the bacterial strains by transformation to prototrophy by natural competence (Anagnostopoulos & Spizizen, 1961). This induces uptake of foreign DNA by the bacterial strains, which creates four separate gfp containing Bacillus sp. lines. Plasmids were also introduced in these bacterial lines through high osmolarity electroporation (Xue et al., 1999). These novel transformed bacterial lines were plated on LB plates containing chloramphenicol antibiotics (10µg/ml) and incubated at 30°C for 1 day to screen for positive transformants. Any positive transformants were purified, and the presence of gfp-containing plasmid was confirmed using fluorescence microscopy under UV light (Eijlander & Kuipers, 2013).
Results

Selection of antifungal bacteria.
Over 120 Solanum lycopersicum derived bacteria were tested against B. cinerea in vitro. In the preliminary multiculture hyphal growth assays around twenty of the tested bacteria showed inhibition of hyphae. The amount of inhibiting bacteria decreased to sixteen, when the experiment was repeated in monocultures. Eleven bacteria were able to inhibit the germination of B. cinerea in a multiculture plate; this amount is lowered to eight bacteria showing signs of inhibition on monoculture plates. The experimental set-up and in vitro inhibition of B. cinerea by bacterial strain AC59 is shown in Figure 3.

Figure 3 In vitro screening of antifungal properties. A) Multiclude plates in which AC58, AC59, AC60, AC61, AC62 and AC63 are tested against the hyphal growth of B. cinerea. B) Plates with selected strains, which show inhibition in the multiclude plates are tested in monoculture with the fungal pathogen; in this case AC59. C) The inhibition of germination of also tested in monoculture and multiclude. This is the monoculture plate of AC59.

Spectrophotometric germination assay: inhibition of Botrytis cinerea.
Outgrowth of B. cinerea spores was monitored in a 96-wells plates. Hourly measurements revealed a growth curve defined as an increase in optical density over time, this is shown in Figure 11 in the appendices. The supernatant of several tested bacteria was able to suppress the growth of B. cinerea in a liquid culture. Strains, which showed to be most promising in in vitro assays, were checked in more detail. Adding the supernatant of one of four Bacillus amyloliquefaciens strains: AC35, AC59, AC60 and AC116 showed slight to no increase in optical density over time. After filtration through a 30,000 MWCO centrifugal filter units (Millipore Corporation) the optical density did not show significant differences compared to unfiltered supernatant (Figure 12 in the appendices). Filtration through a 3,000 MWCO centrifugal filter unit (Merck KGaA) revealed a significantly increased optical density after 120 hours of growth, for all tested bacterial strains, in comparison to unfiltered supernatant (Figure 13 in the appendices).

Heat-treating the supernatant at 95°C for 20 minutes prior to addition of B. cinerea spores revealed to have a divergent effect on each tested bacterial supernatant. The optical density is significantly higher 142 hours post inoculation (HPI) for the heat-treated supernatants of B. amyloliquefaciens strains: AC35, AC59, AC60 and AC116 (P<0.001) (Figure 14 A, B and C in the appendices). The heat-treated bacterial supernatant of B. amyloliquefaciens AC116 showed a significant decrease in optical density (P=0.003) in comparison to untreated supernatant (Figure 14 D in the appendices).

A dilution series of bacterial supernatant was created to ascertain a dose-response curve. The threshold was crossed for B. amyloliquefaciens AC35 at a level in which 20% of the solution was comprised of bacterial supernatant. The optical density was significantly higher at 25% when compared to 50% (P=0.009) (Figure 15 in the appendices). For B. amyloliquefaciens AC116 the threshold was crossed at a level in which 15% of the solution was comprised of bacterial supernatant. The optical density was significantly higher at 15% when compared to 50% (P<0.001) (Figure 16 in the appendices). Optical density was significantly higher for all concentrations of B. amyloliquefaciens AC35 at 142 HPI, when compared to similar concentrations of B. amyloliquefaciens AC116.
Zonal *Botrytis cinerea* inhibition on TLC plates.

