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Insights into the lifestyle of *Kosakonia* rice endophytes

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ABSTRACT

Endophytes are microorganisms that live inside plants and are often beneficial for the host. *Kosakonia* is a novel bacterial genus which includes several species which are diazotrophic and plant-associated. In this thesis, plant-bacteria studies on two rice endophytic *Kosakonia* beneficial strains were performed. Three experimental chapters are presented, the first includes comparative genomics, secretome profiling, *in-planta* tests and a field release trial. Secretomics revealed 80 putative secreted proteins including type VI secretory system (T6SS) proteins. A *Kosakonia* T6SS genomic knock-out mutant showed a significant decrease in rhizosphere and endosphere colonization ability. A field trial using rice seeds inoculated with *Kosakonia* sp. showed no effect on plant growth promotion upon nitrogen stress and its microbiome studies revealed that *Kosakonia* was significantly more present in the inoculated rice. Comparative genomics evidenced that several protein domains were enriched in *Kosakonia* plant-associated strains. The second experimental chapter presents an enrichment strategy for the isolation of endophytes and for the identification of *Kosakonia* bacterial co-inhabitants in rice root endosphere. The last experimental chapter is a study on two LuxR solos from *Kosakonia* KO348 which are involved in cell-cell signaling and discusses their possible involvement in bacterial interspecies and plant-bacteria interkingdom signaling. One of these LuxR solos designated as LoxR, responds to exogenously signals produced by bacterial neighbors whereas the other, designated as PsrR, is important for plant-bacteria signaling. This thesis highlights that *Kosakonia* is an important recently classified genus having strains that possess good root endosphere colonization ability and undergoing signaling in the microbiome and with the plant host.

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Chapter I.

General Introduction

Plant-bacteria associations in the soil are well recognized and have been studied for many years; however our understanding about the interkingdom and interspecies signaling in plant associated microbes is still very limited (Santoyo et al. 2016). Bacterial communities associated with plants form the plant microbiota which is linked to plant fitness and health (Compant, Clement, and Sessitsch 2010). Part of the plant microbiota are microbial communities that live inside plant tissues and these microorganisms are called "endophytes". In recent years, endophytes are generating special interest due to their ability to colonize and adaptability to reside in the endosphere and also because of their desirable plant beneficial traits like nitrogen fixation and phytohormones production which make them good candidates as microbial biofertilizers to be used in modern agriculture (Hardoim et al. 2015; Thakore 2006).

The interest on biofertilizers has recently increased due to the pressure to produce more crops for a growing population as well as the need to reduce chemical additives in agriculture. Rice is a staple food for many different populations around the world, and with the development of low cost "OMICs" technologies, studies on the rice microbiota are considerably increasing (Edwards et al. 2015; Sessitsch et al. 2012). Our current knowledge on rice endophytes, their lifestyle, traits, interkingdom signaling (plant-bacteria) and plant growth promoting (PGP) mechanisms are still at large unknown.

This introduction describes the importance and members of the plant microbiota followed by the role of endophytes with a focus on the rice plant.

1.1 Plant microbiome

Like humans and animals, plants are complex organisms that co-exist with microbial communities originating from soil, air or water. These microbial communities associated with plants form the "plant microbiota" (Müller et al. 2016) which includes several thousands of bacterial and fungal species. Factors such as soil type, host genotype and vertically transferred microorganisms influences the plant microbiota (Edwards et al. 2015; Johnston-Monje and Raizada 2011). The totality of microbial genomes and its genetic information which is co-living with the plant is also called the "plant microbiome" and influences many different functions in the plant host and it is a key determinant for plant health and productivity (van der Heijden and Hartmann 2016; Müller et al. 2016). An important role in the establishment of the plant microbiome is the signaling taking place between plants and microorganisms as well as among microorganisms (Moe 2013; Schenk et al. 2012; Venturi and Keel 2016). Although, the study of the plant microbiome has not received the same attention as the human microbiome, this trend is changing as the number of reports is rapidly increasing (Hacquard et al. 2015).

1.1.1 Microbial networks inside the plant microbiome and its importance to host fitness

The plant microbiome represents thousands of diverse microorganisms that interact between each other in order to occupy and colonize the different plant associated niches. They interact in competitive, mutualistic, synergistic, commensalistic, ammensalistic or parasitic ways resulting in stable mixed populations (Müller et al.

2016; van der Heijden and Hartmann 2016). Adaptation of plant-associated (PA) bacteria to its environment was evident when analyzing over 3800 bacterial genomes including 1100 PA genomes. Results evidenced that PA genomes presented more carbohydrate metabolism functions with respect to non-PA; as well as 64 protein domains that potentially mimic plant domains (Levy et al. 2018). Moreover, the plant microbiome, can positively affect the plant status by providing nutrients, increasing bioavailability or enhancing acquisition capacity at the root level (Edwards et al. 2015; Müller et al. 2016).

Inside the plant microbiota community some members have a strong influence in the structure of the microbial community, these are called "keystone" microorganisms (Niu et al. 2017). For example, in maize roots when removing a *Enterobacter cloacae* strain from a synthetic consortium of seven different bacterial species, the other six where significantly decreased indicating the *E. cloacae* role as a keystone member (Niu et al. 2017). These complex interspecies microbial interactions highlight the importance of studying microbial communities under controlled laboratory conditions for assembling microbial communities that reflect the host-microbial homeostasis (Hassani et al. 2018).

Plant microbiome "core members" are a set of microbial taxa that are found in most samples of a particular set of plants (Busby et al. 2017; Lundberg et al. 2012). Meanwhile, the non-core, variable or "peripheral members" are transient members that enter into the community for a limited period of time depending on different factors, often due to changes in the environment and in the host immune state (Blaustein et al. 2017). Currently, one of the main aims of studying the plant

microbiomes is the identification and characterization of these core members for possible future uses as microbial additives/inoculants for a more sustainable agriculture (Gopal and Gupta 2016; Schlaeppi and Bulgarelli 2015).

1.1.2 The three plant compartments inhabited by microorganisms

The plant microbiota inhabits different plants compartments which are colonized differently by microorganisms (Gopal and Gupta 2016; Müller et al. 2016) (**Figure 1.1**). The compartment with highest diversity and distribution of microbial life is the rhizosphere; this is the root-surrounding soil. It is affected by root exudates being an area rich in carbohydrates, amino acids and secondary metabolites that together drive plant selection/recruitment of the microbial communities (Berg et al. 2005; Hartmann et al. 2008). The phyllosphere on the other hand is the aboveground/aerial surface compartment of different parts of the plant including leaves, stems, flowers and fruits. It is the largest part of the plant and it is exposed to air-borne pathogens and insects (herbivores) (Lindow and Brandl 2003). The microbial phyllosphere communities help the plant to keep air-borne pathogens away and also stimulate/protect the plant to overcome herbivore related biotic stresses (Saleem et al. 2017). Finally, the endosphere consists of the inner tissues of the plant, and the group of microorganisms able to colonize it, are called endophytes.

The first definition of endophytes was made in 1866 by De Bary as “any organism occurring within plant tissues“ (Berg et al. 2014). Colonization of internal tissues is a key step for endophytic lifestyle and the rhizosphere is the main source for endophytic entry via the lateral roots (Compant et al. 2010; Berg et al. 2014; Marquez-Santacruz et al. 2010) (**Figure 1.2**).

Studies in 30 phylogenetically diverse species of angiosperms confirmed that the rhizosphere associated microbiome was more diverse and abundant than the

endosphere; nearly 90% of bacterial phyla presented significant differential abundance between both plant compartments (Fitzpatrick et al. 2018). The analysis of more than 600 plants of *Arabidopsis thaliana* evidenced the strong influence of the bulk soil in the composition of the rhizospheric and endospheric microbial community (Lundberg et al. 2012). In summary, most endophytes among different plants and cultivars originate from the rhizospheric microbiota which in turn are recruited from the bulk soil.

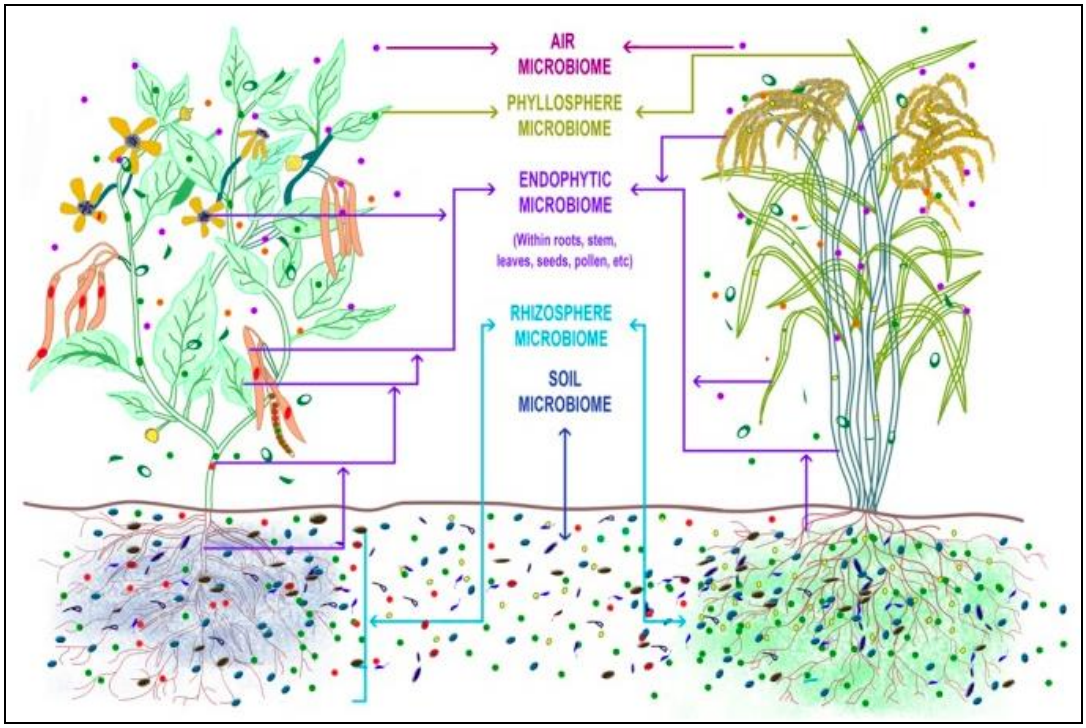


Figure 1.1. Plant microbiota components.
The rhizosphere, endosphere, phyllosphere constitute the major compartments in which the microbial communities reside in the plant and that in permanent interaction with the air and soil microbiome. Front Microbiol. 2016; 7: 1971.

1.1.3 Plant Growth Promoting Bacteria (PGPB) in the rhizophere and endosphere

Many members of the plant microbiota have a beneficial effect for their hosts. Microbial plant growth promoting (PGP) activities include; induction of plant immunity, acquisition of nutrients and resistance to biotic or abiotic stresses (Compant et al. 2010; Lugtenberg and Kamilova 2009). The majority of PGP studies from the last 30 years have been based on beneficial bacteria from the rhizosphere which are called plant growth promoting rhizobacteria (PGPR), a term that was coined in 1978 (J.W. Kloepper and Schroth 1978). Rhizospheric colonization occurs via host-microbe interactions signaling and recognition processes which ensures selection/recruitment of beneficial microorganisms (Benizri et al. 2001; Nelson and Sadowsky 2015) (**Figure 1.2**). PGP activities of endophytes are described in the following section.

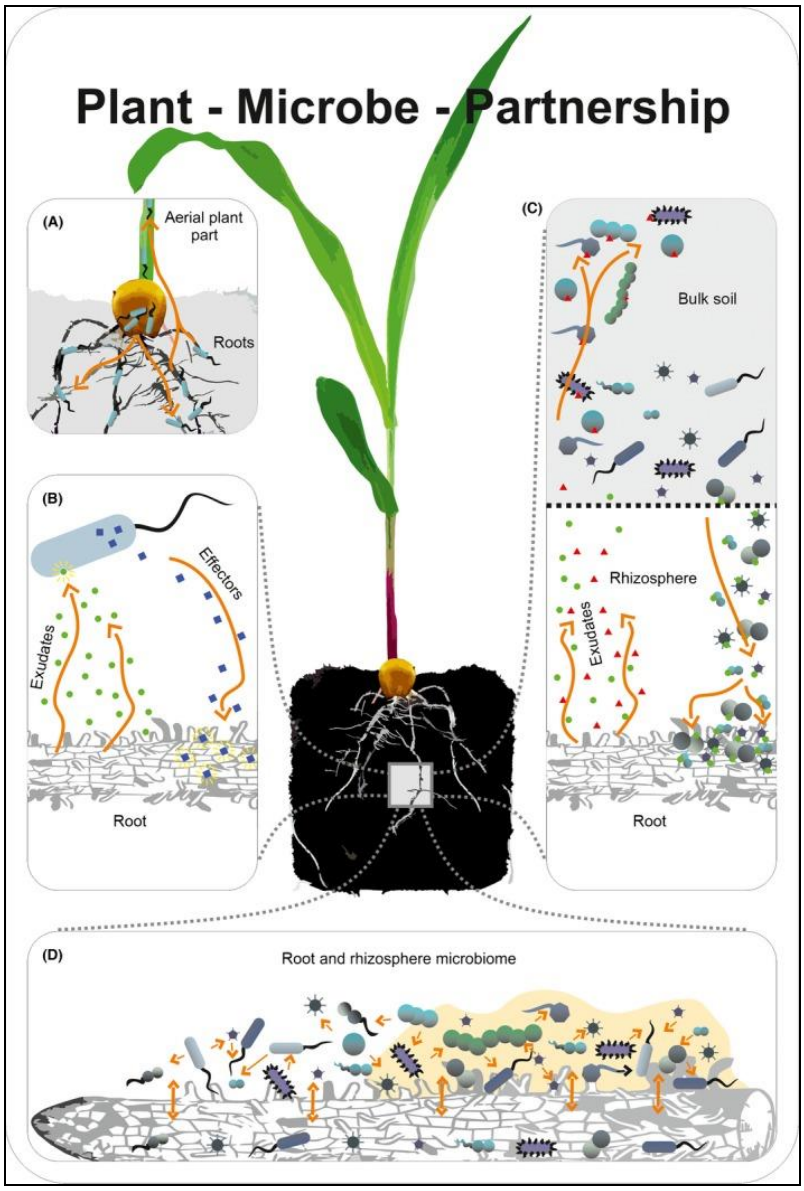


Figure 1.2.- Plant-microbe partnership at root level.
A. The first colonizers at rhizosphere and endosphere level are the seed-related microorganisms. **B, C.** For colonizing the rhizosphere there is high competence between bacteria where the exudates from the plant play an active role in the selection. **D.** The endosphere is colonized by a subset of rhizosphere bacteria. There is a dynamic interaction between the host exudates, the rhizosphere microbiota and root endosphere microbiota.
Microb Biotechnol. 2016 Sep; 9(5): 635–640

1.2. Endophytes

There are more than 300 000 species of plants on earth and the majority of them are thought to be harbored endophytes which colonize intercellular internal tissues (Smith et al. 2008; Heil 2015). A plant colonized by beneficial endophytes is more fit to fight pathogens and to overcome environmental stress conditions (Timmusk et al. 2011) and this is a reason why endophytes are of special interest given their potential to be used for a more sustainable agriculture (Thakore 2006). The definition of endophytes is under debate and many definitions have been postulated since the one given by Bary (1866). Hardoim et al. 2015 suggested that the term endophyte should address "only habitat and not function", and thus should include all the microorganisms that during part or all their lifetime colonize internal plant tissues. Instead, for Bulgarelli et al. 2013, the term endophyte is defined as microbial genome located inside plant organs. Le Cocq et al. 2017 recently defined endophytes as microorganisms that occur within plant tissues for at least part of their life cycle without causing disease under any known circumstances. In addition, Robinson et al. 2016 importantly stress that all isolated strains should be called "putative endophytes" unless they have been positively identified within the host by microscopy or by recovery from a gnobiotic plant. All these definitions of endophytes include bacteria, archaea, viruses, fungi and other unicellular eukaryotes; however this introduction is only focused on bacterial endophytes.

1.2.1 Transmission and diversity of bacterial endophytes

Endophytes are transmitted to the plant host through horizontal transmission via the soil, atmosphere or insects or by vertical transmission from the parent to the offspring via the seeds or pollen (Frank et al. 2017). Many bacterial endophytes are transmitted horizontally; the diversity of bacteria in rice seeds and seedlings grown under gnobiotic conditions is much lower than the plants grown in soil (Hardoim et al. 2012). Bacterial endophytes are "generalists" as often their beneficial properties are not limited to a specific host, but to distantly related plant species (Ma et al. 2011; Compant et al. 2005). Endophytes with larger genomes colonize a wider number of hosts in comparison to endophytic isolates with smaller genomes (Mitter et al. 2013) **(Figure 1.3**).

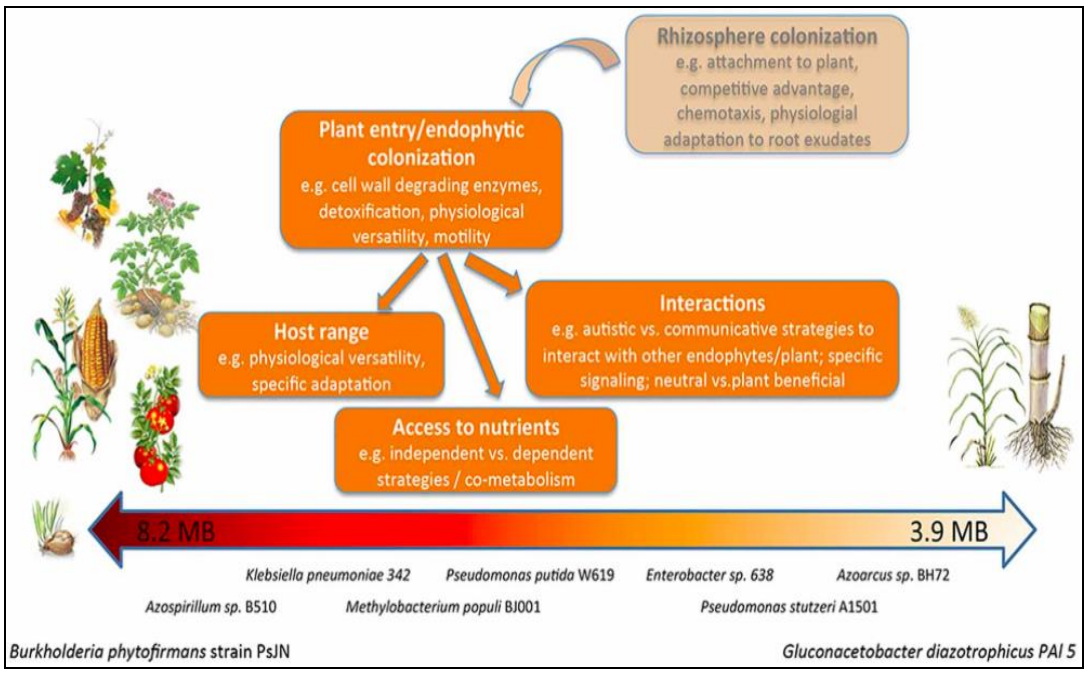


Figure 1.3.- Scheme of the study Mitter et al, 2013.
Differences in genome size of the studied endophytic strains and their reported hosts range. Summary of the main described features of the endophytic lifestyle.
Front Plant Sci. 2013 Apr 30;4:120

The most common route of colonization of endophytes is via the primary and lateral root cracks (Sørensen J 2015) (**Figure 1.4**); there are other less used routes of endophytic colonization such as through stem, leaf surfaces, flowers and fruits (Sessitsch et al. 2012; Berg et al. 2005; Okunishi et al. 2005). Once inside the plant, endophytes establish populations at inter- or intracellular level (Hurek et al. 1994; Zakria et al. 2007) and some can colonize other plant tissues by entering into vascular tissues and reaching other organs (Johnston-Monje and Raizada 2011) (**Figure 1.4**).

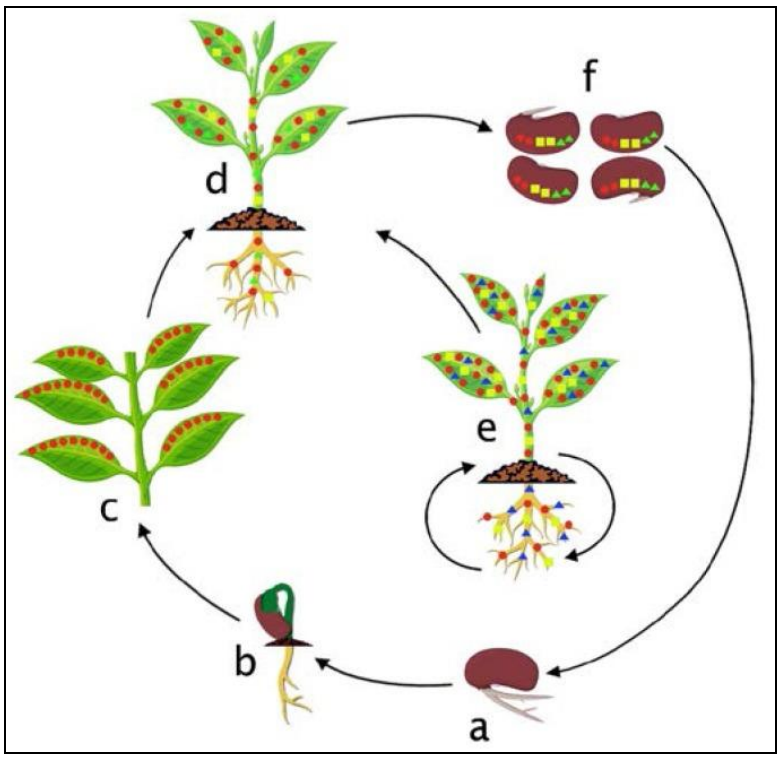


Figure 1.4.- Suggested colonization cycle of bacterial endophytes in the host plant.
(a) Mobilization of seed endophytes in germinating seedlings. (b) Recruitment endophytes from the soil in developing seedlings. (c) Colonization by horizontally and verticallly transmitted endophytes (d) Whole plant tissues colonization by various endophytes. (e) Variation of endophyte communities in the host plant in response to different biotic and abiotic stresses. (f) Vertical transfer of endophytes into seeds.
Microorganisms. 2017 Dec; 5(4): 77.

According to their lifestyle, endophytes are obligate, opportunistic or facultative. The obligates are unable to proliferate outside of plants and are probably transmitted via vertical seed transmission (Hardoim et al. 2015). The opportunistic are primarily epiphytes (living in the surface of the plants) that sporadically enter to the endosphere (Hardoim et al. 2015). The facultative are mutualists as colonization of the endosphere is beneficial for the bacteria and sometimes also for the host (Hardoim et al. 2015, 2008).

Endophytes from different plant hosts, including crops, belong mainly to four phyla; Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Bulgarelli et al. 2013; Hardoim et al. 2015) (**Figure 1.5**). This limited phylogenetic diversity of endophytes could be related to their generalistic ability to colonize different types of plants/crops.

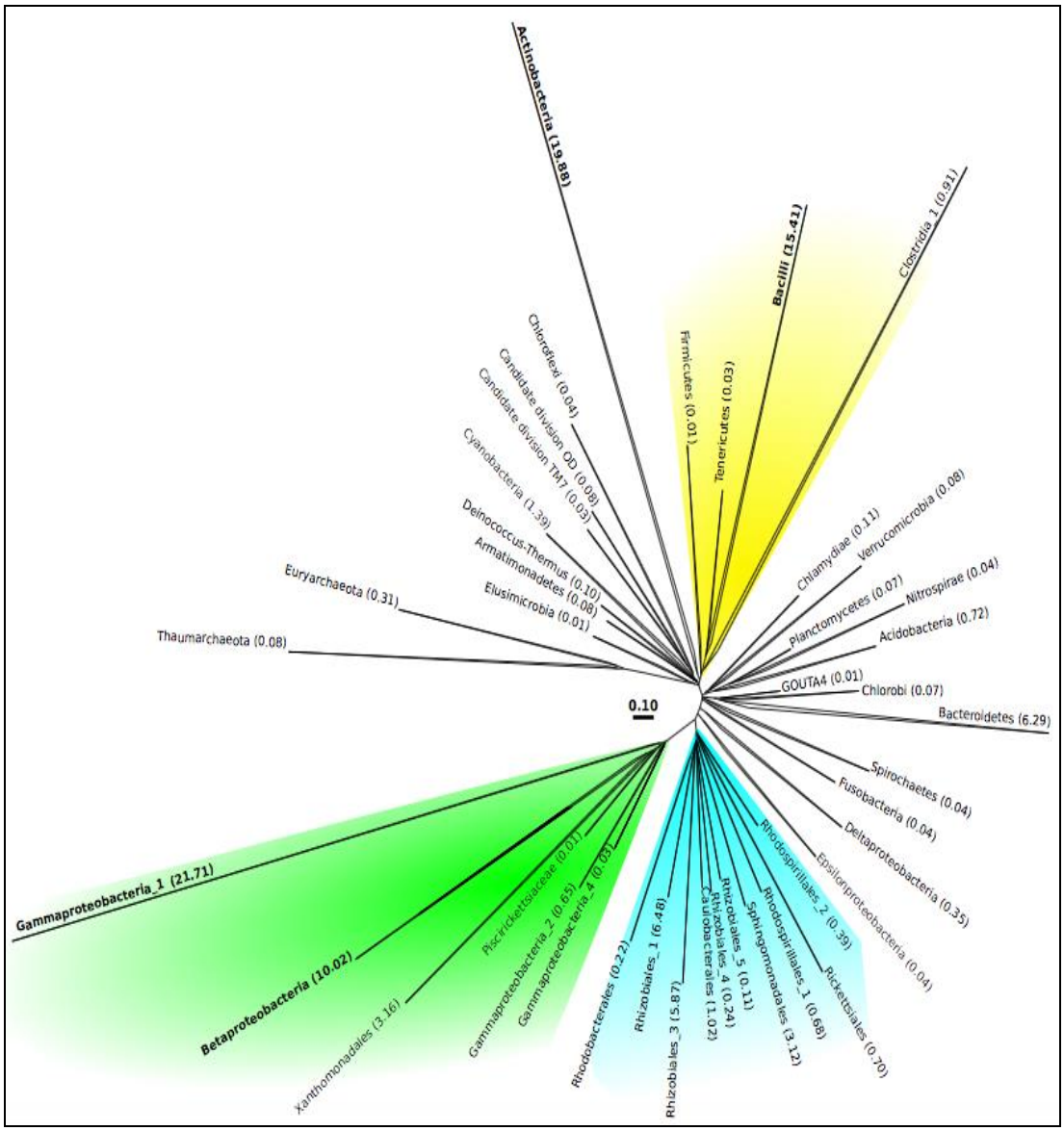


Figure 1.5. Phylogenetic analysis of 16S rRNA genes from prokaryotic endophytes.

The relative abundance is indicated as percentages within round brackets. Taxa with more than 10% of the total are shown in bold. The (sub)phyla Gamma-/Betaproteobacteria, Alphaproteobacteria and phyla Firmicutes are shown within green, blue and yellow shaded area, respectively. Scale bar indicates 10% sequence divergence. **Microbiol. Mol. Biol. Rev. September 2015 vol. 79 no. 3 293-32**

1.2.2 Bacterial endophytic traits.

The mechanism for endophytism is at large currently unknown, however some traits are thought to play an important role in this lifestyle (Ali et al. 2014) are described below:

a. Entry and early colonization

Two important phenotypes/mechanisms for the entry and colonization of the endosphere are motility and chemotaxis. Chemotaxis response regulator proteins CheBR and CheC and the flagellum-related mechanisms are more abundant among endophytes than among phytopathogens (Hardoim et al. 2015). As an example, the bacterial strain *Azoarcus* sp. BH72 requires its flagella for efficient endophytic colonization of rice roots and initial flagella-contact with the plant does not activate plant defense responses (Buschart et al. 2012) (**Figure 1.6**).

Adhesion to the surface of the plant is a key step and adhesion-related metabolites are polysaccharides and surface proteins (Danhorn and Fuqua 2007) (**Figure 1.6**). Genetic loci for proteic curli fibers, agglutination proteins and hemagglutinin genes are well distributed among endophytic genomes (Mitter et al. 2013). Furthermore, hemagglutinins traditionally considered as bacterial pathogenicity factors (Balder et al. 2007; Gottig et al. 2009), are widespread among bacterial endophytes indicating a probable role in the colonization (Mitter et al. 2013)

The presence of hydrolytic enzymes that soften plant cell walls are a common trait found in soft-rot pathogenic bacteria, however they have also been reported to be present in endophytes (Reinhold-Hurek et al. 2006; Mitter et al. 2013). Enzymes

such as cellulases and pectinases play a role for internal colonization (Compant et al. 2005) and in systemic spreading inside the plant (Compant et al. 2010) (**Figure 1.6**). For example, the endophytic strain *Azoarcus* sp. BH72 mutant lacking endogluconase, an enzyme that loosens larger cellulose fibers, is significantly less effective in colonizing rice plants (Reinhold-Hurek et al. 2006).

Six types of protein secretion systems (SS) have thus far been described for Gram-negative bacteria (Tseng et al. 2009), and three of them, Type III, Type IV, and Type VI can transport proteins across the host cell membrane delivering proteins directly into the cytosol of the target cell (Green and Mecsas 2016). The distribution of the different SS widely varies among the different endophytic strains. Type IV SS are more abundant in endophytes compared to rhizospheric bacteria. The Type III SS on the other hand is more distributed in nodule-forming symbionts than in endophytes (Reinhold-Hurek and Hurek 2011; Hardoim et al. 2015). The TypeVI SS, is commonly found among endophytes as observed in a metagenomic study (Mitter et al. 2013; Sessitsch et al. 2012), which could indicate that it plays a role in endophyte-plant interaction (**Figure 1.6**).

b. Adaptation to the endosphere

The transition from the rhizosphere to the plant endosphere involves a quick adaptation to a different environment where endophytes must overcome/detoxify plant defense responses like the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Zeidler et al. 2004; Mitter et al. 2013; Wu et al. 2011) (**Figure 1.6**). In presence of pathogens, plants induce systemic resistance (ISR), however some non-pathogenic bacteria such as rice endophyte *Azospirillum*

sp. B510 activates host ISR without the expression of major-defense related genes. This type of ISR is known as "priming" and it helps to protect the plant host against phytopathogens (Fujita et al. 2017; Yasuda et al. 2009).

