Unearthing *Bacillus* endophytes from desert plants that enhance growth of *Arabidopsis thaliana* under abiotic stress conditions

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ABSTRACT

Unearthing *Bacillus* strains that exhibit biofertilizer potential

Ameerah Bokhari

Here, we embarked a bioprospecting project that focuses on the isolation and characterization of plant root endophytes, collected from the Thar Desert. A total of 381 endophytes were isolated and based on their 16S rRNA gene sequences, genus *Bacillus* (58 strains) was identified as the major taxon and only endophytes from this genus were isolated from all plant types. Of the 58 *Bacillus* strains, only 16 strains were selected for screening of plant growth promotion traits such as P and Zn solubilization, indole-3-acetic acid and siderophore production, and antimicrobial activity. Based on the presence of specific plant growth promotion traits 10 strains were shortlisted for further *in vitro* screening with *A. thaliana*; to confirm that these bacteria can confer resilience to plants under salt stress conditions. *B. circulans* (PK3-15 and PK3-109), *B. cereus* (PK6-15) *B. subtilis* (PK3-9) and *B. licheniformis* (PK5-26) displayed the ability to increased the fresh weight of *A. thaliana* under salt stress conditions by more than 50 % compared to the uninoculated control.

An interesting observation was that *B. circulans* (PK3-109) (shown to produce IAA exopolysaccharide) and *B. circulans* (PK3-138) (shown to produce IAA) *in vitro* results were substantially different as *B. circulans* (PK3-138) decreased the total fresh weight of *A. thaliana* by 47 %, whilst *B. circulans* (PK3-109) was one of the best performing strains. Thus, the genomes of these two strains were sequences to unravel the molecular
versatility of *B. circulans* strains, specifically with respect to their interaction with plants. Most of the genome of these strains is identical but the most interesting feature was the presence of 1/ the DegS–DegU two-component system that is known to mediate the salt stress response and DegU also represses toxin *wapA* similar to antitoxin *wapI*, and 2/ *YxiG*, a gene in the unique orthogroup of PK3-109 was found to be linked to WapI. Thus, PK3-138 substantially decreasing the total fresh weight of *A. thaliana* under salt stress conditions suggests that the toxic activity of a toxin such as WapA is not effectively ameliorated by the antitoxin such as WapI in the absence of a functional YxiG gene.
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LIST OF ABBREVIATIONS

PGPB  Plant growth-promoting bacteria
ST-PGPB Stress tolerance-plant growth-promoting bacteria
PK  Pakistan collection
TSA  Tryptic Soy Agar
TY  Tryptone Yeast extract
LB  Luria-Bertani agar
R2A  Reasoner’s 2A agar
PGA  Potato Glucose Agar
P  Phosphate
IAA  Indole acetic acid
PVK  Pikovskya’s agar
QS  Quorum Sensing
BLAST  Basic Local Alignment Search Tool
PCR  Polymerase Chain Reaction
ExoSAP-IT™  PCR Product Cleanup Reagent
GTG₅  Repetitive DNA element primer for bacterial identification
½ MS  1/2 strength Murashige & Skoog (MS) medium
PEG  Polyethylene Glycol (P.E.G.) 8000
EtOH  Ethanol
TAE Buffer  Tris Base, Acetic Acid and EDTA Buffer
NaCl  Sodium Chloride
NaClO  Sodium hypochlorite
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Chapter 1: Overview

Plant-related research is focused on developing effective and low cost biofertilizers that can gradually replace the commonly used chemical fertilizers. However, this approach is not new, as it was common practice for farmers to mix soil in which leguminous plants grew with soil in which non-legume crops grew, as experience shows this process increases crop yield. This observation along with, 1/ the German agricultural chemist Hermann Hellriegel, in collaboration with Hermann Wilfarth, discovering that leguminous plants assimilate the free nitrogen of the atmosphere [1] and 2/ the Dutch microbiologist Martinus Willem Beijerinck, being the first to isolate root nodule bacteria in pure culture from nodules of Leguminosae plants, which were later classified as *Rhizobium leguminosarum* [2], were capable of fixing atmospheric nitrogen [3], lead to bacteriologists Friedrich Nobbe and Lorenz Hiltner applying for a patent with respect to inoculating seeds with cultivated bacteria. This patent was used in 1905 by the Nitragin Company to start production of nitrogen-fixing biofertilizers in the United States. Since then several biofertilizer products (including QuickRoots®, Nitragin Gold®, JumpStart®, TagTeam®, Cell-Tech®, Optimize®, TagTeam® LCO, TagTeam® LCO XC, and Optimize® XC) has been introduced by the Nitragin (now owned by Liphatech) and other companies. However, new formulations of biofertilizers are required to cope with multiple abiotic stresses as, 50% of arable land is expected to be affected by both drought and salinity by 2050 [4, 5]. Thus, studies are increasingly being focused on identifying biofertilizers that can alleviate these stresses in crops. However, very few studies have focused on the identification of biofertilizers that can alleviate both salt and drought stress, even though drought stress in often combined with salt stress in arid climates [6,
1.1 Endophytes

The term ‘endophytes’ have been commonly used to describe microbes that can internalize host tissues for at least part of their life cycle without causing pathogenic symptoms. They benefit their hosts in many ways such as supplementing essential nutrients, promoting host growth and conferring host resilience to different biotic and abiotic stresses [8, 9]. A wide range of bacterial endophytes have been isolated from different types of plants including tomato (including genera *Pseudomonas, Enterobacter* and *Micrococcus*) [10], rice (including genera *Pseudomonas, Sphingomonas* [11], *Corynebacterium* and *Bacillus* [12]) and soybean root nodules (including genera *Bacillus* [13]).

The plant-bacteria interaction starts with a cross-talk between the potential endophytes and host plant through several processes. The plant host usually produces root exudates that contains different organic compounds, sugars, and volatiles that attract specific type of rhizobacteria. Plant selection for a certain rhizobacterial group is dependent on the plants needs, e.g. relieve from biotic (phytopathogens) or abiotic stress (salinity, drought and high temperatures) stresses [14]. The targeted rhizobacteria subsequently move towards the potential host plant either passively via soil water fluxes or actively via the specific induction of flagella activity by chemotaxis [14]. These host plants play a key role in shaping the microbial community structure in the rhizosphere [15, 16]. Moreover, Lemanceau *et al.* (1995) showed that the endophyts selected from this rhizosphere community are plant-specific [17]. That is, they showed a difference in the bacteria isolated from uncultivated soil and from root tissue of both flax and tomato plants and the
use of 10 different substrates allowed for the discrimination between flax and tomato isolates. In fact, most of the bacteria isolated from the flax and tomato plants were classified as *P. putida* bv. A and to *P. fluorescens* bv. II, respectively [17].

Another example is found in *Rhizobium*-plant interactions which have been well studied and showed high specificity in species-specific communications [16]. From the side of bacteria, one of the signaling mechanism used for colonization is chemotaxis-induced motility coupled with their ability to attach and colonize the roots efficiently [18]. Another important signaling mechanism is via “quorum sensing (QS)” which allows bacteria to interact with both plants and other bacteria from the same species. QS is a cell-to-cell communication process that regulates the bacterial colonization and biocontrol effects. Factors that modulate levels of QS include glucose concentrations, availability of carbon sources in the rhizosphere, and abiotic stress factors such as salt. In Gram-negative bacteria, the key signaling molecule in QS mechanism is acylated homoserine lactones (AHLs) [19], while for Gram-positive bacteria, peptides are the common signaling molecules used in QS [18].

### 1.2 Plant growth-promoting rhizobacteria

These bacteria that colonize plants root and promote plant growth are commonly referred to as plant growth-promoting rhizobacteria (PGPR) [20]. PGPR primarily isolated from plants or their rhizosphere exhibit the ability to increase plant growth and yield when associated with plant roots or as a result of seed-inoculation [21]. Several bacteria from genera such as *Bacillus* [22], *Pseudomonas* [23, 24], *Acinetobacter* [24], *Azospirillum* [25], *Burkholderia* [26] and *Klebsiella* [27] have been identified as PGPR of crops
through direct (phytohormone production, biological nitrogen fixation (BNF), phosphate and zinc solubilisation) and indirect mechanisms (antibiotic production, induced systemic resistance, siderophore production and hydrogen cyanide).

1.2.1 Phytohormones

Several structurally diverse phytohormones have been characterized including auxin, abscisic acid, cytokinin, strigolactone, ethylene, gibberellin, salicylic acid, brassinosteroid and jasmonate. Auxin activity in particular was classically defined as the ability to stimulate plant growth. However, phytohormones were also found to play essential roles in response to pathogens [28, 29], fruit formation [30], and abiotic stress [31]. The most abundant and first identified endogenous auxin is indole-3-acetic acid (IAA) [32]. Only three other compounds with auxin activity have been described including indole-3-butyric acid (IBA) [33], 4-chloroindole-3-acetic acid (4-Cl-IAA) [34], and phenylacetic acid (PAA) [35]. IBA is considered the storage form of IAA that ensures the active hormone (IAA) arrives at the site of activity, as IBA is produced and converted to IAA [36]. In fact, exogenously applied IBA induces rooting more efficiently than IAA itself [37] and is widely used as a rooting agent in agricultural applications [38]. However, bacterial isolates also exhibit the ability to synthesize IAA. [39] and [40] showed rhizobacteria such as Pseudomonas spp. and Rhizobium spp. are often associated with the potential to stimulate plant growth via the production of IAA. These bacteria were shown to produce and degrade IAA thereby manipulating IAA concentrations, that is a critical function, as high levels of IAA were shown to inhibit the growth of roots rather than promote it [40].
The same effect was observed with the gaseous phytohormone ethylene that is produced by both plants and PGPR, that is, overproduction of this hormone inhibits growth of roots and may lead to abnormal growth and development of plants [41]. Ethylene production is induced under stress conditions including wounding, drought and pathogen attack [42, 43]. It is synthesized when methionine is converted into ethylene via 1-aminocyclopropane-1-carboxylate (ACC) [44]. However, some PGPR (from genera *Alcaligenes*, *Burkholderia*, *Bacillus*, *Pseudomonas*, *Variovorax*, *Azoarcus*, *Azorhizobium*, *Azospirillum*, *Gluconacetobacter* and *Herbaspirillum*) have an ACC deaminase [45], that can cleave ACC into α-ketobutyrate and ammonia, thereby lowering ethylene concentrations in a developing seedling or stressed plant, which eliminates the inhibitory effect of high ethylene concentrations [46].

1.2.2 Nitrogen fixation

Nitrogen is an essential nutrient for plant growth and development that is readily available as atmospheric nitrogen. But plants cannot use atmospheric nitrogen as is as atmospheric nitrogen needs to be converted to other plant-utilizable forms such as ammonia and nitrate. These forms can be synthesized industrially or naturally, through a process of biological nitrogen fixation (BNF) wherein bacteria use their innate enzyme systems to convert nitrogen to ammonia [47]. Thus, for sustainable agriculture, developing a climate-smart biofertilizer that can replace the industrially produced nitrogen fertilizers that cause coastal dead zones is of interest. Specifically, BNF by PGPR has been reported to contribute up to 70% of total N uptake in field crops such as sugarcane [48], maize [25] and oil palm [22]. Moreover, its estimated that up to 65% of N in agriculture is contributed by BNF [49].
Next to nitrogen, the second most important element for plant nutrition and growth is phosphate. Phosphate is a structural component of many important molecules like nucleic acids, adenosine triphosphate (ATP) and phospholipids [50, 51] that are key components in plant metabolic processes like photosynthesis, signal transduction, energy transfer and respiration [52]. Similar to nitrogen, most natural phosphate cannot be utilized by plants as at least 95% of the phosphate occurs as insoluble and precipitated forms [53]. PGPR were shown to solubilize phosphate through multiple mechanisms [54]. Thus, phosphate solubilizing bacteria from several genera including *Bacillus, Arthrobacter, Burkholderia, Flavobacterium, Microbacterium, Pseudomonas* etc. are being focus upon to improve plant growth and yield [55]. It has also been reported that increasing the zinc content in plants can facilitate efficient nutrient uptake that translates into improved plant growth [56]. Thus far, zinc solubilizing bacteria have been isolated from genera including *Bacillus* [57], *Azospirillum, Pseudomonas* and *Rhizobium* [56], *Pseudomonas, Ralstonia, Burkholderia* and *Klebsiella* [58]. Inoculating plants with these bacteria showed significant plant growth compared to uninoculated seedlings. Potassium (K) is another macronutrient essential for plant growth that exists in the soil, in the form of silicate minerals and insoluble rocks [59] and is consequently also considered a major constraint in crop production. Thus an alternative source that can increase potassium availability is required [60].
1.2.4 Antibiotic production, systemic acquired resistance and Induced systemic resistance

Initially farmers extensively used copper and antibiotic sprays (specifically Streptomycin) that primarily targeted *Xanthomonas* spp., *Pseudomonas* spp. and *E. amylovora*, for plant-related bacterial disease management [61]. As an alternative to Streptomycin, Oxytetracycline was used to control fire blight caused by *E. amylovora* (on pome fruit trees), bacterial spot caused by *Xanthomonas arboricola* pv. *Pruni* (on peach and nectarine) and target *Pseudomonas* spp. and *Xanthomonas* spp. (on on vegetable crops) [61]. Gentamicin, oxilinic acid and Kasugamycin have also been used in to control fire blight [62, 63]. However, multiple applications of such sprays were found to correlate with the selection of resistance in pathogens and other plant-associated bacteria. Horizontal gene transfer (HGT) has been implicated in the transfer of copper resistance genes that are encoded by a copper-inducible operon (*copABCD*) and related variants in *P. syringae* [64, 65], *Xanthomonas campestris* pv. *juglandis* [66], *Xanthomonas citri* ssp. *citri* and *Xanthomonas alfalfae* ssp. *citrumelonis* [67, 68]. Also, the *strAB* genes which encode aminoglycoside phosphotransferase enzymes that modify streptomycin to a nontoxic form were shown to impart streptomycin resistance in *E. amylovora, P. syringae* and *X. campestris* pv. *Vesicatoria* [69-71]. Similarly, oxolinic acid-resistant *E. amylovora* and gentamicin-resistant bacteria were isolated from the crops treated with these antibiotics [72, 73]. HGT is increasing the prevalence of such resistance in bacterial genera globally [74], thus, antibiotic resistance due to evolution is an eventuality with any new antibiotic deployed.
Because the use of antibiotics to manage crop diseases is not sustainable; biological control, antibacterial peptides and the use of inducers of systemic acquired resistance (SAR) are being considered as possible alternatives. For example, *Agrobacterium radiobacter* strain K84 was shown to protect woody plants from crown gall disease caused by *Agrobacterium tumefaciens* [75].