Under ultra-violet light the TLC-plates reveal the chromatographic distribution of secreted bacterial metabolites in the supernatant. All four plates reveal a similar overall distribution of metabolites, yet the intensity and the placement on the plates differed. Zones of growth inhibition were visible on plates which contained *Bacillus amyloliquefaciens* AC116 supernatant. These inhibition zones were similar in size, placement on TLC-plate, and morphology for both duplicates. The $R_f$-value of the centre of the inhibition zone is 0.26, with an overall width and length of respectively 1.75 and 3.00 cm. Upon replication of the experiment, inhibition was not perceived.

**Verticillium dahliae** symptom reduction in inoculated plants.

Fourteen bacterial strains showed a high level of *in vitro* growth inhibition of *V. dahliae*. Eleven of these fourteen bacteria showed a significant increase in fresh weight of *Lepidium sativum* (Ruaud, unpublished data). A further selection of twelve bacteria were used to monitor growth induction and possible *V. dahliae* reduction. Of all bacterial strains tested, only the mock inoculated plants and *Bacillus subtilis* AC47 revealed to have a significant increase in fresh weight, when compared to control, respectively $P=0.005$ and $P=0.036$.

All treatments were characterised by large standard deviations in the fresh weight of similar plants. These deviations are also noticed between replicates; relative fresh weight is depicted in Figure 4 to correct for possible discrepancies between replicates. Visual signs of *V. dahliae* wilting were absent in every replicate. Nearly all plants looked healthy, with the exception of severely desiccated plants, which occurred randomly throughout every inoculated bacterial treatment. *V. dahliae* outgrowth was observed when surface sterilised leaf petioles were plated on PDA.

![Figure 4 Pooled relative fresh weight of bacteria treated *A. thaliana* plants, inoculated with *V. dahliae*. Each bar represents the mean relative fresh weight of three replicates of eight plants. Red asterisks indicate a significant difference in comparison to the control treatment ($P<0.05$; Student’s t test). Error bars indicate standard error.](image)

Low *Bacillus* sp. transformation yields

Both transformation through natural competence and electroporation yielded no to very few colonies on selective media. *Bacillus amyloliquefaciens* AC116 did not show any colony growth on chloramphenicol after transformation attempts. Plates with selective media did reveal colony formation for *Bacillus amyloliquefaciens* AC35. Control treatments, were plasmids were absent, showed an equal amount of colonies in comparison to transformed strains. Phenotypic differences in colony formation was observed between LB plates with and without chloramphenicol. Fluorescence was not observed with microscopy under UV-light.
**Botrytis cinerea lesion reduction and plant growth induction.**

The relative fresh weight is depicted in Figure 5 to correct for possible discrepancies between replicates. Matching colours indicate matching replicates. *B. amyloliquefaciens* AC35 reveals a significant increase in above ground fresh weight every replicate; 1.60 ($P=0.002$), 1.47 ($P=0.005$) and 1.59 ($P=0.006$) fold increase compared to control plants. *M. luteus* AC06 treated plants did not differ in aboveground fresh weight, when compared with control plants. *B. amyloliquefaciens* AC60 treated plants had a significant increase in fresh weight for two of the three replicates. This was similar for *B. amyloliquefaciens* AC116 (Figure 5).

![Figure 5](image_url)

**Figure 5 Relative fresh weight of bacteria inoculated A. thaliana plants.** Each bar represents the mean relative fresh weight of eight plants per replicate. Red asterisks indicate a significant ($P<0.05$; Student’s t test) difference in mean of sample compared to the mean of control plants of the same replicate. Error bars indicate standard error.

The mean of the three lesions per plant is shown in Figure 6. The infection assay was executed in triplo. Control and *M. luteus* inoculated plants show similarities between mean lesion size, with *M. luteus* having a larger standard deviation. A large difference between means of similar treatments was observed for *B. amyloliquefaciens* AC35, AC60 and AC116. *B. amyloliquefaciens* AC35 significantly reduced the incidence and lesion size in replicate one and three (both; $P<0.001$). *B. amyloliquefaciens* AC116 reduced lesion size in replicate two and three (respectively; $P<0.001$ and $P=0.002$). The first replicate of AC116 yielded significantly larger lesions ($P=0.002$). *B. amyloliquefaciens* AC60 was unable to reduce the disease symptoms of *B. cinerea* and showed a significant increase in lesion size in replicate two ($P<0.001$). Pooled lesion size of all three replicates did not show significant differences between treatments.