Mutualistic endophytes have the ability to metabolize plant-stress-related compounds ameliorating environmental effects (Karpinets et al. 2014). Genes encoding for catalases, superoxide dismutases, peroxidases, hydroperoxide reductases and glutathione-S-transferases (GST) are present among many endophytic genomes analyzed (Mitter et al. 2013 and Ali et al. 2014). Similarly, Hardoim et al. 2015 correlated a higher number of genes encoding glutathione peroxidase (*btuE*), glutathione *S*-transferase (*gst*), catalase (*katE*), and nitric oxide reductase (*norR*) with the bacterial endophytic group compared to the pathogenic or nodule-forming symbionts groups. Additionally, some endophytes can protect the plant from abiotic stresses, like *Burkholderia phytofirmans* PsJN which increases chilling tolerance in grapevine plantlets (Ait Barka, Nowak, and Clement 2006) as well as elevating drought tolerance levels in maize (Naveed et al. 2014). This resistance to harsh environments is thought to be related to several detoxifiying enzymes (Sessitsch et al. 2012). Plants produce many low molecular weight secondary compounds that endophytes often can degrade and utilize as sources of carbon and energy. Bacterial endophytes possess mono- or dioxygenases, enzymes which are involved in oxidation of organic compounds (Mitter et al. 2013). For example, rice endophytes are equipped to degrade a large number of compounds as they present high abundance of genes involved in degradation of aliphatic and aromatic compounds (Sessitsch et al. 2012).

Transport through membranes is also believed to be a key process for the endophytic life inside plants (Gelfand and Rodionov 2008). Transport systems were the most abundant category of genes; for example they were 11.3% of the total analyzed bacterial rice-endophytic metagenomic sequences (Sessitsch et al. 2012). The ATP-binding cassette (ABC) family transporters is the most abundant type and the number and distribution of other type of transporters including porins, major facilitator superfamily (MFS), phosphotransferase system (PTS), solute carrier family (SLC) vary largely among endophytic strains (Hardoim et al. 2015; Mitter et al. 2013). Experimental validation in the future will be necessary to associate these comparative genomic studies to endophytism.

c. Inter-cellular signaling in endophytes

Comparative genomics of rice endophytes showed a regular prevalence of different inter-cellular signaling systems, however their role in endophytism has not yet been investigated (Sessitsch et al. 2012).

Quorum sensing (QS) is a type of cell-cell signaling mechanism which is cell density dependent (Fuqua et al. 2001). QS in Gram-negative proteobacteria is most commonly mediated by *N*-acyl-homoserine lactones (AHL) signals. An AHL QS system is formed by a genetically linked gene pair; a *luxI* family gene (AHL synthase) and a cognate *luxR* transcription factor which interacts with AHLs and affects target gene expression (Fuqua and Greenberg 2002) (**Figure 1.6**). Some plants have been shown to respond to bacterially produced AHLs resulting in primary root elongation, lateral root formation and shoot growth (Von Rad et al. 2008; Bai et al. 2012; Götz et al. 2007; Hartmann et al. 2014). Plants are also able to interact/interfere with the bacterial QS system by making AHL mimics, however

the type of molecule(s) and mechanism(s) remain unknown (Bauer and Mathesius 2004; Chalupowicz et al. 2009).

QS via AHLs has been reported in several endophytes, for example in *Acidovorax radicis* which is involved in the barley root colonization and plant-bacteria interkingdom signaling (Han et al. 2016). In *Pantoea agglomerans*, promoting bacterial growth and symplasmata formation (Jiang et al. 2015) and in the thoroughly studied *Paraburkholderia phytofirmans* PsJN strain which has two AHL QS systems producing four different types of AHL which play a role in plant colonization (Zúñiga et al. 2013, 2017). AHL QS signaling occurs in olive plants between pathogenic *Pseudomonas savastanoi* pv. savastanoi which causes the olive-knot disease and the harmless endophytic *Erwinia toletana*. Both bacteria produce the same type of AHL which they share and establish a mixed population which results in a more aggressive olive-knot disease (Caballo-Ponce et al. 2018; Buonaurio et al. 2015). Another type of QS signal molecule is the 3-hydroxypalmitic acid methyl ester (3-OH-PAME) firstly discovered in plant pathogenic *Ralstonia solanacearum* in which is responsible for regulating a phenotypic switch (Flavier et al. 1997; Von Bodman et al. 2003). 3-OH-PAME QS is also found in the genome of endophytic *P. phytofirmans* PsJN however its role is currently unknown (Mitter et al. 2013). Another type of QS signal is DSF (diffusible signal factor) which is widely studied as a virulence gene regulation system in pathogenic *Xanthomonas* (Ryan et al. 2015). DSF is also present in the phytopathogen *Burkholderia cepacia* being implicated in inter-species signaling with *Candida albicans* and *Francisella novicida* (Boon et al. 2008; Dean et al. 2015) and in endophyte *Stenotrophomonas maltophilia* playing a role in plant colonization

(Alavi et al. 2013). Some endophytes are being studied as for their ability to inhibit/interfere with the QS systems of plant- pathogenic bacteria thus inhibiting their colonization ability (Mookherjee et al. 2018; Alagarasan et al. 2018).

Many proteobacteria possess an AHL-type QS-family *luxR* gene which is not coupled to a cognate *luxI* gene, these are called *luxR* solos or orphans(Subramoni and Venturi 2009). Some of these have the role to detect and respond to exogenous AHLs produced by neighboring bacteria, a phenomenon known as eavesdropping (Chandler et al. 2012). A well studied LuxR solo, is SdiA of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, which responds to AHLs produced by other bacteria (Smith and Ahmer 2003; Sabag-Daigle and Ahmer 2012, Sperandio 2010). A sub-family of QS LuxR solos do not respond to AHLs but have evolved to respond to a plant signal hence being part of a plant-bacteria interkingdom signaling circuit (González and Venturi 2013). Recently, a member of this family called PipR from endophytic *Pseudomonas* strain GM79 has been shown to respond to an ethanolamine derivative called N-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide (HEHEAA) (Coutinho et al. 2018; Schaefer et al. 2016). It is unknown whether this is the signal for all or many members of this sub-family.

In summary, the presence and role of cell-cell signaling in plant endophytes is at the very early stages and future studies will most probably determine an important role since endophytic communities are rich and diverse and are therefore likely to form communities synchronizing their behavior as well as communicating with their host plant.

d. Plant Growth Promoting (PGP) activities of endophytes

PGP by beneficial endophytic bacteria has been linked to various phenotypes like the production of phytohormones such as auxins and also to stress management/ammeloriation as for example the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme (Arshad and Frankenberger 1997; Glick 2004, 2014) (**Figure 1.6**), or 2-keto-4-methylthiobutyric acid for modulating the plant host ethylene pathway (de Zélicourt et al. 2018). Another direct PGP effect is the production of volatile compounds, such as acetoin and 2,3-butanediol which act as plant growth stimulators (Ryu et al. 2004) (**Figure** 1.**6**). PGP phenotypes can also be indirect by making available to the host nutrients such as nitrogen, phosphate and iron (Reinhold-Hurek and Hurek 2011; Baldani et al. 1997; Verma et al. 2001). Endophytes produce siderophores which are secreted, chelate iron and are then transported back via specific membrane receptors. Endophytes can monopolize the iron with their siderophores and plants in some cases also utilize the ferric siderophores directly (Reinhold-Hurek and Hurek 2011) (**Figure 1.6**). Some endophytes are diazotrophic as they able to fix atmospheric nitrogen into ammonia which can then be taken up by the plant. Endophytic bacterial strains belonging to the genera *Acetobacter*, *Diazotrophicus*, *Herbaspirillum* and *Azoarcus* are diazotrophic and have the potential to provide fixed nitrogen to economically important plants (Baldani et al. 1997; Reinhold-Hurek and Hurek 1998) (**Figure 1.6**). Different strains of endophytes including several members of the Enterobacteriaceae family are able to mineralize insoluble phosphates making them available to the plant (Verma et al. 2001).

Endophytes can also antagonize pathogens either by producing secondary metabolites with antimicrobial properties (antibacterial, antifungal and antiprotozoan) (Martinez-Klimova et al. 2017) or by niche exclusion by outcompeting pathoens (Griffin 2014). Endophytes can also induce plant systemic resistance (ISR) which allows a higher tolerance to pathogens (Zamioudis and Pieterse 2012). The most common bacterial genera displaying ISR are *Pseudomonas* and *Bacillus* (Chanway 1998; Kloepper and Ryu 2007). Among the bacterial factors implicated in ISR induction are flagella, antimicrobials, AHLs, salicylic acid, jasmonic acid, siderophores, volatile compounds and lipopolysaccharides (Bordiec et al. 2011; van Loon et al. 2008; Hardoim et al. 2015). In summary, endophytes can possess many PGP properties making them suitable biofertilizers and biocontrol agents (see below).

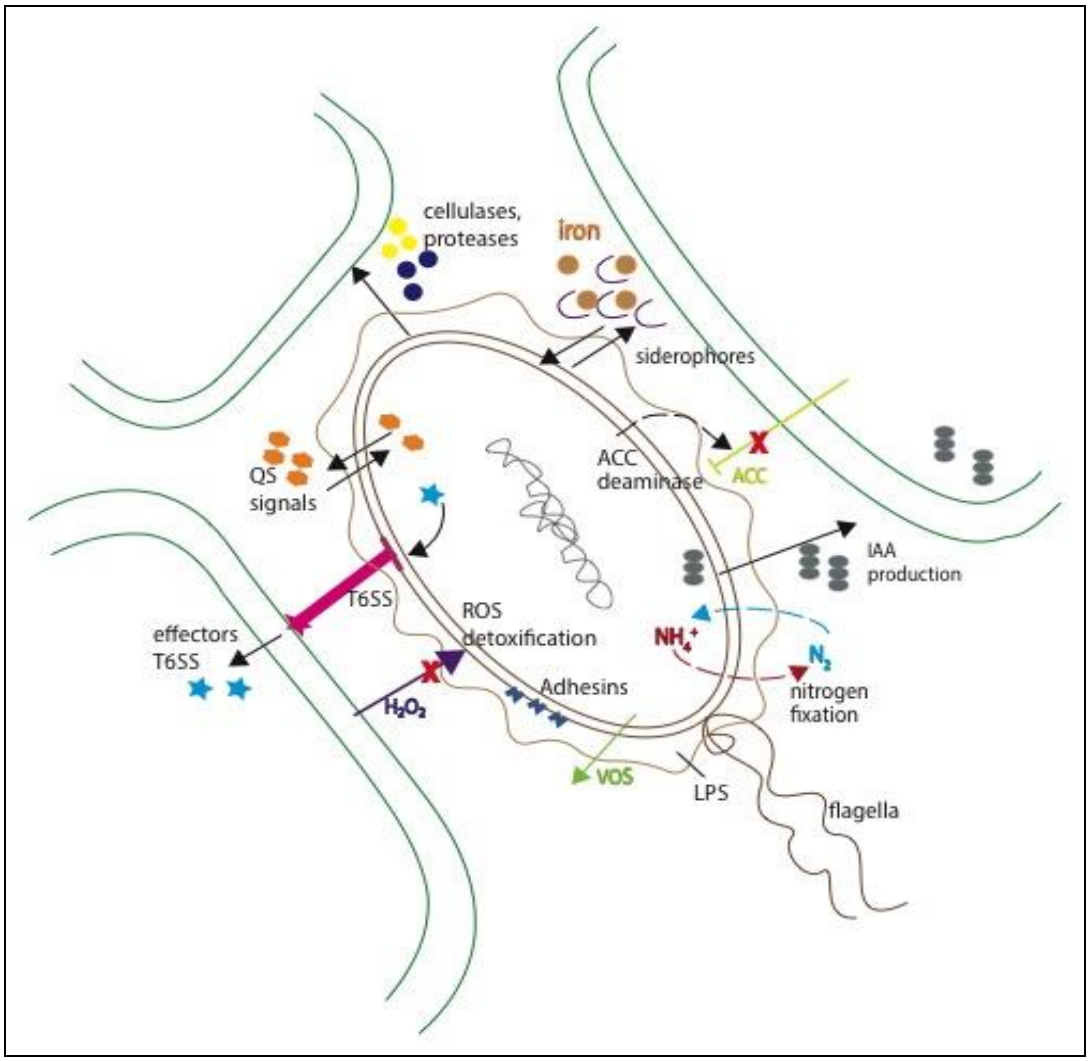


Figure 1.6.- Endophytic entry and adaptation mechanisms and described PGP properties.

Endophytes possess different mechanisms to allow the entrance, spreading and colonization of the host including inter-cellular signaling. Additionally they present PGP properties like phytohormones and IAA production.

Microbially based biofertilizers are most commonly bacterial or fungal inoculants applied to plants for increasing nutrients availability and/or increase abiotic stress tolerance. In the recent years the term "biostimulant" is also used and this refers to any substance applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and other crop quality traits (du Jardin 2015). The biostimulant market to date is estimated at about $ 2.0 billion, expecting to reach $ 3.0 billion by

2021 at an annual growth rate of 10–12% (Rouphael and Colla 2018). Biofertilizers are a subgroup of biostimulants (du Jardin 2015) and *Azospirillum*, *Azotobacter*, and *Rhizobium* spp. and mycorrhizal fungi are promising biofertilizers as they promote yield stability under nitrogen and/or phosphorus deficiency or other environmental constraints (Rouphael and Colla 2018).

Endophytes, represent a promising group biofertilizers since they colonize the internal tissues and possibly have less competition than rhizospheric microbes. Moreover, their beneficial metabolites act directly into the host having a minimal leakage into the environment (Khare et al. 2018). Currently not many commercial biofertilizers based on endophytes are on the market, one example is a cocktail of three different endophytic diazothropic strains of *Herbaspirillum seropedicae* strain, *Herbaspirillum rubrisubalbicans*, and *Gluconacetobacter diazotrophicus* which is used for increasing sugar cane yield (da Silva et al. 2017). It is expected that more biofertilizer products for agriculture based on endophytes will be introduced into the market in the near future.

1.2.3 Rice endophytic microbiome and plant health

Rice is a staple food for 50% of the people worldwide and is approximately 80% of the diet for Asians populations (Zeigler and Barclay 2008); the estimated required rice yield by 2025 is 8000 million tons (Kubo and Purevdorj 2004). One of the major limitations of rice cultivation is the availability of nitrogen; the use of biofertilizers will reduce the use of chemical nitrogen fertilizers being an important contribution to a more sustainable agriculture.

Just like other economically important crops, rice is inhabited by complex microbial communities that play an important role in plant health (Okubo et al. 2014; Turner et al. 2013). Various rice microbiomes in different environments and under stress conditions have been determined (Ikeda et al. 2014; Hardoim et al. 2012; Sessitsch et al. 2012; Okubo et al. 2014). In the endosphere, the phyla Proteobacteria represents more than 50% of bacterial life with Gammaproteobacteria being the most prevalent class (Edwards et al. 2015; Hardoim et al. 2015; Okubo et al. 2014; Moronta-Barrios et al. 2018). There is lower microbial diversity in the endosphere and it increases through the rhizoplane and rhizosphere (Edwards et al. 2015). The endosphere has a greater proportion of Proteobacteria and Spirochaetes whereas Acidobacteria, Planctomycetes and Gemmatimonadetes are less represented in comparison with the rhizoplane and rhizosphere (Edwards et al. 2015).

It is likely that there is a coevolutionary trend between rice and its seed endophytic microbiome. The rice seed microbiome composition is affected by different factors such as abiotic stress, crossbreeding, repeated inbreeding, human selection and planting in different ecogeographical locations. The genera *Microbacterium*, *Curtobacterium*, *Stenotrophomonas Xanthomonas* and *Enterobacter* are constantly found, suggesting they are part of the rice seed core microbiota (Walitang et al. 2018a, 2017).

1.3 Scope of this thesis

The aim of this thesis is to characterize two diazotrophic endophytic strains belonging to the genus *Kosakonia* which were selected as good endosphere colonisers and PGP from a large collection of endophytes isolated from rice grown in Italy (Bertani et al. 2016). *Kosakonia* is a recently described genus, previously associated with *Enterobacter* and named in honor to Y. Kosako, a scientist that contributed to bacterial taxonomy (Brady et al. 2013; Kämpfer et al. 2016b). *Kosakonia* is currently composed of eight different species, seven are plant-associated (PA) most of which are diazotrophic (Y. Li et al. 2017). Comparative genomics in the genus *Kosakonia (*analyzing 31 available genomes) evidenced common features such as flagella, the presence of type VI secretion system (T6SS), loci encoding proteins involved in nitrogen fixation, siderophore production, phosphate solubilization and glycine-betaine production (Becker et al. 2018). *Kosakonia* PA members are generalists and colonize economically important plants such as maize, rice, wheat, sweet potato, sugarcane and crop cotton; in addition to peanut, pineapple, tomato and yerba mate (Becker et al. 2018). *K. radicincitans* strain DSM 16656T is becoming a model endophyte (Kämpfer et al. 2005; Berger et al. 2015); it enters the host through the cracks of newly emerging lateral roots and colonizes intercellular spaces of root parenchyma (Becker et al. 2018).

The two *Kosakonia* strains studied in this thesis are efficient plant tissue colonizers and PGP, therefore being promising candidates to investigate their plant-associated features via genomics, proteomics and genetics studies.

The experimental work of this thesis is divided into three experimental chapters (chapter II – IV); chapter II describes and characterizes both *Kosakonia* strains, including their colonizing abilities, localization by microscopy and comparative genomics. The secretome profile and type VI secretion system involvement in rhizosplane and endosphere colonization were also studied. Additionally, it includes the results of a rice field trial supplementing rice with a *Kosakonia* strain under nitrogen-limiting conditions. Chapter III is aimed to perform an enrichment strategy for the isolation of endophytes and to identify *Kosakonia* bacterial co-inhabitants for rice root endosphere colonization. Chapter IV presents studies on two LuxR solos found in the genome of *Kosakonia* strain KO348 and discusses their possible involvement in interspecies and interkingdom signaling. In summary, this thesis presents results on the plant lifestyle of endophytic *Kosakonia.*

Chapter II.

Characterization of two *Kosakonia* rice endophytes

2.1 Introduction

Rice is the most important food crop in the developing world, being a staple food for over two billion people in Asia and for many millions in Africa and Latin America (Khush 2003; Zeigler and Barclay 2008). The challenge in the future will be to increase rice yields for a growing world population and to decrease the use of chemical pesticides and fertilizers for more sustainable approaches (Schütz et al. 2018; Mano and Morisaki 2008). The use of microbially-based biopesticides and biofertilizers is currently believed to be a promising way to render agriculture more sustainable by reducing the chemical input (Berg 2009; Gupta and Dikshit 2010; Mahanty et al. 2017; Schütz et al. 2018).

Plant-associated microbiota constitutes the plant microbiome playing a fundamental role in plant growth promotion (PGP) and health (Okubo et al. 2014; Turner et al. 2013; Schlaeppi and Bulgarelli 2015). PGP activities by plant-associated microbes include induction of plant immunity, acquisition of nutrients and resistance to biotic or abiotic stresses (Compant et al. 2010; Lugtenberg and Kamilova 2009; Glick 2014). The plant microbiome represents many diverse microorganisms that interact and colonize different plant-associated niches (Müller et al. 2016; van der Heijden and Hartmann 2016). One of these compartments is the rhizosphere, which consists of the root-surrounding soil being influenced by root exudates and has a high diversity and distribution of microbial life (Berg et al. 2005; Hartmann et al. 2008; Lundberg et al. 2012). Some microbiome members do not only colonize the rhizosphere but also thrive as endophytes inside plant tissues (Berg et al. 2014; Reinhold-Hurek and Hurek 2011). Endophytes mostly enter via the roots and have

evolved an intimate relationship with the plant host; many of them do not elicit a plant immune response and some display PGP properties (Glick 2014; Hayat et al. 2010; Reinhold-Hurek and Hurek 1998; Sessitsch et al. 2012; Garrido-Oter et al. 2018). Endophytes constitute therefore an important class of beneficial bacteria now considered to be a potentially important group that can be used as microbial inoculants for a more sustainable agriculture. However, more information is needed on the endophytic lifestyle and mechanisms of plant entry and colonization.

General features of the rice microbiome, and plant microbiomes in general, include less species richness in the plant endosphere than on the rhizoplane (surface of the root) and in the rhizosphere (Edwards et al. 2015; Lundberg et al. 2012; Bulgarelli et al. 2012). Microbial communities of the rice rhizoplane and root endosphere stabilize after seven to eight weeks from germination due to the plant life cycle (Edwards et al. 2017). The rice endophytic bacteriome has a prevalence of Proteobacteria representing more than 50% of the bacterial community with Gammaproteobacteria being the most abundant class. Many rice endophytes possess nitrogen fixation genes as well as genes related to nitrification and denitrification processes which suggest they are involved in the entire nitrogen cycle (Sessitsch et al. 2012). Examples of PGP rice endophytes include *Pantoea agglomerans* YS19 (Feng et al. 2006; Yang et al. 1999; Jiang et al. 2015) and *Pseudomonas stutzeri* A15, a rhizospheric and endospheric diazotrophic root colonizer (Pham et al. 2017). Other rice endophytes with potential use as nitrogen biofertilizers include *Gluconacetobacter diazotrophicus* LMG7603, *Herbaspirillum seropedicae* LMG6513, *Azospirillum lipoferum* 4B (LMG4348), and *Burkholderia vietnamiensis* LMG10929 (Govindarajan et al. 2008; Trân Van et al. 1996; Baldani

et al. 1986; Rouws et al. 2010).

A large study in Italian rice cultivars isolated and characterized bacterial endophytes that resulted in a collection of over 1300 putative isolates (Bertani et al. 2016). Several *in vitro* and *in planta* selection steps resulted in a smaller set of putative endophytes, which displayed efficient *in planta* colonization levels as well as having PGP traits. Among these, were two strains that belong to the recently described *Kosakonia* genus (Brady et al. 2013; Alnajar and Gupta 2017), which consists mostly of plant-associated diazotrophs (Kämpfer et al. 2016; Y. Li et al. 2017). Some *Kosakonia* strains, as for example *K. radicincitans* DSM 16656, are generalists endophytes and promote plant growth in different plants including wheat, maize, tomato, pea, and cruciferous vegetables (Berger et al. 2013; Höflich and Ruppel 1994; Schreiner et al. 2009). In recent years, members of this genus have gained attention and several genome sequences have been reported (Chen et al. 2015; Shinjo et al. 2016; Kämpfer et al. 2016b; Bergottini et al. 2015; Li et al. 2017; Mohd Suhaimi et al. 2014; Becker et al. 2018).

This chapter presents the characterization of two *Kosakonia* strains previously isolated as promising PGP endophytes of rice in the study mentioned above (Bertani et al. 2016). In order to begin to study the features that make them efficient endophytic colonizers, studies on plant colonization, as well as genomic and protein secretome studies were performed. In this chapter it is also reported a rice field release study of one *Kosakonia* strain and its effect on plant yield and on the composition of the rice root endophytic microbiome.

2.2. Materials and methods

2.2.1 Bacterial strains and growth conditions

The *Kosakonia* strains used in this chapter KO348 and KO774 were previously isolated from the root endosphere from rice grown in Italy (Bertani et a 2016). Strains KO774, KO348, KO348(pBBRgfp), KO348hcp and KO348hcp(pBBRhcp) were routinely grown in Luria-Bertani (LB) broth at 30 °C. In order to obtain spontaneous rifampicin resistant KO348 and streptomycin resistant KO774, strains were grown in 1/6 TSB (Tryptic Soy Broth) medium supplemented with gradually increasing amounts of rifampicin (Rif) or streptomycin (Sm) respectively, ranging from 15 to 100 µg ml^{-1}. Finally, cultures were plated on TSA (Tryptic Soy Agar) and single colonies were re-inoculated in TSB containing Rif 100 µgml^{-1} or Sm 100 µg ml^{-1}. When required, antibiotics for *Kosakonia* strain growth were added at the following concentrations: rifampicin, 50 µg ml^{-1}, gentamicin, 25 µg ml^{-1} and kanamycin, 100 µg ml^{-1}. *Escherichia coli* DH5α and S17 were grown at 37 °C in LB broth and when appropriate antibiotics were added at the following concentrations: ampicillin, 100 µg ml^{-1}, and gentamicin, 15 µg ml^{-1}.

2.2.2. Genome sequencing of *Kosakonia* strains

The genome of *Kosakonia* sp. KO348 was previously sequenced and is deposited at DDBJ/EMBL/GenBank under the accession no. JZLI00000000 (Meng et al. 2015) and in IMG/M (U.S. Department of Energy- Joint Genome Institute) as genome ID 2651869662. The genome of KO348 was re-sequenced using Illumina HiSeq technology at 230x sequencing depth. The genome was assembled using spades v. 3.10.1. and annotated via IMG Annotation Pipeline v.4.16.0. The draft genome

sequence of *Kosakonia* sp. KO774 was also determined. For KO774, the genomic DNA was obtained by Sarkosyl-Pronase lysis protocol as described by Better et al. (1983) and then used to prepare a sequencing-ready library. Sequencing was performed on Illumina MiSeq platform using 150-bp paired-end reads. The genome of *Kosakonia* sp. KO774 was deposited in IMG/M as genome ID 2758568389. Automated annotation of *Kosakonia* sp. KO774 draft genome sequence was performed using IMG/M (U.S. Department of Energy- Joint Genome Institute).

2.2.3 *Kosakonia* genome analysis

In order to analyze the genomes for a phylogenetic analysis, 15 *Kosakonia* genome sequences were retrieved from the Integrated Microbial Genomes database IMG/M (U.S. Department of Energy- Joint Genome Institute).

Escherichia coli K12 MG1655 served as an outgroup. The list of single copy marker genes was retrieved for all genomes and consisted of mainly ribosomal proteins. Only genes that were present in all 15 genomes were used and these included the following Clusters of Orthologous Groups (COGs): COG0012, COG0016, COG0052, COG0087, COG0090, COG0091, COG0092, COG0094, COG0096, COG0097, COG0098, COG0099, COG0102, COG0103, COG0124, COG0186, COG0197, COG0200, COG0201, COG0522, COG0525, COG0533, COG0541. The genes of each COG in all 15 genomes were aligned separately using MAFFT multiple aligner version 7.221 (Kazutaka Katoh and Daron M. Standley) using default parameters. The multiple sequence alignment was trimmed with trimAl v1.3 using default parameters. Next, the different COG alignments were concatenated together using a custom script to yield 15 sequences of all 23 single copy genes. RAxML version 7.6.3 (The Exelixis Lab - Heidelberg Institute for

Theoretical Studies) was used to construct the tree using the following parameters: raxmlHPC-PTHREADS-SSE3 -f a -p 12345 -x 12345 -# 1000 -m PROTGAMMALG -T 8 with the outgroup being *E. coli*. The Best-scoring ML tree with support value was visualized using iTOL (Letunic I and Bork P).

All proteins (n=70) located within and adjacent to the T6SS operons in the KO348 genome were retrieved and blasted against the proteins found in the secretome (see below) searching for possible hits of putative T6SS effectors.

2.2.4 Plant colonization experiments

For all the rice endosphere colonization experiments, the inoculation protocol described previously by Bertani et al. (2016) was followed, with a few modifications. *Kosakonia* strains Rif or Sm resistant were grown on LB media to an OD_{600} of 0.8 and 7 days-old germinated rice plantlets cv. "Baldo" were then submerged in this bacterial suspension for 1 hour and transferred independently to a tube containing Hoagland´s semi-solid solution (Steindler et al. 2009). Plantlets were then watered and grown for a number of days; *Kosakonia* strains were then re-isolated from roots and/or the green aerial part of the plant after surface sterilization and sterility controls were performed as previously reported (Bertani et al. 2016). Plant material was finally macerated in PBS solution and serial dilutions of this macerate were plated in TSA containing the appropriate antibiotics, then incubated at 30 °C for 24 hours and counted for CFU/g calculation.

In the case of rhizospheric colonization: roots were rinsed with sterile water removing all remaining Hoagland´s semi-solid solution and then vortexed in 5ml of PBS solution for 1 min. Serial dilutions of this PBS solution were then plated on the appropriate selection media for CFU/g calculation.

Plasmid-loss calculation of *Kosakonia* plant colonization strains was performed by plating complemented *Kosakonia* cells KO348hcp(pBBRhcp) isolated from the rhizosphere and root endosphere in the following selective media: LB supplemented with kanamycin 100 μg ml^{-1} plus gentamicin 25 μg ml^{-1} for plasmid complemented cells and LB supplemented with only kanamycin 100 μg ml^{-1} for cells which lost the plasmid. CFU/g and percentage of plasmid loss was calculated.

For comparing the rhizosphere and endosphere colonization ability between KO348 and KO774 strains, analysis of variance (ANOVA) was performed with Prism 7 (Graphpad Software, Inc). For each group analyzed three biological replicates were used. In the analysis of the rhizospheric and endophytic colonization of KO348 and, on the effect of T6SS in colonization ability of KO348 Kruskall-Wallis test was used for specific pairs of data. For each group analyzed in all plant colonization studies at least three biological replicates were used. All statistical analyses were performed with Prism 7 (Graphpad Software, Inc).

2.2.5 Visualization of *Kosakonia* sp. KO348 in rice roots by confocal microscopy

To further describe colonization process by *Kosakonia* strains, rice plantlets were inoculated with strain KO348(pBBRgfp) harboring plasmid pBBR2GFP, which constitutively expressed the autofluorescent GFP protein (da Silva et al. 2014), as described above. Colonization assessment of rice by strain KO348 harboring the pBBR2GFP was performed at several time points (5, 10, 30 and 50 days post inoculation). For surface visualization, samples (roots and shoots) taken from 10 plants at each time point were rinsed with distilled water and directly observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 HeNe(G) and laser FV10-LAHEG230-2). For internal

colonization, samples were surface sterilized, after being rinsed, with ethanol 75% for 2 minutes and rinsed thrice with distilled water. Then samples were treated with sodium hypochloride (7%) solution for 2 minutes and then rinsed followed by two 75% ethanol treatments for 1 minute and finally rinsed thrice with distilled water. Samples were then cut with a razor transversally or longitudinally and observed under the confocal microscope. X, Y, Z pictures were taken at 405, 488, 633 nm and with 10X, 20X or 40X objectives. Z stacks were observed using Imaris software or with Image J (National Institute of Health, U.S.A.). Pictures were cropped and due to the convolution process in the microscope, whole pictures were sharpened and the light/contrast balance improved to better observe the image details, as seen when samples are observed in the dark under the microscope (as described in Glassner et al. 2015).