However, an extended window of infection, significantly reduces the efficacy of biological controls. In these situations, success may be achieved by combining biological control with SAR and/or ISR. SAR is the application of a chemical inducer, such as acibenzolar-S-methyl (ASM), to pre-condition plants to pathogen attack [76]. This has been applied in several pathosystems, including bacterial canker of tomato, bacterial wilt of tomato and fire blight [77-79]. Similarly, ISR involves plant hormone-mediated signaling from plant growth-promoting rhizobacteria such as *Bacillus* spp., *Pseudomonas* spp., *Trichoderma* spp., etc. pre-conditioning plant defence [80]. ISR was proven to be an effective strategy against bacterial pathogens such as *P. syringae*, *R. solanacearum* and *Xanthomonas oryzae* pv. *oryzae* [81].

### 1.2.5 Hydrogen cyanide (HCN) production

The volatile HCN is a secondary metabolite that is produced from bacterial membrane-bound flavoenzyme, HCN synthase, oxidizing glycine [82]. It has been shown that HCN suppresses the growth and development of both microbes and plants [83, 84]. Rudrappa *et al.* (2008) demonstrated that HCN produced by *Pseudomonas* inhibits the primary growth of *Arabidopsis thaliana* roots due to the suppression of an auxin responsive gene [85]. However, Voisard *et al.* (1989) demonstrated *Pseudomonas fluorescens* strain
CHA0 suppresses black root rot disease caused by the fungus *Thielaviopsis basicola* in tobacco plants [86]. They showed, using a gnotobiotic system containing iron-rich soil, that wild-type CHA0 more effectively suppresses black root rot disease in tobacco plants than a cyanidenegative (*hcn*) mutant CHA5. They further demonstrated that inserting an artificial transposon carrying the *hcn*<sup>+</sup> genes of strain CHA0 (Tn*hcn*) into the genome of another *P. fluorescens* strain P3, which naturally does not produce cyanide and gives poor plant protection, improved its ability to suppress black root rot disease [86]. Siddiqui *et al.* (2006) demonstrated that the *P. fluorescens* strain CHA0 applied in unsterilized sandy-loam soil as drench, caused marked suppression of root-knot disease caused by *Meloidogyne javanica* in tomato seedlings [87]. They further demonstrated that increasing bioavailability of iron, through EDTA application, in soil substantially improved suppressive effects of CHA0 against *M. javanica*. This corroborates in part Castric *et al.* (1975) reporting that iron was stimulatory to cyanogenesis in concentrations above 1 μM, while copper, zinc, cobalt, and manganese at concentrations of 20 μM had no effect. Devi *et al.* (2007) also demonstrated that hydrogen cyanide-producing rhizobacteria are able to kill the subterranean termite *Odontotermes obesus* (rambur) by cyanide poisoning under in vitro conditions [88].

### 1.2.6 Siderophore production

In soil, ferrous iron is readily oxidized to the ferric form that occurs as a poorly soluble iron hydroxide that cannot be used by biological systems [89, 90], thus causing an iron deficiency problem in soil. To survive in such an environment, plant-associated PGPR secrete iron chelators, termed siderophores, into the environment to form ferric-siderophore complexes that can move by diffusion and be returned to the cell via specific
membrane receptors [91-93]. Several siderophores have been identified, some of which are widely recognized and used by different microorganisms, while others are species-specific [94, 95]. Thus, PGPR can use siderophores to sequester the available iron in the area surrounding the root to prevent the proliferation of pathogenic microorganisms [87, 96]. Potent pyoverdine siderophores produced by Pseudomonads were also shown to suppress, 1/ Fusarium oxysporum known to cause wilt diseases in potato plant [83], 2/ the Gaeumannomyces graminis known to inhibit plant growth [86], and 3/ Macrophomina phaseolina, Fusarium moniliforme and Fusarium graminearum known to cause maize root disease in maize plants [97]. Kloeper et al. (1980), similarly showed that the pseudobactin siderophore produced by the P. putida B10 strain was also able to suppress F. oxysporum and further demonstrated that this suppression was lost when iron is added to the soil [98]. Thus when siderophores function involves antimicrobial activity or scavenging for iron, it directly and indirectly shapes microbiome diversity.

Here it should be noted that plants also utilize their own mechanisms to acquire iron; dicots via a root membrane reductase protein and monocots via production of phytosiderophores [99] and it is not known if bacterial siderophores contribute substantially to plant iron uptake. This suggests that the role of siderophores should for now not be viewed as a plant growth promotion trait but rather as a plant protective trait.

1.3 Goal of the present study

The goal of the present study is to isolate and characterize PGPR endophytes from desert plants and screen them for plant growth promotion traits (Chapter 2), then to confirm their ability to confer such traits to plants expose salt stress (Chapter 3). Then to examine computationally at the genomic level why identical species do not confer similar
resilience to plants exposed to abiotic stress (Chapter 4). The identified strains may be helpful in increasing the production of different crops in an eco-friendly manner.
Chapter 2: Bioprospecting desert plants for their *Bacillus* endophytes with biofertilizing potential

2.1 Background

Plants are largely considered to be metaorganisms due to their dependence (in specific habitats such as rhizosphere, phyllosphere and endosphere) on plant-specific growth promoting bacterial communities for: 1/ increasing plant nutrient uptake (nitrogen fixation and phosphate solubilization), 2/ stimulating plant growth through the production of phytostimulators (auxins, indole-3-acetic acid, gibberellins), 3/ suppressing biotic stressors (hydrogen cyanide, siderophores, antibiotics and insecticidal agents), and 4/ conferring tolerance to plants against abiotic stresses such as drought, salinity and extreme temperatures [100-104]. These characteristics led to several initiatives to identify plant growth-promoting rhizobacteria (PGPR) that can increase crop growth and yield. One such initiative is associated with biofertilizers replacing or at least reducing the use of chemical fertilizers, reason being, the availability of expensive chemical fertilizers limits crop production worldwide and more so in developing countries. Moreover, they negatively impact the agro-ecosystem and the environment as nitrogen fertilizers are made from ammonia and their continuous application result in pollution of water sources through leaching and emission of ammonia gas [105]. Also, phosphate fertilizers that are imperative to crop production have efficacy issues owing to ~80% of phosphorous applied as fertilizer precipitating in the soil [105, 106]. Considering these limitations of chemical fertilizers, microbial consortia that are capable of promoting plant growth,
fixing nitrogen, solubilizing phosphate and alleviating biotic stresses should be appropriate options as biofertilizers.

Thus in this chapter, we aim to screen *Bacillus* endophytes isolated from diverse desert plants growing in the Thar desert in Pakistan for their ability to: 1/ support plant growth, and 2/ provide protection against biotic stresses.

### 2.2 Materials and Method

#### 2.2.1 Isolation and cultivation of endophytes

Nine plants including three *Panicum antidotale* (PK3, PK3b, PK9) *Tribulus terrestris* (PK5, PK7), *Zygophyllum simplex* (PK1, PK6), *Euphorbium officinarum* (PK4) and *Lasiurus scindicus* (PK8) plants were collected from the Thar desert in Pakistan (24°45'00.4"N 69°56'00.8"E), then placed on ice to be transported to the Desert Agriculture laboratory at King Abdullah University of Science and Technology (KAUST), Saudi Arabia. Bacteria were isolated from the endorhizosphere using the serial dilution method. Specifically, roots were surface sterilization by dipping in 70% ethanol for 30s, then 2% sodium hypochlorite for 5 minutes, followed by washing with sterilized distilled water (Elbeltagy et al., 2000) [1]. The sterilized roots were then macerated with 0.8% saline solution and subjected to serial dilution. Luria-Bertani (LB), Tryptic Soy Agar (TSA), Tryptone-Yeast extract (TY) and Reasoner’s 2A (R2A) media were used for the isolation and purification of bacteria. In addition, 1.5% NaCl was added to R2A as a fifth media to enable the isolation of endophytes that can grow in high salt concentrations.
2.2.2 Taxonomical identification

Isolated strains were identified through the sequencing of their 16S rRNA genes. Due to the large number of bacterial isolates to be identified (381 isolates), bacterial DNA was extracted using cell lysis method. It is a quick high-throughput method in which 10µl of each pure isolate were diluted in 90µl distilled water (MilliQ) and exposed to cell lysis in PCR thermocycler (95°C for 5 min, then 20°C for 1 min, then 95°C for another 5 min). PCR amplification of 16S rRNA genes was performed using the universal primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-TACGGYTACCTTGTTAGCACTT-3’). Thermocycler conditions started with initial denaturation at 95°C for 1 min, followed by 30 cycles at 95°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1.5 minutes, and a final polymerization extension at 72°C for 5 minutes. PCR amplification were verified by 1% agarose gel electrophoresis (see appendix XXX) and those showing positive signal, were purified using EXOSAP IT (Invitrogen) then sent for Sanger sequencing.

For (GTG)5-rep-PCR fingerprinting [2], we used the GTG5 primer (5’-GTGGTGCTGGGTG-3’), that are strain specific. Bacterial DNA was extracted using GenElute™ Bacterial Genomic DNA Kits (Sigma). Thermocycler conditions started with Initial temperature at 94°C for 5 minutes, followed by 30 cycles at 94°C for 1 minute, 40°C for 1 minute, and 65°C for 8 minutes, ending by 65°C for 16 minutes.

2.2.3 Phylogenetic analyses

The 16S rRNA gene sequences were compared with those in the GenBank database using NCBI BlastN. The T-Coffee multiple sequence aligner version 11 [107] was used to align
the 16S rRNA sequences using the parameter ‘t_coffee –mode rcoffee’. Subsequently, to identify conserved blocks from the multiple sequence alignment (MSA), the Gblocks 0.91b [108] was applied onto the MSA by using minimum sequence for flank position at 85%, maximum contig nonconserved position at 8, and minimum block length at 10. Next, we employ Phyml version 20120412 [109], a widely used phylogeny tool based on maximum-likelihood principle. For building the phylogeny, the bootstrap was set to SH-like branch supports, HKY85 [110] was used as the nucleotide-based model and parameter optimization was implemented for the tree topology, branch length and rate parameters. Finally the newick output from phyml was used as input for the tree-drawing tool, TreeDyn 198.3 [111] where all branches with branch support values smaller than 50% was collapsed.

2.2.4 Screening for plant growth-promoting and protective traits

For all biochemical assays, *Bacillus* isolate suspensions were adjusted to an optical density (OD) of 1.0 at 600 nm, and 30 μl of suspension was used to inoculate all media plates, unless otherwise stated. Also, all screening were done in triplicate. In cases where the *Bacillus* isolates produced high amounts of exopolysaccharides in LB broth, strains were alternatively grown in the media (either TSA, TY, R2A, or R2A+salt) that they were originally isolated with in order to obtain homogenous liquid culture for the biochemical and *Arabidopsis* seedling-inoculation assays.
2.2.4.1 Solubilization of phosphate and zinc oxide

Isolates were tested for their ability to solubilize phosphate (P) using the Pikovskaya et al. (1948) [112] protocol. Briefly, bacteria were grown on Pikovskya’s (PVK) Agar (M520, Himedia) plates at 28°C for 48 hours. Plates were observed for zone of clearance around the bacterial inoculum to identify bacteria capable of solubilizing P.

Similarly, modified PVK Agar [113] was used to screen isolates for their ability to solubilize zinc oxide (ZnO). Here too, Plates were observed for zone of clearance around the bacterial inoculum to identify bacteria capable of solubilizing ZnO.