![Figure 6](image_url)

**Figure 6 B. cinerea lesions of bacteria inoculated A. thaliana plants.** The mean of three lesions per plant are grouped per replicate. All lesions are measured in millimetres. Red asterisks indicate a significant ($P<0.05$; Student’s t test) difference in mean of sample compared to the mean of control plants of the same replicate. Error bars indicate standard error.
**De novo Bacillus amyloliquefaciens assembly and analysis.**

In total from one Nanopore run, 54,472 reads were obtained. These showed an N50 of 6,733 bp and a total sequence data of 272.5 Mb, representing ~68× theoretical coverage of the 4.0-Mb *B. amyloliquefaciens subsp. plantarum* reference genome.

So that all continuous long reads (CLRs) from the sequencing data generated by the Nanopore are used, the longest reads were corrected using the shorter reads. The length cut off for the seed reads was selected such to target about 30× genome coverage. After the first error correction step, we retrieve 12,655 reads with an N50 of 7,856 bp. The total amount of corrected reads was equivalent to ~22× genome coverage. Using all the corrected reads, we performed a new round of error correction. All the corrected reads were used as a seed reads. The second round of correction generated 12,314 reads with an N50 of about 7.8 Kb. The assembly of the corrected Nanopore reads resulted in 41 contigs with an N50 of ~282.9 Kb. The assembled genome was aligned to a similar *B. amyloliquefaciens subsp. plantarum* genome deposited at NCBI (GeneBank: CP007165.1) using Nucmer software (Kurtz et al., 2004). The alignment showed a rearrangement between the two strains (Figure 7A and B).

In order to improve the genome assembly quality, the sequences derived from genome assembly of the corrected of the Nanopore sequences were scaffolded using the original Nanopore reads. After scaffolding, 34 contigs were retrieved with and N50 of ~356 Kb. In order to remove additional errors insert during the assembly of the scaffolding, the genome was polished by using the total amount of Nanopore reads. The final assembly resulted in 34 contigs with an N50 of 362 Kb and the largest contig of ~670 Kb. Using the *B. amyloliquefaciens subsp. plantarum* as reference genome, we could estimate the number of indels (deletions and insertions) between the different assemblies and the reference *B. amyloliquefaciens subsp. plantarum* genome. Comparing the different assemblies to the reference genome, we could observe a decrease of indels after the polishing step (Table 2 in the appendices). The alignment between the reference genome and the final assembly showed almost a perfect alignments except for one translocations (Figure 7B).

**Figure 7** Dot plot comparing the alignment of de novo assembled Bacillus amyloliquefaciens AC35 with the reference genome of Bacillus amyloliquefaciens subsp. plantarum NJN6. Alignment of genomes prior (A) and post (B) improvement with PBjelly2 and Nanopolish.

RAST, which annotates genes of the sequenced genome, identified over 8000 features in *Bacillus amyloliquefaciens* AC35. Features are the total amount of protein encoding genes and RNAs found in the sequenced genome. 114 of these features encoded for RNAs, the remaining 7975 features encode for proteins. By using SEED Viewer it is possible to identify the subsystem of these protein encoding genes. Around 42% of all proteins were annotated with SEED Viewer as being part of a subsystem. “Amino Acids and Derivatives” is the most highly represented subsystem, with 839 feature counts.
Anti-SMASH identified twelve antibiotic or secondary metabolite gene clusters in *Bacillus amyloliquefaciens* AC35. Of which four are nonribosomal peptide synthetase (NRPS) gene clusters, which encode for the biosynthesis pathway of surfactin, bacillibactin, and bacillaene. Three bacillaene biosynthesis clusters were found, with a gene similarity of 35% (contig 32), 35% and 71% (both on contig 25) related to known gene clusters. Similarly two surfactin gene clusters were found with Anti-SMASH, gene similarity of 13% and 91%, located on respectively contigs 13 and 32. The gene clusters encoding for the biosynthesis of bacilysin, bacilliomyccin and macrolactin were matched to homologous genes, with high levels similarity (Figure 8). Gene clusters encoding for difficidin, amylocyclicin and an unknown terpene were also found using Anti-SMASH.