2.2.6 Determination of the *Kosakonia* sp. KO348 protein secretome

In order to determine the proteins which were secreted by *Kosakonia* sp. KO348, the strain was grown in 200 ml plant mimicking AGF liquid media (Ryan et al. 2007) at 30 °C for 16 hrs. The culture was then centrifuged at 3,800 ×g at 4 °C for 15 min and the spent supernatant filtered through a 0.45 μm membrane in order to remove any residual bacterial cells. Trichloric acetic acid (TCA) was then added to a final concentration of 10% w/v and incubated 16 hours at 4 °C. Samples were then centrifuged for 60 min at 15,000 ×g at 4 °C. Pellets were washed with acetone and air dried. Protein pellets were then resuspended in NuPage LDS buffer 1x (ThermoFisher Scientific Inc., Waltham, MA, USA), boiled for 5 min and then run 3 cm in a precast NuPAGE 12% Bis-Tris gel (ThermoFisher Scientific Inc., Waltham, MA, USA). The gels were stained with colloidal commasie brilliant blue

(Sigma-Aldrich Inc., San Louis, MS, USA). The stained area of the gel was cut into five bands and processed for in-gel digestion with trypsin using standard procedures (Wysocka et al. 2003). LC-MS/MS of the digests was performed using an Easy-nLC II coupled to an Amazon ETD mass spectrometer (Bruker Daltonics, Hamburg, Germany). The resulting spectra were searched using the X!tandem (The Global Protein Machine Organization) search engine and a *Kosakonia* sp. KO348 protein database and filtered at a 5% false discovery rate.

2.2.7 Construction of the *Kosakonia* strain KO348 *hcp* genomic knock-out mutant and its genetic complementation

A genomic knockout mutation of the *hcp* gene was constructed, using genomic DNA as template, by amplifying the 5′ DNA flanking regions with primers pEXhcp1Fw 5'-AGGATCCTTTAATTTCTACCCGCCTGG3-´ and pEXhcp1Rv 5´-ACTCGAGTTTGCAGACAGACAGCTCAAC-3´) and 3′ DNA flanking regions with primers pEXhcp2Fw 5´-AGAATTCAGGTGTGACCTATGCATTCCA-3´and pEXhcp2Rv 5´-AGGTACCTTGTTTGACAGCCATTTCGG-3´). The 5′ and 3′ fragments were then ligated on either side of a kanamycin resistance gene and the final fragment cloned in gene replacement vector pEX19Gm (Hoang et al. 1998) generating pEX19Kmhcp. This latter plasmid was then electroporated into strain *KO348* and following selection (Km^R Gm^S) resulted on the generation of an *hcp* knock-out mutant strain which was named *Kosakonia* KO348hcp.

The *hcp* full-length gene (including its gene promoter) was amplified with the primers prom+hcpFW 5´-AGGTACCTGTTTCTGAAGGTCGATGGAG-3´ and prom+hcpRv 5´-AGGATCCTGTTTGACAGCCATTTCGGT-3´, the sequence was verified via DNA sequencing and the 802 bp fragment was cloned in the

gentamicin resistant pBBR1MCS-5 vector (Kovach et al. 1995). This plasmid was electroporated in the mutant strain KO348hcp, and selected for Km^R and Gm^R, and the resulting KO348hcp complemented strain was named KO348hcp(pBBRhcp).

2.2.8 Rice field trial using seeds inoculated with a *Kosakonia* strain

A rice field trial using seeds inoculated with *Kosakonia* sp. KO774 was carried out between May and October 2016 at Catarroja, Valencia-Spain (39,3859292°N 0.376225411°W). It consisted in 16 experimental plots of wet-seeded paddy rice cv. "J. Sendra" divided in four groups of treatment as follows; (i) eight plots received 100% nitrogen/urea recommended fertilization with four of these planted with seeds inoculated with *Kosakonia* sp. KO774 and the other four plots with the seeds not inoculated and (ii) eight plots received 50% of urea/nitrogen recommended fertilization with four of these planted with seeds inoculated with *Kosakonia* sp. KO774 and the other four plots with the seeds not inoculated. The seed inoculation with *Kosakonia* sp. KO774 was performed by soaking rice seeds in a solution containing 10^8 CFU/ml of the strain for 24 hrs. Rice plants were harvested at day 100 post rice-sowing and measurements including germination/plot, weight (1000 grains/plot), (25 panicles/plot), yield (m^2) and yield (kg ha^{-1}) were performed. Statistical analysis of variance (ANOVA) was performed for analyzing the phenotypic differences between groups using Prism 7 (Graphpad Software, Inc).

2.2.9 Microbiome studies

Microbiome analysis was performed on rice roots grown in two plots of the following two treatment groups of the field trials, (i) rice seeds soaked in *Kosakonia* and the soil fertilized with 50% of the recommended nitrogen (treated group) and (ii) rice seeds which were not inoculated with *Kosakonia* and the soil fertilized only with 50% of the recommended nitrogen (untreated group). Rice plants were collected at 30, 60 and 90 dpi and rice roots were washed and surface sterilized. In order to maintain the variability but decreasing the number of samples, one sample was considered as the sterilized roots of three different plants of rice derived from the same plot and collected at the same time point (**Figure 2.1**).

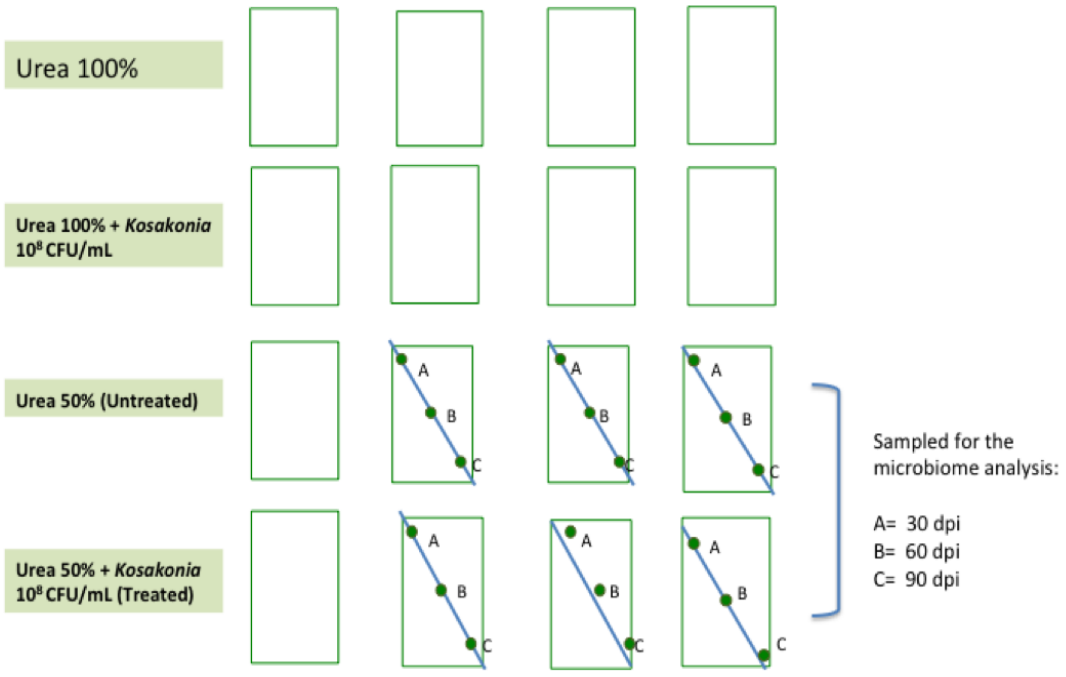


Figure 2.1. Rice field trial layout.
A total of 16 parcels of paddy rice were followed during 100 days divided in 4 groups of treatment. Urea 100% fertilized (225 kg/ha) with and without the addition of KO774, and urea 50% fertilized (125 kg/ha) with and without the addition of KO774. For the microbiome analysis, 2 groups were tested, both urea fertilized 50% with (Treated) and without (Untreated) the addition of KO774. 3 samples were taken from 3 different parcels at 3 time points (30, 60 and 90 days after rice seeds were sowed).

DNA from sterilized roots was extracted using PowerMax Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol and using 0.5 grams of each sample. The 16S rRNA gene amplicon library was prepared following the manufacturer's protocol (15044223 B) (Illumina Inc., San Diego, CA, USA). Briefly, samples were amplified in the V3 and V4 regions using denaturated primers (Klindworth et al. 2013) in a limited cycle PCR, followed by an AMPure XP bead clean-up (A63880l; Beckman Coulter Inc., Brea, CA, USA). A second PCR reaction was then performed to attach dual index and Illumina sequencing adapters using the Nextera XT Index Kit; followed by a final AMPure XP bead clean-up. 16S rRNA gene concentration was measured by fluorimetric quantification using Qubit 2 (Invitrogen Inc., Carlsbad, CA, USA). Sequencing was performed using the Illumina Miseq technology. The sequences of raw data were filtered out and the reads were trimmed to a consistent length. Then the data was denoised, chimera filtered, and taxonomically assigned using DADA2 v1.1.5 (Callahan et al. 2016). For the taxonomic analysis, the sequencing reads were clustered into operational taxonomic units (OTUs) defined as groups of sequencing reads that differ by less than a fixed dissimilarity threshold (97%) generated in DADA2 using the Greengeenes database v13.5 (McDonald et al. 2012) modified for including sequence "CTACGGGTGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGA CGGAGCAATGCCGCGTGGAGGTGGAAGGCCCACGGGTCGTCAACTTCT TTTCTCGGAGAAGAAACAATGACGGTATCTGAGGAATAAGCATCGGCT AACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGATGCAAGCGTTATCC GGAATGATTGGGCGTAAAGCGTCTGTAGGTGGCTTTTCAAGTCCGCCGT CAAATCCCAGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTG

GAGTCTCGTAGAGGGAGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT
AGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCTCCTGGACGAAGA
CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
CTGTAGT" as Bacteria Proteobacteria Gammaproteobacteria Enterobacterales Enterobacteriaceae Kosakonia_S (belonging to our strain KO774).

2.3.1 Genome sequence and analysis of the two *Kosakonia* strains

The genome sequences of the two rice beneficial *Kosakonia* sp. KO348 and KO774 endophytic strains which were previously identified (Bertani et al. 2016) were analyzed. The genome sequence of strain KO348 was previously reported (Meng et al. 2015) and for this chapter was re-sequenced; the assembly yielded a higher quality genome compared to the previous version. The new sequence version gave 26 scaffolds of average size 192.7Kbp vs. 56 scaffolds of average size 89.3 Kbp in the old genome version. In the case of strain KO774, its genome was for first time sequenced which was performed on an Illumina MiSeq platform (2x300 bp) by *de novo* assembly using Velvet 1.2.09. *Kosakonia* sp. KO774 assembly using IMG/M yielded 12 scaffolds giving a total of 4,875,574 bp, including 4,530 putative protein coding genes and 153 RNA genes; this assembly also revealed a putative plasmid (74Kbp). The contig containing the putative plasmid included loci for plasmid replication, conjugation, segregation genes, few toxin-antitoxin systems and a large cellobiose phosphorylase gene (**Figure 2.2**).

The bacterial genomes, KO348 and KO774, had 3,853 orthologous genes identified as bidirectional best hits (BBH). Namely, 82.5% of the genes in each genome display at least 70% sequence identity over at least 70% of the length of the shorter sequence in each BBH pair. The genomic average nucleotide identity (gANI) between the BBH pairs is 83.69% (Varghese NJ NAR 2015). The two genomes demonstrate overall high genome similarity at the scaffold level when compared using the progressive Mauve aligner **(Figure 2.3).**

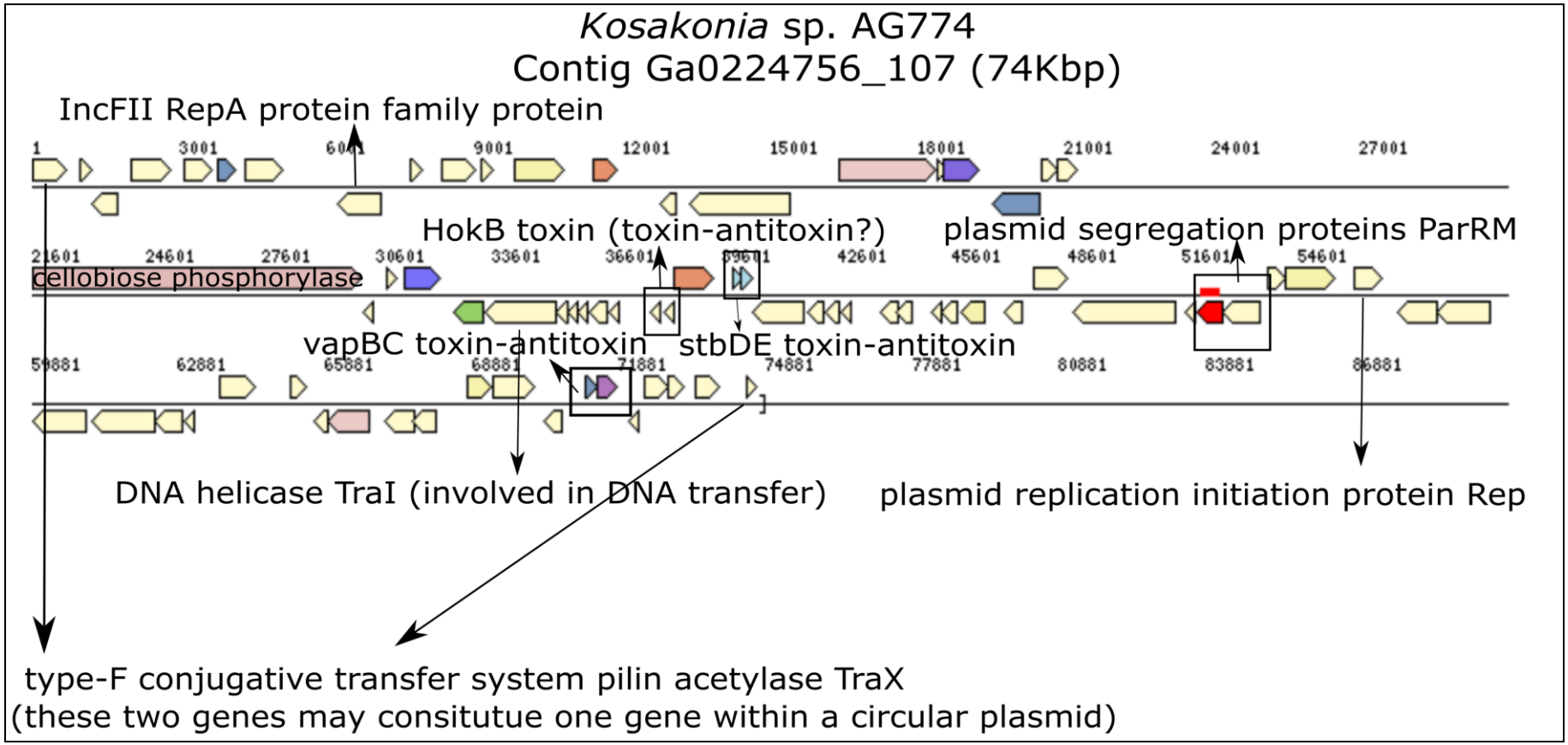


Figure 2.2 Putative plasmid sequence KO348.
KO348 genome assembly revealed a putative plasmid (74Kbp). The figure shows the contig containing the putative plasmid included loci for plasmid replication, few toxin-antitoxin systems and a large cellobiose phosphorylase gene among others.

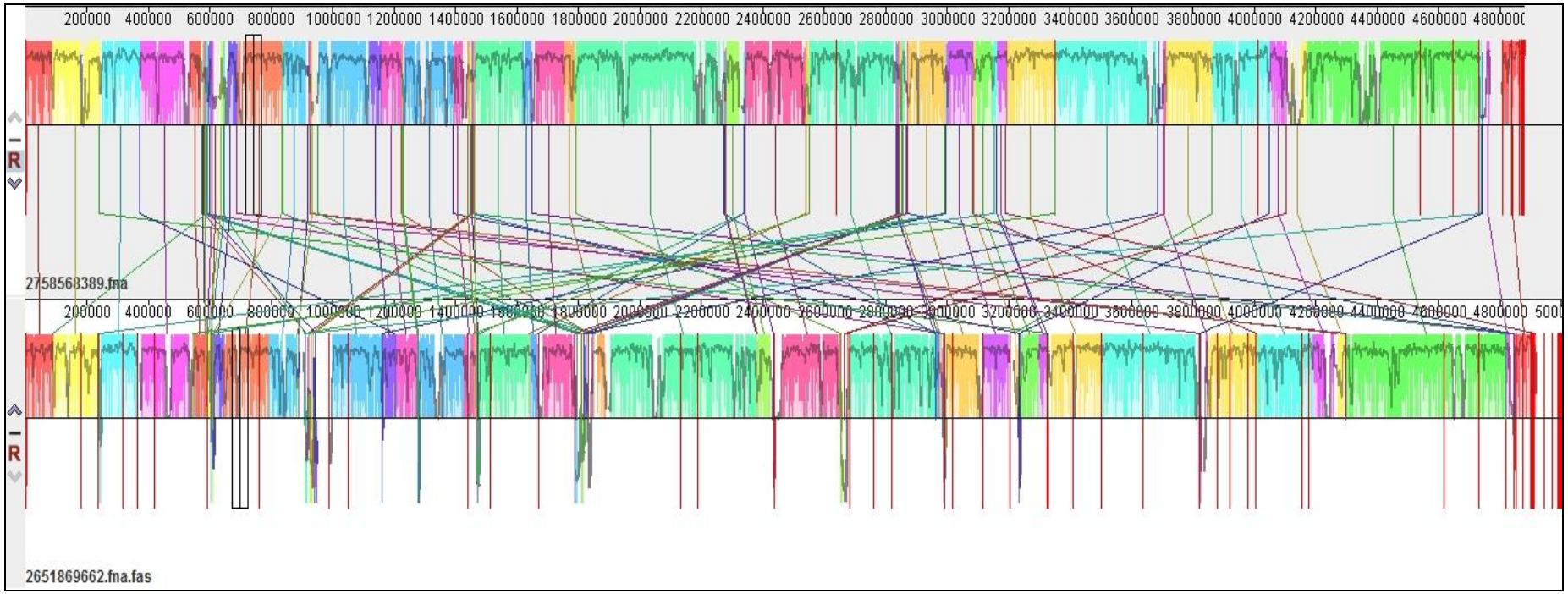


Figure 2.3 Genome similarity among both *Kosakonia* strains.
Genome alignment was done using progressive Mauve aligner. In the figure, KO348 is 2651869662 (the lower and longer genome).

Among the open reading frames (ORFs) analyzed, both strains share some PGP related genes involved in siderophore production (enterobactins), phosphate solubilization (phytase), flagellar motility, plant tissue degrading enzymes (cellulase) and the *nif* gene cluster for nitrogen fixation. The genome size of KO774 is approximately 100 kb smaller compared to the one of KO348. The KO348 strain has extra phage-related proteins while KO774 has a higher copy number of flagellin-related proteins **(Table 2.1).**

Table 2.1 Protein domains (PFAM) with different copy number among both *Kosakonia* strains

Function ID	Name	KO348	KO774	copy number difference
pfam00589	Phage integrase family	15	8	7
pfam00959	Phage lysozyme	7	2	5
pfam02413	Caudovirales tail fibre assembly protein, lambda gpK	4	1	3
pfam05840	Bacteriophage replication gene A protein (GPA)	4	1	3
pfam03374	Phage antirepressor protein KilAC domain	3	0	3
pfam09669	Phage regulatory protein Rha (Phage_pRha)	3	0	3
pfam12571	Phage tail-collar fibre protein	5	3	2
pfam05930	Prophage CP4-57 regulatory protein (AlpA)	4	2	2
pfam04717	Type VI secretion system, phage-baseplate injector	3	1	2
pfam04865	Baseplate J-like protein	3	1	2
pfam04985	Phage tail tube protein FII	3	1	2
pfam05489	Phage Tail Protein X	3	1	2
pfam06891	P2 phage tail completion protein R (GpR)	3	1	2
pfam07022	Bacteriophage CI repressor helix-turn-helix domain	3	1	2
pfam09684	Phage tail protein (Tail_P2_I)	3	1	2
pfam16080	Bacteriophage holin family HP1	3	1	2
pfam16452	Bacteriophage CI repressor C-terminal domain	3	1	2
pfam03589	Antitermination protein	2	0	2
pfam04860	Phage portal protein	2	0	2
pfam05069	Phage virion morphogenesis family	2	0	2
pfam05106	Phage holin family (Lysis protein S)	2	0	2
pfam05125	Phage major capsid protein, P2 family	2	0	2
pfam05926	Phage head completion protein (GPL)	2	0	2
pfam05944	Phage small terminase subunit	2	0	2
pfam05973	Phage derived protein Gp49-like (DUF891)	2	0	2
pfam06892	Phage regulatory protein CII (CP76)	2	0	2
pfam09003	Bacteriophage lambda integrase, N-terminal domain	2	0	2
pfam09524	Conserved phage C-terminus (Phg_2220_C)	2	0	2
pfam16872	Putative phage abortive infection protein	2	0	2
pfam00669	Bacterial flagellin N-terminal helical region	4	8	-4
pfam00700	Bacterial flagellin C-terminal helical region	4	8	-4

2.3.2 Comparative genomics in the *Kosakonia* genus

A phylogenetic tree was constructed comprising all the *Kosakonia* complete genomes publicly available in the Integrate Microbial Genomes & Microbiomes database (IMG/M) of the Joint Genome Institute (Nordberg et al. 2014), including strains KO348 and KO774 (n=15), the analysis was performed having *Escherichia coli* K12 MG1655 as an outgroup. The phylogenetic analysis showed that the strain *Kosakonia* sp. KO348 is most closely related to the strain *K. sacchari* CGMCC.1.12101, while *Kosakonia* sp. KO774 is most closely related to the strain *K. pseudosacchari* NN143 **(Figure 2.4).** The hierarchical clustering in the phylogenetic tree showed a clear separation between two groups, the *Kosakonia* strains isolated from plants (phytophyla group) and the *Kosakonia* isolated from human/animal samples **(Figure 2.4)**.

The enriched protein domains between the genomes of the phytophyla group and the human/animal associated group were analyzed and some clear differences were found; in the *Kosakonia* phytophyla group, nitrogen fixation, cobalamin biosynthesis, ethanolamine and phosphonate metabolism domains were enriched, while in the human/animal associated strains, the domains for host adaptation and virulence such as immunoglobulin A1 protease and Haem utilization were enriched **(Figure 2.4).**

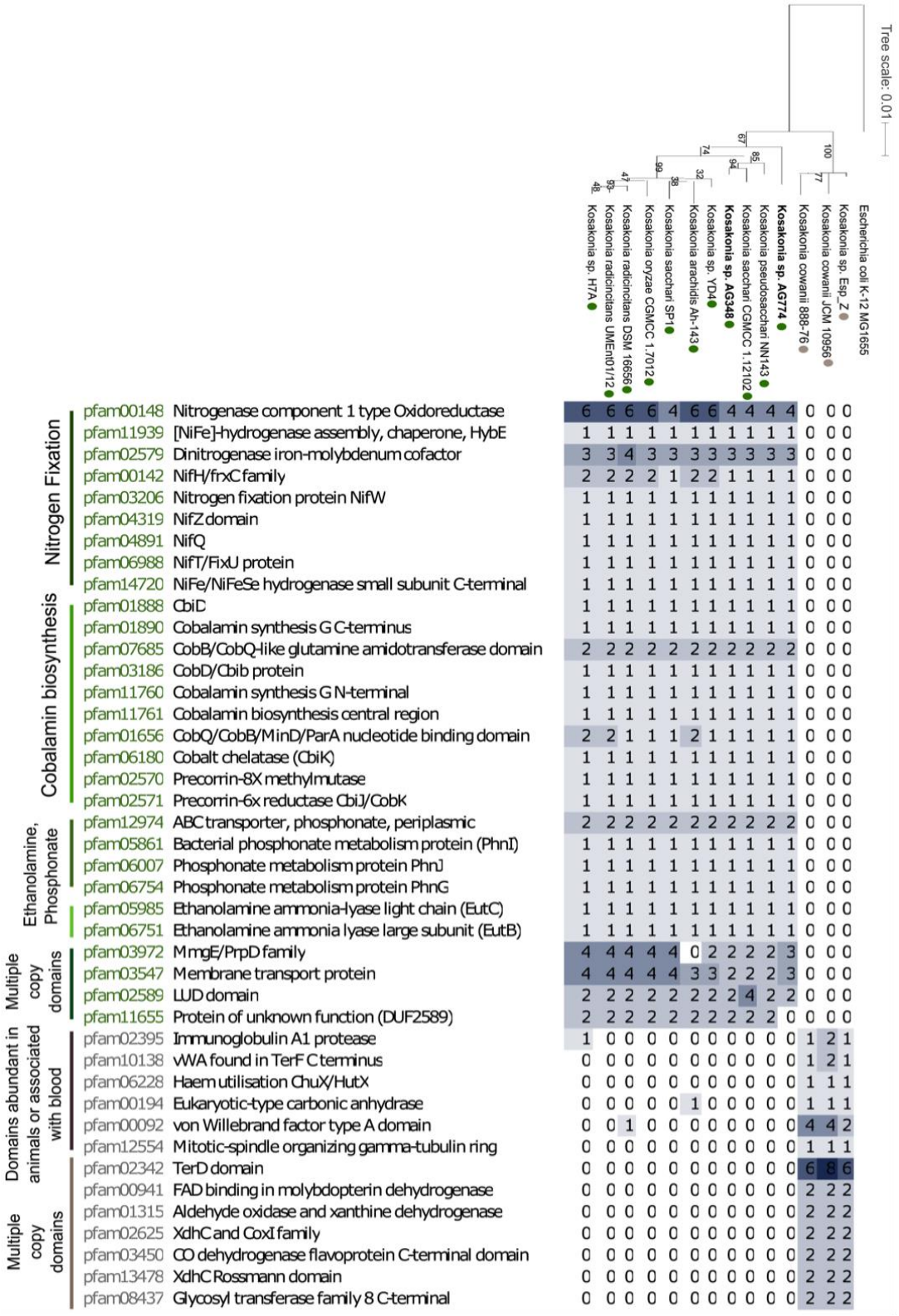


Figure 2.4. *Kosakonia* phylogenetic tree showing enriched protein domains by source of isolation (plants vs. human/animals)

Phylogenetic tree showing the phylogenetic position of the Kosakonia sp. strains KO348 and KO774 based on 23 single single copy genes found among the 14 complete Kosakonia genomes from IMG dataset. Escherichia coli K-12 MG1655 was used as an outgroup. Enriched protein domains in the plant-associated Kosakonia vs. human/animal isolated Kosakonia strains are shown by genome.

2.3.3 Plant colonization assays on rhizosphere and endosphere

Colonization studies were conducted to determine the colonization ability of the rice rhizosphere and endosphere of both *Kosakonia* strains and also determine if they out-competed each other. Single-inoculation and co-inoculation using both *Kosakonia* strains were performed **(Figure 2.5).** Both *Kosakonia* sp. KO348 and KO774 strains were able to colonize the rice rhizosphere and endosphere efficiently and at very similar levels when inoculated independently. Interestingly, when both strains were co-inoculated on rice, both were able to equally colonize the two plant compartments without out-competing each other and likely forming stable and mixed communities.

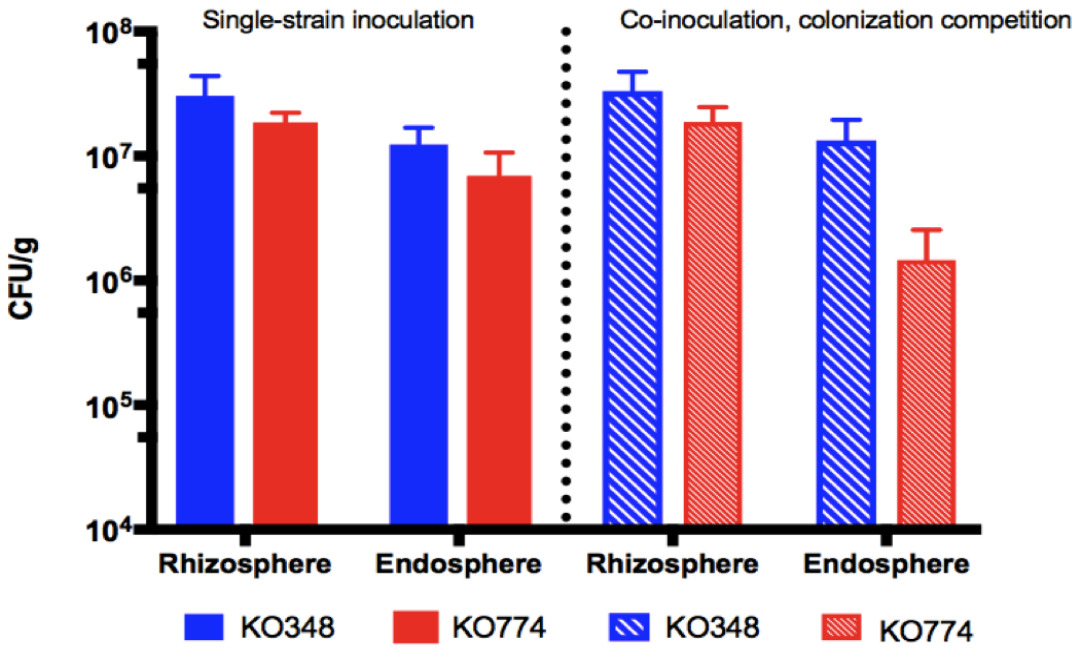


Figure 2.5 Rhizosphere and root endosphere colonization by the two *Kosakonia* strains.
Rhizosphere and root endosphere colonization by *Kosakonia* strains was evaluated in rice plants at 14 dpi by antiobiotic selection (KO348 RifR and KO774 SmR). Three treatment groups were evaluated, KO348 and KO774 in single inoculation ($1x10^8$ CFU/ml) and a third group of plants co-inoculated with 0.5x108 CFU/ml of each strain. Each sample was considered as the total roots of 4 rice plants, for each treatment group 3 samples were analyzed. No statistical difference was found between both strains at rhizosphere or endosphere level.