2.2.4.2 Production of indole acetic acid (IAA), ammonia (NH₃) and siderophores

Production of IAA was assessed using the Brick et al. (1991) [114] protocol. Briefly, IAA production was detected qualitatively by adding two drops of orthophosphoric acid and 2 ml of the Salkowski reagent to bacterial supernatant, then incubating the tube at room temperature for 30 min. Development of pink color was used as an indicator of IAA production.

NH₃ production was assessed using the Cappuccino and Sherman (1996) [115] protocol. Briefly, 50 µl of bacterial suspension was inoculated in 5 ml of peptone broth and incubated at 28°C for 48–72 hours. Subsequently, 250 µl Nessler’s reagent was added to each tube. Development of brown-orange color was used as an indicator of ammonia production.

Siderophore production was detected qualitatively using the Blue Agar CAS assay as described by Louden et al. (2011) [116]. Formation of an orange-yellowish zone of clearance was used as an indicator of siderophore production.
2.2.4.3 Screening for antimicrobial activity

Antimicrobial activity against pathogens (including *Pseudomonas syringae* DC3000 and two pathogenic fungal strains *Botrytis cinerea* and *Alternaria brassicicola*) were detected using dual culture assay. Briefly, the fungal strains were grown at 24°C for 20-25 days on Potato Dextrose Agar (PDA) plate, and *P. syringae* DC3000 were grown at 28°C, 200 rpm, overnight on LB broth. The pathogens and *Bacillus* isolates suspensions were adjusted to an optical density (OD) of 1.0 at 600 nm. The spread plate technique was used to inoculate LB agar plates with 100 μL aliquots of each individual *Bacillus* culture, and then sterile disks were soaked with the pathogenic bacterial suspension and added to each inoculated plate. Formation of a zone of clearance was used as an indicator of a potential biocontrol agent.

2.3 Results and Discussion

2.3.1 Isolation and characterization of endophytes from diverse desert plants

We isolated 381 endophytes from various desert plants (*Panicum antidotale, Tribulus terrestris, Zygophyllum simplex, Euphorbium officinarum* and *Lasiurus scindicus*). Then determined their taxonomic classification based on their 16S rRNA gene sequences, we found 146 of the 381 strains were unique at species level. These 146 species belonged to four major phyla Firmicutes (42 strains; 8 genera), Actinobacteria (54 strains; 16 genera), Proteobacteria (45 strains; 24 genera) and Bacteroidetes (5 strains; 3 genera) (Figure 2.1, see also Appendix_1). In accordance with current knowledge, most of the isolates were
from the Actinobacteria and Proteobacteria phyla, but we also found isolates from the Firmicutes phylum similarly dominant. Surprisingly, of these culturable bacteria, the Firmicutes phylum showed more diversity at the species level within much less genera (Appendix_1). Also, the genus Bacillus (Firmicutes) was identified as the major taxon (17.12 %), followed by genus Microbacterium (Actinobacteria, 13.01 %). More than 69 % of the endophytic bacteria were isolated from Panicum antidotale (52.17 %) and Tribulus terrestris (17.39 %). Moreover, only Bacillus endophytes were isolated from all plant types (Figure 2.2).

**Figure 2.1:** Un-rooted phylogenetic tree showing the bacterial diversity, at both phylum and genus level, isolated from all plants.
Actinobacteria, at both phylum and genus level, Proteobacteria and Firmicutes in *Mes*, while non-

It is interesting to note that Koberl *et al.* (2011) [117] showed that bacteria isolated from

Marasco *et al.* (2012) [118] further reported that bacteria isolated from the endosphere, rhizosphere, and root-surrounding soil from desert farmed *Capsicum annum* L. plants (growing in the desert region in Egypt, near El-Tawheed Village) were primarily Proteobacteria and Firmicutes, while non-cultivated arid soil harbored more Actinobacteria (genus *Cellulosimicrobium*) and Firmicutes (genus *Bacillus*) than Proteobacteria. This result was corroborated by Dai *et al.* (2013) [119] in part, whom reported the dominance of endophytes Proteobacteria and Firmicutes in *Carragana microphylla* growing in a plantation in the desert-region of Ningxia Hui, China. El-Deeb *et al.* (2013) [120] also reported the isolation of endophytic bacteria from *Plectranthus tenuiflorus* growing in the Saudi Arabian desert that primarily included Firmicutes (genus

**Figure 2.2:** Bar graph showing bacterial diversity, at both phylum and genus level, isolated from all plants.
Bacillus), Actinobacteria and Proteobacteria using only Tryptic Soy Agar (TSA) and Potato Dextrose Agar (PDA) media. While Zhao et al. (2016) [121] reported the use of only low nutrient media Reasoner's 2A (R2A), designed to facilitate the growth of stressed slow growing bacteria, to isolate endophytes from the Salicornia europaea plant growing in arid saline land (in Gurbantunggut desert, China), and reported the dominance of Actinobacteria, Proteobacteria and Firmicutes. Li et al. (2017) [122] similarly reported a search for endophytes associated with desert plant Lepidium perfoliatum L. and reported the isolation of 62 Bacillus strains only, despite the use of general-purpose non-selective beef extract peptone medium. Taken together, these studies reported Firmicutes counts to be higher than Actinobacteria counts in the endorhizosphere of both cultivated and noncultivated desert plants, while Actinobacteria counts were higher in desert soil. Moreover, this result shows the consistent isolation of Firmicutes despite differences in plant types and only one or two media types being used in specific studies.

Here we report the dominance of these same phyla (Actinobacteria, Proteobacteria and Firmicutes) but based on a less biased approach that utilizes five different media (including rich or low nutrient media, and salt-containing media) (see Materials and Methods). Moreover, in our study, Firmicutes counts, especially those of the Bacillus genus, are more pronounce in the endorhizosphere of noncultivated desert plants. Note that Firmicutes counts in desert soil has been previously reported to be half that of Actinobacteria [28], whereas here, we show that Firmicutes counts from the endorhizosphere of desert plant are similar to those of both Actinobacteria and Proteobacteria (see Figure 1).
From published data, it seems noncultivated desert soil is generally dominated by Actinobacteria and Proteobacteria, followed by Firmicutes. However, from our study and other endorhizosphere studies, the Firmicutes (especially genus *Bacillus*) counts become more competitive with host interaction. Taking these aspects into account and *Bacillus* diversity at the species level being more pronounced than *Microbacterium* (Appendix_1), we focused our search for biofertilizers on the 64 strains belonging to the *Bacillus* genus. Strains were randomly selected based on their clustering (see Figure 3). The phylogenetic tree illustrates that the 58 strains can be categories into eight clusters based on their 16S rRNA gene sequences. Out of 58 strains, 25 were selected based on their GTG5 clustering (see Figure S1) and tested for their ability to growth in liquid media. Some strains were unable to grow in liquid media, thus only 16 strains that are placed in five of the eight clusters were screened for their potential as biofertilizers.
Figure 2.3. Phylogenetic tree showing the relationship of the 58 newly isolated strains. The sequence alignment was performed using the T-Coffee online tool and trees were built using Phyml. The strains used in this screening process are marked with an asterisk.
2.3.2 Identification of potential biofertilizers

2.3.2.1 Screening for select plant growth promotion traits

The 16 Bacillus strains were screened for their ability to solubilize phosphorous (P) and zinc oxide (ZnO), produce indole-3-acetic acid (IAA), ammonia and siderophores, as well as their antimicrobial effects against known plant pathogens (Table 2.1). Among them, only Bacillus licheniformis (PK5-26) and Bacillus cereus (PK6-15) could solubilize P and ZnO, respectively. Also, Bacillus badius (PK3-68) and three Bacillus circulans (PK3-15, PK3-138, and PK3-109) were the only strains capable of producing IAA. However, most of the strains (13 out of 16) were capable of producing ammonia.

Noteworthy, B. licheniformis (PK5-26), B. cereus (PK6-15) and B. badius (PK3-68) that exhibit some of the other plant growth-promoting traits (such as solubilizing P and ZnO, and production of IAA) were amongst the strains capable of producing ammonia. Also, such PGP traits have been reported for other B. cereus [123, 124], B. licheniformis [125, 126] and B. circulans [127] strains as well, but our study seems to be the first to report plant growth promoting B. badius strain.

When screening bacteria for PGP traits, it is common to screen for bacteria capable of solubilizing phosphate and IAA owing to the proof-of-concept provided through several field studies. However, screening for zinc solubilizing bacteria (ZSB) is less common, even though this trait has been shown to positively facilitate plant growth. Specifically, Sharma et al. (2011) reported zinc-solubilizing Bacillus strains recovered from soybean rhizosphere soil significantly increased Zn content in soybean seeds as compared with the
uninoculated controls [57]. Naz et al. (2013) recently reported a field study that demonstrated wheat treated with zinc solubilizing bacteria (ZSB) from genera *Azospirillum*, *Pseudomonas* and *Rhizobium* significantly increased the zinc contents in different parts of the wheat plant at specific growth stages, thereby facilitating efficient nutrient’s uptake that translates into improved plant growth [56]. Gontia-Mishra et al. (2017) bioprospected for ZSB from the rhizosphere of rice and reported that rice seedlings inoculated with ZSB from genera *Pseudomonas*, *Ralstonia*, *Burkholderia* and *Klebsiella* showed significant plant growth compared to uninoculated seedlings [58]. So, it seems the search for ZSB is on the rise, but to our knowledge, our study is the first to report *B. cereus* (PK6-15) as a zinc-solubilizing *Bacillus* recovered from desert plants.

### 2.3.2.2 Screening for plant protective traits

We also screened for siderophore production, however, only the *B. subtilis* strains (PK3-9 PK1-2 and PK1-3) and *B. licheniformis* (PK5-26) exhibited siderophore production. Thus, *B. licheniformis* PK5-26 was the only strain capable of solubilizing phosphate, and producing ammonia and siderophores.

Several studies reported siderophores function in scavenging of iron from the host or environment [128], and their function in the biological control of pathogens as several siderophores exhibit antimicrobial activity [129, 130]. For plant-related research, pyoverdine siderophores produced by Pseudomonads were shown to be involved in the control the wilt diseases of potato caused by *Fusarium oxysporum* [83], the inhibition of plant growth caused by *Gaeumannomyces graminis* [86], and maize root diseases caused by *Macrophomina phaseolina*, *Fusarium moniliforme* and *Fusarium graminearum* [97].
Also, bacillibactin siderophore producing *B. subtilis* CAS15 significantly reduced the incidence of wilt disease caused by *Fusarium* in pepper, and that iron supplementation reduced this biocontrol effect [131]. This result suggests that bacillibactin, in this case, may be responsible for the biocontrol effect. Recently, Ruiz *et al.* confirmed one of the siderophore-associated mechanisms involved with the biocontrol of plant pathogens. That is, they demonstrated the plant disease suppressing *Pseudomonas protegens* strain survives in toxic environment created by the metal chelating mycotoxin, Fusaric acid (produced by *Fusarium strains*) by producing metal scavenging siderophores including pyoverdine and pyochelin. On point, Butaite *et al.* [132] recently demonstrated that non-producers of siderophores, with the appropriate siderophore-receptors, can exploit foreign siderophores, while siderophore-producers producing exclusive siderophore types renders the iron acquisition inaccessible to competing strains that lack the appropriate receptor. These finding suggest that the functions of siderophores (scavenging of iron and antimicrobial activity) are both involved in shaping microbiome diversity and community dynamics. However, siderophores produced by these and other *Bacillus* strains have generally not been screened for their antimicrobial effects.