**A**) *Bacillus amyloliquefaciens* AC35, contig 11

Bacilysin biosynthetic gene cluster (100% similarity)

**B**) *Bacillus amyloliquefaciens* AC35, contig 32

Bacilliomyccin biosynthetic gene cluster (100% similarity)

**C**) *Bacillus amyloliquefaciens* AC35, contig 6

Macrolactin biosynthetic gene cluster (100% similarity)

*Figure 8 Anti-SMASH schematic depiction of homology in genes clusters.* The de novo sequenced gene cluster is shown above their found homologous known gene clusters. Near perfect similarity is found for antibiotic biosynthetic gene clusters: A) Bacilysin, B) Bacilliomyccin and C) Macrolactin.

Using prefectBLAST, it is possible to see the placement of the contigs in comparison with the gene of interest in more detail. PrefectBLAST shows that part of the macrolactin biosynthetic gene cluster found in the database has a high similarity (98% identical to database) to a gene cluster found on contig 6 of the de novo assembled genome of *Bacillus amyloliquefaciens* AC35 (Figure 9A). Parts of the bacillaene biosynthetic gene cluster is found on two separate contigs of the AC35 genome. Over 97% of both contig 32 and contig 25 are identical to parts of bacillaene gene cluster found in the NCBI nBLAST database. Large parts of contigs 32 and 25 overlap, when viewing the bacillaene biosynthetic gene cluster with prefectBLAST in more detail (Figure 9B).

**A**) Macrolactin biosynthetic gene cluster

**B**) Bacillaene biosynthetic gene cluster

*Figure 9 prefectBLAST schematic depiction of gene similarity.* Reference gene cluster is indicated with the crossed line. The matching contigs are revealed as blue bars. Similarity shown for A) macrolactin and B) bacillaene biosynthetic gene cluster.
Discussion and future prospects

In vitro inhibition experiments pooled reveals that a multitude of tomato derived bacteria inhibit one or several tested fungal pathogens. These results were acquired by Christina Papazlatani, Hesham Gibriel and Albane Ruaud (unpublished data). Of all plant-related bacteria tested, 33% of them showed some signs of plant pathogen inhibition on plates (Figure 10A). This indicates that endophytic and plant-related bacteria have a high potential as source of novel antifungal metabolites (Tan & Zou, 2001). 90% of these inhibiting bacteria are Gram-positive species. Over 65% of pathogen inhibiting species were Bacilli (Figure 10B), and over 11% of all Solanum lycopersicum isolated Bacillus species revealed to be a fungal inhibiting strain. Bacilli are well-documented to be producers of large quantities of enzymes; which include antifungal proteins and secondary metabolites (Alvarez et al., 2012), (Han et al., 2014).

Figure 10 Pathogen inhibition by plant-related bacteria. A) Percentages of in vitro pathogen inhibiting bacteria, derived from Solanum lycopersicum. B) The percentage of bacterial species, which reveal an in vitro pathogen inhibiting effect.

Several bacteria like Bacillus amyloliquefaciens AC35, AC60 and AC116 and Bacillus subtilis AC47 stimulates plant growth, as shown in A. thaliana, but also in L. sativum (unpublished work by Albane Ruaud). Several B. amyloliquefaciens related strains are known to be root colonising rhizobacteria, and PGPBs. Their potential as biocontrol agents is known and some strains are even commercially available as bio fertilizer (Borriss, 2011). AC35 is a competent plant growth promoting bacteria, or PGPB. Next to the induction of plant biomass growth, the development speed of A. thaliana was also increased. Inflorescence emergence was spotted in several AC35 inoculated plants, when only eight rosette leaves (greater than 1mm) were present. This induction of plant development and growth could potentially be explained with data from the SEED Viewer. Genomic analysis revealed that AC35 posses the genes encoding for the production of auxin. Anti-SMASH revealed the production of bacillibactin, which is a catechol type siderophore. Siderophores are often used in the sequestering of iron in the rhizosphere, making this nutrient more accessible for host plants. These metabolites could explain why plant growth is stimulated when plants are inoculated with AC35.