Bacterial CFU of strain KO348 attached to the surface of the seedling root after one hour of inoculation was $2.8x10^7$ CFU/g and the number of bacterial cells recovered from the root endosphere at the different time-points analyzed (5-50 dpi) was on average of $3.1x10^4$ CFU/g **(Figure 2.6A)**. The re-isolation in the endosphere of the aerial-green-part of the plant displayed a lower colonization, at three and six dpi being $2.2x10^4$ CFU/g and $1x10^4$ CFU/g respectively. At 10 dpi it decreased to $0.7x10^3$ CFU/g and at 30 dpi only 80 CFU/g were counted. It was therefore observed that unlike what occurs in the root endosphere, a significant difference in the aerial endosphere was detected between day 3 and 30 post-inoculation **(Figure 2.6B)**.

It was concluded that *Kosakonia* sp. KO348 is a good rice root endosphere colonizer and can also colonize the aerial endosphere, albeit with considerable less efficiency.

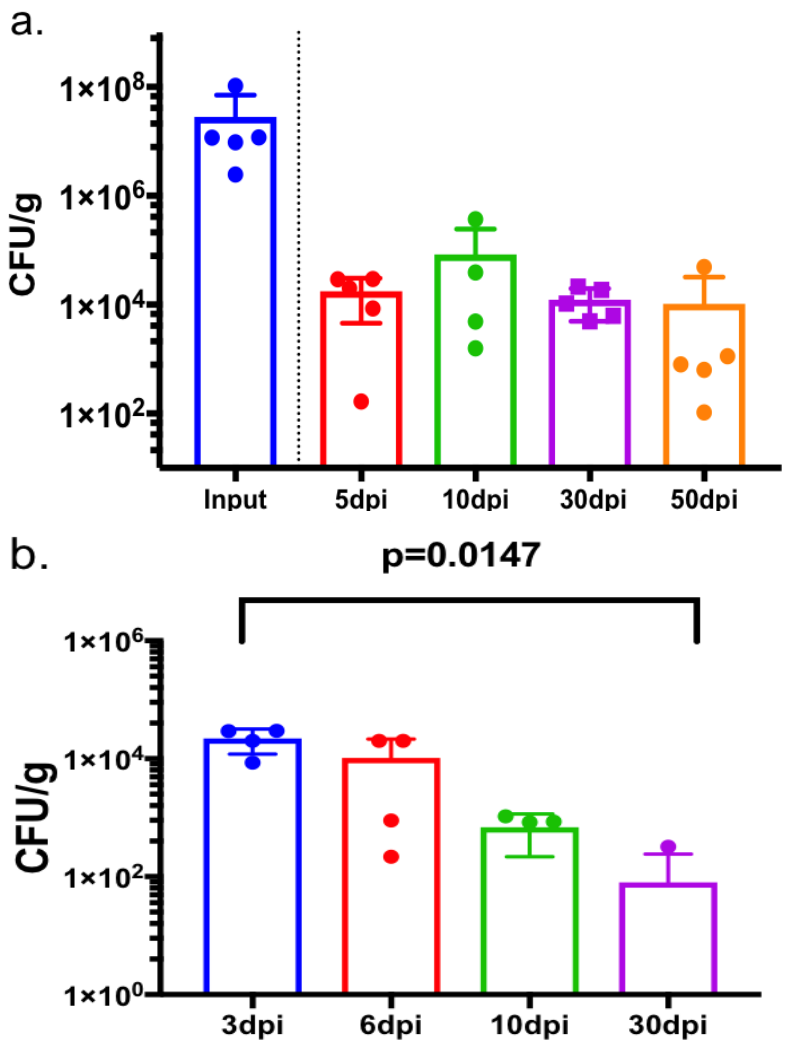


Figure 2.6 Endophytic colonization by Kosakonia strain KO348.
The endosphere colonization of the strain KO348 was evaluated in roots (a) and in green-part (b) of rice plants at different time-points by plating serial dilutions from previously sterilized plant tissues.

2.3.4 Visualization by confocal microscopy in the rice rhizosphere and endosphere of strain *Kosakonia* sp. KO348

Internal plant colonization by *Kosakonia* sp. KO348 was unequivocally determined by confocal microscopy localization. Location of the strain on the rhizoplane (including root hair zone, secondary root emergence and grain surface) and inside the root endosphere (transversal sections of the root) of rice roots were determined within rice plants inoculated with *Kosakonia* strain KO348(pBBRgfp) at different time-points of 5, 10, 30 and 50 dpi.

Strain KO348 presented higher densities in the rhizoplane than in the root endosphere at the four time points analyzed **(Figures 2.7 -2.8)**. At 5 and 10 dpi it was mainly found at the root hair zone, secondary root mergence, and on grain surface, presenting at 10 dpi clear bacterial aggregation, especially at the root hair zone **(Figure 2.7)**. At 30 dpi high densities of bacterial aggregation on the rhizoplane were also observed that was less evident at 50 dpi **(Figure 2.8)**. *Kosakonia* sp. KO348 was also observed as endophyte in the transversal root sections, up to the parenchyma, in all the four time points determining that the strain was able to colonize the root endosphere **(Figure 2.7 -2.8)**. The un-inoculated plants did not present fluorescence at any time point analyzed neither in the rhizoplane nor in the root endosphere **(Figure 2.8 -2.9)**. It was therefore concluded that KO348 was able to attach and colonize the rhizoplane of rice plants and as endophyte forming communities observable until 50 dpi.

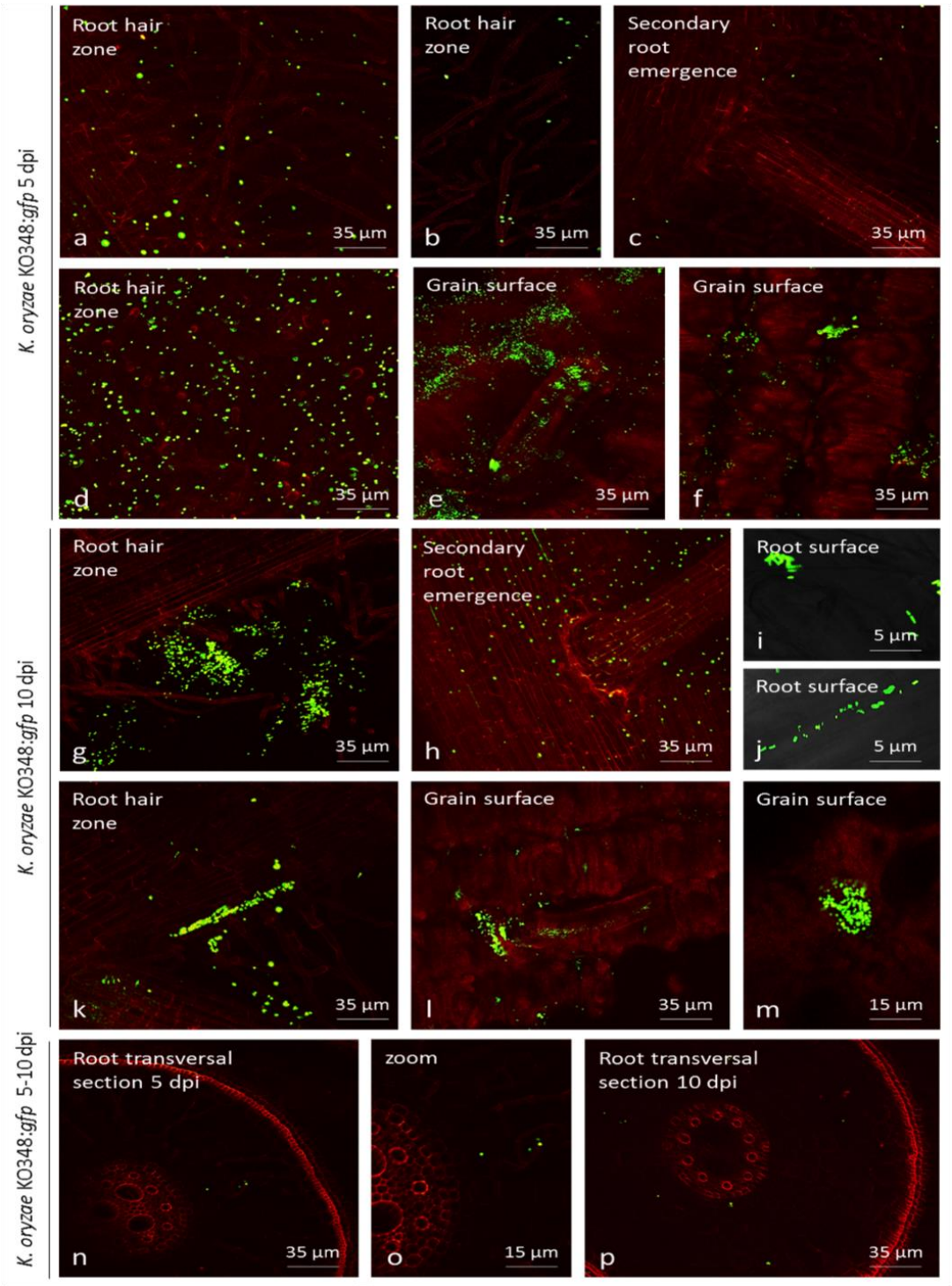


Figure 2.7 Microscopic visualization of *Kosakonia* strain KO348(pBBRgfp) in rice roots at rhizosphere and root endosphere level at 5 and 10 dpi.
KO348(pBBRgfp) was visualized by fluorescence microscopy at 5 and 10 dpi in the root hair zone (rhizosphere) and in the root aerenchyma (endosphere). A total of 10 plants were analyzed for each time point.

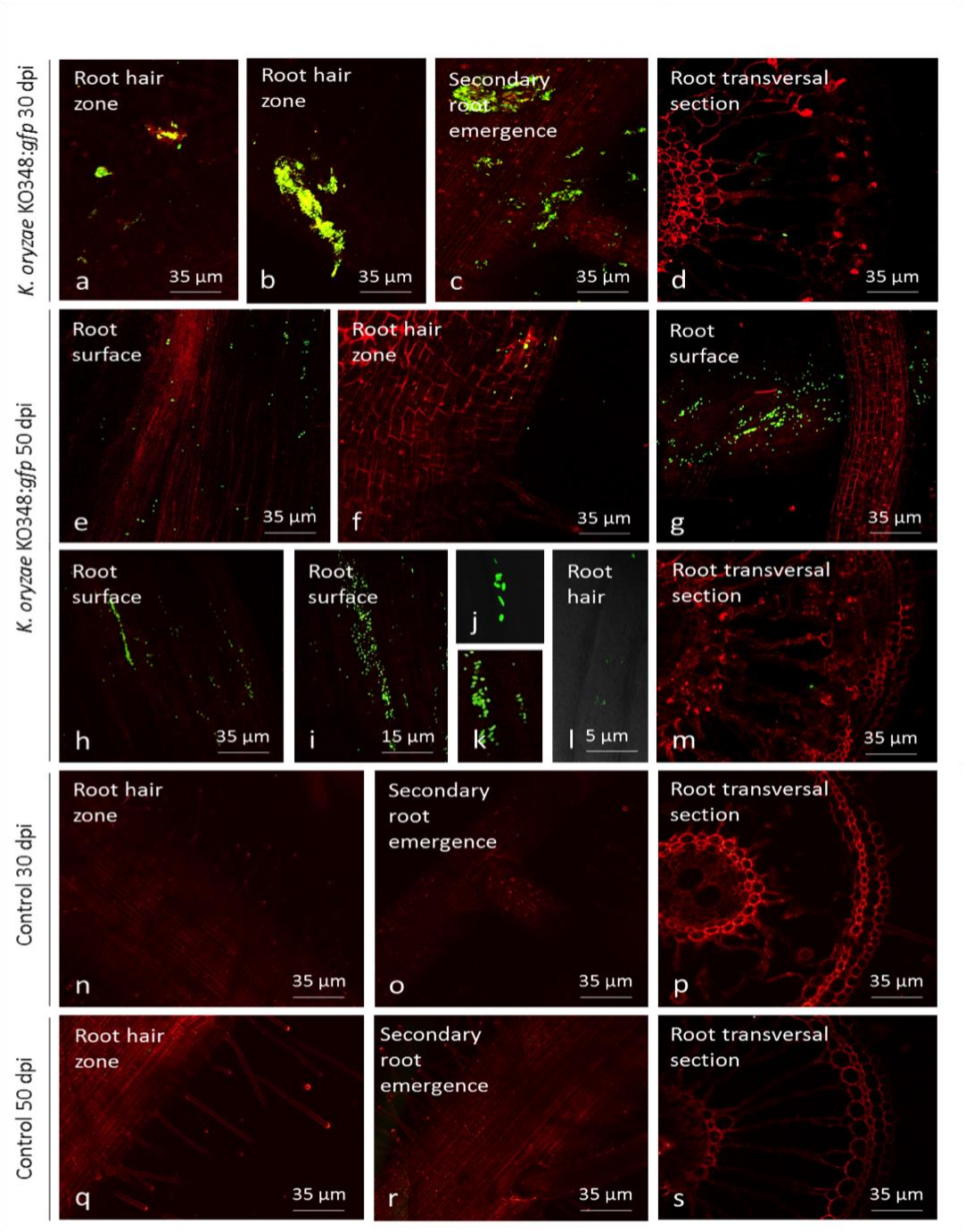


Figure 2.8 Microscopic visualization of *Kosakonia* strain KO348(pBBRgfp) in rice roots at rhizosphere and root endosphere level at 30 and 50 dpi.
KO348(pBBRgfp) was visualized by fluorescence microscopy at 30 and 50 dpi in the root hair zone (rhizosphere) and in the root aerenchyma (endosphere) and compared to contol plants. A total of 10 plants were analyzed for each time point.

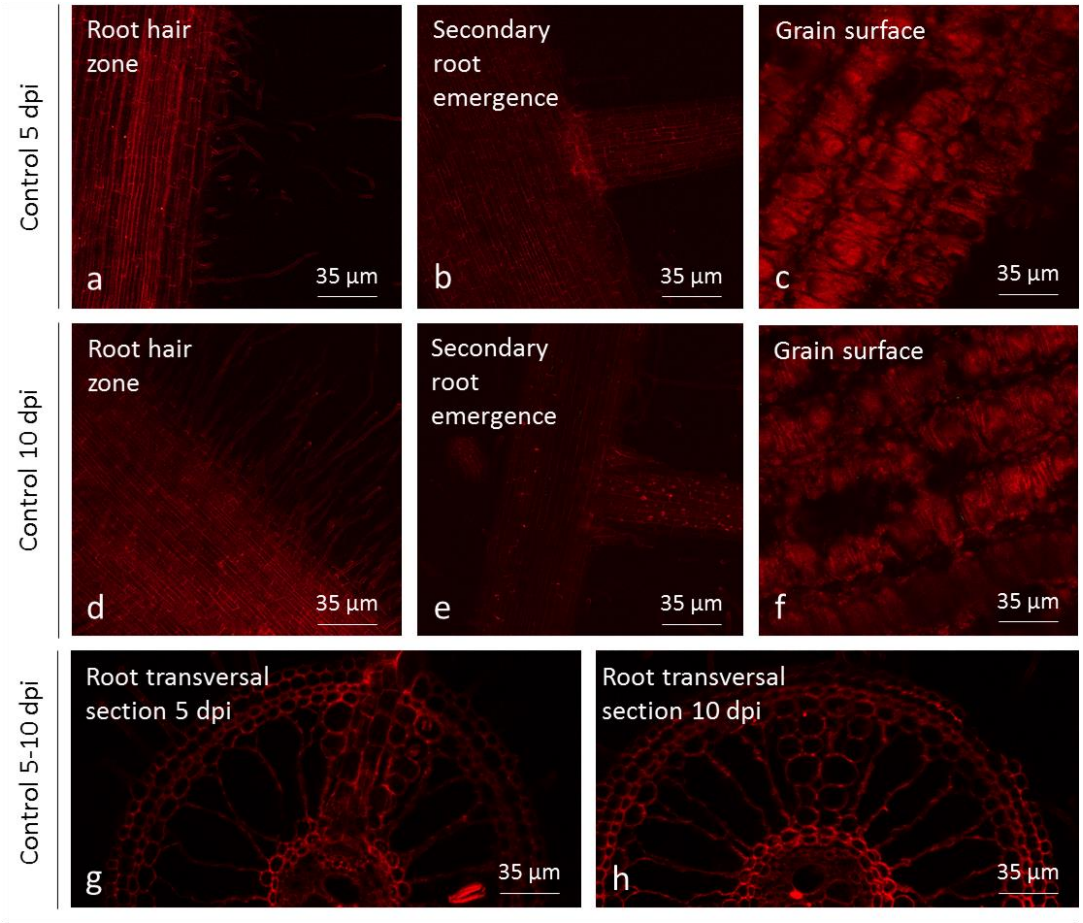


Figure 2.9 Visualization of control un-inoculated rice plants at 5 and 10 dpi by confocal microscopy.
Fluorescent colonies containing *Kosakonia* KO348(pBBRgfp) were not detected in the control plants.

2.3.5 Secretome profile determination of *Kosakonia* KO348

Since the secreted proteins of *Kosakonia* could play a role in the endophytic colonization process. The produced and secreted proteins in the extracellular medium by *Kosakonia* sp. KO348 were determined. A total of 80 putative secreted proteins were detected when strain KO348 was grown in a minimal plant-mimicking medium **(Table 2.2).** Among the ones found were nine flagella-related proteins (FliD, FLiK, FlgK, FlgL, FlgE and 3 different proteins of FliC) five

proteins belonging to the type VI secretory system (Hcp, TssH and three different proteins of VgrG) **(Table 2.2)**.

Table 2.2 Secretome profile of KO348 in plant minimal mimical media

Putative proteins	**Gene identifier**
Flagella-related proteins	
flagellin FliC [*Kosakonia oryzae*]	gi|780195958
flagellar filament capping protein FliD [*Enterobacteriaceae*]	gi|521289916
flagellar hook-associated protein FlgK [*Enterobacteriaceae*]	gi|521293169
flagellin FliC [*Kosakonia oryzae*]	gi|780195958
flagellin FliC [*Enterobacteriaceae*]	gi|521292903
flagellar hook length control protein FliK [*Kosakonia oryzae*]	gi|780195928
flagellar hook-filament junction protein FlgL [*Kosakonia oryzae*]	gi|780189038
flagellar biosynthesis protein FlhA [*Kosakonia*]	gi|780196051
flagellar assembly peptidoglycan hydrolase FlgJ [*Enterobacteriaceae*]	gi|516025827
Type VI secretion systems	
type VI secretion system tip protein VgrG [*Kosakonia oryzae*]	gi|780193605
type VI secretion system tip protein VgrG [*Enterobacteriaceae*]	gi|521291363
type VI secretion system tip protein VgrG [*Kosakonia oryzae*]	gi|780193664
Hcp1 family type VI secretion system effector [*Enterobacteriaceae*]	gi|512737854
type VI secretion system-associated FHA domain protein TagH [*Kosakonia oryzae*]	gi|780193691
Hypothetical proteins	
hypothetical protein [*Kosakonia oryzae*]	gi|780197120
hypothetical protein [*Kosakonia oryzae*]	gi|919124150
hypothetical protein [*Kosakonia oryzae*]	gi|780188037
hypothetical protein [*Kosakonia*]	gi|780197434
hypothetical protein [*Kosakonia*]	gi|1175529147
hypothetical protein [Enterobacteriaceae]	gi|512738009
hypothetical protein [*Kosakonia oryzae*]	gi|780194185

Membrane/ transport

ATP-dependent Clp protease ATP-binding subunit ClpA [*Kosakonia*]	gi|516025582
F0F1 ATP synthase subunit alpha [*Enterobacteriaceae*]	gi|516027463
MFS transporter [*Enterobacteriaceae*]	gi|764913067
porin [*Kosakonia oryzae*]	gi|780188241
ABC-F family ATPase [*Enterobacteriaceae*]	gi|521292875
multidrug transporter subunit MdtB [*Kosakonia*]	gi|780195173
inorganic phosphate transporter [*Kosakonia*]	gi|780190310
idonate transporter [*Enterobacteriaceae*]	gi|521291292
electron transport complex subunit RsxC [*Kosakonia oryzae*]	gi|780199521
ABC transporter ATP-binding protein [*Kosakonia oryzae*]	gi|780200498
multidrug transporter [*Enterobacteriaceae*]	gi|512737926
ATP-dependent DNA helicase DinG [*Kosakonia oryzae*]	gi|780187568
ABC transporter ATP-binding protein [*Enterobacteriaceae*]	gi|521290803
ATP-independent periplasmic protein-refolding chaperone [*Kosakonia oryzae*]	gi|780196453
copper-exporting P-type ATPase CopA [*Kosakonia oryzae*]	gi|780192654
penicillin-binding protein 1C [*Kosakonia oryzae*]	gi|780194667
TonB-dependent siderophore receptor [*Kosakonia oryzae*]	gi|780192080

Cellular processes/metabolism

molybdopterin-guanine dinucleotide biosynthesis protein B [*Kosakonia oryzae*]	gi|780197869
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase [*Kosakonia oryzae*]	gi|780197054
cytidine deaminase [*Enterobacteriaceae*]	gi|521290110
DNA helicase RecQ [*Kosakonia*]	gi|780198727
peptidase T [*Kosakonia oryzae*]	gi|780189257
3-deoxy-7-phosphoheptulonate synthase [*Kosakonia oryzae*]	gi|780200115
1-deoxy-D-xylulose-5-phosphate synthase [*Kosakonia oryzae*]	gi|780192820
3-deoxy-8-phosphooctulonate synthase [*Enterobacteriaceae*]	gi|512738011
arabinose-5-phosphate isomerase GutQ [*Kosakonia oryzae*]	gi|780195548
FtsH protease activity modulator HflK [*Enterobacteriaceae*]	gi|521291963
protein NifY [*Kosakonia oryzae*]	gi|780191615
DNA repair protein RecN [*Kosakonia*]	gi|780199998
methyl-accepting chemotaxis protein [*Enterobacteriaceae*]	gi|764911836

bifunctional uridylyltransferase/uridylyl-removing protein GlnD [*Enterobacteriaceae*]	gi|516027410
DUF1176 domain-containing protein [*Enterobacteriaceae*]	gi|521290398
nitrate reductase subunit beta [*Kosakonia oryzae*]	gi|780199604
phosphoribosylglycinamide formyltransferase [*Kosakonia*]	gi|780194593
filamentous hemagglutinin N-terminal domain-containing protein [*Kosakonia oryzae*]	gi|780192260
molecular chaperone GroEL [*Enterobacteriaceae*]	gi|521291941
alanine--glyoxylate aminotransferase family protein [*Kosakonia oryzae*]	gi|780199232
glycoside hydrolase family 2 [*Kosakonia oryzae*]	gi|780191839
2-isopropylmalate synthase [*Kosakonia oryzae*]	gi|780192256
HDOD domain-containing protein [*Enterobacteriaceae*]	gi|512738353
glycerophosphodiester phosphodiesterase [*Kosakonia oryzae*]	gi|780200713
tRNA dihydrouridine synthase DusB [*Enterobacteriaceae*]	gi|764912401
DUF968 domain-containing protein [*Kosakonia oryzae*]	gi|780201375
2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase [*Kosakonia oryzae*]	gi|780192125
50S ribosomal protein L7/L12 [*Enterobacteriaceae*]	gi|504694962
phenylalanine--tRNA ligase subunit beta [*Kosakonia oryzae*]	gi|780196404
1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase [*Kosakonia oryzae*]	gi|780195039
MULTISPECIES: GNAT family N-acetyltransferase [*Enterobacteriaceae*]	gi|521291624
elongation factor G [*Kosakonia oryzae*]	gi|780194933
HAMP domain-containing protein [*Kosakonia*]	gi|780199610
flavocytochrome c [*Enterobacteriaceae*]	gi|512737894
oxidoreductase [Kosakonia oryzae]	gi|780200926
molecular chaperone DnaK [*Kosakonia oryzae*]	gi|780194002
exodeoxyribonuclease V subunit gamma [*Kosakonia oryzae*]	gi|780197912
cellulose biosynthesis cyclic di-GMP-binding regulatory protein BcsB [*Kosakonia oryzae*]	gi|1114033417

Regulators

LysR family transcriptional regulator [*Kosakonia oryzae*]	gi|780190664
HTH-type transcriptional regulator CysB [*Enterobacteriaceae*]	gi| 512738072
TetR family transcriptional regulator [Kosakonia oryzae]	gi|780195860
LysR family transcriptional regulator [*Kosakonia oryzae*]	gi|780196901

2.3.6 Role of Type VI secretory system in rhizosphere and root endosphere colonization in *Kosakonia* sp. KO348

The secretome profile of strain KO348 demonstrated that several proteins of the Type VI secretion system were present (see section above). A database search for type VI secretion systems (T6SS) domains among the *Kosakonia* available genomes at the IMG/M showed that the T6SS was present among all genomes, including the two strains of this study **(Table 2.3)**. When analyzing the genome of KO348 for T6SS known annotations two loci were found and in one of them a complete gene cluster of T6SS was identified **(Figure 2.10)**. For further search for putative T6SS effectors, 70 proteins found in the T6SS loci **(Appendix 1)** were aligned against the proteins identified in the secretomic profile and it was then evidenced that three proteins matched; two were T6SS tip proteins VrgG (gi|780193605 and gi|780193664) and one secretion system-associated FHA domain protein TagH (gi|780193691). Since T6SS component proteins and candidate effectors were expressed in plant-mimicking medium, it was of interest to determine the possible role of T6SS in rhizosphere and root endosphere colonization. A knock-out mutant of the T6SS *hcp* gene (responsible for the formation of the needle-like structure for the passage of the effectors) called KO348hcp and its complement KO348hcp(pBBRhcp) (carrying a plasmid with the complete *hcp* gene) were generated. In order to assess rice colonization ability of the *hcp* knock-out mutant of *Kosakonia* sp. KO348, the four following inoculation groups were performed; the KO348 wild-type (WT), the KO348hcp mutant, the complemented *hcp* mutant KO348hcp(pBBRhcp) and the KO348 WT and mutant KO348hcp together in a competition experiment. For all the four groups the same

amount of total bacteria 1.3 x10^7 CFU/ml was used for plant inoculation, in the case of the competition experiment 0.65 x10^7 CFU/ml of each strain were used.

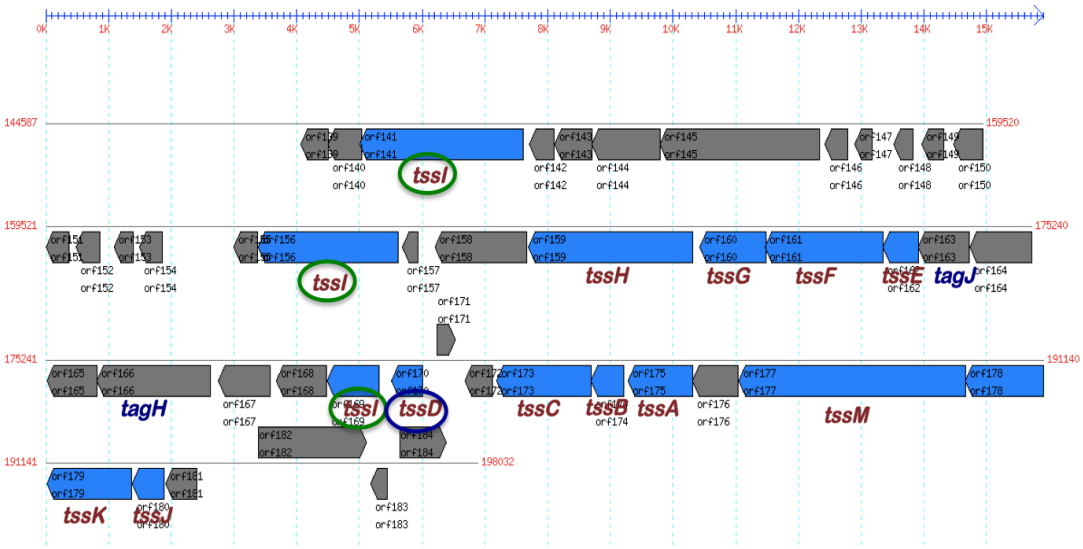


Figure 2.10 Cluster of Type VI Secretion System of KO348
T6SS cluster was identified using the online database SecreT6 (Microbial Bioinformatics Group, SKMML, SJTU), the genes are named according to the most used nomenclature of Shalom et al. 2007, where ***hcp*** is named ***tssD*** and ***vgrG*** as ***tssI.*** The 3 copies of ***vgrG*** that belong to this cluster are the ones found on the secretome profile of KO348.

Table 2.3. Type VI secretion system-related genes among *Kosakonia* genus

Function ID	pfam12790	pfam06744	pfam05943	pfam05638	pfam04717	pfam05947	pfam05591	pfam06996	pfam06812	pfam05936
Kosakonia sp. KO774	1	1	1	1	1	1	1	1	1	1
Kosakonia oryzae KO348	1	1	2	1	3	1	2	1	2	2
Kosakonia arachidis Ah-143	2	1	3	4	3	2	3	2	2	2
Kosakonia cowanii Esp_Z	1	1	2	1	1	1	1	1	1	1
Kosakonia cowanii JCM 10956	1	1	1	1	1	2	1	1	2	1
Kosakonia diazotrophica S29	1	1	3	2	0	1	3	1	3	3
Kosakonia oryzae CGMCC 1.7012	3	2	3	2	2	3	3	3	4	3
Kosakonia oryzae D4	3	1	2	2	2	2	3	2	3	2
Kosakonia oryzendophytica REICA_082	1	1	1	1	2	1	1	1	1	1
Kosakonia oryziphila REICA_142	3	3	4	5	3	3	3	3	4	3
Kosakonia radicincitans DSM 16656	3	2	3	2	3	3	3	3	4	3
Kosakonia radicincitans UMEnt01/12	3	2	3	2	2	3	3	3	4	3
Kosakonia radicincitans YD4	3	2	3	2	1	3	3	3	4	3
Kosakonia sacchari CGMCC 1.12102	2	2	2	2	2	2	2	2	3	2
Kosakonia sacchari SP1	3	2	3	2	2	3	3	3	4	3
Kosakonia sacchari SP1	2	2	2	2	2	2	2	2	3	2

Pfam name legends:

PFAM	Protein domain name
pfam12790	Type VI secretion lipoprotein, VasD, EvfM, TssJ, VC_A0113
pfam06744	Type VI secretion protein IcmF C-terminal
pfam05943	Type VI secretion protein, EvpB/VC_A0108, tail sheath
pfam05638	Type VI secretion system effector, Hcp
pfam04717	Type VI secretion system, phage-baseplate injector
pfam05947	Type VI secretion system, TssF
pfam05591	Type VI secretion system, VipA, VC_A0107 or Hcp2
pfam06996	Type VI secretion, TssG
pfam06812	ImpA, N-terminal, type VI secretion system
pfam05936	Bacterial Type VI secretion, VC_A0110, EvfL, ImpJ, VasE

In planta experiments determined that at 14 dpi the colonization of the rhizosphere by WT strain KO348 was 40-fold higher than the colonization of the *hcp* mutant KO348hcp ($8x10^5$ CFU/g of root vs. $1.9x10^4$ CFU/g of root respectively, **Figure 2.11**). A significant difference was also observed between the WT KO348 and KO348*hcp* mutant when they where co-inoculated ($2.8x10^5$ CFU/g of root vs. $2.5x10^4$ CFU/g of root respectively). Complementing the mutant with the *hcp* gene harbored in a plasmid, resulted in restoration of its ability to colonize the rhizosphere ($5.1x10^5$ CFU/g) back to WT levels **(Figure 2.11A)**. In this complementation experiment the percentage of bacterial strains that retained the plasmid was 89% indicating a low incidence of plasmid loss.