Al-amoudi *et al.* (2016) [133, 134] reported the Firmicutes phylum (specifically strains from the *Bacillus* genus) to be better targets for antimicrobial bioprospecting than Actinobacteria due to selection pressure in environments exposed to high salinity and hydrocarbon contamination. For that reason, we further verified if these select *Bacillus* strains are capable of producing antimicrobial effects that will confer disease resistance to the plant, despite only four strains showing siderophore production potential. All strains were screened against known plant pathogens, *Pseudomonas syringae* that causes
bacterial speck disease [135], and fungal strains *Botrytis cinerea* and *Alternaria brassicicola* that cause grey mould disease [136] and rot disease [137], respectively. Most strains showed potential as biocontrol agents except *B. licheniformis* (PK5-26), *B. subtilis* (PK1-2) and *B. subtilis* (PK5-68). Specifically, 12 of the 16 strains exhibit antimicrobial effects against *A. brassicicola*. However, only *B. circulans* (PK3-109) exhibit antimicrobial effects against *P. syringae*, while *B. cereus* (PK6-15) and *B. circulans* (PK3-109) exhibit antimicrobial effects against *B. cinerea*. Thus, only *B. subtilis* (PK3-9) exhibit siderophore production and antimicrobial effects against *A. brassicicola*. Moreover, *B. circulans* (PK3-109) and *B. cereus* (PK6-15) were the only strains that exhibit antimicrobial effects against two of the plant pathogen used in this screening process. Also, *B. subtilis* (PK1-3, PK3-2, PK5-52 and PK3-9) and *B. circulans* (PK3-15 and PK3-138) strains displayed the most effective clearing of *A. brassicicola*. However, this finding is not surprising as *B. subtilis* isolate B7 [138] and *B. subtilis* OTPB1 [139] were reported to be effective in exhibiting antimicrobial effects against *A. brassicicola*. 
Table 2.1: *Bacillus* strains screened for plant growth promotion traits. In this table, potential biocontrol agents against, 1/ *Pseudomonas syringae* DC3000 is indicated using *, 2/ *Botrytis cinerea* is indicated using √, and 3/ *Alternaria brassicicola* is indicated using ∞. Elsewhere positive activity is indicated using (-) and negative activity is indicated using (+).
<table>
<thead>
<tr>
<th>Strain code</th>
<th>Identification based on 16S rRNA sequencing</th>
<th>Nutrient uptake traits</th>
<th>Growth promoting traits</th>
<th>Disease suppression traits</th>
<th>Anti-microbial effects</th>
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<tr>
<td></td>
<td></td>
<td>Phosphate solubilization</td>
<td>Zinc solubilization</td>
<td>IAA production</td>
<td>Ammonia production</td>
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</table>
2.4 Concluding Remarks

Molecular characterization of the bacterial isolates from the nine desert plants indicates that our isolates (146 bacteria) belonged to four major phyla including Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes. Thus, we report the same dominant phyla as in other published plant-related literature. However, in this study, Firmicutes counts are shown to be more pronounced in the endorhizosphere of noncultivated desert plants which is in agreement with previous reports for cultivated desert plants, but are not in agreement with desert soil findings that show Firmicutes counts to be approximately half that of Actinobacteria counts. Specifically, in this study, the Firmicutes phylum showed more diversity at the species level within much less genera and the genus Bacillus (Firmicutes) was identified as the major taxon, followed by genus Microbacterium (Actinobacteria). Moreover, only Bacillus endophytes were isolated from all plant types. Thus the findings in this study, and other endorhizosphere studies, suggest that the Firmicutes (especially those from the Bacillus genus) counts become more competitive with host interaction.

Moreover, when screening Bacillus strains to identify those with biofertilizing potential, most of these strains were shown to be capable of ammonia production and antibacterial effects against A. brassicicola. Noteworthy, the newly isolated B. licheniformis (PK5-26), B. cereus (PK6-15) and B. badius (PK3-68) strains that exhibit some of the other plant growth-promoting traits (such as solubilizing P and ZnO, and production of IAA) were
amongst the strains capable of ammonia production. Thus, in this study these characteristics were not used as selection criteria, as majority of strains showed these characteristics. All other potential plant growth promotion traits and disease suppression capabilities assessed in this study allowed for the shortlisting of the most promising strains including B. licheniformis (PK5-26), B. cereus (PK6-15), B. badius (PK3-68), B. circulans strains (PK3-15, PK3-138 and PK3-109), B. subtilis strains (PK3-9, PK1-2 and PK1-3) and B. endophyticus (PK5-39). To the best of our knowledge, this is the first B. badius strain reported to have PGP traits, and only B. circulans (PK3-109) exhibit antimicrobial effects against P. syringae.
Chapter 3: *In vitro* screening of *Bacillus* endophytes for their resilience promoting potential in *A. thaliana*

3.1 Introduction

Several research initiatives focus on finding means to alleviate both biotic and abiotic stresses as these factors are the cause of erratic agricultural yields and significant crop losses [140-142]. Abiotic stresses also influence the spread of pathogens and insects [143-145], and the co-occurrence of multiple abiotic stresses has been shown to exert a more devastating effect on crop production [146, 147]. In this regard, at present, ~20% of total cultivated and 33% of irrigated agricultural lands are affected by salt stress [148] and more than 50% of arable land is expected to be affected by both drought and salinity by 2050 [4, 5]. Both stresses have been shown to affect the plant water potential and turgor pressure, thereby resulting in a reduction of plant growth. Thus, several studies have focused on identifying appropriate biofertilizers that can alleviate these stresses in several crops such as rice [149, 150], maize [151, 152], tomato [153, 154] and wheat [155]. However, very few studies have focused on the identification of biofertilizers that can alleviate both salt and drought stress. This is problematic as an estimated 50% increase in major crops such as rice (*Oryza sativa* L.), maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) is required to fulfill food supply requirements for the projected population by 2050 [156, 157]. This knowledge coupled with the expectation that more agricultural land will be exposed to climate change volatilities and severity of these stresses would increase, highlights the urgency for developing biofertilizers that can confer crop resilience to multiple abiotic and biotic stresses as climate-smart agriculture.
To identify the type of bacteria that can provide such resilience to multiple abiotic stresses, bacteria from desert plants were isolated as those are expected to provide resilience at least against drought stress, then to further screen these bacteria to identify those that also confer resilience to salinity stress. Thus in this chapter, we aim to assess the resilience of the desert plant endophytes identified in Chapter 2 against abiotic stresses such as heat and salt, and assess their effects on plant growth.

### 3.2 Materials and Methods

#### 3.2.1. Inoculation methods: direct-contact (drop) vs indirect (plug) inoculation

The effect of inoculating *Arabidopsis thaliana* Col-0 with each of the identified strains (*Bacillus licheniformis* (PK5-26), *Bacillus cereus* (PK6-15), *Bacillus badius* (PK3-68), *Bacillus circulans* (PK3-15; PK3-138; PK3-109), *Bacillus subtilis* (PK3-9; PK1-2; PK1-3) and *Bacillus endophyticus* (PK5-39)) was evaluated. Seeds of *Arabidopsis thaliana* Col-0 were surface sterilized, stratified on ½ Murashige and Skoog (½MS) media for 2 days and then germinated for an additional 5 days at 22°C, long day photoperiod (16h light/8h dark), 70 Lux. Germinated seedlings were then transferred to fresh ½MS and ½MS +100mM NaCl and inoculated with 10 µl (for Drop method, see below) and bacterial plugs (for Plug method, see below) of individual strains (*Enterobacter* spp. (SA187) and *B. subtilis* (PK3-9). In the Drop method, the bacterial suspension was washed and resuspended in ½MS media that was adjusted to an optical density (OD) of 0.2, 0.02 and 0.01 at 600 nm. Inoculated seedlings were incubated at 22°C, 16h light/8h dark, 70 Lux, for a total of 15 days post-inoculation. On day 9, lateral root density was
calculated. Fresh weight was measured for plants growing in ½ MS on day 12 and for those growing in ½ MS +100mM NaCl on day 15.

The cell counts at OD of 1.0 at 600 nm were assessed via FACS. Briefly, 10 µl of *Bacillus* isolates (OD of 1.0 at 600 nm) were diluted in 1 ml dH2O. As a cleaning step, cells were centrifuged at 13000 rpm for 10 min, then the supernatant was removed and the pellet resuspended in 1 ml dH2O. From this suspension, a total volume of 50 µl of unstained cells was acquired with a BD LSRFortessa™ and the cell count was determined using BD FACSDiva™ software gating on the side scatter (SSC) vs forward scatter (FSC) profiles of cells.

**Indirect (Plug) method**

On LB agar, 100 µl of each liquid culture was spread as a bacterial lawn with an L-shaped cell spreader (VWR) and incubated overnight at 28°C. Rectangular-shape plugs were cut out of the plate cultures and then placed beside each seedling without touching the seedling. The control used for this method was LB plugs without bacterial.

**Direct (Drop) method**

Bacterial cultures were grown overnight in LB broth at 28°C and 220 rpm. Culture concentrations were then adjusted to an optical density (OD) of 0.2, 0.02 and 0.01 at 600 nm for each bacterial strain. Bacterial cultures were then pelleted and washed with ½ MS media to remove all bacterial media from the bacterial suspension. A final inoculum of 10 µl of washed bacterial suspension was inoculated to each of the 5-days-old seedlings. The control used for this method was uninoculated seedlings.
3.2.2. Screening strains for their resilience against abiotic stress

Isolates were screened for their resilience against heat and salt stresses. In brief, the *Bacillus* isolates inoculated on LB agar plates were incubated at multiple temperatures (28°C, 37°C, 42°C and 50°C). These *Bacillus* isolates suspensions were also inoculated on LB agar plates containing various salt concentrations (0.5, 1, 1.5, and 2 M NaCl) that were incubated at 28°C.

3.2.3. Assessing the newly isolated strain effects on plant growth

The effect of inoculating *Arabidopsis thaliana* Col-0 with each of the identified strains (*Bacillus licheniformis* (PK5-26), *Bacillus cereus* (PK6-15), *Bacillus badius* (PK3-68), *Bacillus circulans* (PK3-15; PK3-138; PK3-109), *Bacillus subtilis* (PK3-9; PK1-2; PK1-3) and *Bacillus endophyticus* (PK5-39)) was evaluated in the following way. Seeds of *Arabidopsis thaliana* Col-0 were surface sterilized, stratified on ½ Murashige and Skoog (½MS) media for 2 days and then germinated for an additional 5 days at 22°C, long day photoperiod (16h light/8h dark), 70 Lux. Germinated seedlings were then transferred to fresh ½MS and ½MS +100mM NaCl and inoculated with 10 µl of individual identified strains (suspended in ½MS media adjusted to an optical density (OD) of 1.0 at 600 nm). Inoculated seedlings were incubated at 22°C, 16h light/8h dark, 70 Lux, for a total of 15 days post-inoculation. On day 9, lateral root density was calculated. Fresh weight was measured for plants growing in ½ MS on day 12 and for those growing in ½MS +100mM NaCl on day 15.
3.3 Results and Discussion

3.3.1. Bacillus strain resilience towards abiotic stresses

Here, the 16 strains screened in Chapter 1 were also evaluated for their resilience against low (0.5M NaCl), mild (1M NaCl), high (1.5M NaCl) and severe (2M NaCl) salt stress conditions (see Table 3.1). All the strains grew well on LB media with no added NaCl and with added 0.5M NaCl. However, *B. subtilis* (PK5-16) was the only strain that grew exclusively on low salt media (0.5M NaCl), while all other strains displayed higher resilience, growing on media of high salt concentrations (1.5M NaCl). Moreover, *B. subtilis* (PK3-2), *B. licheniformis* (PK5-26), and *B. endophyticus* (PK5-39), were the only isolates able to grow under high salt stress conditions (2M NaCl). These results suggest that most of the bacteria are likely moderate halophiles (growing best in media containing 0.5–2.0 M NaCl) indicating that they do not have an absolute requirement for salt to grow, but can often also grow at very high salt concentrations [158, 159].

The 16 strains were also screened for their thermotolerance (Table 3.1). All strains grew at 37°C and 42°C on LB media. The majority of strains were also able to grow at 50°C, except *B. badius* (PK3-68), *B. circulans* (PK3-15 and PK3-109) and *B. cereus* (PK6-15). Thus, even though we have insufficient data to classify these strains precisely, the data suggest that most of the strains may be simple/moderate thermophiles that can thrive at moderately high, but also at lower temperatures.

Thus, all 10 shortlisted strains (Chapter 1) exhibited resilience to temperature up to at least 42°C and mild salt stress conditions (1.5M NaCl).
Table 3.1: *Bacillus* strains evaluated for their resilience against abiotic stresses. The 10 strains shortlisted in Chapter 1 are denoted in blue font.