AC35 and AC116 differed in metabolite secretion, as the supernatant of AC116 was more potent in inhibiting the growth of B. cinerea. The supernatant of both B. amyloliquefaciens strains showed a reduced inhibiting effect when centrifuged through 3 kDa filter. The active component which inhibit growth is greater than 3 kDa, but smaller than 30 kDa. As filtrate of a 30 kDa did not lose this growth inhibiting effect. The inhibiting metabolite or metabolites produced by AC116 are more thermostable, than those of AC35. The supernatant of AC116 was able to inhibit growth of B. cinerea at lower concentrations in comparison to AC35.
We have shown \textit{B. amyloliquiefaciens} AC35 and AC116, and \textit{B. subtilis} AC47 are not only plant growth promoting bacteria: capable of inducing plant growth, but they could potentially be used as candidates for viable biocontrol agent against fungal plant pathogens. AC35 and AC116 against foliar pathogen \textit{B. cinerea} and AC47 against the soilborne pathogen \textit{V. dahliae}. AC35 and AC116 inoculated plants showed a reduction or complete absence of lesions when treated with \textit{B. cinerea}, whilst control plants and plants inoculated with \textit{B. amyloliquiefaciens} AC60 and \textit{M. luteus} AC06 were infected and macerated. This protecting effect of \textit{Bacillus amyloliquiefaciens} strains has been observed with \textit{B. amyloliquiefaciens} UCMB-5113, which inhibit colonisation of \textit{B. cinerea} in \textit{Brassica napus}. According to Sarooh et al. this was solely due to differential systemic gene expression in the leaves (Sarosh et al., 2009). The priming of defensive genes by \textit{B. amyloliquiefaciens}, which colonised the root system, can have an extended effect on foliar and systemic diseases (Kloepper et al., 1999).

The second replicate (second column of Figure 5) for \textit{B. amyloliquiefaciens} AC35 and the first replicate (first column of Figure 5) of AC116 did not show a reduction in lesion size, this coincided with a slightly lower mean fresh weight. Arguably, the colonisation by the bacteria was not as robust as in the other replicates.

\textit{V. dahliae} infection assay resulted with \textit{A. thaliana} having a higher fresh weight when inoculated with \textit{B. subtilis} AC47. The significant difference in fresh weight between mock inoculated and \textit{V. dahliae} inoculated control plants, indicate that \textit{V. dahliae} did have a pathogenic effect on the plants. Outgrowth of \textit{V. dahliae} hyphae from surface sterilized petioles indicate a systemic colonization, but the pathogen was not able to create visual disease symptoms. AC47 was able to reduce the disadvantageous effect of \textit{V. dahliae} on the growth of the plants.

The majority of the bacteria used in these experiments were extracted internally from surface sterilised \textit{Solanum lycopersicum}, with the exception of \textit{B. amyloliquiefaciens} AC116. There is a probability that these strains are not only a root colonizing \textit{Bacillus amyloliquiefaciens} species, but they could potentially be representatives of tomato-specific endophytic bacteria, which are able to systemically colonise an entire plant. The endophyte-host species interaction is a highly complex mechanism. Host-species, soil type, abiotic and biotic stresses all have an impact on the abundance, concentration, and diversity of endophytic species. Siciliano et al. showed that the colonisation success of endophytes was even regulated on a genotypic level within bacterial species (Siciliano et al., 2001). \textit{B. amyloliquiefaciens} strains are documented as being endophytic, and highly host specific. White et al. hypothesized that a \textit{B. amyloliquiefaciens} strain similar to FZB42 was an endophyte and defensive mutualist of vanilla orchids (White et al., 2014).

If this were the case a more direct inhibition of pathogens is possible, in contrary to the systemic indirect defence due to priming. Whether a direct inhibition of \textit{B. cinerea} by AC116 and AC35 is the case should be validated in further experiments. It is however difficult to identify whether direct or indirect interaction is causing inhibition (Kloepper & Ryu, 2006). Direct inhibition can be the result of lipopeptide production. Cyclic lipopeptides are divided in three families: fengicins, iturins, and surfactins. Via a modified drop-collapse assay on parafilm it was shown that all \textit{Bacillus amyloliquiefaciens} strains tested produced a surface tension lowering substance (data not shown), (Bodour & Miller-Maier, 1998). This effect can be caused by a surfactin.