Studies of root endosphere colonization were also performed and the results showed a significant difference between the colonization ability of WT and the *hcp* mutant in plants inoculated independently ($6.4x10^4$ CFU/g of root vs. $2.2x10^4$ CFU/g of root respectively). This significant difference between WT and mutant was maintained when plants were co-inoculated ($2.7x10^4$ CFU/g of root vs. $1.4x10^4$ CFU/g of root respectively) **(Figure 2.11B)**. However, unlike in the rhizosphere experiment, the complemented mutant did not result in the restoration of endophytic colonization to WT levels ($4.9X10^2$ CFU/g). In this case the percentage of bacterial cells which retained the plasmid harboring the *hcp* gene was only 23%; this is the most likely the reason for the lack of complementation. In summary, these results suggest a significant role for T6SS in the rhizosphere colonization and to a much lesser extent in the root endosphere colonization by *Kosakonia* sp. KO348.

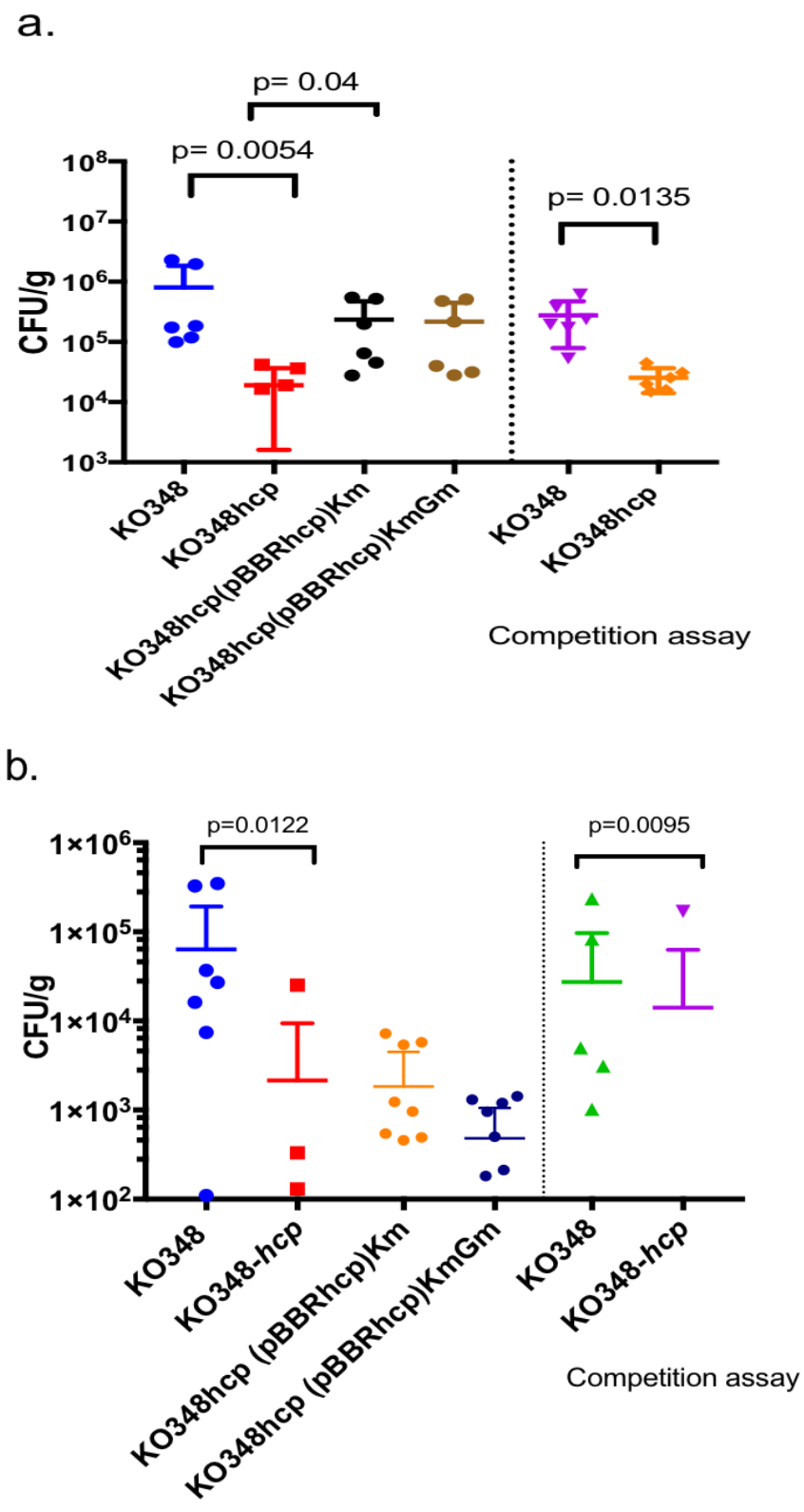


Figure 2.11 Role of the Type VI secretion system of *Kosakonia* KO348 in rhizosphere and endosphere rice root colonization.
The effect of type VI secretion system was tested in the rhizosphere (5a) and in the endosphere (5b) colonization of rice root plants at 10 dpi. KO348hcp mutant was inoculated independently and in competition assays (in the same rice plant) with KO348 WT.

2.3.7 Field rice inoculation with *Kosakonia* sp. KO774

As it was previously determined that both diazotrophic *Kosakonia* strains studied in this chapter displayed plant growth promoting properties (Bertani et al. 2016), it was performed a field rice experiment using *Kosakonia* KO774 in order to assess if it could compensate a reduction in nitrogen fertilization. Between May and October 2016 in Valencia, Spain, a rice field trial was conducted with diazotrophic *Kosakonia* sp. KO774 with the aim of testing if rice seeds inoculated with the bacterial strain can compensate a 50% reduction in nitrogen fertilization. A total of 16 growth plots were grown which were divided in four groups, (i) eight plots receiving 100% nitrogen/urea fertilization, four planted with seeds inoculated with strain KO774 and the other four plots with the seeds not inoculated and (ii) eight plots receiving only 50% of urea/nitrogen fertilization, four of these planted with seeds inoculated with strain KO774 and the other four plots with the seeds not inoculated **(Figure 2.1)**.

All the plots were harvested 100 days post sowing and different growth parameters such as germination/plot, weight of 1,000 grains/plot and 25 panicles/plot and yield (kg/ha) were then assessed/measured **(Table 2.4)**. No significant differences in any of the measured parameters were found between the plots of the same set. This indicated that inoculation with *Kosakonia* did not result in any plant growth promotion and/or nitrogen biofertilization under the conditions tested **(Table 2.4)**.

Table 2.4. Field trial biological parameters by group

	Weigth (1,000 grains/plot)	Weight (25 panicles/plot)	Yield (m^2)	Yield (kg/ha)
Nitrogen 100%	**35**	**96,8**	**958,8**	**9587,5**
Nitrogen 100% + KO774	**35**	**90**	**943,8**	**9437,5**
Nitrogen 50%	**34,3**	**98,8**	**853,5**	**8585**
Nitrogen 50% + KO774	**35,3**	**101,3**	**828,5**	**8285**

2.3.8 Rice microbiome analysis of the rice field trial using seeds inoculated with *Kosakonia* sp. KO774

Following the rice seed inoculation with *Kosakonia* sp. KO774 in the field trial (see above), the colonization of strain KO774 and its effect on the total endospheric microbial community of rice plants were determined. The root endomicrobiome was analyzed at three different time points (30, 60 and 90 post-sowing) in plants which have been fertilized with 50% of the recommended amount of nitrogen and have been inoculated with KO774 as well as with ones that were not.

In the endomicrobiome; firstly it was determined the presence of bacterial sequences with 100% identity to the 16S rDNA gene fragment of *Kosakonia* sp. KO774. This sequence was present in all three samples in the *Kosakonia*-seed inoculated group at 30 dpi, however at 60 dpi it was only observed at much lower levels in one of the three samples. At 90 dpi this 16S rRNA gene sequence of *Kosakonia* sp. KO774 was not detected (**Figure 2.12**). In the group which was not seed inoculated with strain KO774, only in one sample at 90 dpi and low abundance was observed the 100% identity *Kosakonia* DNA sequence (**Figure 2.12**).

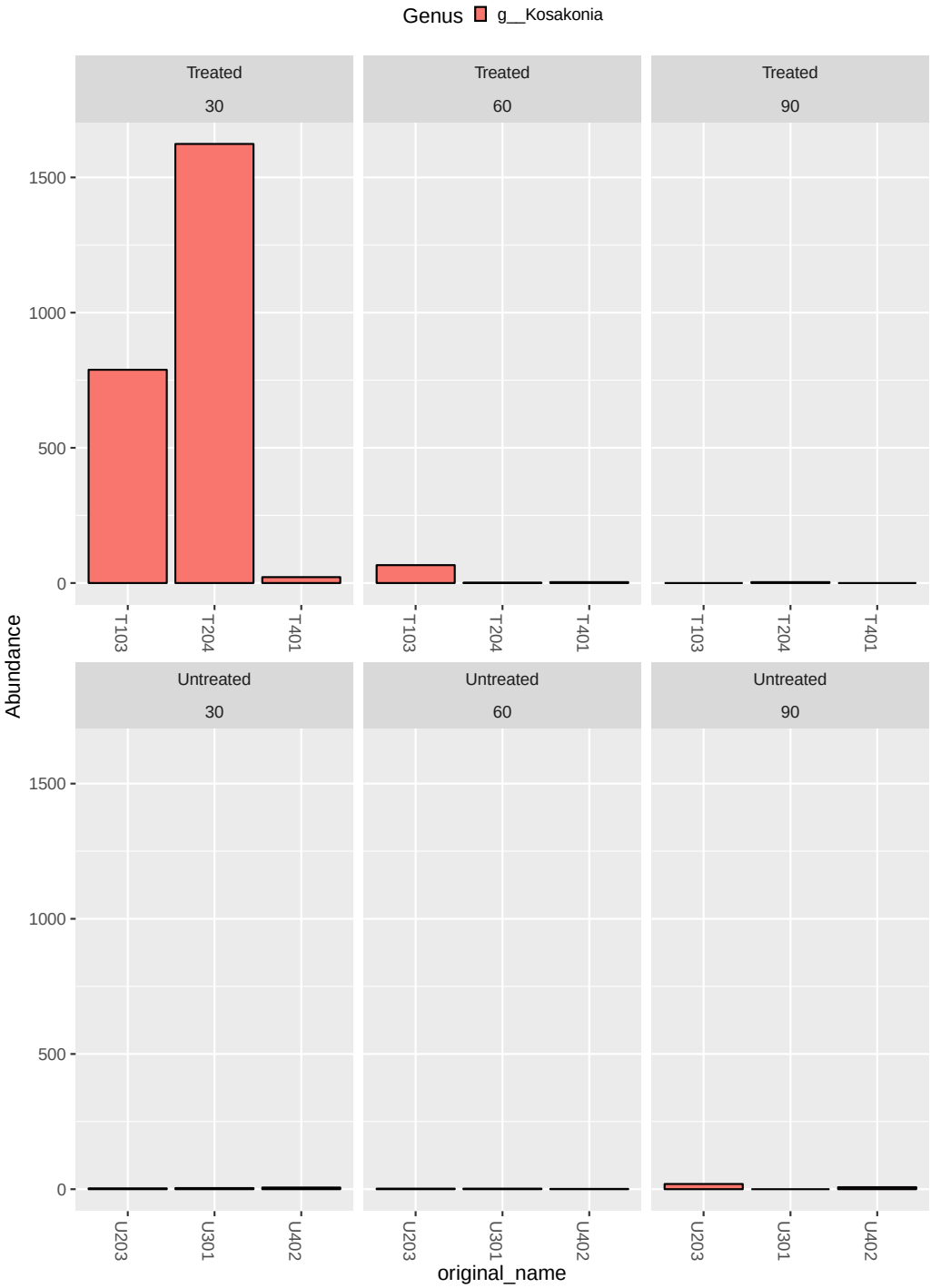


Figure 2.12 Abundance of 16S rDNA gene sequence 100% identical to the KO774 strain.

Using a modified version of the data base greengenes gg_13_8_99 that includes the sequence belonging to the V3-V4 16S rDNA fragment of *Kosakonia* strain KO774, the abundance by sample by treatment and time point of KO774 was determined (T= inoculated with Kosakonia, U=control).

A total root endomicrobiome analysis was also performed analyzing the main bacterial genera (>1% abundance) at 30 dpi, when *Kosakonia* sp. KO774 was still present. It was observed that the hierarchical clustering positioned closer the samples within each treatment group, indicating that all seed-inoculated samples were clustered together and all the un-inoculated samples were closer among them (**Figure 2.13**). However, when observing the Z-scores based on distribution and relative abundance of each genus, it was observed a significant difference between the two group-sets (inoculated vs. un-inoculated) in only a few genera like *Kosakonia* (as expected), *Rhodospirillum*, *Asticcacaulis* and *Enterobacteriaceae* NA (**Figure 2.13**).

When analyzing the clustering and patterns of distribution of all samples by treatment and time point by non-multidimensional scaling analysis (NMDS), it was observed that all samples were mainly clustered by time point, with seed inoculation of *Kosakonia* sp. KO774 not being a major factor for clustering. One sample of 30 dpi belonging to the untreated group can be clearly identified as an outlier (**Figure 2.14**).

These findings suggested that *Kosakonia* sp. KO774 was able to colonize the rice root endosphere at the given conditions only in the first time point analyzed (30 dpi) after seed sowing and that the endosphere microbial community was not significantly affected by the inoculation.

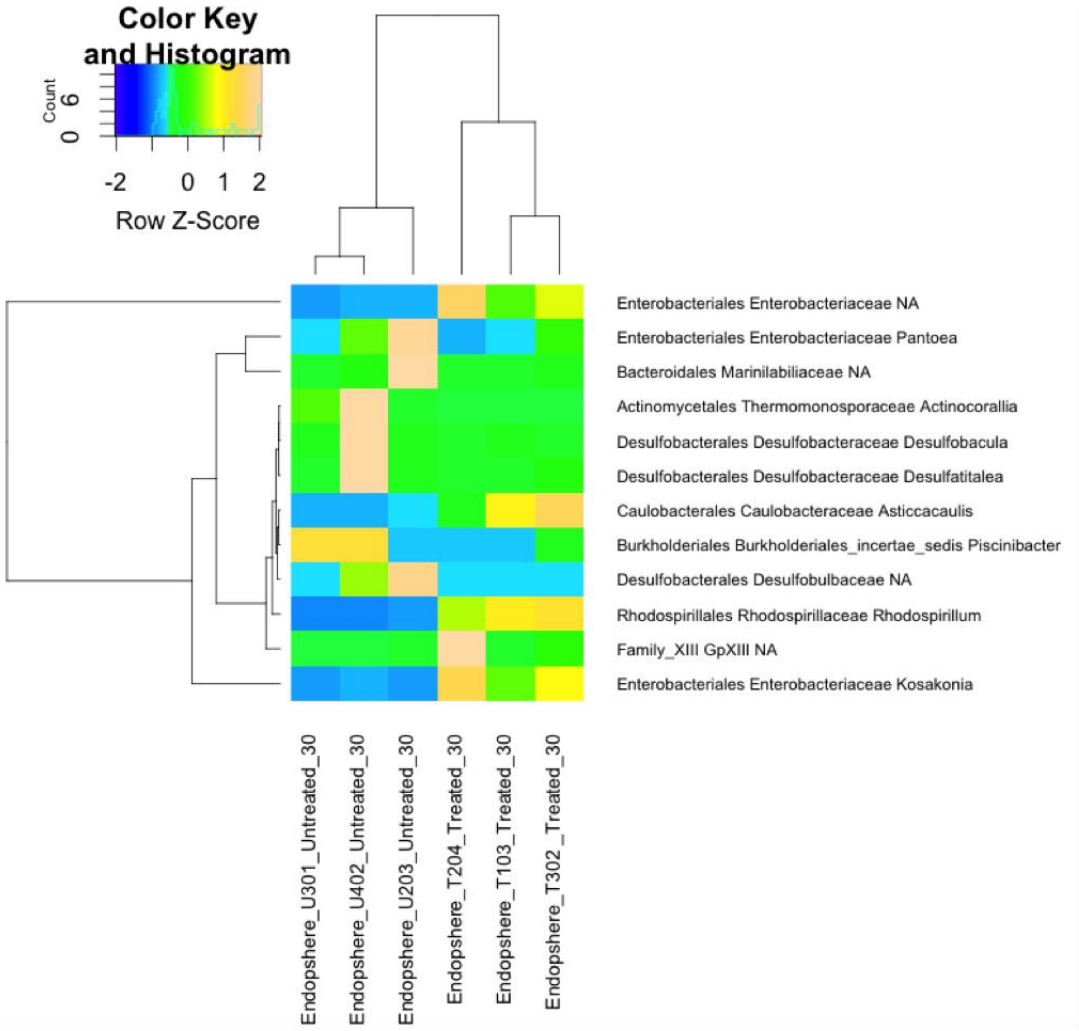


Figure 2.13. Heatmap of the most abundant genus (>1%) by sample and treatment at 30 days post inoculation in the field trial.

The clustering of the most abundant bacterial genera and OTUs present in rice root endosphere at 30 dpi among different samples is showed in the heatmap. The heat map scale displays the row Z score. Where the Z score is calculated as ([relative abundances of a genus in one sample − mean relative abundance of the same genus among total samples]/standard deviation).

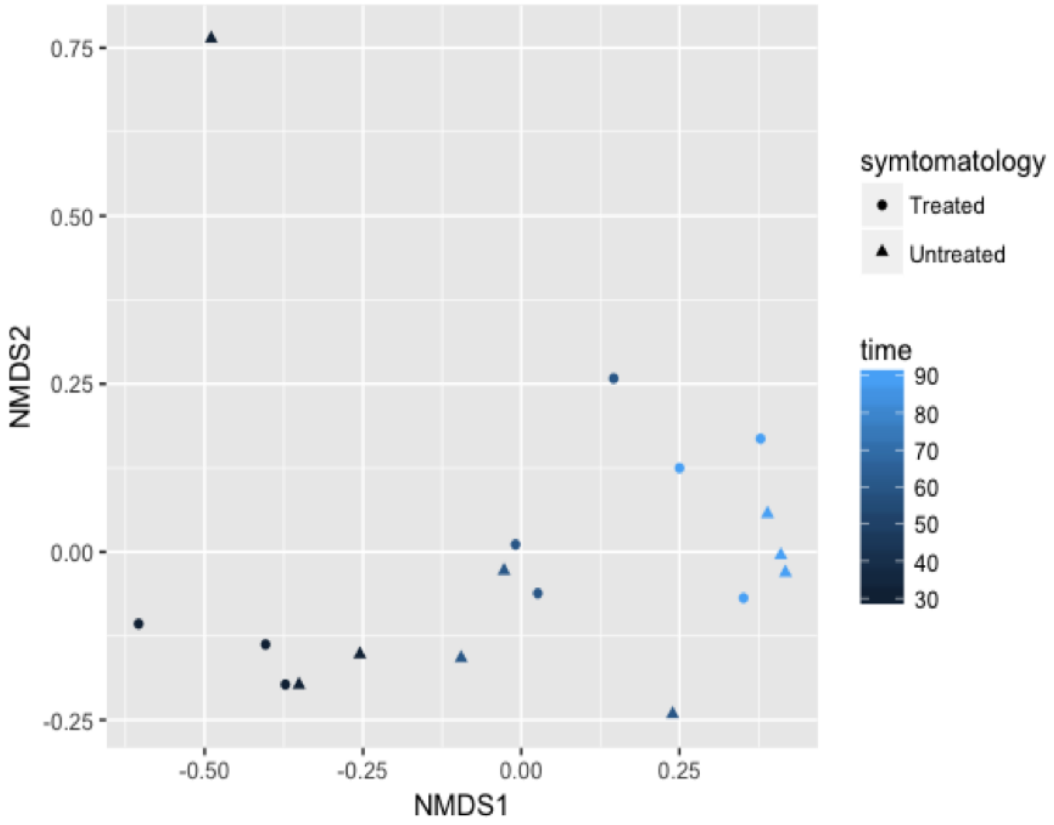


Figure 2.14 Distribution patterns analysis by NMDS of the microbiomes by time and treatment.

Non-multidimensional scaling analysis plot showing clustering (NMDS) plot showing clustering of samples of rice endophytic microbial communities by time and treatment based on Bray-Curtis dissimilarity.

2.4 Discussion

Different strains of the recently described *Kosakonia* genus have been isolated from crops and vegetables (Shinjo et al. 2016; Kämpfer et al. 2016b; Bergottini et al. 2015; Witzel et al. 2012; Berger et al. 2018) and many possess plant beneficial phenotypes such as nitrogen fixation and phosphate solubilization. This chapter was aimed to characterize two endophytic diazotrophic *Kosakonia* strains.

A recent comparative genomics study based in *Kosakonia radincincitans* DSM 16656 described multiple flagellar and secretion systems contributing to high motility and high competitiviness thus increasing bacterial fitness (Becker et al. 2018). Comparative genomics also revealed that enriched protein domains include the nitrogen fixing cluster (*nif* regulon) which has been reported in diverse *Kosakonia* plant- associated strains (Becker et al. 2018). Other enriched protein domains include functions related to cobalamin biosynthesis; this is an enzyme cofactor synthesized only by prokaryotes which in *Sinorhizobium meliloti* involved in simbiosis and nodule formation (Taga and Walker 2010). Phosphonate metabolism is also enriched in plant- associated *Kosakonia* strains; phosphonate is a rich source of soil phosphate which plays a role in plant-bacteria interaction (Kamat and Raushel 2013). Phosphonate utilization strains of *Stenotrophomonas rhizophila*, *Cupriavidus basilensis*, *Caulobacter segnis*, among others have been isolated from the rhizosphere of *Lolium perenne* (Fox et al. 2014). Finally some ethanolamine utilization protein domains were also enriched; ethanolamine can be used in some bacteria as a valuable source of carbon and nitrogen (Kaval and Garsin 2018). Ethanolamine utilization is important for bacterial pathogens of animals and plants as for example in the plant pathogen *Erwinia chrysanthemi*

(Kaval and Garsin 2018). Interestingly, recently ethanolamine and derivatives have been linked to plant bacterial interkingdom signaling (Coutinho et al. 2018).

In the particular case of KO774, the genomic analysis revealed a plasmid containing a putative large cellobiose gene, enzyme possibly involved in cellulose degradation; a similar enzyme has been previously reported in other endophyte *Enterobacter* sp. 638 (Taghavi et al. 2015).

Previous studies have shown that good endosphere colonizers are recovered *in vitro* in the order of 10^4-10^6 CFU/g after more than one week post-inoculation (Schmidt et al. 2011; Luna et al. 2010). Colonization studies described in this chapter, indicate that the two *Kosakonia* strains are very efficient root endosphere and rhizosphere colonizers. Fluorescence microscopy visualization also confirmed the KO348 ability to colonize; the rice roots endosphere. *K. radicincitans* DSM 16656 has also been recently observed in the root cortex of cucumber by confocal microscopy (Sun et al. 2018). Additionally, by transmission electron microsscopy (TEM), bacterial diazotrophs in rice have been localized within apoplastic locations (Gyaneshwar et al. 2001; Hurek et al. 1994; Egener et al. 1999).

Endophytes are likely to have evolved an intimate relationship with their plant host probably involving interkingdom signaling (Reinhold-Hurek and Hurek 2011; Lòpez-Fernàndez et al. 2017). Approximately 80 putative secreted proteins of *Kosakonia* sp. KO438 were detected and many of these can be involved in plant-bacteria interactions; flagellar and T6SS proteins were among the most abundant found. A similar secretome analysis has been performed in the endophyte *H. seropedicae* SmR1 and 41 secreted proteins have been reported including 19

flagella-related proteins (Chaves et al. 2009). No proteins belonging to the T6SS have been found in the secretome of *H. seropedicae* however, the presence of T6SS in genomes is very common among plant-associated bacteria (Levy et al. 2018) and in endophytes (Frank 2011). Interestingly, T6SS genes have been found enriched in the rhizosphere of barley (Bulgarelli et al. 2015). The T6SS is a phage-like secretion system found in approximately 25% of Gram- negative bacteria, mainly in Proteobacteria including many plant-associated bacteria (Boyer et al. 2009; Bernal et al. 2018). Interestingly, it is a host-specificity factor in the symbiont *R. leguminosarum* (van Brussel, A. A.Zaat et al. 1986). *K. radicincitans* possesses 3 different types of T6SS, however their function and /or mechanism of action have not been studied (Becker et al. 2018). T6SS in endophytes can be involved in host interaction and/or antagonizing other microbes in the endosphere (Frank 2011). In this chapter it is reported that a T6SS mutant of *Kosakonia* sp. KO348 displayed a significant decrease in rice rhizosphere and root endosphere colonization thus suggesting a role in the host-bacteria colonization/interaction.

Nitrogen is one of the mostly used fertilizers worldwide for all cereals including rice with an annual utilization growing rate of 1.9 and it is expected that 201.66 million tonnes will be used in 2020 (FAO 2017). Biofertilizers are considered an alternative to decrease the use of chemicals, however only few reports of greenhouse and field trials using rhizospheric or endospheric diazotrophic strains have been performed thus far. Most of these are in wheat or maize and reporting an increase between 6 and 33% in total yield (Santi et al. 2013). Furthermore, plant-associated microorganisms applied in agriculture as biofertilizers or biopesticides are usually subject to a rigorous risk assessment which requires a better

understanding of the mechanisms involved in the mutualism to facilitate and promote the development and application of sustainable microbial solutions in crop production (Brader et al. 2017). The associative microbial nitrogen fixation supplied by microbes in rice is predicted to be between 20-25% of the total nitrogen needed by the plant (Ladha et al. 1987; Saikia and Jain 2007). Inoculation experiments using *H. seropedicae* or *Burkholderia* sp. revealed that 11-20% of the total nitrogen accumulated in rice plants can be attributed to the bacterial strains (Divan Baldani et al. 2000). Similarly, inoculation studies using *K. radicincitans* (DSM 16656) increased plant root or shoot dry weight by 150% under high nitrogen conditions (350 mg per plant) and 130% in low nitrogen conditions (150 mg per plant) (Berger et al. 2013). This latter study also showed that plants with low nitrogen supplementation increased the pathogen defense-related markers and suggested this plant response could negatively affect/inhibit the PGP effect of *Kosakonia*. A recent report using the AbiVital product (67% *K. radicincitans* DSM 16656^{T} and 37% cryopreservation additives) in maize resulted in an increase in yield of around 30% in field trials including organic and conventional cultivation systems (Berger et al. 2018). In the field trial reported in this chapter, the nitrogen fertilization was decreased by 50% hoping that by supplementation via seed-inoculation of the *Kosakonia* strain could at least in part overcome nitrogen deficiency; this was not the case in any of the measurements performed. This experiment could have benefited from knowing the nitrogen concentration in the soil used for the field trial. It was observed that under the used conditions the colonization of the inoculated strain was rather inefficient over a longer period of time (more than 30 days) and that it did not affect the root endosphere microbiome composition; thus a possible limiting factor was likely to be the establishment of

the strain in the plant endosphere. This could have been due to inoculation method, soil microbial community or abiotic factors which are not favorable for the *Kosakonia* strain that was used. Interestingly, Becker et al. 2018 reported a significant impact on the bacterial community composition of tomato following inoculation of *K. radicincitans* DSM 16656^{T} .

Due to the recent description of the genus *Kosakonia* (Brady et al. 2013), there are only a few reports on the presence of *Kosakonia* sp. in rice microbiome. *Kosakonia* sp. is a dominant colonizer in seeds of three salt-tolerant rice varieties (Walitang et al. 2017, 2018b). In addition, when inbreeding seed varieties containing *Kosakonia,* it was then present with a similar abundance or even at higher levels within the offspring, suggesting that is part of the core microbiota of some rice varieties (Walitang et al. 2018a). *Kosakonia* sp. has been isolated from different rice varieties, largely representing approximately one third of the total isolates (Hardoim 2015). This indicates that members of *Kosakonia* are common endophytes of rice.

This chapter has characterized two *Kosakonia* strains giving some highlights of their interaction with the plant host and its colonization. Further studies on the genus *Kosakonia* are important for understanding the mechanisms which allow members of this genus to be successful endophytic colonizers and be part of the microbiome of economically important crops.

Chapter III.

Enriching for rice bacterial endophytes and *Kosakonia* co-inhabitants

3.1 Introduction

Endophytes are microorganisms that live in the plant endosphere which is an ecologically protected and stable environment (Senthilkumar et al. 2011). Endophytes develop a intimate relationship with their host and do not elicit an immune responses and in some cases display plant growth promoting (PGP) activities (Glick 2014; Hayat et al. 2010; Sessitsch et al. 2012; Garrido-Oter et al. 2018). Moreover, endophytes are commonly generalists, being able to colonize the internal tissues of different hosts, including distantly related plant species (Ma et al. 2011; Compant et al. 2005). Using microbial endophytes as biofertilizers for a sustainable agricultural system is therefore of growing interest.