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Identification based on 16S rRNA sequencing</th>
<th>Media</th>
<th>Temperature (°C)</th>
<th>NaCl (M)</th>
<th>1 or 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK3-68</td>
<td><em>Bacillus badius</em></td>
<td>LB</td>
<td>(+) (+) (-)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK3-9</td>
<td><em>Bacillus subtilis</em></td>
<td>LB</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (+)</td>
<td></td>
</tr>
<tr>
<td>PK3-2</td>
<td><em>Bacillus subtilis</em></td>
<td>LB</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (+)</td>
<td></td>
</tr>
<tr>
<td>PK3-15</td>
<td><em>Bacillus circulans</em></td>
<td>LB</td>
<td>(+) (+) (-)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK3-138</td>
<td><em>Bacillus circulans</em></td>
<td>TSA</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK5-26</td>
<td><em>Bacillus licheniformis</em></td>
<td>TSA</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (+)</td>
<td></td>
</tr>
<tr>
<td>PK5-39</td>
<td><em>Bacillus endophyticus</em></td>
<td>TSA</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (+)</td>
<td></td>
</tr>
<tr>
<td>PK1-2</td>
<td><em>Bacillus subtilis</em></td>
<td>TSA</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK1-3</td>
<td><em>Bacillus subtilis</em></td>
<td>TSA</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK5-17</td>
<td><em>Bacillus subtilis</em></td>
<td>TY</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK5-16</td>
<td><em>Bacillus subtilis</em></td>
<td>TY</td>
<td>(+) (+) (+)</td>
<td>(+) (-) (-)</td>
<td></td>
</tr>
<tr>
<td>PK5-68</td>
<td><em>Bacillus subtilis</em></td>
<td>TY</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK5-52</td>
<td><em>Bacillus subtilis</em></td>
<td>TY</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK6-15</td>
<td><em>Bacillus cereus</em></td>
<td>R2A</td>
<td>(+) (+) (-)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK3-109</td>
<td><em>Bacillus circulans</em></td>
<td>R2A+1.5%NaCl</td>
<td>(+) (+) (-)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
<tr>
<td>PK3-4</td>
<td><em>Bacillus subtilis</em></td>
<td>LB</td>
<td>(+) (+) (+)</td>
<td>(+) (+) (-)</td>
<td></td>
</tr>
</tbody>
</table>
3.3.2. Establishing the ability of these strains to facilitate growth of *Arabidopsis thaliana* under salt stress conditions

3.3.2.1. Determining the suitability of the inoculation method: direct contact (drop) versus indirect (plug) inoculation

In Chapter 2, the most promising potential plant growth promoting strains including *B. licheniformis* (PK5-26), *B. cereus* (PK6-15), *B. badius* (PK3-68), *B. circulans* strains (PK3-15, PK3-138 and PK3-109), *B. subtilis* strains (PK3-9, PK1-2 and PK1-3) and *B. endophyticus* (PK5-39), were shortlisted based on their plant growth promotion traits and disease suppression capabilities. Before commencing to assess the 10 strains for their ability to facilitate growth of *A. thaliana* under salt stress conditions, the suitability of the inoculation method was determined. The drop method is well-established [30] wherein the bacterial inoculants are added directly to the seedlings at a specific concentration. While the plug method was only recently developed [29], wherein bacterial inoculants grown on agar are cut into rectangular shaped plugs that are placed next to each seedling root thereby avoiding a direct contact between plant and bacteria. These two methods were used to assess the efficacy and usefulness in testing potential PGPR effects on plants, using *A. thaliana* with the known PGPR *Enterobacter* spp. (SA187) [29] and *Bacillus subtilis* (PK3-9) that was chosen randomly from this ‘Pakistan collection’ (see Figure 3.1, 3.2 and 3.3).
Figure 3.1: Comparison of Indirect (Plug) vs Direct contact (Drop) inoculation methods applied for *A. thaliana* using two strains *Enterobacter spp.* (SA187) and *Bacillus subtilis* (PK3-9) under non-salt (1/2 MS) and salt stress conditions (1/2 MS+100 mM NaCl).
Figure 3.2: *Arabidopsis thaliana* inoculated with *Enterobacter* spp. (SA187) and *Bacillus subtilis* (PK3-9) using the indirect (Plug) assay under (A) non-salt (1/2 MS) and (B) salt stress (1/2 MS+ 100 mM NaCl) conditions.

![Image](image1)

(A) (B)

Figure 3.3: *Arabidopsis thaliana* inoculated with *Enterobacter* spp. (SA187) and *Bacillus subtilis* (PK3-9) using the direct (Drop) assay under (A) non-salt (1/2 MS) and (B) salt stress (1/2 MS+ 100 mM NaCl) conditions.

**Control in plug vs. drop:** When comparing the uninoculated controls in the two methods under non-salt condition (Figure 3.1), the fresh weight in the plug method was decreased by 28.4% compared to the drop method. However, the fresh weight in the salt stress conditions did not show a difference in both methods. This latter result excludes the possibility that the 5g/L (85.6 mM) of NaCl in the LB plug (see method) might negatively affect plant growth and hence the plug and drop method provide equivalent results under salt stress conditions.
Inoculation of *A. thaliana* with *Enterobacter spp.* (SA187): Using the plug method, *Enterobacter* spp. (SA187) increased the total fresh weight of *A. thaliana* grown under non-salt condition by only 10% more than the uninoculated control. In contrast, *Enterobacter* spp. (SA187) increased the total fresh weight of *A. thaliana* exposed to salt stress by 250% compared to the uninoculated control. Whilst using the drop method, *Enterobacter* spp. (SA187) (OD$_{600}$ = 0.02) increased the total fresh weight of *A. thaliana* grown under non-salt by 18% and by 280% under salt conditions. These results show that the plant growth promotion induced by *Enterobacter* spp. (SA187) was more or less equivalent by the two methods.

Inoculation of *A. thaliana* with *Bacillus subtilis* (PK3-9): Using the two methods, and when compared with the uninoculated control, *Bacillus subtilis* (PK3-9) decreased the fresh weight of *A. thaliana* under non-salt conditions to 74%, and to 16% under salt stress conditions using the plug method. In contrast, under salt stress conditions, *Bacillus subtilis* (PK3-9) increased the fresh weight of *A. thaliana* grown by 62% under salt conditions using the drop method.

Even though *Enterobacter spp.* (SA187) showed comparable results in both methods, as it increased the total fresh weight of *A. thaliana* compared to the uninoculated control. However, this was not the case for *Bacillus subtilis* (PK3-9) as it showed very different behavior in the two different methods. Both strains showed more effective plant growth promotion using the drop method irrespective of differences that may exist in modes of mobility and plant growth promotion traits.

This suggests that the drop method is more effective in such in vitro studies than the plug method. The observed differences of the behavior of the two strains may be a
consequence of the motility required to reach the potential host plant either passively via
diffusion in the medium or actively via the flagellar or other movement systems [14].
Also, when using *Bacillus subtilis* (PK3-9) as an inoculant with concentrations
corresponding to an OD600 of 0.2, 0.02 and 0.01, we determined that the inoculant at
concentration OD600 of 0.01 showed a better increase in total plant fresh weight under
salt stress conditions (Figure 3.4). In contrast, no major differences were found for
SA187 using different concentrations (Axel de Zelicourt, pers. comm.). In summary, the
drop and the plug assays result in different for plant growth and hence cannot be easily
compared between different bacterial species.

![Figure 3.4: Inoculation efficacy of different concentrations (OD600 of 0.01, 0.02, and
0.2) of *Bacillus subtilis* (PK3-9) inoculated with *A. thaliana* under non-salt (1/2 MS) and
salt stress conditions (1/2 MS+100 mM NaCl).](image)
3.3.2.2. *In vitro* screening for plant growth promotion Bacillus endophytes

*A. thaliana* seedlings were inoculated with strains showing PGP traits and antimicrobial capabilities against both bacterial and fungal plant pathogens. In Table 3.2 (also see Supplementary Material_S3.1), we list the strains based on their ability to increase fresh weight of *A. thaliana* compared to the uninoculated control under salt stress conditions (100 mM NaCl). Bacteria were categorized as plant growth-promoting bacteria ((+)PGPB) and/or salt tolerance plant growth-promoting bacteria ((+)ST-PGPB) if their presence conferred at least a 10% increase in fresh weight of *A. thaliana* compared to the uninoculated control in the absence and in the presence of NaCl, respectively. *B. cereus* (PK6-15), *B. licheniformis* (PK5-26) and *B. circulans* (PK3-109) increased in lateral root density (LRD) of *A. thaliana*, that was associated with the primary root length (RL) decreasing by 17%, 20%, and 35%, respectively, compared to uninoculated controls. However, these strains did not enhance plant growth under non-salt conditions, as indicated by no increases in total, aerial or root fresh weight of the plants (Figure 3.5, also see Supplementary Material_S3.1). However, all strains strongly enhanced growth of *A. thaliana* under salt stress conditions, when compared to the uninoculated control plants, except *B. endophyticus* (PK5-39) and *B. circulans* (PK3-138) (Figure 3.5, also see Supplementary Material_S3.1). Interestingly and in contrast to non-salt conditions, the LRD and RL did not increase although, both root and aerial fresh weight as well as total fresh weight increased. These results suggest that the interaction of these *Bacillus* strains with *A. thaliana* regulates quite different responses during non-salt and salt stress conditions and further investigations are needed to clarify the molecular processes.
Although *B. licheniformis* (PK5-26) slightly increased plant growth, this effect was minimal in comparison to other strains like *B. badius* (PK3-68), which exhibited the ability to support plant growth under non-salt conditions (denoted as (+)PGPB) as well as under salt stress conditions (denoted as (+)ST-PGPB). However, *B. endophyticus* (PK5-39) and *B. circulans* (PK3-138) did not positively affect the growth of *A. thaliana* despite the fact that these strains had the ability to fix nitrogen and produce IAA, respectively (see Supplementary Material_S3.2). These results indicate that strains displaying PGP traits in the biochemical assays will not necessarily function as a PGPB during the interaction with a plant. Reasons for this discrepancy might be that the levels of PGP factors produced by the bacteria may not be sufficient to enhance plant salt stress tolerance. The communication between specific *Bacillus* strains and *Arabidopsis* might be more complicated as evidenced by the results of the test with *B. circulans* (PK3-15 and PK3-109), *B. cereus* (PK6-15) and *B. subtilis* (PK3-9) which did not display growth enhancement under non-salt stress conditions (denoted as (-)PGPB), but these strains surprisingly increased the fresh weight of *A. thaliana* by at least more than 50% when compared to uninoculated control plants under salt stress conditions (see Supplementary Material_S3.1). For the strains that do not show PGP traits, possible reasons might be an inhibitory effect of salt or that *A. thaliana* may not be the optimal host.

Upadhyay *et al.* (2009) [160] reported that majority of IAA-producing strains could not produce IAA at 8% (1.4 M) NaCl and that levels of IAA produced by bacteria decreased in all isolates with increasing levels of NaCl. However, many salt tolerant strains in this study did not display a decrease in facilitating plant growth, but rather showed an increase in plant growth in the presence of salt, that is, *A. thaliana* (+)PGPB fresh weight
was approximately half that of (+)ST-PGPB fresh weight. This suggests that either the salt stress is activating \textit{Bacillus} to produce beneficial factors that plants can benefit from to survive in the salt stress or that the stressed plants are producing factors to stimulate the bacteria to produce beneficial factors for the plants to survive in the salt stress. However, in our assays, the salt stress conditions for \textit{A. thaliana} growth were chosen at 100 mM NaCl, which already showed a strong growth inhibitory effect on the plants. In contrast, all bacterial strains could grow without any negative effects at 0.5 M NaCl, suggesting that the trigger for the induction of bacterial PGP factors might most likely originate in the stressed plants. At present, the molecular events during the interaction under these two conditions are not clear and require further investigations.

Nonetheless, in comparison to the \textit{Bacillus} PGPR in published literature [161-164], \textit{B. cereus} (PK6-15), \textit{B. licheniformis} (PK5-26) and \textit{B. circulans} (PK3-109) increasing the fresh weight of \textit{Arabidopsis} exposed to salt by 78%, 71% and 62%, respectively, shows the potential of these strains to be biofertilizers based on increase in total fresh weight of the plant under salt stress conditions. That is, Barriuso \textit{et al.} (2008) reported \textit{Bacillus} sp. L81, originally isolated from the rhizosphere of \textit{P. pinea}, when used as an inoculant, increased \textit{A. thaliana}’s fresh weight by 95 % under salt stress conditions using the direct contact method [161]. Siddikee \textit{et al.} (2010) demonstrated inoculating canola seedlings with \textit{Brevibacterium epidermidis} RS15, \textit{Micrococcus yunnanensis} RS222, and \textit{Bacillus aryabhattachi} RS341 (isolated from coastal soils), all increased plant root elongation and dry weight by more than 40% when compared with uninoculated control under salt stress condition using a direct contact method [162]. Liu \textit{et al.} (2017) also used a direct contact method to demonstrate that \textit{B. amyloliquefaciens} FZB42 as inoculant facilitates the
growth of *A. thaliana* in the presence or absence of 100 mM NaCl. They reported an increase of 31.2% and 24.7% in the plant fresh weight at 0 and 100 mM NaCl, respectively [164]. This strain was initially isolated from the rhizosphere soil of lettuce and it has been used widely in commercial applications to support the production of a broad range of economically important plants [165]. Chen *et al.* (2017) also reported the use of another *B. amyloliquefaciens* strain (SQR9) as an inoculant. SQR9 increased *A. thaliana* lateral root numbers but not primary root length under salt stress conditions and enhanced plant growth associated with an increase in total fresh weight of approximately 100% using the direct contact method [163].

The differences in the effectiveness of the bacteria may also be due to the fact that plants respond to different stresses uniquely and these processes are highly complex and involve changes at the transcriptome, cellular, and physiological levels, and due to the bacteria’s metabolic make-up might be unique and further studies are needed to unravel this complex network of interacting factors in the PGPR strains identified in this study.
Table 3.2: *Bacillus* strains screened for their ability to confer plant growth promotion under salt stress conditions. Strains that increase the fresh weight of *A. thaliana* at least more than 50% compared to the un-inoculated control are indicated using blue font.