RAST annotated over 8,000 features in the genome of \textit{Bacillus amyloliquiefaciens} AC35. This is extremely high, when compared to the genome of \textit{Bacillus subtilis} which contains 4,100 protein encoding genes (Kunst et al., 1997). This increase in amount of protein-encoding-genes can be explained by the small inaccuracies of the genomic sequencing. Where several genes are annotated as multiple genes by RAST. This could be the case with bacillaene biosynthesis clusters, Anti-SMASH found three clusters encoding for bacillaene. But with closer examination this could be one cluster on two separate contigs. The prefixBLAST revealed a shared part of the gene cluster on both contigs. These contigs are located near each other, shown on the dotplot (Figure 7B). Making this a possible explanation.
The annotation tools used in this experiment are capable of detecting gene clusters in fragmented assemblies. Using multiple of these annotation tools enabled us to uncover a multitude of the secondary antimicrobial metabolites. Metabolites known to inhibit the growth of fungal pathogens, such as bacilysin, bacillomycin, macrolactin and surfactin, were present in AC35.

The introduction of a gfp-containing plasmid was tried on several occasions, both via natural competence as through electroporation. *B. subtilis* 168 was used as control during all transformation processes as a positive control. However, AC35 and AC116 were not able to be transformed, through these methods. Using chloramphenicol in a selective media, transformants could be selected with relative ease, as both plasmids contained a chloramphenicol resistance cassette. However AC35 revealed to have a natural tolerance to chloramphenicol; making selection of transformants a difficult task. This natural tolerance to antibiotics can be explained with data from the SEED Viewer. Genomic analysis revealed that AC35 posses the genes to encode for a multidrug efflux systems or parts of it (Zgurskaya & Nikaido, 2000). Making it possible for AC35 to tolerate certain levels of chloramphenicol.

TLC-bioautography is a tried and tested method of revealing antimicrobial and antifungal compounds (Poole, 2014). Inhibition on plates was visualised once, but reliable replication was not obtained. Concentrating the supernatant prior to TLC could yield better and consistent results.

**Future prospects**

The inhibition of *B. cinerea* by *B. amyloliquefaciens* AC35 and AC116 and the inhibition of *V. dahliae* by *B. subtilis* AC47 should be further examined. Several of these bacteria were isolated from *S. lycopersicum*, so testing it on this species should give a clear view of pathogen protection. Testing this inoculum on several other plant species could indicate if an endophytic relationship is species specific. It should be tested whether these bacteria can be used as a broad scale biocontrol agents against *B. cinerea* and *V. dahliae* in cash crop. This could result in using one or several of these bacteria as application for agricultural means. Comparing genomes of several bacteria could reveal certain important question about the genes necessary for being endophytic or a potent biocontrol agent.

The alignment showed a rearrangement between the AC35 and the reference strain. This rearrangement between strains should be looked at with closer detail. Whether this is a error in assembly or a true large scale rearrangement within the genome.

Using multiple bacterial strains together could potentially result in a synergistic effect of biocontrol, and could be further examined. Several bacterial strains revealed to be capable of plant-growth promoting properties. Genomic evidence shows that AC35 has the genes capable of producing bacillibactin and auxin. Further experiments should reveal whether these genes are expressed, and at which level.
References


Appendices

Figure 11 Optical density of *B. cinerea* spore suspensions in 96-Wells plate reader.

Figure 12 Difference in optical density over a period of 120 hours, after 30kDa filtration. 30kDa filtration of supernatant of four *Bacillus amyloliquefaciens* strains with *B. cinerea* inhibition properties.

Figure 13 Difference in optical density over a period of 120 hours, after 3kDa filtration. 3kDa filtration of supernatant of four *Bacillus amyloliquefaciens* strains with *B. cinerea* inhibition properties.
Figure 14 Inhibition of growth by supernatant over time, heat treated and untreated. Inhibition properties of Bacillus amyloliquefaciens supernatant: A) AC35, B)AC59, C)AC60 and D)AC116. Red indicates heat treatment (20 minutes at 95°C)

Bacillus amyloliquefaciens AC35

Figure 15 Dose-response curve over time for Bacillus amyloliquefaciens AC35. Percentage supernatant in well shown on x-axis, optical density shown on y-axis.

Bacillus amyloliquefaciens AC116

Figure 16 Dose-response curve over time for Bacillus amyloliquefaciens AC116. Percentage supernatant in well shown on x-axis, optical density shown on y-axis.
Table 2 Results from QUAST when comparing the draft assembly with the polished assemblies. A polished assembly with only PBJelly2 and an assembly with both PBJelly2 and Nanopolish. *Bacillus amyloliquefaciens* subsp. plantarum NJN6 is the reference genome.

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