The bacterial endophytic community is a variable population shaped by interkingdom and microbe-microbe interactions, by the soil bacterial population, by environmental parameters and by the host genotype (Dimkpa et al. 2009; Bulgarelli et al. 2012; Kandel et al. 2017; Ryan et al. 2008). The plant beneficial properties of the endosphere microbiome can be mainly associated to a few microbial species and their additive or synergistic effects (Timm et al. 2016; Herrera Paredes et al. 2018). Some endophytic strains belonging to different genera including *Azoarcus*, *Burkholderia*, *Azospirillum*, *Pseudomonas* and *Kosakonia* have PGP properties in important crops such as rice, maize, wheat and barley (Krause et al. 2006; Isawa et al. 2010; Fukami et al. 2016; Divan Baldani et al. 2000; Pham et al. 2017; Berger et al. 2018). Deciphering the composition of the microbial communities, the identification of specific endophytes and the design of endophytic synthetic bacterial communities can potentially devise ways to improve plant health and yield.

Endophytic microbiota properties are not limited to PGP features but can also play a role in abiotic stress tolerance including high saline conditions (Yaish et al. 2015; Ali et al. 2014). Soil salinity is an increasing worldwide problem in agriculture that affects plant health and decreases yields of crops like wheat, maize, rice and barley (Coleman-Derr and Tringe 2014; Acquaah 2007). Root microbiome studies under different salinity concentrations have shown that it results in a shift of the abundance of microbial members of the community (Szymańska et al. 2018). Bacterial endophytes have been shown to improve plant fitness under saline stress (Yuan et al. 2016); consequently, the use of bacterial endophytes under salinity conditions is a possible way for enhancing abiotic stress resistance and improve plant yield.

In this chapter an enrichment strategy for the identification of root endophytes by microbiome studies under non-stressful and saline conditions is presented. Additionally, the same enrichment approach was used in order to identify the most compatible bacteria which can form communities with the *Kosakonia* sp. strain KO348, which was previously isolated as en efficient endosphere colonizer with PGP properties (Chapter 2, Bertani et al. 2016).

3.2 Materials and Methods

3.2.1 Bacterial Strain KO348

The *Kosakonia* strain used in this study KO348 was previously isolated from the root endosphere from a rice cultivar grown in Italy (Bertani et a 2016). Strain KO348 was routinely grown in Luria-Bertani (LB) broth at 30 °C.

3.2.2 Endophytic enrichment strategy

Two sets of experiments were performed; set I was aimed to enrich for rice root endophytes under non-stressful and increasing saline conditions (**Figure 3.1**) and set II was aimed to enrich for the best endophytic microbial partners for *Kosakonia* KO348 strain under non-stressful and saline conditions (**Figure 3.2**).

Preparation of the initial inoculum of microbial endophytes: For experiments of set I, rich wild organic soil (not previously used for agricultural purposes) was collected from the Padriciano area, Trieste – Italy (45,660091°N 13,825205°W). This soil was placed on pots (n=6) (**Figure 3.1a**) and used to grow *Oryza sativa* c. Baldo plants for 30 days in order to allow the plants to recruit endophytes from the soil.

Four rice seedlings (surface bleach-sterilized and one-week old germinated) were sowed in each pot (**Figure 3.1b**). The plants were watered every other day with sterile water for four weeks (**Figure 3.1c**). The roots were recovered, washed, weighed and surface-sterilized following Bertani et al. 2016 methodology. The sterilized roots were macerated using 400 mL of PBS and filtered for solid particles resulting in a suspension

containing endophytes which was considered as the initial inoculum of endophytes (**Figure 3.1d**).

Inoculation passages of endophytes under non-stressful and saline conditions: The 50mL suspension constituting the initial endophytic inoculum, was used to inoculate a new group of one-week old rice seedlings (n=48) (surface-bleach sterilized and one-week old germinated) by submerging the seedling roots in the suspension for one hour; this new group of inoculated seedlings constitute the first inoculation passage of endophytes (**Figure 3.1e**). The plants inoculated in the first passage (**Figure 3.1f**) were individually transferred to a tube containing 35mL of Hoagland´s semi-solid solution (Steindler et al. 2009), one half of the seedlings (n=24) were grown under non-stressful conditions; the other half (n=24) were grown under salinity-stress group for which NaCl was added to the preparation of the Hoagland´s solution. The plants were grown for 14 days (watered every two days) after which the roots were recovered, sterilized and macerated in 180mL of PBS and filtered as described above. From this resulting suspension containing endophytes, 50mL were used as inoculum of a new group of seedlings (which was considered the second passage (**Figure 3.1g**), the same strategy was used for obtaining the third (**Figure 3.1h**) and the fourth inoculation passages (**Figure 3.1i**). The NaCl concentrations added in the Hoagland´s solution of the saline group were increased within each passage; 30mM, 45mM, 60mM and 75mM were used from the first to the fourth passages respectively.

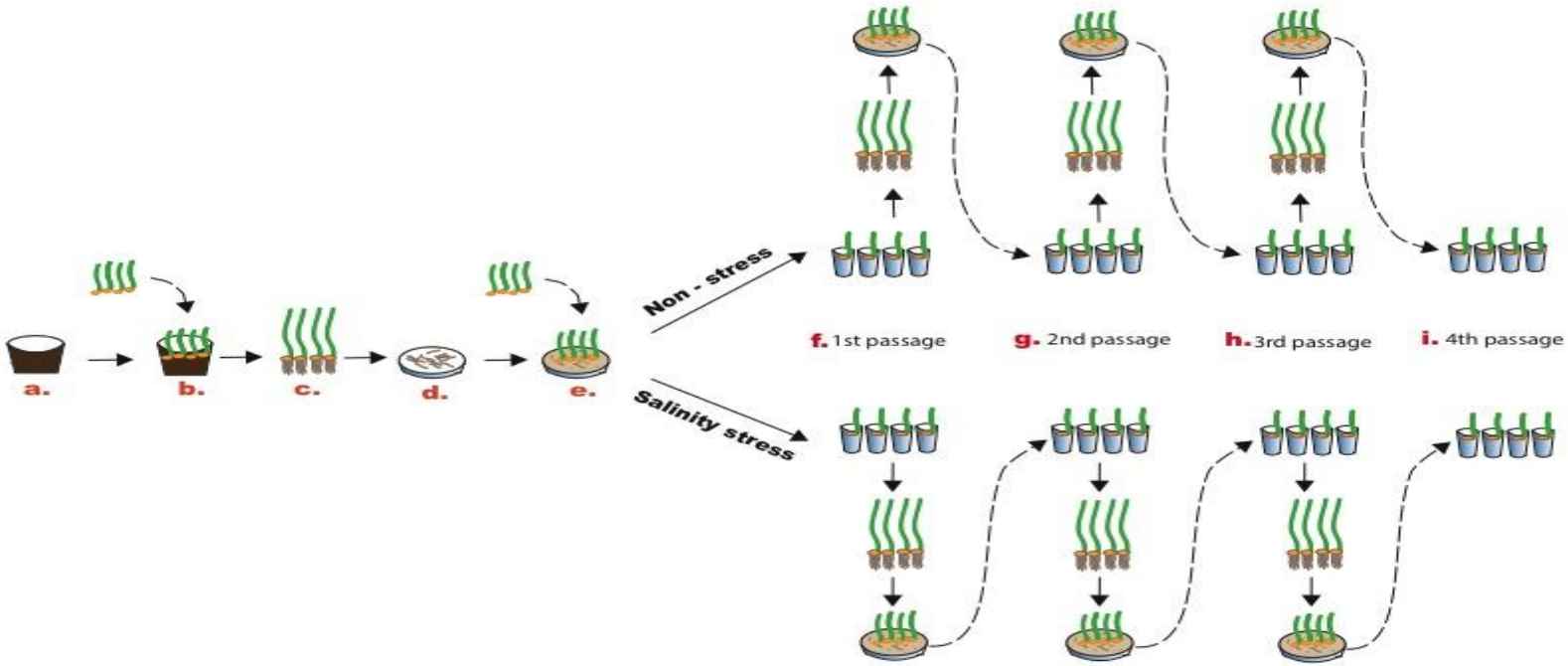


Figure 3.1. Set I: Experimental strategy for enrichment of endophytes under non-stressful and saline conditions through inoculation passages.

(**a**) Rich soil was put in pots for (**b**) rice seedlings to grow for 4 weeks after which (**c**) the roots were recovered, sterilized and macerated resulting in a (**d**) suspension containing endophytes used for the (**e**) inoculation of a new group of seedlings. The inoculated seedlings constituted (**f**) the first inoculation passage of endophytes, these seedlings were grown for 2 weeks individually in tubes containing Hoagland´s solution (containing NaCl in the salinity-stress group) for the later recovery of their roots, which were sterilized and macerated resulting in a new suspension of endophytes used for inoculating a new group of seedlings forming (**g**) the second passage of endophytes. The same methodology was used for (**h**) the third and (**i**) fourth inoculation passages of endophytes.

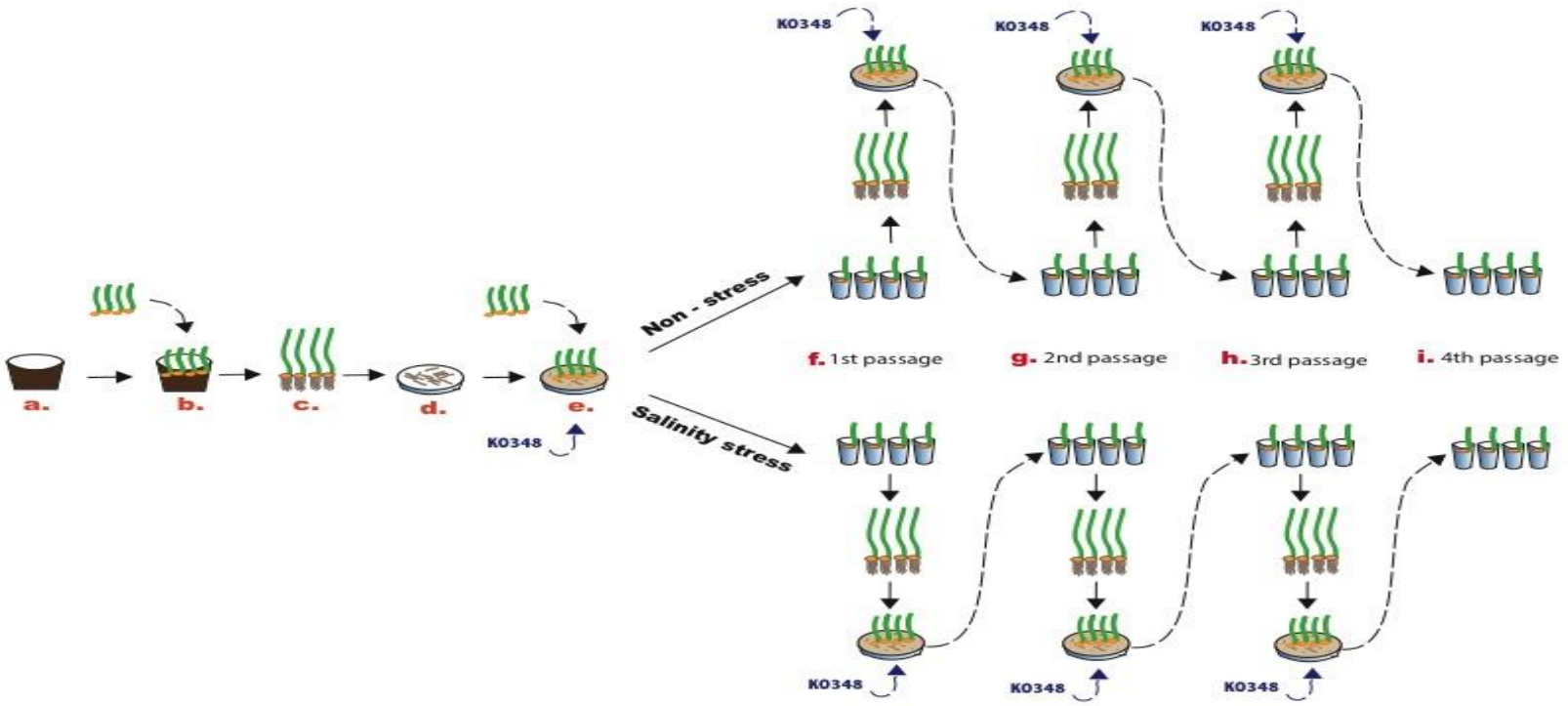


Figure 3.2. Set II: Experimental strategy for enrichment of endophytic *Kosakonia* partners under non-stressful and saline conditions through inoculation passages

(**a**) Rich soil was put in pots for (**b**) rice seedlings to grow for 4 weeks after which (**c**) the roots were recovered, sterilized and macerated resulting in a (**d**) suspension containing endophytes plus *Kosakonia* strain KO348 used for the (**e**) inoculation of a new group of seedlings. The inoculated seedlings constituted (**f**) the first inoculation passage of endophytes, these seedlings were grown for 2 weeks individually in tubes containing Hoagland´s solution (containing NaCl in the salinity-stress group) for the later recovery of their roots, which were sterilized and macerated resulting in a new suspension of endophytes (to which KO348 was added) used for inoculating a new group of seedlings forming (**g**) the second passage of endophytes. The same methodology was used for (**h**) the third and (**i**) fourth inoculation passages of endophytes.

Enrichment for Kosakonia bacterial compatible partners: The set II experiments was set up in the same way as set I experiments (**Figure 3.2**) with the only difference from the experimental design depicted in Figure 3.1 being the addition of the strain KO348 in the root filtered suspension containing endophytes. *Kosakonia* was added in a proportion of 1:20 of the total culturable bacterial community present in the suspension (the bacterial CFU/gr of root was calculated by serial dilutions of the suspension and plated in Tripticase Soy Agar medium). *Kosakonia* KO348 was added to each inoculation passage (**Figure 2 f-i**), for both groups of plants grown in non-stressful and saline conditions.

Endophyte sample preparation for microbiome determination: The remaining filtered suspension containing endophytes (approximately 90-100mL), from the initial inoculum and from each inoculation passage (from both groups of treatment, non-stressful and saline conditions) and from both sets of experiments I and II (without and added *Kosakonia* KO348 respectively*)* were individually concentrated (10min at 48000rpm) in aliquots and used for micriobiome determination. For standarizing the quantity of starting material before the DNA extraction, the aliquots of concentrated solution corresponded to 1 gram of root; each of these aliquots was considered a sample. All samples were stored with 18% glycerol at -80ºC for subsequent the DNA extraction and 16S rDNA amplicon library preparation.

3.2.3 DNA extraction, 16S rDNA gene library preparation and NGS sequencing

DNA purification from samples: Three samples corresponding to technical replicates of each root suspension containing endophytes were used. DNA was extracted using the PowerMax Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. DNA concentration was analyzed by using a Nanodrop^{TM} spectrophotometer (Thermofisher Scientific Inc., Waltham, MA, USA) and diluted to a concentration of 5-7 ug/uL for the preparation of the 16S rRNA amplicon DNA library.

16S rDNA amplification library preparation: The 16S rDNA amplicon library was prepared following the manufacturer's protocol (15044223 B) (Illumina Inc., San Diego, CA, USA). Briefly, samples V3 and V4 regions were amplified using denaturated primers (Klindworth et al. 2013) in a limited 25 cycles PCR, followed by an AMPure XP beads clean-up (A63880l) (Beckman Coulter Inc., Brea, CA, USA). A second PCR reaction will then performed to attach dual index and Illumina sequencing adapters using the Nextera XT Index Kit; followed by a second AMPure XP bead clean-up. 16S rRNA gene concentration was measured by fluorimetric quantification using Qubit 2 (Invitrogen Inc., Carlsbad, CA, USA).

NGS sequencing of amplicon libraries: NGS Sequencing was performed using the Illumina Miseq technology. The resulting sequences of raw data were filtered out and the reads were trimmed to a consistent length. Then the data was denoised, chimera filtered, and taxonomically assigned using DADA2 v1.1.5 (Callahan et al. 2016). For the taxonomic analysis, the sequencing reads were clustered into

operational taxonomic units (OTUs) defined as groups of sequencing reads that differ by less than a fixed dissimilarity threshold (97%) generated in DADA2 using the Ribosomal Project Database (RDP) v.11 (Cole et al. 2014).

Statistical analysis: For the comparison between the passages among the groups, it was performed a differential analysis using a quasi-likelihood (QL) F-test, with a p-value threshold of 0.05 this test was implemented in edgeR, R package (McCarthy et al. 2012).

3.3 Results

3.3.1 Enrichment for rice root bacterial endophytes (Set I Experiments)

The set I of experiments was set up with the aim to enrich for rice root endophytes by using a strategy of inoculation passages under non-stressful and increasing saline conditions (**Figure 3.1**). The purpose of these several purification and re-inoculation steps was to identify the most efficient bacterial endophytes which could repeatedly enter rice roots and colonize the endosphere under non-stressful and saline conditions. The root bacterial microbiome after each inoculation passage was determined in triplicate by amplification of a variable region of the 16S rDNA gene.

The set I of experiments had, after quality control and filtering, a total of 202,932 high-quality sequencing reads with a median read count per sample of 5,637. The high-quality reads were clustered using >97% sequence identity into 3,114 bacterial OTUs (Operational Taxonomic Units). Low-abundance OTUs were discarded as well as chloroplast and mitochondrial-related sequences, resulting in 1,431 OTUs. The OTUs were clustered at Genus taxonomic level obtaining a final number of 165 different bacterial taxa.

The cumulative relative abundance of the most abundant genera in the microbial population (>1%) from the initial inoculum and all the inoculation passages was obtained in order to analyze their microbiome composition. The microbiome analysis of the initial inoculum, which contained the root endophytes after growing

surface sterilized seeds for 30 days in rich soil, contained at phylum level, Proteobacteria, Acidobacteria and Actinobacteria at 66,3%, 23,8% and 9,83% levels respectively. The main genera of endophytes present in this initial inoculum with a higher cumulative relative abundance of 1% were *Sphingomonas* (63,97%), *Methylobacterium* (11,4%), *Arthrobacter* (6,7%), *Aurantimonas* (4,27%), *Nocardiodes* (2,98%) *and Pedomicrobium* (2,87 %) (**Figure 3.3**).

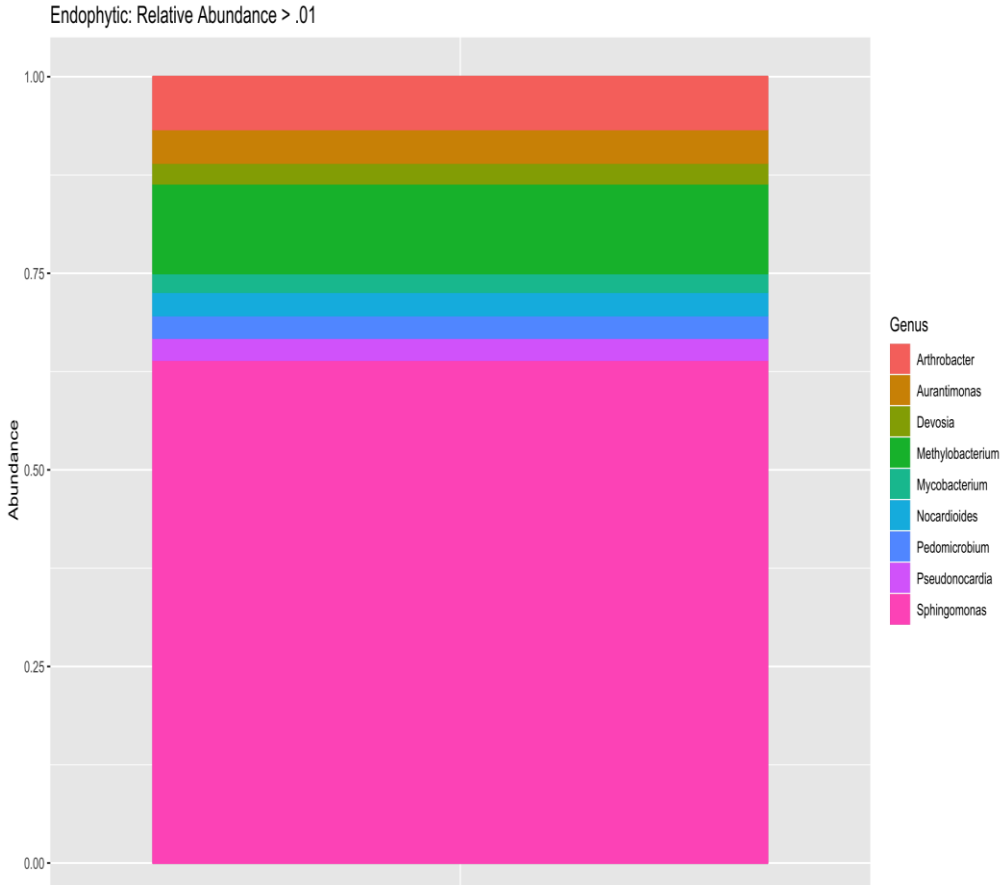


Figure 3.3. Cumulative relative abundance found in the initial inoculum of the set I of experiments (without added *Kosakonia*).
The bar chart shows the genera with a cumulative relative abundance >1% of three technical replicates.

The microbiome composition of the set I of experiments of the four inoculation passages of endophytes under non-stressful conditions showed that Proteobacteria and Firmicutes were the two phyla mainly present along all inoculation passages, reaching approximately the same cumulative relative abundance at the end of the

fourth inoculation passage (**Table 3.1**). Two genera were the most abundant along all the inoculation passages; these were *Azospirillum* and *Clostridium* (**Table 3.1**, **Figure 3.4)**. In the fourth inoculation passage some genera started to increase in abundance as for example is the case for *Roseomonas* (4,58%), *Propionibacterium* (2,67%) and *Pleomorphomnas* (2,29%) (**Figure 3.4**). *Caulobacter* and *Sphingomonas* on the other hand, decreased in abundance in each inoculation passage (**Table 3.1**) (**Figure 3.4**).

Table 3.1. Cumulative relative abundance of the main phyla and genera (>1%) found in the set I of experiments (without added *Kosakonia*) under non-stressful conditions along the four inoculation passages.

Passage	**1st (%)**	**2nd (%)**	**3rd (%)**	**4th (%)**
Phyla				
Proteobacteria	60,80	38,26	45,78	46,26
Firmicutes	38,20	53,05	52,03	46,23
Actinobacteria	0,80	3,31	0,00	5,85
Genera				
Clostridium_sensu_stricto	33,77	42,15	35,69	46,22
Azospirillum	26,48	16,73	27,58	25,11
Caulobacter	17,86	8,68	3,98	1,83
Sphingomonas	7,47	1,37	2,02	2,39
Clostridium_XlVa	6,46	12,70	17,85	3,70
Rhizobium	4,44	6,31	2,77	3,05

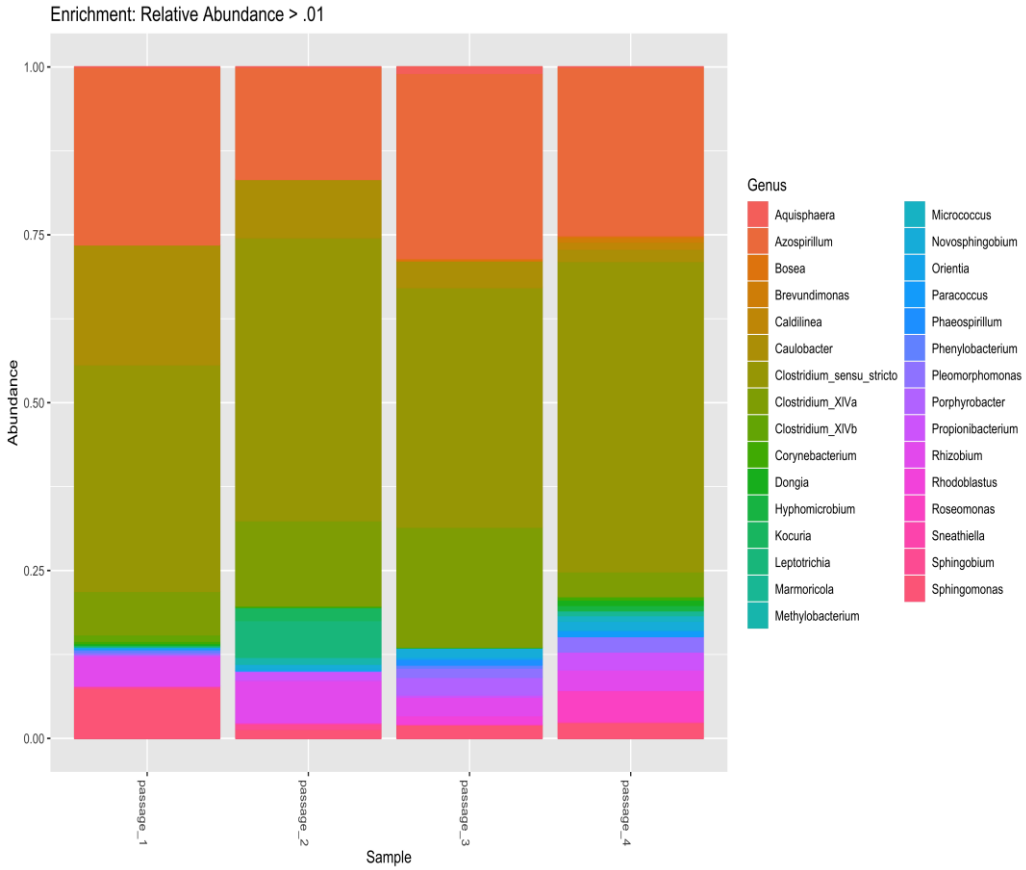


Figure 3.4 Most abundant genera found in the set I of experiments (without added *Kosakonia*) under non-stresful conditions along the four inoculation passages.
The bar chart present the genera with a cumulative relative abundance >1%.

In order to determine the genera that were either significantly enriched or decreased along all the inoculation passages under non-stressful conditions, a differential analysis was performed using as a threshold a p value of 0.05 (**Figure 3.5**). All the genera found in each sample were included, i.e. not only the genera with high abundance. *Rhizobium* was the genus most enriched, followed by *Novosphingobium* and *Brevundimonas*, these three genera belong to the phyla *Proteobacteria*. In addition, *Micrococcus* was also enriched which belongs to the phyla *Actinobacteria* (**Figure 3.5**). Of these, only *Rhizobium* had a higher cumulative relative abundance than1%.

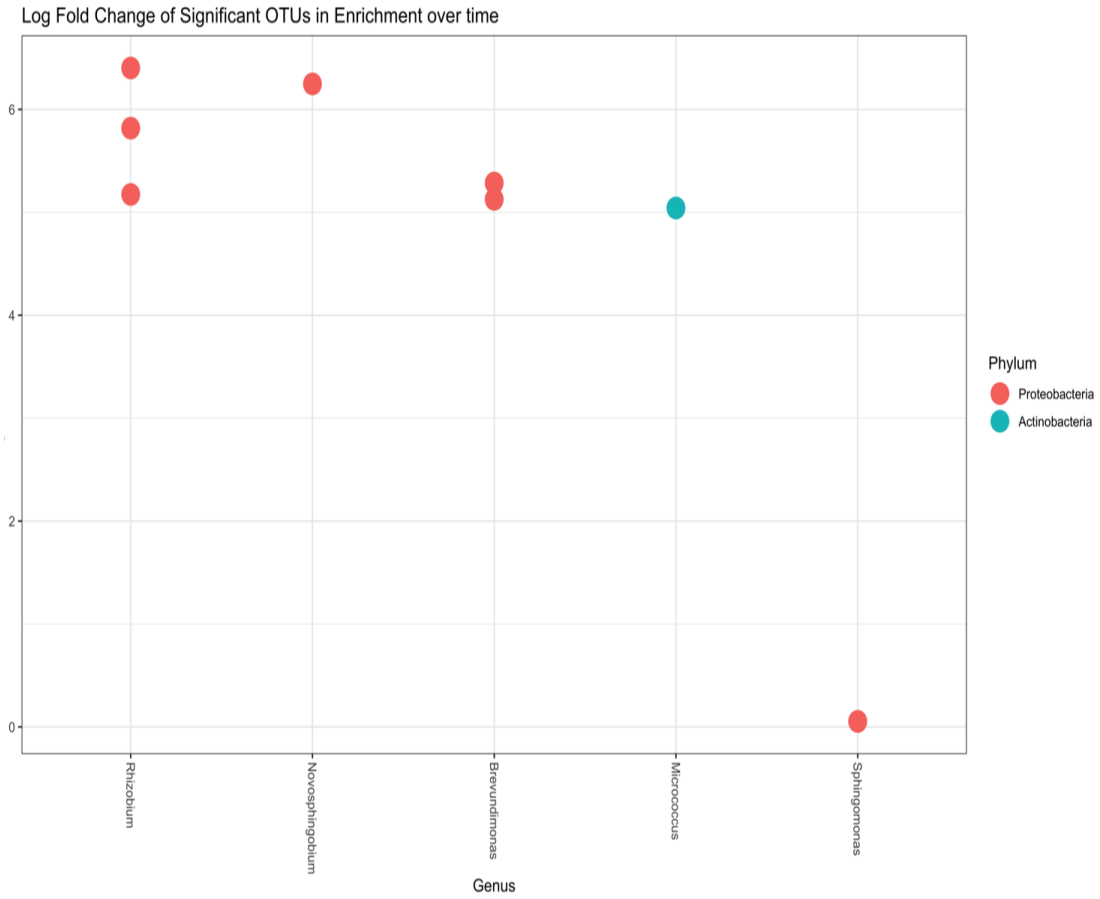


Figure 3.5 Differential analysis among the four inoculation passages of endophytes under non-stressful conditions in the set I of experiments (without added *Kosakonia*).
The differential analysis was performed using a quasi-likelihood (QL) F-test, with a p-value threshold of 0.05

The microbiome composition of the set I experiments under increasing saline conditions showed that the phylum Firmicutes increased in abundance through all passages, especially in the fourth inoculation passage reaching 96,8% and out-competing Proteobacteria (**Table 3.2**). As was the case above (non-stressful conditions), *Clostridium* and *Azospirillum* were the most abundant genera through all inoculation passages; however at the fourth inoculation passage *Azospirillum* disappeared probably being out-competed by *Clostridium*. *Rhizobium* increased until the third passage, in the fourth passage it was probably also out-competed by

Clostridium. Caulobacter and *Sphingomonas* decreased through the inoculation passages as they did under non-stressful conditions (**Table 3.2**; **Figure 3.6**).