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Identification based on 16S rRNA sequencing</th>
<th>Conferring PGP traits and salt tolerance</th>
<th>Production and Solubilization Capabilities</th>
<th>Bacterial Resilience</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK6-15</td>
<td><em>Bacillus cereus</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>Zinc &amp; Ammonia</td>
<td>1.5 42</td>
</tr>
<tr>
<td>PK5-26</td>
<td><em>Bacillus licheniformis</em></td>
<td>(+)PGPB &amp; (+)ST-PGPB</td>
<td>Phosphate, Siderophore &amp; Ammonia</td>
<td>2 50</td>
</tr>
<tr>
<td>PK3-109</td>
<td><em>Bacillus circulans</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>IAA, Exopolysaccharide</td>
<td>1.5 42</td>
</tr>
<tr>
<td>PK3-9</td>
<td><em>Bacillus subtilis</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>Siderophore &amp; Ammonia &amp; Exopolysaccharide</td>
<td>1.5 50</td>
</tr>
<tr>
<td>PK3-15</td>
<td><em>Bacillus circulans</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>IAA</td>
<td>1.5 42</td>
</tr>
<tr>
<td>PK3-68</td>
<td><em>Bacillus badius</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>IAA &amp; Ammonia &amp; Exopolysaccharide</td>
<td>1.5 42</td>
</tr>
<tr>
<td>PK1-3</td>
<td><em>Bacillus subtilis PY79</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>Siderophore &amp; Ammonia &amp; Exopolysaccharide</td>
<td>1.5 50</td>
</tr>
<tr>
<td>PK1-2</td>
<td><em>Bacillus subtilis</em></td>
<td>(-)PGPB &amp; (+)ST-PGPB</td>
<td>Siderophore &amp; Ammonia</td>
<td>1.5 50</td>
</tr>
<tr>
<td>PK5-39</td>
<td><em>Bacillus endophyticus</em></td>
<td>(-)PGPB &amp; (-)ST-PGPB</td>
<td>Ammonia &amp; Exopolysaccharide</td>
<td>2 50</td>
</tr>
<tr>
<td>PK3-138</td>
<td><em>Bacillus circulans</em></td>
<td>(-)PGPB &amp; (-)ST-PGPB</td>
<td>IAA</td>
<td>1.5 50</td>
</tr>
</tbody>
</table>
Figure 3.5. Screening assay of A. thaliana inoculated with (A) Bacillus cereus (PK6-15) and (B) Bacillus subtilis (PK5-26) in non-salt (1/2 MS, 12 days) and in salt (1/2 MS+100mM NaCl, 15 days) conditions. The total fresh weight of Arabidopsis is represented as the mean of three biological experiments. Asterisks indicate statistical
differences compared with WT under the same conditions based on Mann-Whitney U Test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.4 Concluding Remarks

Before *in vitro* screening for plant growth promoting bacteria, the suitability of the inoculation method, as well as the resilience of the used *Bacillus* strains to heat and salt stress was determined. The direct contact (drop) inoculation method showed more effective plant growth promotion versus the indirect (plug) inoculation method despite differences that may reflect differences in the mobility and plant growth promotion traits in *Enterobacter* spp. (SA187) and *B. subtilis* (PK3-9). Moreover, the differences in the inoculation methods were also prominent under salt stress conditions, excluding a direct comparability of the two methods. When examining the resilience of the 10 strains against heat and salt stress, it was determined that they are likely mesophiles as well as moderate halophiles indicating that they can grow in high salt environments. This categorization of these strains based on their abiotic resilience is further supported by the results obtained when screening 10 *Bacillus* strains for their ability to facilitate plant growth promotion under salt stress conditions. That is, *A. thaliana* inoculated with strains such as *B. circulans* (PK3-15 and PK3-109), *B. cereus* (PK6-15) and *B. subtilis* (PK3-9) (see Supplementary Material_S3.1) only exhibited an increase in fresh weight under salt stress conditions suggesting that salt stress is a requirement for optimal growth, a trait displayed by moderate and extreme halophiles. Thus, the fitness of the bacterial strains in salt might be a determinant of its ability to facilitate growth in salt stressed environments.
Nonetheless, in comparison to the published literature, top ranked *Bacillus* strains reported in this study show the potential to be better biofertilizers based on the increase in total fresh weight under salt stress conditions. Further investigations to unravel the molecular mechanisms of the strain-specific plant-*Bacillus* interactions are needed to better understand the communication mechanisms in terms of plant and bacterial production of factors that alleviate salt stress.
Chapter 4: Comparative genomic analysis of ST-PGPB (PK3-109) and non-ST-PGPB (PK3-138) *Bacillus circulans* strains

### 4.1 Background

*Bacillus* species have adapted and continues to adapt to various niches including human, animal and plant hosts, food, soil, marine and fresh water environments etc. These adaptations are represented in their diverse genomic and physiological features [166, 167]. That is, *Bacillus thuringiensis* (Bt) was first isolated as the causative agent of sudden-collapse disease that killed silkworms, then from the Mediterranean flour moth, and both were found to produce unique insecticidal agents (genes that encode unique toxic crystals) that are key to the effectiveness of the commercially produced Bt spray used in agriculture [168]. *Bacillus amyloliquefaciens* FZB42 isolated from *Beta vulgaris* (sugar beet) roots is also used commercially as a biocontrol agent and biofertilizer [169], but *B. amyloliquefaciens* LL3 isolated from fermented vegetables is used for the commercial production of poly-\(\gamma\)-glutamic acid production [170]. *Bacillus dipsosauri* is also used in industry as a source of halophilic amylases [171], and *B. licheniformis* and *Bacillus stearothermophilus* as sources of thermostable \(\alpha\)-amylase and pullulanase, repectively [172]. Thus, understanding the molecular versatility of these *Bacillus* strains in different environments and how they can be used is of interest.

To unravel the different molecular features, several studies reports the *in silico* comparison of whole-genome sequencing data [173-175]. To mention a few, Alcaraz *et al.* (2010) reported that genomes of soil bacteria such as *B. subtilis* are enriched with carbohydrate metabolism genes, while genomes of pathogenic bacteria such as *B. cereus*
are enriched with genes involved in defense mechanisms [176]. Less generally, Hossain et al. (2015) used comparative genomics to identify the conserved genetic features in *B. amyloliquefaciens subsp. plantarum* associated with disease control in plants [177]. Likewise, Zhang et al. (2016) reported a genomic based *in silico* comparison of *Bacillus* strains isolated from plant-associated (PA) habitats to those isolated from non-plant-associated (nPA) niches. They showed that the genomes of *Bacillus* isolated from PA are enriched in genes involved in the utilization of plant derived substrates and for the biosynthesis of antimicrobial compounds when compared to the genomes of *Bacillus* isolated from nPA niches [178].

Here too we attempt to unravel the molecular versatility of *B. circulans* strains, specifically with respect to their interaction with plants. As shown in chapter 3, *B. circulans* (PK3-109) was shown to be a PGPR with several desirable traits (such as the production of IAA and exopolysaccharides, and antimicrobial effects against *P. syringae* DC3000 and *B. cinerea*), while *B. circulans* (PK3-138) was shown not to be a PGPR, despite exhibiting the ability to produce IAA and both strains being isolated from the same plant *P. antidotale*. That is, *B. circulans* (PK3-109) increased total fresh weight of *A. thaliana* under salt stress conditions by 62 %, while *B. circulans* (PK3-138) decreased the total fresh weight of *A. thaliana* by 47 %. Here, we report the sequenced genomes and annotation of both strains, to understand the genomic basis for plant growth promotion, as well as the possible causes for the lack of plant growth promotion exhibited by PK3-138.
4.2 Material and Method

4.2.1 DNA extraction and sequencing

The biomass of PK3-109 and PK3-138 was determined after growth under optimal conditions (as described Chapter 2). Genomic DNA was extracted using the Sigma GenElute Bacterial Genomic DNA Kit (Sigma, USA) following the manufacturer’s protocol followed by a second purification step using MO BIO PowerClean Pro Clean-Up Kit (USA). As a quality control, overnight gel electrophoresis and NanoDrop (Thermo Fisher Scientific, USA) were used to assess purity of DNA, while Qubit 2.0 (Life Technologies, Germany) was used to quantify the DNA. Whole genome sequencing was performed at the Core Lab sequencing facility at KAUST using the PacBio RS II sequencing platform (Pacific Biosciences, USA). The large-insert libraries were sequenced in single-molecule real-time (SMRT) sequencing cells using P6-C4 chemistry.

4.2.2 Genome assembly

Raw data from PacBio’s RS II were assembled using PacBio’s SMRT Analysis pipeline v2.3.0. The following SMRT Analysis pipeline modules were used with default parameter: P_Fetch, P_Filter, P_FilterReports, P_PreAssemblerDagcon, P_AssembleUnitig (genomeSize=6Mb), P_ReferenceUploader, P_Mapping, P_MappingReports and P_AssemblyPolishing. Assembly produced a single contig per library. We visually checked for overlapping ends using Gepard v1.40 [179] which would indicate circular genomes. To circularize both genomes, one end of each contig was trimmed to reduce the amount of overlap, then each contig was split into two halves.
which were then rejoined and overlap removed using minimus2 [180]. After circularization, multiple rounds of polishing were performed using the SMRT Analysis Resequencing protocol until convergence. The following modules were used with default parameters: P_Fetch, P_Filter, P_FilterReports, P_Mapping, P_MappingReports, P_AssemblyPolishing (Quiver) and P_ConsensusReports. After each polishing round, we count the number of variant positions that were generated from mapping filtered subreads to the polished genome. We repeated the polishing steps until the number of variant positions is reduced to the minimum possible (convergence).

4.2.3 Genome functional annotation and analysis

The complete genome sequences for PK3-109 and PK3-138 were submitted to the Automatic Annotation of Microbial Genomes pipeline (AAMG) [181] with default parameters (BLAST bit score of 30) and Prodigal [182] for gene prediction. For details about the annotation pipeline, tools and databases used, refer to [181]. The overall genome similarities between PK3-109 and PK3-138 were inspected using a dot plot that was generated with Gepard v1.40 [179]. Genome variation and synteny between the two strains were performed using AliTV (https://doi.org/10.7717/peerj-cs.116).

4.2.4 Phylogenetic tree construction

To perform a whole genome phylogenetic analysis, we require a representative dataset of complete genomes. Next, the core genome must be determined and genes in the core genome are then used to build a phylogenetic tree. Due to the all-versus-all nature of the
pangenome analysis, the bigger the dataset the more computationally intensive the analysis is and the longer it would take. To minimize compute time, we followed a guided approach to building the tree. First, we assumed that our two strains are in fact *B. circulans* based on the 16S rRNA phylogenetic placement as well as NCBI’s average nucleotide identity (ANI) analysis that is now carried out by default for all genome submissions. Second, we used www.timetree.com to curate a short list of bacterial species that are closely related to *B. circulans*. Working our way upward through the tree, we searched for available annotated genomes of closely related branches until we reached a species group that is distant enough to act as an outgroup (*B. endophyticus* in this case). Using this approach, we retrieved 13 bacilli genomes belonging to four different *Bacillus* species. Third, we calculated the pangenome of the 13 public strains plus our two strains using the Orthofinder tool v2.2.1 [183]. An all-vs-all BLASTp [184] annotation was performed for the protein sets of all 15 organisms included. Based on Orthofinder’s normalized BLAST bitscore, gene pairs were selected to build a graph which was then resolved into orthologous groups (orthogroups) using the MCL tool [185]. Gene trees were built for each orthogroup using the alignment-free Dendroblast tool [186]. The species tree was reconstructed by reconciling the gene trees into a single consensus tree using STAG [https://github.com/davidemms/STAG] which is then rooted using STRIDE [187]. Support values for the tree branches were calculated using STAG. Versions for DendroBLAST, STAG and STRIDER are those included in Orthofinder release v2.2.1. The final tree was visualized using FigTree v1.4.3 [http://tree.bio.ed.ac.uk/software/figtree/].
4.2.5 The pangenome of PK3-109 and PK3-138

To determine the core and accessory genes that are shared between or unique to either strains, we used panX [188]. PanX uses DIAMOND [189] for protein-protein alignments and MCL (hidden Markov clustering, [190]) to predict orthologous groups. We visualized the pangenome analysis results with a Venn diagram using jenvn [191].

4.3 Results and Discussion

4.3.1 Assembly and annotation of the B. circulans (PK3-109) and (PK3-138) genomes

The genome of B. circulans (PK3-109) and B. circulans (PK3-138) were sequenced using the SMRT sequencing platform. The sequencing generated 56,495 subreads with a mean length of 6,022 bp (58x genome coverage) for PK3-109 and 151,332 subreads with a mean length of 7,580 bp (141x genome coverage) for PK3-138. The assembly produced a single circular chromosome without plasmids for both PK3-109 and PK3-138. PK3-109’s circular chromosome is 5,175,203 bp in length containing 5,017 predicted open reading frames (ORFs), whilst PK3-138’s circular chromosome is 5,274,417 bp in length containing 5,073 predicted ORFs. Both genomes have 33 rRNAs and 75 tRNAs genes (Table 4.1). The assembled genome size is within the expected range compared to other B. circulans strains (see Table 4.1). Moreover, the genome size is closest to the endophyte B. circulans RIT379, isolated from the Insulin Plant Costus igneus [192].
The genome alignment indicated several regions of inversion and rearrangement. However, it seems the chromosomal alignments of these strains are approximately identical, as shown by the existence of large segments of high similarity when most portions of the two chromosomes are mapped onto each other (Figure 4.1).

Table 4.1: Genomic features for *B. circulans* (PK3-109) and (PK3-138), as well as six other sequenced *B. circulans* strains.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Genome size (Mb)</th>
<th>Genes</th>
<th>Proteins</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK3-109</td>
<td>5.175203</td>
<td>5249</td>
<td>5017</td>
<td>Desert plant <em>P. antidotale</em></td>
<td>this project</td>
</tr>
<tr>
<td>PK3-138</td>
<td>5.274417</td>
<td>5307</td>
<td>5,073</td>
<td>Desert plant <em>P. antidotale</em></td>
<td>this project</td>
</tr>
<tr>
<td>NBRC 13626</td>
<td>5.09725</td>
<td>4951</td>
<td>4791</td>
<td>NBRC culture collection</td>
<td>not available</td>
</tr>
<tr>
<td>RIT379</td>
<td>5.45427</td>
<td>5294</td>
<td>5088</td>
<td>Insulin Plant Costus igneus (endophyte)</td>
<td>[192]</td>
</tr>
<tr>
<td>7524</td>
<td>5.01172</td>
<td>4756</td>
<td>4615</td>
<td>Heroin provided by a Criminal Police Office</td>
<td>[193, 194]</td>
</tr>
<tr>
<td>7521-2</td>
<td>5.09634</td>
<td>4868</td>
<td>4748</td>
<td>Heroin provided by a Criminal Police Office</td>
<td>[193, 194]</td>
</tr>
<tr>
<td>7520-T</td>
<td>5.03287</td>
<td>4790</td>
<td>4646</td>
<td>Heroin provided by a Criminal Police Office</td>
<td>[193, 194]</td>
</tr>
<tr>
<td>7506</td>
<td>5.03451</td>
<td>4757</td>
<td>4611</td>
<td>Heroin provided by a Criminal Police Office</td>
<td>[193, 194]</td>
</tr>
</tbody>
</table>
Figure 4.1: Whole genome alignment of the genomes of PK3-109 and PK3-138. Overall, both genomes seem to be largely similar with large structural differences in the form of two inversions.

4.3.2 Phylogenetic placement

To further confirm the phylogenetic position of PK3-109 and PK3-138, we constructed a genome-wide SNP-based species tree using the core genome (xxx core genes) that is shared between our two strains and 14 other Bacillus species (Figure 4.2). Consistent with the phylogenetic tree that was built using the 16S rRNA genes, our two strains are closely related to other B. circulans strains. Whole genome phylogenetic trees suffer from the limited availability of sequenced genomes. All but one of the genomes that we used to construct the tree in figure 4.2 are at the draft contig or scaffold level. Therefore, any pangenomic analysis may not capture the entire picture; however, considering that the
number of genes annotated for all genomes are comparably similar, this strongly indicates that such analysis is in fact representative.

Figure 4.2: Genome-wide species tree of the two *B. circulans* PK3-109 and PK3-138 strains. The red box encloses the *B. circulans* cluster that includes PK3-109 and PK3-138. Within the *B. circulans* sub-cluster, PK3-109 and PK3-138 form a group with *B. circulans* 7521-2 (blue box).

4.3.3 *B. circulans* (PK3-109) and (PK3-138): Genome features

Functional analysis for these strains performed using KEGG tool BlastKOALA [195] allowed for the identification of 2602 genes (51.9 % of all CDSs) for PK3-109 and 2609 genes (51.4 % of all CDSs) for PK3-138 (Table S4). The KEGG pathway metabolism categories showed most of the metabolism-related genes to be involved in metabolism of carbohydrates (13.9 % for PK3-109, 14.4 % for PK3-138), amino acids (9.5 % for PK3-109, 8.4 % for PK3-138) and cofactors and vitamins (6.3 % for PK3-109, 5.8% for PK3-
138). While most of the remaining genes were shown to be classified as ABC transporters (4.7% for PK3-109, 5.3% for PK3-138), phosphotransferase system (PTS) (1.9% for PK3-109, 2.8% for PK3-138), two-component system (3.2% for PK3-109, 3.5% for PK3-138), quorum sensing (2.3% for PK3-109, 2.5% for PK3-138), bacterial chemotaxis (0.7% for PK3-109, 0.8% for PK3-138) and flagellar assembly (1.1% for PK3-109, 1.1% for PK3-138). Some endophytic bacteria are transferred to the next generation of plants as a consequence of their presence in the seeds [196, 197], while others have to survive in the soil prior to colonizing the plant [198]. Based on the genome features, both PK3-109 and PK3-138, seem capable of surviving in the plant rhizosphere because their genomes encode many transporters involved in carbohydrate, amino-acids and iron uptake, as well as some resistance genes.

4.3.3.1 Surviving in the rhizosphere

Both strains are unable to grow autotrophically, but can use a large variety of plant-derived compounds as carbon sources including glucose, galactose, galactitol, mannitol, sucrose, arbutin, trehalose, mannose, maltose, fructose, cellobiose, fructoselysine, glucoselysine and ascorbate. To facilitate the use of these carbon sources both strain genomes encode complete Embden-Meyerhof and pentose-phosphate pathways. Taking into account that phosphorylated carbohydrates such as glucose-6-P or fructose-6-P are used as precursors of glycolysis to produce two phosphoenolpyruvate molecules (one of which is used to drive the transport and initial phosphorylation of the carbohydrate) and that PTS functions in the transport and phosphorylation of carbohydrates [199], the presence of multiple PTS (such as glucose-specific II component, maltose-specific II component, beta-glucosides-specific II component, trehalose-specific II component,
arbutin-specific II component, sucrose-specific II component, fructose-specific II component, mannitol-specific II component, cellubiose-specific II component, galactitol-specific II component, mannose-specific II component, ascorbate-specific II component, glucitol/sorbitol-specific II component, and the fructoselysine and glucoselysine-specific II component) in both strains genome supports this process. Also, the gene ptsI that encodes Enzyme I of the PTS, positively regulates transcription of sodA2 that encodes manganese-containing superoxide dismutase (MnSOD2) required for colonization of wheat rhizosphere by B. cereus 905 [200, 201]. Gao et al. (2017) recently showed loss of ptsI caused a 70% reduction in sodA2 expression, 1000-fold reduction in colonization of wheat roots, as well as a reduced growth rate, thus PTS also plays an important role in rhizosphere colonization by regulating sodA2 expression in B. cereus 905 [201]. Thus PTS is recognized as a key factor in efficient uptake of plant synthesized nutrients and the plant associated lifestyle [202].

Another genome characteristic that contributes to the plant associated lifestyle/nutrient uptake is a large diversity of transporters. For PK3-109, a total of 340 ORFs encode putative transporter proteins: among them 225 coded for membrane transporters (ABC transporters, PTS and bacterial secretion system), 44 coded for metallic cation, iron-siderophore and vitamin B12 transport, 65 coded for phosphate and amino acid transport system and 6 encoded drug efflux transporter/pump (see complete list of putative transporters in Table S4). For PK3-138, a total of 325 ORFs encode putative transporter proteins: among them 217 coded for membrane transporters (ABC transporterS, PTS and bacterial secretion system), 45 coded for metallic cation, iron-siderophore and vitamin B12 transport, 57 coded for transporters from the phosphate and amino acid transport
system and 6 encoded drug efflux transporter/pump (see complete list of putative transporters in Table S4). These bacterial genomes also harbor several two-component regulatory systems (TCRS) that enable the two strains to detect physical and/or chemical changes and then relay these signals through the cytoplasm to the bacterial nucleoid, where modulation of gene expression occurs. Both strains have the complete set of genes for TCRS such as PhoR-PhoB (phosphate starvation response), VicK-VicR (cell wall metabolism), BceS-BceR (bacitracin transport), DegS-DegU (multicellular behavior control), DesK-DesR (membrane lipid fluidity regulation), LiaS-LiaR (cell wall stress response), MalK-MalR (malate transport), YesM-YesN, NisK-NisR (lantibiotic biosynthesis), ResE-ResD (aerobic and anaerobic respiration), LytS-LytR. However, PK3-138 genome also harbors more that 70 % of the genes needed for the CheA-CheYBV (chemotaxis) and KinABCDE-Spo0FA (sporulation control) TCRSs. In particular, the DegS–DegU two-component system was shown to mediate the salt stress response in Bacillus intermedius via salt-specific induction [203]. This DegS–DegU signal transduction system was also shown to be involved in the transition from the exponential to the stationary growth phase under growth-limiting conditions [204]. This provides an idea of how both these strains should be capable of surviving in the rhizosphere and able to survive in salt-stress conditions.

4.3.3.2 Moving towards the roots for colonization

To colonize the roots, endophytes require a means of motility. Bacteria can follow water fluxes for passive movement but the port of entry in the plant does not always correspond to the plant part colonized by endophytes. Thus, they require active motility via flagellar and chemotaxis for the bacterial colonization process at plant surfaces [205]. Both PK3-
109 and PK3-138 genomes have the same flagellar biosynthesis genes (see Figure 4.3) including those involved in Type-III secretion (fliHIOZPQRAB), motor/switch (motAB), C-ring (fliGMNY), M,S,P and L rings (fliF), regulation (fliAMFG), Chaperones (fliJSTWY) and formation of rod, hook and filament (flgBCDGEKL). They also have pili biosynthesis genes including those involved in twitching motility (pilT) and pilus assembly (pilBCONM) and genes for chemotaxis including two component system proteins (cheAWCDRBYVX) and methyl-accepting chemotaxis proteins (MCPs) (mcp and hemAT).

The chemotactic responses of these bacteria are determined by the root exudates that serve as chemoattractants for these bacteria. In this respect, the genomes of PK3-109 and PK3-138 harbor transport systems for exudates such as spermidine/putrescine, proline, glycine betaine/proline etc. Firiedman et al. (1986) reported an increase in salt stress applied to sunflower plants results in an increase in levels of putrescine and spermidine in the exudate [206]. Also, Marin et al. (2010) reported an increase in proline levels in root tissue and exudates from rootstocks exposed to salt stress. Glycine betaine was also shown to be an exudate and its accumulation in transgenic rice seedlings enhanced their tolerance to salt and heat stress [207]. These findings indicate that both of these B. circulans strains likely have the flagella required for active motility and they can be attracted to the root of plants exposed to salt stress.

Moreover, some of the two-component systems that help their survival in the rhizosphere have also been shown to affect colonization. That is, respiration in B. subtilis using oxygen or nitrate requires the ResD-ResE signal transduction system and this system is induced at the stationary phase of aerobic growth or when exposed to oxygen limitation.
This suggests that this system is likely induced upon colonization due to changes in oxygen availability in the plant. Also, the LytSR two-component regulatory system was shown to regulate the cid and lrg operons, which affect murein hydrolase activity, stationary-phase survival, antibiotic tolerance, and biofilm formation [209]. Moreover, disruption of this lytSR was shown to alter murein hydrolase activity and induce spontaneous cell lysis [210]. LiaR was also shown to regulate expression of genes involved in microbial defence against host antimicrobial systems, cell wall synthesis, pili formation and cell membrane modification [211]. Also, Xu et al. showed that in Bacillus amyloliquefaciens SQR9, DegU regulates genetic competence, swarming motility, biofilm formation, complex colony architecture, and protease production [212].
Phosphorus limitation enhances biofilm formation of the plant pathogen *Agrobacterium tumefaciens* through the PhoR-PhoB regulatory system [213], which is the same one
present in our strains. However, PhoP/PhoR is one two-component regulatory system present in *B. subtilis* that responds to phosphate deficiency [214].

Both genomes also harbor the FLOT gene that encodes the membrane microdomain-associated proteins flotillin that localize to the peribacteroid membrane (PBM) and is required for nodule organization and function [215, 216]. Specifically, it plays a role in infection thread (IT) initiation and elongation, and the entry of the bacteria [215-217]. It is known that rhizobia associate with plants by developing this IT, through hydrolysis of the plant cell wall and invagination of the plasma membrane, to guide them through the root cell layers to the nodule primordium where it is released by an endocytosis-like process to form the symbiosome [217].

These genomic features suggest that both strains have the ability to move towards the roots and potentially enter the plant for colonization via IT.

4.3.3.3 Plant microbe interactions that support plant growth promotion

Both strains exhibit the ability to produce IAA (Chapter 2), a major property of rhizosphere bacteria that stimulate and facilitate plant growth. According to the genes present in their genomes this can be accomplished via amidase [EC: 3.5.1.4] or aldehyde dehydrogenase (NAD+) [EC: 1.2.1.3]. Wittenmayer & Merbach (2005) reported a 52 % increase in carbohydrates released from P-deficient plants treated with IAA, whereas no significant change was observed in P-deficient plants supplied with P [218]. This suggests that bacterially produced IAA could also induce the release of increased levels of carbohydrate exudates from plants, that is essentially an increased nutrient supply for bacteria [219].
However, several other mechanisms may also be contributing to the bacterial ability to support plant growth such as the regulation of ethylene. Both strains had all the genes that code for the enzymes required to produce ethylene either from L-Cystathionine (via 1-aminocyclopropane-1-carboxylate synthase [EC: 4.4.1.14] and aminocyclopropanecarboxylate oxidase [EC: 1.14.17.4] or from L-Methionine (via S-adenosylmethionine synthetase [EC: 2.5.1.6]). However, 1-aminocyclopropane-1-carboxylate (ACC) deaminase [EC: 3.5.99.7] is absent from both genomes, which suggests that they are unable to metabolize ACC as a way to moderate the host plant’s ethylene stress response. PGPR such as *B. subtilis* and *B. amyloliquefaciens* were also shown to emit a blend of volatile compounds such as acetoin and 2,3-butanediol that enhance plant growth [220]. PK3-109 and PK3-138 also have all the genes necessary to produce volatile compounds such as acetoin and 2,3-butanediol from pyruvate. However, acetylactate decarboxylase [EC: 4.1.1.5] that is able to convert 2-acetylactate directly to acetoin was absent from both genomes. Thus these strains have other characteristics that can contribute to plant growth promotion beyond IAA.