Table 3.2. Cumulative relative abundance of the main phyla and genera (> 1%) found in the set I of experiments (without added *Kosakonia*) under saline conditions along the four inoculation passages.

	1st(%)	**2nd(%)**	**3rd(%)**	**4th(%)**
Phylum				
Proteobacteria	50,75	43,84	46,25	1,24
Firmicutes	33,81	44,79	44,72	96,80
Actinobacteria	12,87	7,79	6,05	1,87
Genera				
Clostridium_sensu_stricto	24,73	31,37	36,42	95,83
Azospirillum	24,43	13,77	25,67	0,11
Caulobacter	10,44	4,48	5,42	0,16
Sphingomonas	7,24	3,49	2,18	0,41
Clostridium_XlVa	8,59	14,71	12,03	0,69
Rhizobium	3,84	5,87	10,91	0,14

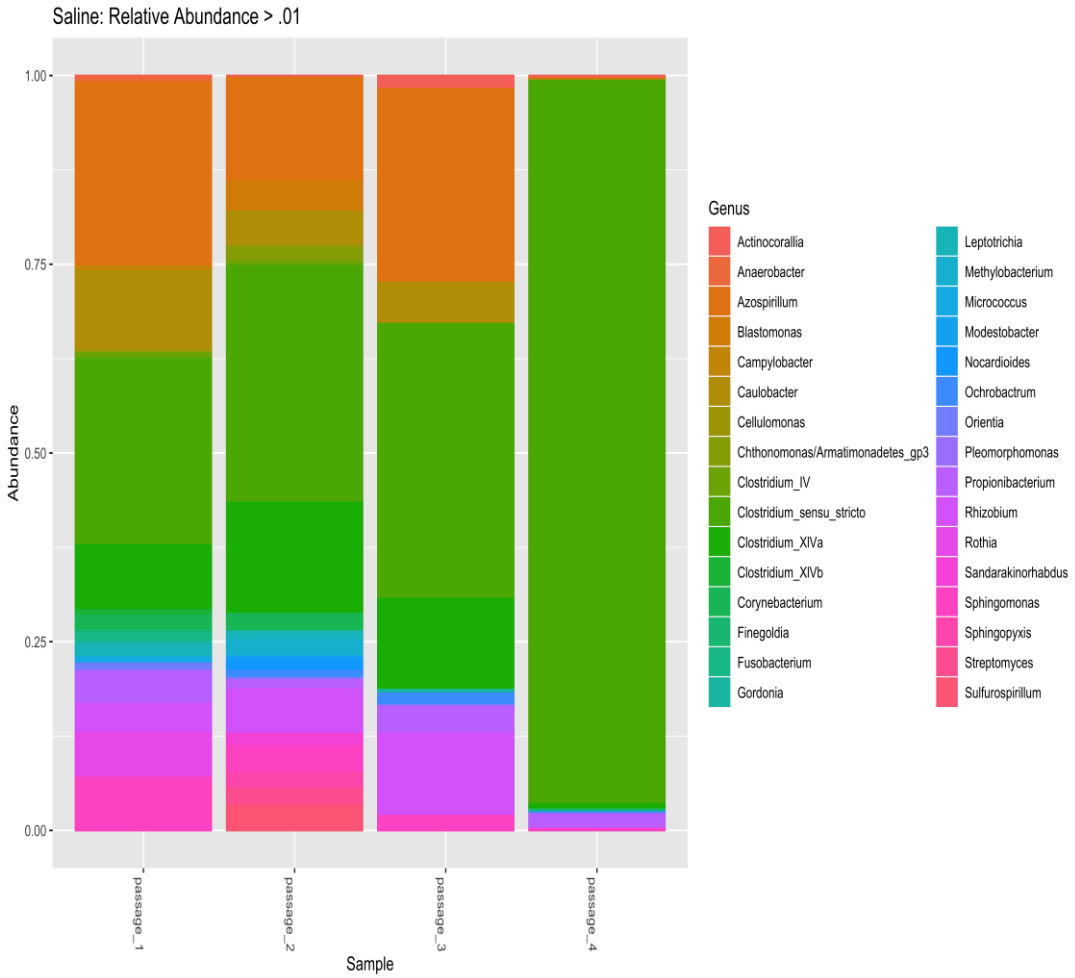


Figure 3.6 Most abundant genera found in the set I of experiments (without added *Kosakonia*) under saline conditions along the four inoculation passages.
The bar chart present the genera with a cumulative relative abundance > 1%.

The differential analysis was performed in order to determine the enriched genera among all the inoculation passages of endophytes under increasing saline conditions. It was established that only genera *Nocardiodes*, belonging to the phyla Actinobacteria, with a cumulative relative abundance higher than 1% was significantly enriched (**Figure 3.7**). Interestingly, *Rhizobium* that was increased more than 4 fold in the inoculation passages in rice under non-stressful conditions, was not significantly increased in the inoculation passages under saline stress (**Figure 3.4**; **Figure 3.7**).

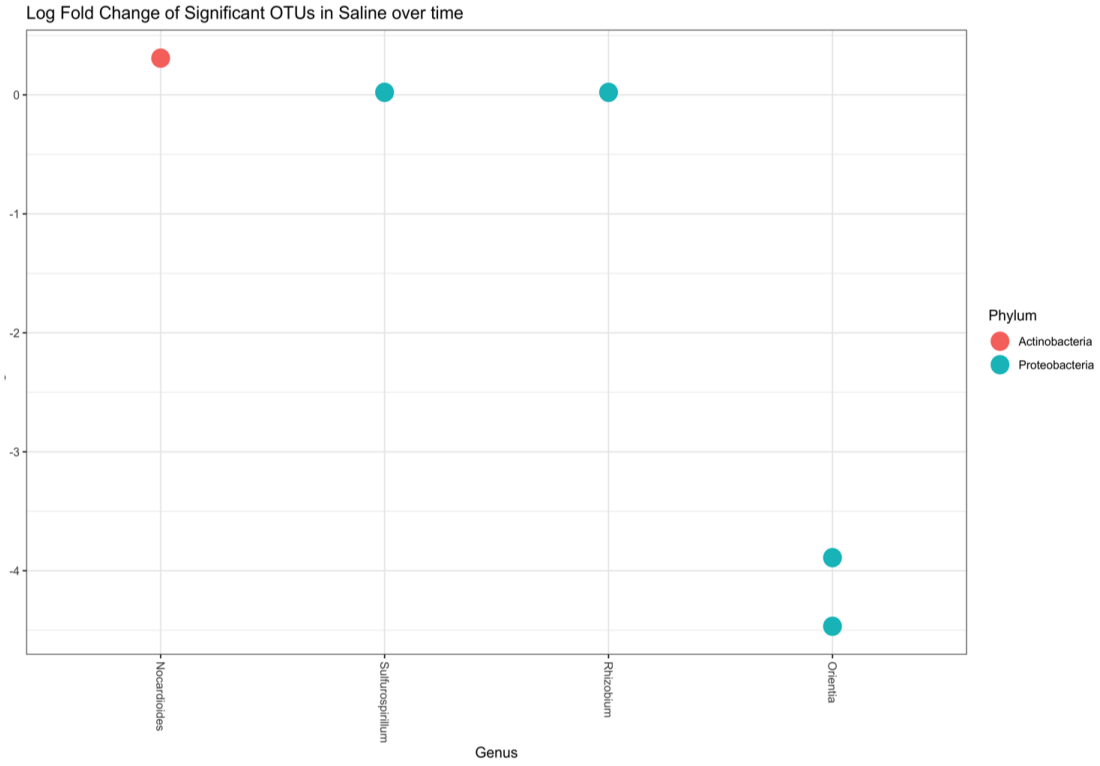


Figure 3.7 Differential analysis among the four inoculation passages of endophytes under increasing saline conditions in the set I of experiments (without added *Kosakonia*).
The differential analysis was performed using a quasi-likelihood (QL) F-test, with a p-value threshold of 0.05.

The biodiversity was analyzed based on the richness of the species within the microbial community by using two indexes, Shannon and the Inverse Simpson **(Table 3.3).** Biodiversity indexes from these set I of experiments **(Figure 3.1),** indicated a high biodiversity from the initial inoculum to the different inoculation passages in both, non-stressful and saline conditions. The higher numbers in the Inverse Simpson index indicated that many members of the microbiome had a very low abundance. Interestingly, both used biodiversity indexes indicated that under non-stressful conditions there was a slight decrease in the values of biodiversity from the first to the fourth inoculation passages that was not observed under saline conditions.

Table 3.3. Biodiversity indexes in the set I of experiments (without added *Kosakonia*)

	Shannon	***Inverse Simpson***
Inoculum	5,82 ± 0,19	245,75 ± 47,64
Standard conditions (passages)		
First	6,26 ± 0,16	299 ± 59,28
Second	5,7 ± 0,14	187,66 ± 26,07
Third	5,73 ± 0,16	171,27 ± 27,98
Fourth	5,64 ± 0,02	142,24 ± 9,99
Saline conditions (passages)		
First	5,65 ± 0,1	166,2 ± 17,04
Second	5,36 ± 0,14	131,25 ± 29,6
Third	5,84 ± 0,13	199,92 ± 29,88
Fourth	5,26 ± 0,19	130,35 ± 27,27

3.3.2 Enrichment for *Kosakonia* bacterial cooperators rice root bacterial endophytes (Set II Experiments)

The set II of experiments were set up with the aim to enrich for rice root endophytes which were compatible and/or cooperate with *Kosakonia* KO348 by using a strategy of passages of endophytes under non-stressful and increasing saline conditions in the continuous presence of large amounts of *Kosakonia* KO348 (**Figure 3.2**). Again, several passages of purification and re-inoculation of endophytes were conducted but this time, *Kosakonia* strain KO348 was added each time. This was performed in order to identify the most efficient bacterial endophytes which in the presence of large amounts of *Kosakonia*, could repeatedly enter into rice roots and colonize the endosphere. The root bacterial microbiome after each inoculation passage was then determined in triplicate.

Kosakonia KO348 was added to each of the four inoculation passages of endophytes in a proportion of 1:20 of the total culturable bacteria for which the

CFU/(g of root) was calculated (**Table 3.4**). The CFU/(g of root) showed a high number of culturable endophytes in all the inoculation passages. The initial inoculum had the lowest number of culturable endophytes and was most likely due to the lack of *Kosakonia* KO348 inoculation in this step (**Table 3.4**).

Table 3.4 Culturable endophytic colonization in the set II of experiments (with added *Kosakonia*) in the inoculation passages (CFU/g of root)

	1st Standard	**2nd Standard**	**3rd Standard**	**4th Standard**
Initial inoculum	1,34E+07	2,45E+07	5,90E+07	3,32E+07
1,44E+04	**1st Saline (30mM)**	**2nd Saline (45mM)**	**3rd Saline (60mM)**	**4th Saline (75mM)**
	1,34E+07	1,96E+08	3,47E+07	2,61E+06

In these set II of experiments (i.e. with added *Kosakonia*) there were a total of 1,946,317 high-quality sequencing reads with a median read count per sample of 58,979. The high-quality reads were clustered using >97% sequence identity into 8,214 bacterial OTUs (Operational Taxonomic Units). Low-abundance OTUs were discarded as well as chloroplast and mitochondrial-related sequences, resulting in 2,635 OTUs. The OTUs were clustered at Genus taxonomic level obtaining a final number of 391 different bacterial taxa.

The analysis of the microbiome composition of the suspension of the initial inoculum obtained after growing rice for 30 days in rich soil and before the adding of the *Kosakonia* strain KO348 (**Figure 3.2e**), revealed that the phyla Proteobacteria, Firmicutes and Bacteroidetes were found at 81,05%, 17,83% and 1,11% respectively. The main genera of endophytes present in this initial inoculum were *Pantoea* (39,81%), *Stenotrophomonas* (10,93%), *Rhizobium* (10,85%),

Clostridium senso stricto (2,92%), *Clostridium III* (9,92%) and *Novosphingobium* (7,71%) (**Figure 3.8**).

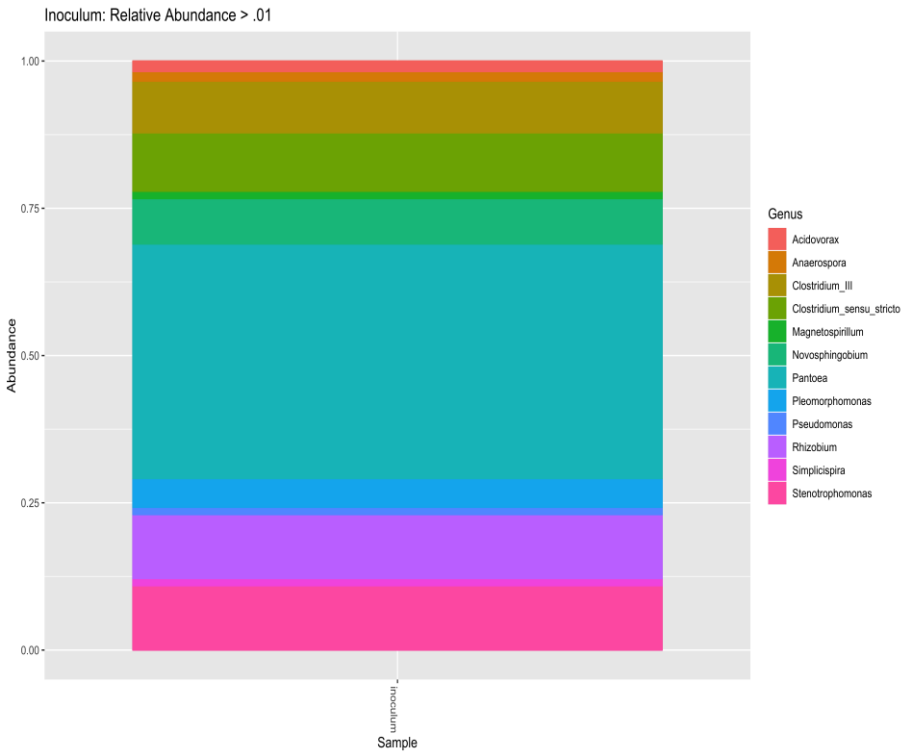


Figure 3.8 Cumulative relative abundance found in the initial inoculum of the set II of experiments (with the added *Kosakonia* strain KO348).
The bar chart present the genera with a cumulative relative abundance >1%.

The microbiome composition of the inoculation passages under non-stressful conditions of the set II of experiments showed that in all the four inoculation passages Proteobacteria was the phyla with the highest abundance (**Table 3.5**). At genus level, *Kosakonia*, which belongs to the Proteobacteria phyla, was as expected the most abundant along all the inoculation passages (**Table 3.5**; **Figure 3.9**). *Stenotrophomonas* increased while *Herbaspirillum* and *Acidovorax* decreased through all the inoculation passages. (**Table 3.5**).

Table 3.5. Cumulative relative abundance of the main phyla and genera (>1%) found in the set II of experiments (with added *Kosakonia*) under non-stressful conditions along the four inoculation passages.

	1st(%)	2nd(%)	3rd(%)	4th(%)
Phyla				
Proteobacteria	97,04	98,11	98,25	97,24
Firmicutes	2,96	1,83	1,71	2,06
Verrucomicrobia	0	0,05	0	0,34
Genera				
Kosakonia	85,09	84,62	87,35	75,91
Herbaspirillum	7,59	1,13	3,3	0,74
Clostridium_sensu_stricto	2,84	0,84	1,46	1,19
Acidovorax	2,1	1,37	1,64	0,52
Azospirillum	0	6	0,65	8,57
Pantoea	0,13	0,19	0,05	4,22
Stenotrophomonas	0	1,63	4,3	3,97

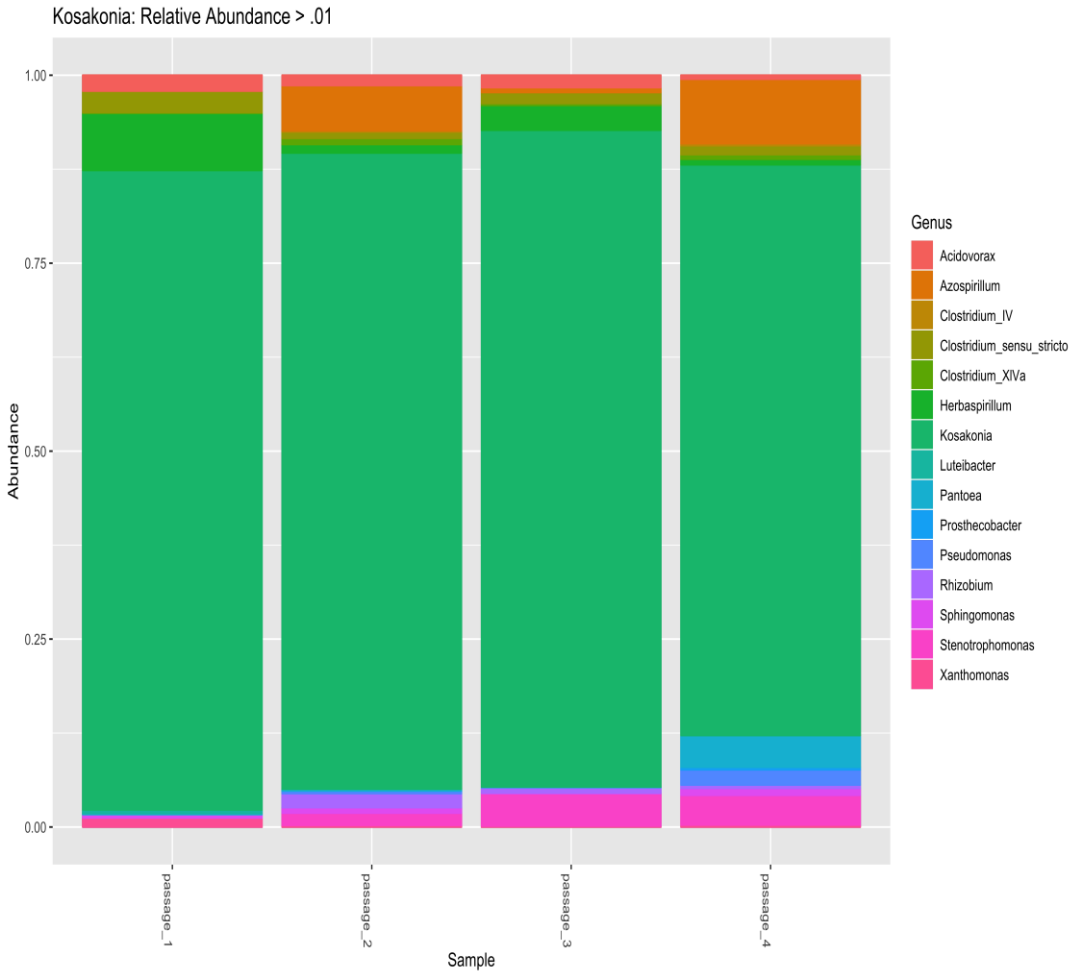


Figure 3.9 Most abundant genera found in the set II of experiments (with added *Kosakonia*) under non-stressful conditions along the four passages.
The bar chart present the genera with a cumulative relative abundance >1%.

The differential abundance analysis of the set II of experiments of rice grown under non-stressful conditions showed that along the four inoculation passages, four genera were significantly enriched; three of them being *Pseudomonas*, *Clostridium* and *Sphingomonas*, interestingly having a cumulative relative abundance of >1%; (**Figure 3.10**).

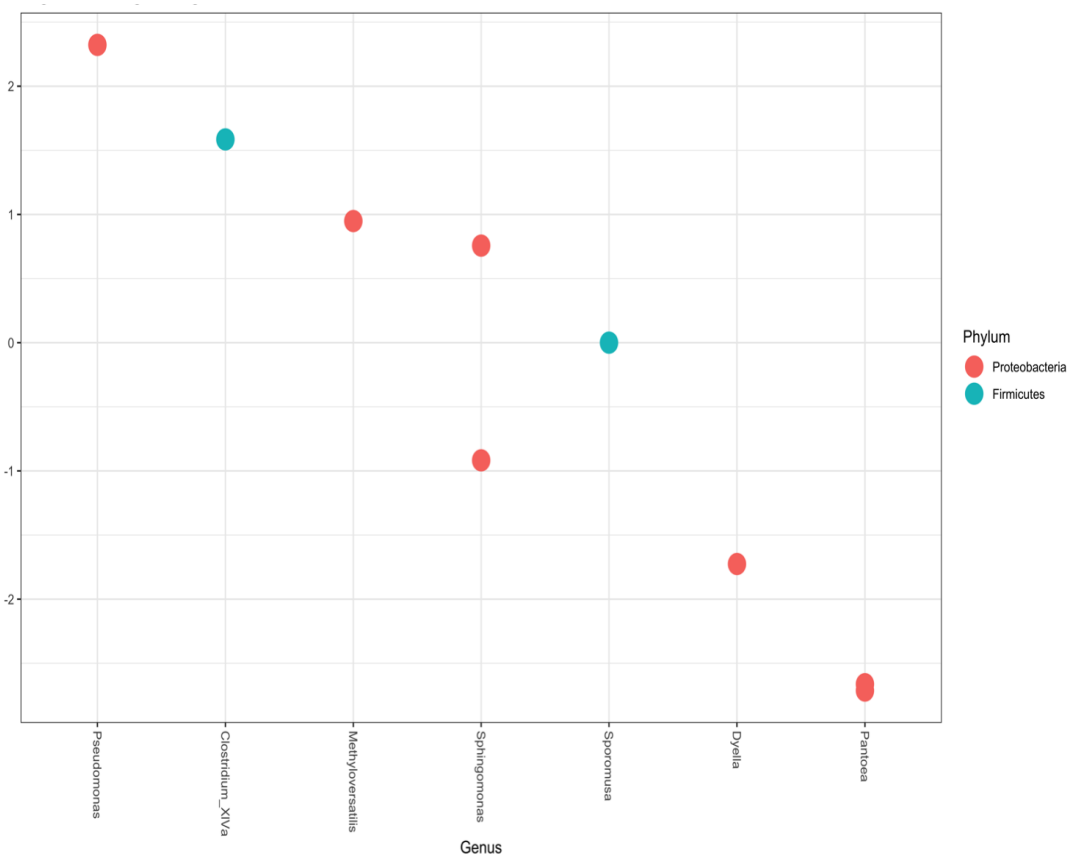


Figure 3.10 Differential analysis among the four passages of endophytes with added *Kosakonia* under non-stressful conditions in the set II of experiments.
The differential analysis was performed using a quasi-likelihood (QL) F-test, with a p-value threshold of 0.05

The microbiome analysis of the group of rice grown under saline conditions belonging to these set II of experiments showed that the phylum Proteobacteria, had values over 90% in all the passages, this was likely to be very much affected by the addition of *Kosakonia* (**Table 3.6**). Interestingly, Bacteroidetes showed an increase

more than twofold when comparing the first and the fourth inoculation passages (**Table 3.6**). At genus level, *Herbaspirillum* decreased considerably through the passages whereas *Pantoea* decreased and almost disappeared in the second and third passage, but at the fourth passage it increased again (**Figure 3.11**). *Luteibacter* and *Acidovorax* also drastically decreased from the first to the second passage and then remained at very low levels (**Table 3.6; Figure 3.11**). Genera *Stenotrophomonas* and *Achromobacter* increased from the second passage and kept increasing until the fourth passage, whereas *Azospirillum* and *Pseudomonas* increased at the third and fourth passages (**Table 3.6; Figure 3.11**).

Kosakonia showed less cumulative relative abundance under saline conditions than under non-stressful conditions and interestingly, under both non-stressful and saline conditions, at the fourth inoculation passage, *Kosakonia* decreased in abundance (**Table 3.2**; **Table 3.6**).

Table 3.6. Cumulative relative abundance of the main phyla and genera (>1%) found in the set II of experiments (with added *Kosakonia*) under increasing saline conditions along the four passages.

	1st(%)	**2nd(%)**	**3rd(%)**	**4th(%)**
Phylum				
Proteobacteria	93,54	96,96	92,64	91,34
Firmicutes	4,11	2,82	3,45	3,30
Bacteroidetes	2,35	0,22	3,91	5,36
Genera				
Kosakonia	45,74	82,75	76,72	64,65
Herbaspirillum	23,11	2,07	1,86	0,60
Pantoea	12,70	0,10	0,28	1,53
Luteibacter	4,22	0,09	0,00	0,09
Acidovorax	3,67	1,40	0,22	0,78
Clostridium_sensu_stricto	2,42	2,10	3,22	2,43
Rhizobium	1,96	0,09	0,96	1,41
Stenotrophomonas	0,12	5,81	3,72	4,51
Achromobacter	0,10	4,28	5,26	7,28
Azospirillum	0,01	0,00	1,66	5,40
Pseudomonas	0,00	0,00	5,00	6,13

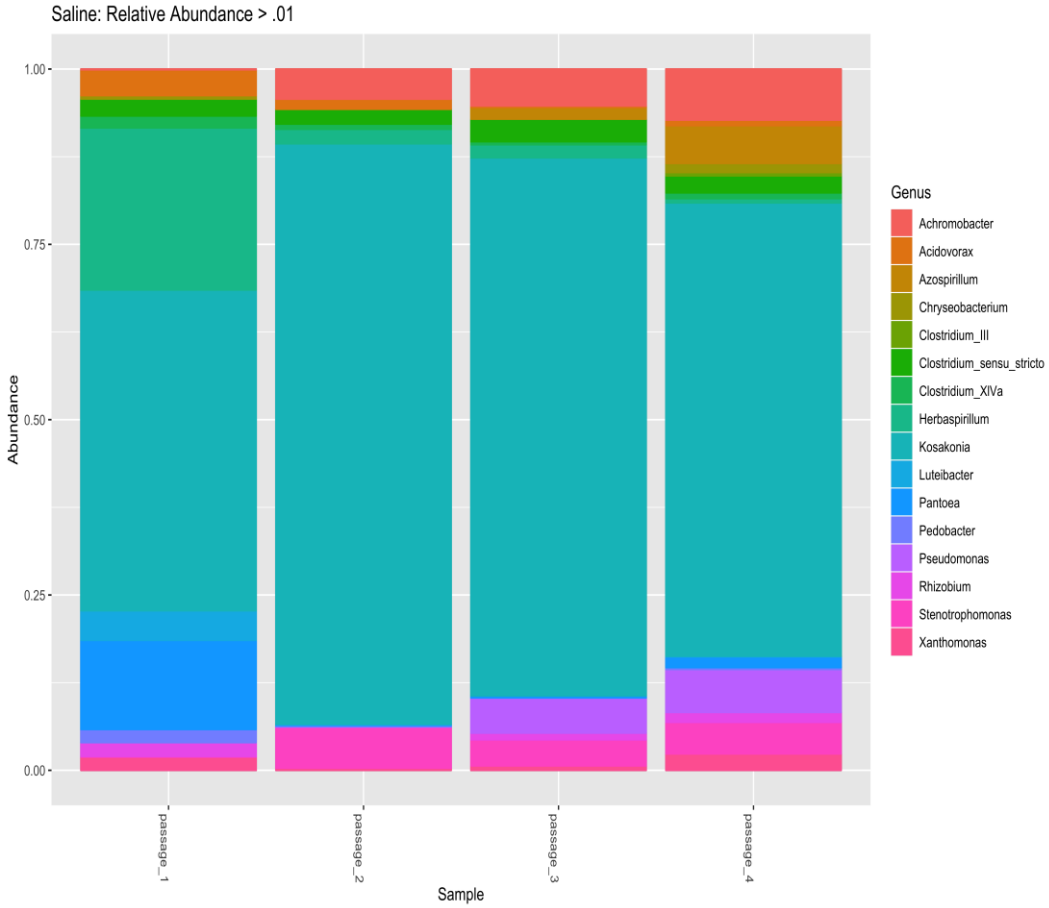


Figure 3.11 Most abundant genera found in the set II of experiments under saline conditions along the four passages.
The bar chart present the genera with a cumulative relative abundance >1%.

The differential abundance of rice grown under increasing saline conditions showed that five genera were significantly enriched. *Caulobacter* and *Brevibacillus* had the highest fold-change but were not among the most abundant genera (>1%). However, *Pantoea* and *Stenotrophomonas* were significantly enriched among the passages and were highly abundant in the microbiome, especially in the fourth passage (**Figure 3.12**).

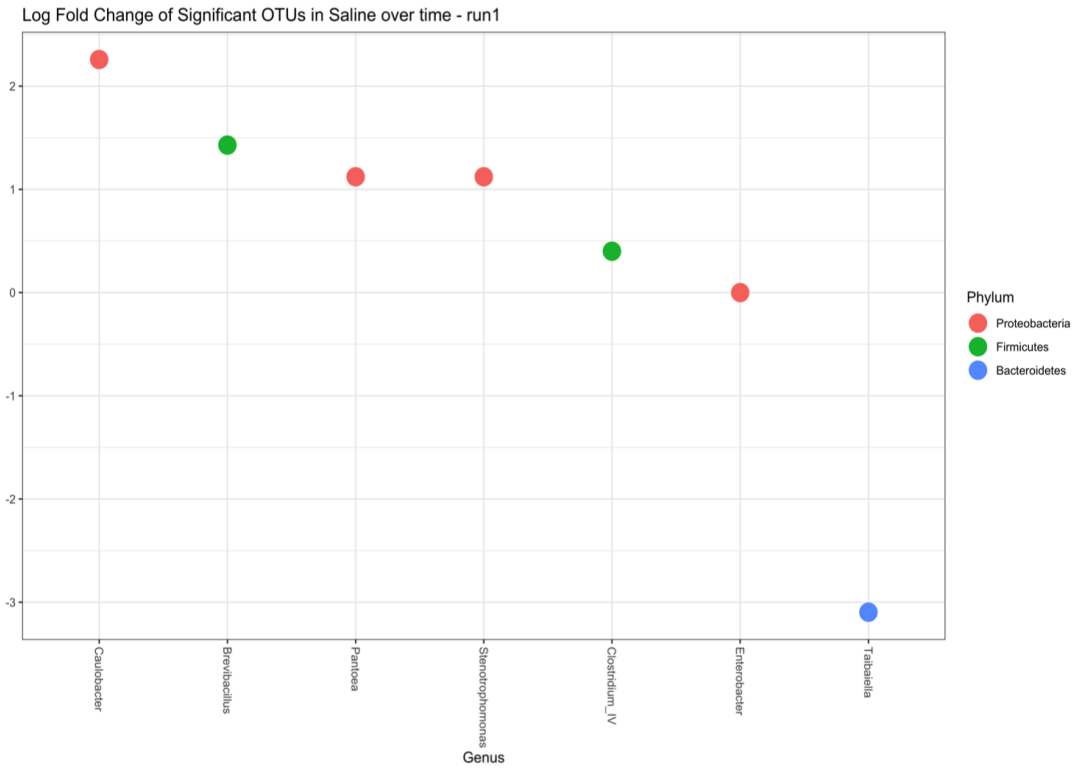


Figure 3.12 Differential analysis among the four passages of endophytes with added *Kosakonia* under saline conditions in the set II of experiments.
The differential analysis was performed using a quasi-likelihood (QL) F-test, with a p-value threshold of 0.05.