### 4.3.4 Comparing the genomes of *B. circulans* (PK3-109) and (PK3-138)

The pangenome analysis revealed 3996 clusters of orthologs (containing 9337 proteins) that are common to both species. A total of 2 clusters of orthologs (containing 4 proteins) and 4 clusters of orthologs (containing 9 proteins) were identified as being specific to the PK3-109 and PK3-138, respectively. Additionally, 366 and 410 singletons were determined to be specific to PK3-109 and PK3-138, respectively.
Figure 4.4: A Venn diagram illustrating the core and accessory genome of PK3-109 and PK3-138 strains. The intersection refers to the core genome with 3,996 orthogroups. Numbers in black bold are orthogroups. Colored numbers between brackets are the actual number of proteins making up the respective orthogroup category. Bracketed numbers outside of the Venn diagram refer to singletons that do not have orthologues or paralogues. Blue refers to PK3-109 while pink/purple refers to PK3-138.

Genes in the unique orthogroups may contribute to PK3-109’s ability to significantly increase the total fresh weight of *A. thaliana* under salt stress conditions compared to the uninoculated controls, while *B. circulans* (PK3-138) substantially decreased the total fresh weight of *A. thaliana* compared to the uninoculated controls. Despite the fact that the genomes of the *Bacillus* strains are largely identical, potential functional differences were searched for unique sequences, excluding the presence of homologous domains and predicted protein-protein interactions (PPR) (see Table 4.2).
### Table 4.2: Potential function of proteins in the unique orthologs.

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>BASED ON SEQUENCE</th>
<th>BASED ON DOMAIN</th>
<th>BASED ON PPI</th>
<th>DESCRIPTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK3_109</td>
<td>N/A</td>
<td>yxiG</td>
<td>YesG interacts with yapl</td>
<td>Proteins interacting together tend to be involved in the same function. yapl protects the cell against the toxic activity of yapA. yapl is an essential gene and mutation on it causes growth defects.</td>
<td><a href="https://string-db.org/network/224308_Boob1_0101000021151">https://string-db.org/network/224308_Boob1_0101000021151</a></td>
</tr>
<tr>
<td>PK3_109</td>
<td>N/A</td>
<td>yxiG</td>
<td>Equivalent to the UmuC subunit of the E. coli DNA polymerase V that is involved in translesion synthesis. Translesion synthesis (TLS) is a DNA damage tolerance</td>
<td>Repair [1] &amp; Defense mechanism [2].</td>
<td></td>
</tr>
<tr>
<td>PK3_138_80</td>
<td>Transposase, IS116/IS110T</td>
<td>IS902</td>
<td>Transposases are needed for efficient transposition of the insertion sequence or transposon DNA. This family includes transposases for IS116, IS110 and IS902. It is often found with the transposase IS111A/IS1328/IS1533 family</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>PK3_138_80</td>
<td>Transposase, Tn5-like, core</td>
<td>This superfamily represents the central core domain of Tn5-type transposase proteins. Prokaryotic Tn5 transposase makes two types of DNA contacts (cis-contacts for DNA recognition and trans-contacts for catalysis) as well as protein-protein contacts.</td>
<td>[3, 4]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK3_138_80</td>
<td>Glyoxylase/B leomycin resistance protein/Dihyd roxybilophenyl dioxygenase</td>
<td>The entry represents a domain containing a beta-alpha</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK3_138_80</td>
<td>Trp repressor/repl icator initiator</td>
<td>The Trp repressor (TrpR) binds to at least five operators in the E. coli genome, repressing gene expression. The TrpR controls the trpEDCBA (trpO) operon and the genes for trpR, aroH, mtr and aroL, which are involved in the biosynthesis and uptake of the amino acid tryptophan.</td>
<td>[PMID: 12234917], [PMID: 12475235]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK3_138_80</td>
<td>Glyoxylase/B leomycin resistance protein/Dihyd roxybilophenyl dioxygenase</td>
<td>The entry represents a domain containing a beta-alpha</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK3_138_80</td>
<td>Transposase IS3/IS911fam ily</td>
<td>Transposase proteins are necessary for efficient DNA transposition.</td>
<td>[5]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The function of most of the proteins in the unique orthogroups is unclear. However, YxiG was shown via a protein-protein interaction network, generated using STRING, to cluster with WapI (Figure 4.5). WapI is an immunity protein component of a toxin-immunity protein module, which functions as a cellular contact-dependent growth inhibition (CDI) system, that is, it protects the bacterial cell against the toxic activity of WapA. Koskiniemi et al. (2013) reported a *Bacillus subtilis* strain that harbors genes that encode a *wapA-wapI* toxin-antitoxin module, and WapA was shown to cause toxicity to neighboring *Bacillus subtilis* cells lacking the protective antitoxin, WapI [221]. Also, Stempler et al. (2017) demonstrated that *Bacillus subtilis* rapidly inhibits *Bacillus megaterium* growth via its tRNase toxin WapA and that this toxin is delivered via the same route as nutrients are received [222]. Moreover, Dartois et al. (1998) demonstrated using mutation/deletion analyses of the wapA promoter region that the putative DegU control site is involved in the repression of wapA transcription at high salt concentrations and revealed a second site of repression located downstream from the transcription start site. Moreover, repression was observed at this second site in the absence of DegU, suggesting that an additional repressor acts on the wapA control region to further downregulate wapA transcription under salt stress conditions [223]. Thus the activity of the toxin WapA is repressed via two mechanisms, 1/ wapA transcription is repressed by DegU that is activated under salt stress conditions, and 2/ via the antitoxin WapI, in both strains. However, the results suggest that this toxin activity may not have been ameliorated in PK3-138 as its presence decreased the total fresh weight of *A. thaliana* by 47% despite salt stress, indicating that the antitoxin may not be functioning in this way. This conclusion is based on the WapA toxin being delivered via the same route as
nutrients are received [222] and Abu Baker et al. (2016) reporting that the toxin from the *Streptococcus pneumoniae* toxin-antitoxin (*yefM-yoeB*) system was lethal in the model plant *A. thaliana* [224]. This means that the WapA activity in PK3-109 has likely been effectively repressed, as its presence does not exert the same toxic effects on *A. thaliana*. This suggests that WapA repression via transcription and antitoxin WapI may be working effectively in PK3-109, and it further suggests that since the YxiG is only present in the PK3-109 genome (that clusters with WapI in the PPI network), it may be involved in the antitoxin activity of WapI.

![Figure 4.5](image_url): Protein-protein interaction clustering generated via STRING showed YxiG to cluster with WapI (in blue) using kmeans clustering.
4.4 Concluding Remarks

Here, we report the sequenced genomes and annotation of two closely related *Bacillus* endophyte strains, to understand the genomic basis for plant growth promotion, as well as the possible causes for the lack of plant growth promotion exhibited by PK3-138 in *Arabidopsis*. As expected, most of the genomes were identical in these strains, with both strains showing traits that allow to survive in the rhizosphere such as being able to utilize several carbon sources, and complementary membrane transporters (ABC transporters, PTS and bacterial secretion system), as well as several two-component regulatory systems (TCRS) that enable them to detect physical and/or chemical changes in the environment. In particular, they have the DegS–DegU two-component system that is known to mediate the salt stress response. They also show traits associated with moving towards the roots for colonization such as flagellar and pili biosynthesis genes and genes for chemotaxis and the FLOT gene that encodes the membrane microdomain-associated protein flotillin that functions in infection thread (IT) initiation and elongation, and the entry of the bacteria into the host plant. Their genomes also show other traits beyond IAA production that help facilitate plant growth including the gene set required for ethylene production, as well as those required for the production of acetoin and 2,3-butanediol from pyruvate. Moreover, we also found that both genomes harbor the *wapA-wapI* toxin-antitoxin system but this system may not be working effectively in PK3-138 as the presence of PK3-138 substantially decreases the total fresh weight of *A. thaliana* under salt stress conditions, which may be due to this toxin. Since PK3-109 is not exerting the same toxic effects on *A. thaliana*, this effect may be linked to the YxiG gene that is only present in the PK3-109 genome.
Taking into account that PK3-109 increased total fresh weight of *A. thaliana* under salt stress conditions by 62% compared to the uninoculated control, it should be viewed as a strong potential biofertilizer that requires further testing in field trials and mutation/deletion studies are required to ascertain the potential role of YxiG in the antitoxin activity of WapI.
Chapter 5: Conclusions

New formulations of biofertilizers are required as substantially more arable land is expected to be affected by both drought and salinity in the near future [4, 5]. However, this process is hampered by studies focusing on identifying potential biofertilizers from locations that have not been exposed to salt stress and research projects that do not use an effective screening process. To overcome this shortfall this project is focused on bioprospecting Thar desert plants (near salt lakes) for their endophytes with biofertilizer potential. In Chapter 2, I showed the isolation and molecular characterization of such endophytes. I report Firmicute counts to be more pronounced in the endorhizosphere of noncultivated desert plants despite Firmicute counts being reported to be approximately half that of Actinobacteria counts in previous studies. These results suggest that Firmicutes become more competitive with host interaction. A subsequent biochemical assay screen for plant growth promotion traits was focused on the Bacillus strains. Here, I showed how the biochemical assays could be used to shortlist the most promising potential biofertilizers by not using traits common to most strains in the shortlisting process. Through this process most promising strains including B. licheniformis (PK5-26), B. cereus (PK6-15), B. badius (PK3-68), B. circulans strains (PK3-15, PK3-138 and PK3-109), B. subtilis strains (PK3-9, PK1-2 and PK1-3) and B. endophyticus (PK5-39) were shortlisted.

Subsequently, in Chapter 3, these strains were tested for their resilience to temperature and salinity stress and shown to be likely mesophiles (20°C (or lower) to 45°C) as well as moderate halophiles (growing best in media containing 0.5–2.5 M NaCl). These results indicate that the Bacillus strains do not show an absolute requirement of salt for their
growth, but can grow well in high salt concentrations. Moreover, *A. thaliana* inoculated with strains such as *B. circulans* (PK3-15 and PK3-109), *B. cereus* (PK6-15) and *B. subtilis* (PK3-9) did not exhibit an increase in fresh weight in the absence of salt but displayed the ability to positively facilitate plant growth promotion in the presence of salt which suggests that salt is a requirement for optimal growth, a trait displayed by moderate and extreme halophiles. Moreover, three of our strains including *B. cereus* (PK6-15), *B. licheniformis* (PK5-26) and *B. circulans* (PK3-109) exhibit the ability to increase the total fresh weight of *A. thaliana* by more than 100 % when compared to the uninoculated control. Interestingly, the *B. circulans* strain that did not exhibit this ability could nonetheless produce IAA.

In Chapter 4, the genomes of *B. circulans* (PK3-109) that increased total fresh weight of *A. thaliana* under salt stress conditions by 62 %, and *B. circulans* (PK3-138) whose presence decreased the total fresh weight of *A. thaliana* by 47 %, were sequenced to unravel the molecular basis for their differential behavior with respect to their interaction with plants. As was expected, the genomes were found to be mostly identical and showed several traits that allow them to survive in the rhizosphere, move towards the roots for colonization and to facilitate plant growth. But the most interesting feature was the presence of the DegS–DegU two-component system that is known to mediate the salt stress response and a *wapA-wapI* toxin-antitoxin module as *wapA* is known to be repressed by DegU, as a gene in the orthogroup of PK3-109 was found to be linked to WapI. This suggests that when observing that PK3-138 substantially decreases the total fresh weight of *A. thaliana* under salt stress conditions, the toxic activity of WapA is not effectively ameliorated by WapI as is the case for PK3-109. Thus in this study, we
suggest that the YxiG gene may be involved in the antitoxin activity of WapI and consequently responsible for indirectly facilitating the plant growth promotion effects of PK3-109.

Overall, several strains have been identified that exhibit strong biofertilizer potential that requires further testing in field trials.


Table S4: BlastKoala results for protein sequences of PK3-109 and PK3-138

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| ABC transporters                            | 138    | 5.28% |
| Phosphotransferase system (PTS)             | 72     | 2.75% |
| Two-component system                        | 91     | 3.48% |
| Quorum sensing                              | 65     | 2.49% |
| Bacterial chemotaxis                        | 20     | 0.76% |
| Flagellar assembly                          | 29     | 1.11% |