The biodiversity indexes showed that the initial inoculum had the higher biodiversity (**Table 3.7**), The values of the Inverse Simpson index of the inoculation passages suggest that the members of the microbiome were not found at very low abundances. Interestingly, at the fourth inoculation passage there was an increase of values by both indexes and for both groups of rice grown under non-stressful and saline conditions (**Table 3.7**). It is evidenced that the indexes of biodiversity in the set II of experiments were much lower when compared to the set I of experiments where *Kosakonia* was not added (**Table 3.3**; **Table 3.7**).

Table 3.7. Biodiversity indexes in the set II of experiments (with added *Kosakonia*)

	Shannon	***Inverse Simpson***
Initial noculum	6,36 ± 0,01	258,41 ± 3,6
Standard conditions (passages)		
First	3,39 ± 0,01	13,92 ± 0,07
Second	3,43 ± 0,006	13,85 ± 0,15
Third	3,17 ± 0,04	12,48 ± 0,31
Fourth	3,7 ± 0,06	18,58 ± 0,68
Saline conditions (passages)		
First	4,5 ± 0,04	40,94 ± 1,94
Second	3,36 ± 0,04	14,08 ± 0,33
Third	3,78 ± 0,05	19,02 ± 0,77
Fourth	4,37 ± 0,02	32,16 ± 0,65

3.4 Discussion

In this chapter, a strategy for the enrichment of endophytes through a series of inoculation passages under non-stressful and increased saline conditions is presented. This methodology was aimed for enriching endophytes which could re-colonize the root endosphere several times; this allowed the enrichment of different cooperative members of the endophytic microbial community. Similarly Timm et al. 2016, by using a different isolation strategy, found beneficial effects by the re-inoculation of a combination of bacterial endophytes in non-host crop plants. This approach could prove important for the development of multispecies/strain biofertilizers since as discussed in Chapter 3, single strain microbial inoculants, often failed to achieve the expected beneficial effects on the plant (reviewed by O'Callaghan 2016) . For this reason, *Kosakonia* KO348 was added in the set II experiments in order to find the most suitable cooperative bacterial members which could then be used in the design of a synthetic community with enhanced PGP properties.

It was found that the initial inocula of endophytes in both set of experiments was different in phyla and genera composition; this was probably due to the soil that was collected at different time points and/or the different batches of seeds.

In the set I experiments including both, the non-stressful and saline conditions groups, the five genera *Clostridium*, *Azospirillum*, *Sphingomonas, Caulobacter and Rhizobium*, were always present in the inoculation passages and only changing their values of relative abundance. It is likely that these genera probably form the core of

the microbial community in this experiment. Endophytes are important for stress amelioration and saline stressful conditions have been affect the root endophytic community (Yuan et al. 2016). In the experiments reported here, only *Nocardiodes* was statistically significantly enriched under saline conditions and therefore merits further study. Interestingly *Nocardiodes* species have been isolated not only as endophytes from important crops as rice and maize but also from halophytes indicating a connection with abiotic resistance to salinity (Wang et al. 2016; Song et al. 2011; Kämpfer et al. 2016a) . Increasing salinity concentrations in the set I of experiments did not affect the biodiversity index values that were comparable with the group of rice grown under non-stressful conditions. This constancy in biodiversity under salinity agrees with a recent report by Szymańska et al. 2018.

The set II of experiments involved the addition of *Kosakonia* KO348 in the inoculation passages and not surprisingly, the microbiome *Kosakonia* represented 70% or more of the bacterial endophytic community. Under non-stressful conditions some genera increased their abundance by the fourth inoculation passage, these were Azospirillum, *Pantoea* and *Stenotrophomonas.* These however did not have a statistical significant increase in the differential analysis, but are worthwhile to study for their possible coexistence with *Kosakonia* KO348 and because strains of these genera have been reported as prevalent endophytes (reviewed by Santoyo et al. 2016). In the case of the set II experiments grown under salinity stress, by the fourth inoculation passage, the genera *Azospirillum*, *Pseudomonas* and *Achromobacter* increased in abundance albeit not significantly. *Achromobacter* is a very interesting genera as it has been connected to salinity stress tolerance in tomato plants (Mayak et al. 2004).

The enrichment strategy addressed in this chapter was aimed at the identification of the bacterial efficient endophyte colonizers as well as the most likely *Kosakonia* bacterial cooperators. Further studies are necessary to validate this strategy via the isolation and characterization of strains of the enriched genera as well as *in-planta* tests.

Chapter IV.

LuxR solos signaling studies in *Kosakonia*

4.1 Introduction

Quorum sensing (QS) is a cell-cell signaling system in bacteria dependent on cell density that modulates cooperative behaviors important for bacterial fitness and host interactions (Fuqua et al. 1994; Whiteley et al. 2017; Venturi and Ahmer 2015). The most common QS system in Gram-negative proteobacteria is mediated by *N*-acyl-homoserine lactone (AHL) signals. The archetypical AHL-QS system is composed by two genes/proteins; the *luxI* family gene encoding for an AHL synthase and its cognate *luxR*-family gene which encodes for a transcriptional factor that detects and responds to the cognate AHL (Fuqua and Greenberg 2002). LuxR-family proteins are modular having an autoinducer (AHL)-binding domain at the N-terminus (Slock et al. 1990; Shadel et al. 1990) and a DNA-binding HTH domain at the C-terminus (Choi and Greenberg 1991, 1992).

Analysis of different genomes of proteobacteria has revealed the widespread presence of uncoupled *luxR* genes which lack a cognate *luxI* gene, these are called LuxR orphans or solos (Case et al. 2008; González and Venturi 2013; Subramoni and Venturi 2009; Fuqua 2006). Surprisingly, only a few *luxR* solos have been studied evidencing a role in intraspecies, interspecies, and interkingdom communication (Venturi et al. 2018; Chugani and Greenberg 2014; Ahmer 2004; Ferluga et al. 2007). One of the most studied LuxR solos is SdiA of the family *Enterobacteriaceae* (Smith and Ahmer 2003; Sabag-Daigle and Ahmer 2012). SdiA of *E. coli* and *Salmonella* can detect exogenous AHLs produced by other species of neighbouring bacteria (Ahmer 2004) and is involved in virulence in part due to the regulation of transcription of the *rck (resistance to killing)* operon (Ahmer 2004; Abed et al. 2014; Smith and Ahmer 2003; Habyarimana et al. 2014). QscR from

Pseudomonas aeruginosa on the other hand, is a solo which responds to an endogenously produced AHL signal by the LasI-LasR system and the role of QscR is therefore to extend the LasI/R regulon (Chugani and Greenberg 2014).

A subfamily of LuxR solos found exclusively in plant associated bacteria (PAB) has evolved to respond to plant low molecular weight molecules thus being an interkingdom signaling circuit (González and Venturi 2013; Venturi and Fuqua 2013). The members of this subfamily have some substitutions among the highly conserved amino acids in the AHL-binding domain (Venturi et al. 2018; González and Venturi 2013) and regulate the adjacently located proline iminopeptidase (*pip*) gene. Members of this PAB LuxR solos are involved in plant virulence in members of *Xanthomonas* genus or in plant beneficial interactions in *Pseudomonas* (Zhang et al. 2007; Ferluga et al. 2007; Chatnaparat et al. 2012; Ferluga and Venturi 2009; Subramoni et al. 2011). Recently, a derivative of ethanolamine present in *Poplar* plant leaf macerate has been implicated to induce PipR activity in *Pseudomonas* (Coutinho et al. 2018).

Kosakonia is a novel genus first described 2013 (Brady et al. 2013); several of its members are diazotrophs and efficient plant colonizers with plant growth promoting properties (PGP) (reviewed by Becker et al. 2018). Most *Kosakonia* strains have been isolated from economically important crops like maize, rice, wheat, sweet potato, sugarcane and cotton (Hardoim et al. 2015; Kämpfer et al. 2016b; Remus et al. 2000; Li et al. 2010; Ho et al. 2015; Magnani et al. 2010; Shinjo et al. 2016). This genus is gaining attention as the analysis of a few recently available genomes has shown interesting features that would support their plant-associated lifestyle and PGP properties (Becker et al. 2018). To our knowledge there are no reports with respect to QS and/or interkingdom signaling in *Kosakonia*.

Kosakonia sp. strain KO348 has been previously isolated from an Italian rice cultivar and shown to possess PGP properties (Bertani et al. 2016). This *Kosakonia* strain is an efficient rhizoplane and rice root endosphere colonizer with a type VI secretion system involved in its plant colonization ability (see Chapter 2). In this chapter it is reported that *Kosakonia* KO348 possesses two LuxR solos; one is an SdiA homolog designated as LoxR while the other one, designated as PsrR, belongs to the sub-family PAB solos that responds to plant signals. Structure-based modeling, putative target gene promoter expression analysis and *in-planta* studies were performed. In addition, LoxR has been purified and shown to bind to AHLs. Their possible involvement of these two LuxR solos in *Kosakonia* plant-associated lifestyle is discussed.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

Kosakonia KO348 was previously isolated from the root endosphere from rice grown in Italy (Bertani et al. 2016) and its genome is published in DDBJ/EMBL/GenBank (JZLI00000000) (Meng et al. 2015). KO348, KO348*loxR*, KO348*psrR* and *Escherichia coli* strains DH5α, S17 and BL21 were routinely grown at 30°C and 37°C, respectively, in Luria–Bertani (LB) broth medium (Miller 1972) or in MME minimal medium (Ryan et al. 2007). When required, antibiotics for *Kosakonia* strain growth were added at the following concentrations: rifampicin, 50 µg ml $^{-1}$, gentamicin, 25 µg ml^{-1} and kanamycin, 100 µg ml^{-1}. *E. coli* DH5α and S17 were grown at 37 °C in LB broth and antibiotics were added when required at the following concentrations: ampicillin, 100 µg ml^{-1}, and gentamicin, 15 µg ml^{-1}. AHLs *N*-hexanoyl-homoserine lactone (C6) and *N*-3-oxododecanoyl-homoserine lactone (OC12), were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2 Structure homology modeling

Five web-based servers were used to build the 3D structure-based homology models of full-length LoxR, PsrR and PipR. The top-score models generated by the servers were then ranked and validated by the protein model quality predictor ProQ (Wallner and Elofsson 2003) and by PSIPRED (Buchan et al. 2010) for the secondary structure prediction. The IntFOLD server (Buenavista et al. 2012) produced the highest quality 3D models for LoxR and PsrR from *Kosakonia*, according with the ranking obtained by ProQ being the predicted LG-score and

MaxSub value of 4.749 and 0.486 respectively for LoxR and 4.125 and 0.706 respectively for PsrR. In the case of the protein model of PipR it was obtained by RaptorX (Källberg et al. 2012), being the predicted LG-score and MaxSub value of 4.039 and 1.010 respectively.

The templates used for LoxR modeling were SdiA from *E. coli* (PDB_ID 4LGW_A (Kim et al. 2014) and QscR from *P. aeruginosa* (PDB_ID 3SZT (Lintz et al. 2011). The PsrR model was obtained using the same templates as for LoxR combined with LasR (PDB_ID 3IX3 (Zou and Nair 2009) from *P. aeruginosa*. The template used for PipR modeling was QscR from *P. aeruginosa* (PDB_ID 3SZT (Lintz et al. 2011)).

4.2.3 Bacterial expression and purification

LoxR was amplified from *Kosakonia* KO348 using the following primers: lox_Fw catatgcaggatacagaattctttacc and lox_Rev actcgagaatcatccctgtcgccgctgc and directionally cloned at the NdeI/XhoI restriction sites into His_6-tagged protein expression vector pET22b (Addgene; Watertown, MA, U.S.A) which was later transformed in *E. coli*. LoxR expression levels were optimized by small-scale expression test using several *E. coli* strains and the BL21 (DE3) pLysS resulted to be the most efficient one. LoxR was expressed by autoinduction following the Studier 2005 methodology, at 16°C overnight in presence of 10 μM AHLs. Cells were harvested, suspended in lysis buffer (50 mM Tris-base buffer [pH 8.5], 300 mM NaCl, 1 mM EDTA, 5 mM imidazole, 5 mM 2-β-mercaptoethanol, 5% glycerol), and lysed by sonication. After extended centrifugation the filtered lysates were loaded on a 5mL HisTrap FF Crude column (GE Healthcare Inc.; Chicago, IL, U.S.A). The column was washed with wash buffer (50 mM Tris-base buffer [pH

8.5], 300 mM NaCl, 0.1 mM EDTA, 10 mM imidazole, 5 mM 2-β-mercaptoethanol, 5% glycerol) and eluted in a linear gradient with the elution buffer (50 mM Tris-base buffer [pH 8.5], 300 mM NaCl, 0.1 mM EDTA, 250 mM imidazole, 5 mM 2-β-mercaptoethanol, 10% glycerol): the elution fractions were collected for SDS PAGE analysis, pooled and concentrated by VivaSpin (GE Healthcare Inc.; Chicago, IL, U.S.A) for size exclusion chromatography on a HiLoad 200 16/60 column (GE Healthcare S200 column) in 50 mM Tris-base buffer [pH 8.5], 300 mM NaCl, 0.1 mM EDTA, 5 mM 2-β-mercaptoethanol, 10% glycerol. Peak fractions were pooled, concentrated, flash frozen and stored at -80°C.

4.2.4 Mass spectrometry

Heterologous expressed LoxR proteins bound with AHLs C6 and OC12 contained in the peak fractions described above were precipitated with acetonitrile 10% and then digested with trypsin. LC-MS/MS of the digestion was performed using an Easy-nLC II coupled to an Amazon ETD mass spectrometer (Bruker Daltonics, Hamburg, Germany). Synthetic AHLs (OC12) were used as standards (Sigma-Aldrich Inc.; San Luis, MO, U.S.A). The resulting spectra were searched using the X!tandem (The Global Protein Machine Organization) search engine.

4.2.5 Construction of KO348*loxR* and KO348*psrR*

Both LuxR solo mutants were generated using the suicide vectors from the pKNOCK series (Alexeyev 1999). To generate KO348*loxR*, an internal fragment (312 bp) of the *loxR* gene was amplified by PCR using the primers pKNloxR.Fw: gatgcagcattatcaggcaga and pKNloxR.Rv: tgatcttccagacgcgttaa and cloned as

a *Bam*HI-*Kpn*I fragment into the corresponding sites of pKNOCK-Km resulting in pKNOCKloxR. To generate KO348*psrR*, an internal fragment (245 bp) of the *psrR* gene was amplified by PCR using the primers pKNpsrR.Fw: ctatcaacgcccggacag and pKNpsrR.Rv: acagcggaaaggcagatt and cloned as a *Bam*HI-*Kpn*I fragment into the corresponding sites of pKNOCK-Km resulting in pKNOCKpsrR. These latter plasmids, pKNOCKloxR and pKNOCKpsrR were delivered to *Kosakonia* KO348 by electroporation and transformants were selected after appropriate antibiotic selection. The *loxR and psrR* full-length genes (including its gene promoter) were amplified with the primers LoxR-comp_Fw: ggatccccgttaacgttggcgttaaa and LoxR-comp_Rv: gaattctttaaatcatccctgtcgcc for *loxR* gene; and PsrR-comp_Fw: ggatccacggtgcatcagcattctcc and PsrR-comp_Rv: gaattcggcgctgaacactagcaaaa for *psrR* gene; the sequences were verified via DNA sequencing and the resulting fragments were cloned in the gentamicin resistant pBBR1MCS-5 vector (Kovach et al. 1995). The plasmids containing the fragments were individually electroporated in the mutant strains KO348*loxR* and KO348*psrR* respectively, and selected for Km^R and Gm^R, the resulting mutant complemented strains were named KO348*loxR*(pBBR*loxR*) and KO348*psrR*(pBBR*psrR*). Mutants and complemented mutants were verified by colony PCR.

4.2.6 Gene promoter studies

Transcriptional activity studies of six gene promoters (*srgE*, hypothetical T6SS gene, three different *vgrG* the *pip* gene promoter) were studied in *Kosakonia*. Gene transcriptional fusion plasmids were constructed in the pMP220 promoter probe vector which harbors a promoterless *lacZ* gene (Spaink et al. 1987). The primers used for the cloning of the gene promoters were as follows: promsrgE_FwBam

ggatccgcttggacaggattgtttattg and promsrgE_RvEco gaattcttccctgttccttagcgtgt (*srgE*PROM; 345 bp); promphyp_FwBam ggatccggcggtaaaggtgctgaa and promhyp_RvEco gaattcatcacccttgcgcataac (PROMhypprot; 287 bp); promVgrG1_FwBglII agatctcgcgtgaaagtgggataata and promVgrG1_RvEco gaattccatcgacgggtaactgaac (*vgrG1*PROM; 381 bp); promVgrG2_FwBam ggatccgccaagccagttcaaagta and promVgrG2_RvEco gaattccccggagagtttccaga (*vgrG2*PROM; 200 bp); promVgrG3_FwBam ggatccaggcacctggcttatca and promVgrG3_RvEco gaattcccctgattgctgtgtgtt (*vgrG3*PROM; 205 bp); and prompip_FwBam ggatccgcgttgcactaccgtct and prompip_RvEco: gaattcgaagggaacgtagcctt (pipPROM; 146 bp). After PCR amplification using genomic DNA as template, and veryfing the fidelity by DNA sequencing, the corresponding fragments were digested using BamHI and EcoRI and cloned in the corresponding sites in promoter vector pMP220.

β–galactosidase, activity of *Kosakonia* transconjugants harboring the transcriptional plasmid fusion constructs was determined as previously described by Miller 1972, with the modifications of Stachel et al. 1985. Determination of each promoter activity was performed in biological triplicates and as control the empty pMP220 promoter probe vector was used. When necessary, AHLs were added at 1 □M. The *pip* gene promoter activity was also performed in presence of rice root extract, ethanolamine (500 μM) obtained from Sigma-Aldrich (St. Louis, MO, USA) and *N*-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide (HEHEAA) (10μM) obtained from Chiron AS (Trondheim, Norway).

4.2.7 Rice root colonization assays

Rice root colonization experiments were performed as described by Bertani et al. (2016) protocol with the following modifications. Briefly, each strain was grown until an OD_{600} of 0.8 and used to inoculate 9 days-old germinated rice plant roots cv. "Baldo" by submerging them in the bacterial suspension for 1 hour. Plant were then transferred to a 50mL tube containing Hoagland´s semi-solid solution (Steindler et al. 2009); when necessary AHLs were added in a concentration of 1μM. All plants were followed for 14 days and then *Kosakonia* strains were re-isolated.

For rhizoplane colonization, rice roots were rinsed with sterile water for removing all adhered Hoagland´s solution and then vortexed in 5ml of PBS solution for 1 min. Serial dilutions of this PBS solution containing bacteria were then plated on the appropriate media with antibiotics for CFU/g calculation. For the determination of endosphere colonization, the roots were sterilized as described by Bertani et al. 2016, macerated in PBS and then plated after serial dilutions in TSA containing the appropriate antibiotics; the plates were then incubated at 30 °C for 48 hours and CFU/g was calculated. For comparing the rhizoplane and endosphere colonization ability, analysis of variance (ANOVA) was performed with Prism 7 (Graphpad Software, Inc). Five biological replicates were used in each group.

4.3 Results

4.3.1 *Kosakonia* sp. contains two LuxR solos

The genome of *Kosakonia* KO348 (Meng et al. 2015) presents two *luxR* solo genes and both have been annotated as single transcriptional units (**Figure 4.1**). One LuxR solo, designated as LoxR displays its highest identity (76-99%) in its primary structure with SdiA from the Enterobaceriaceae family, while the second solo that has been designated as PsrR had highest identity (71-99%) with a sub-family of LuxR solos that responds to plant compounds. Genetically adjacent to the *psrR* gene, is the proline iminopeptidase *pip* gene which is typical of the subfamily of LuxR solos that respond to plant compound(s) (**Figure 4.1B**). Both LoxR and PsrR had two PFAM domains conserved among the LuxR-type family proteins, one autoinducer binding domain (PF03472) and one bacterial regulatory protein, LuxR type DNA-binding HTH domain (PF00196).

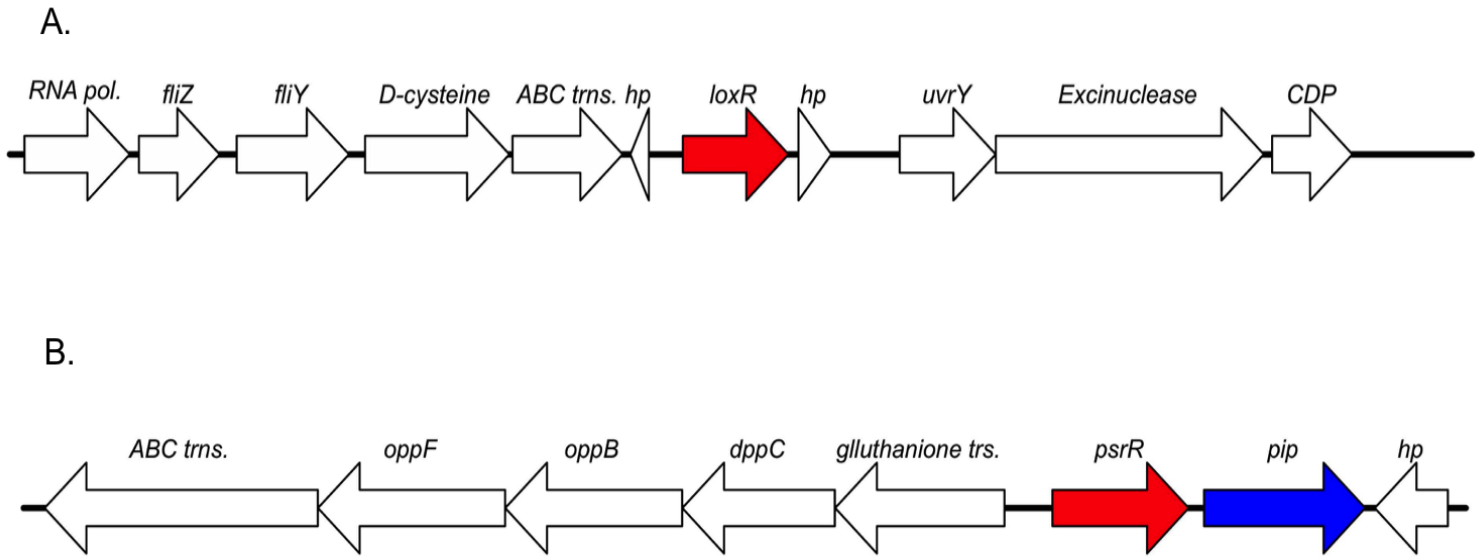


Figure 4.1. Gene maps of the two luxR solos loci present in KO348.
(A) 10kb region of the surrounding genes of the *loxR* gene. RNA pol: RNA polymerase sigma factor, *fliZ*: flagellar biosynthesis protein, *fliY*: periplasmic cysteine binding protein, *D-cysteine:* D-cysteine dessulfhydrase, *ABC trns*.: cysteine ABC transporter, *hp*.: hypothetical protein, *uvrY:* response regulator. *Excinuclease*: excinuclease ABC subunit C, *CDP*: CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase. **(B)** 8kb region of the surrounding genes of the *psrR* gene. *ABC trns*.: Dipeptide binding ABC transporter, *oppF*: oligopeptide transport ATP-binding protein, *oppB*: oligoeptide transport system permease protein, *dppC:* dipeptide transport system permease protein, *gluthatione trs*.: putative gluthatione transporter, *pip*: proline iminopeptidase, *hp.:* hypothetical protein.

4.3.2 LoxR is likely to respond to AHLs whereas PsrR to a plant compound

In order to gain structural insights underlying substrate specificity of LoxR and PsrR, multiple structure-based sequence alignment and structure-based homology modelling were used. The structure-based sequence alignment of the ligand-binding domain of LoxR and PsrR was performed using the program Expresso (Armougom et al. 2006) (**Figure 4.2**). The following primary sequences were included in the multiple alignment: PipR and from *Pseudomonas sp. GM79* and OryR from *Xanthomonas oryzae*, prototypes of the Plant Associated Bacteria (PAB) LuxR solos subfamily, TraR from *Agrobacterium tumefaciens* -TraR_*At*- (PDB_ID 1H0M_A) (Vannini et al. 2002) and from *Sinorhizobium fredii* NGR234 -TraR_*Sf*- (PDB_ID 2Q0O_A) (Chen et al. 2007) prototypes of the canonical QS (Quorum Sensing) LuxR proteins (**Figure 4.2**), and the templates used in the structure-based homology modelling of LoxR and PsrR were SdiA from *Escherichia coli* (PDB_ID 4LGW_A) (Kim et al. 2014), QscR (PDB_ID 3SZT_B) (Lintz et al. 2011) and LasR (Zou and Nair 2009) from *Pseudomonas aeruginosa* (**Figure 4.3- 4.5**).

Structure-based homology modeling of the full-length **LoxR and PsrR** was performed exploiting several approaches and the resulting models ranked according protein models quality assessments based on LG-score and MaxSub (see section 4.2 2). In order to elucidate the molecular determinants of ligand discrimination in LoxR and PsrR, a cartographic analysis of the top-ranked models has been exploited as described in Covaceuszach et al. 2013. The focus was on the pocket residues directly interacting with the ligand that are conserved and belong to the previously described Cluster 1 (Whitehead et al. 2001; Fuqua et al. 2002) and to Cluster 2, colored in green and in cyan respectively (**Figures 4.3– 4.5**) and on

pocket residues identified as variable, belonging to Cluster 3, colored in orange (**Figures 4.3-4.5**).

The comparative structural analysis revealed key differences between the ligand-binding pockets of **LoxR** and PsrR. Striking similarities to the structural determinants of the canonical QS LuxRs family were found in LoxR while the ligand-binding site of the PsrR closely resembles the ones of PAB LuxR solos subfamily.

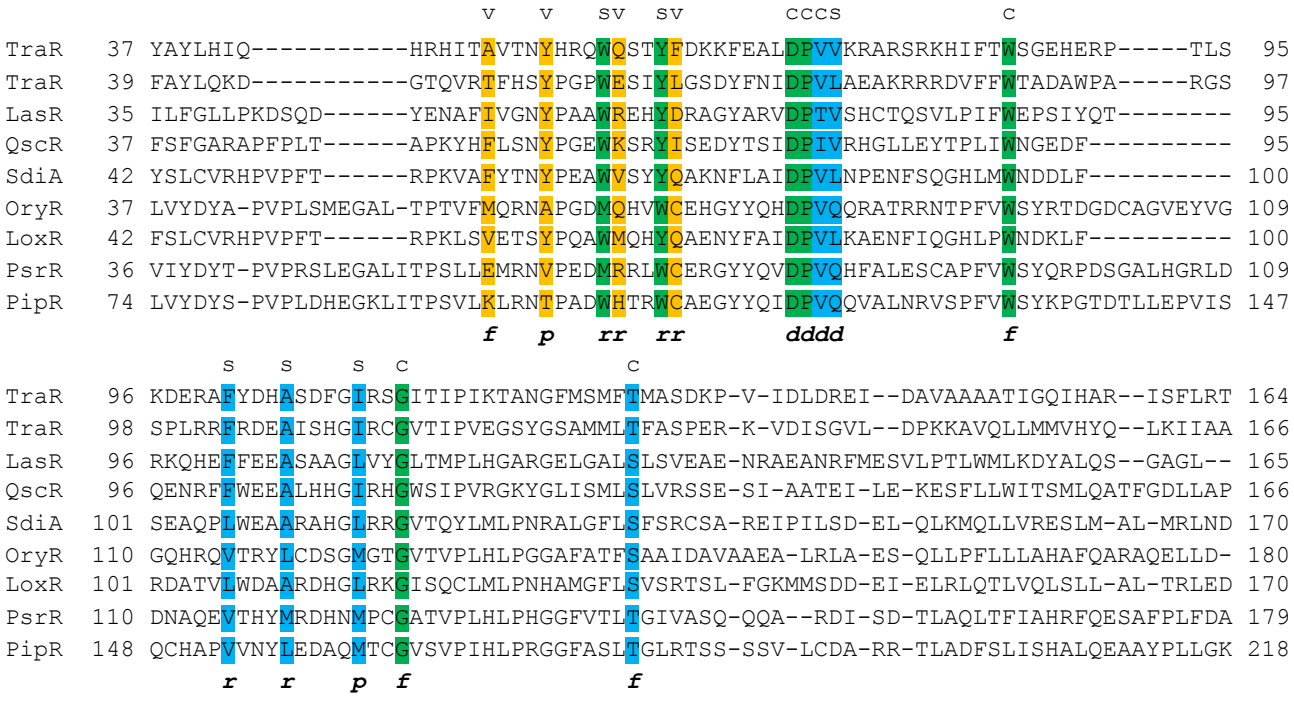


Figure 4.2. Structure-based multiple sequence alignment of the regulatory domains of *Kosakonia* LuxR solos with members of canonical QS LuxRs family and with the prototypes of the PAB LuxR solos subfamily.

The residues belonging to **Cluster 1,** to **Cluster 2** and **Cluster 3** are highlighted in green, cyan and in orange, respectively. The 3D architecture of the boundaries of the ligand-binding site is schematized by ***r*** (roof), ***f*** (floor), ***p*** (proximal wall) and ***d*** (distal wall) and its tripartite topology by ***c*** (conserved core), ***s*** (specificity patch) and ***v*** (variable patch).

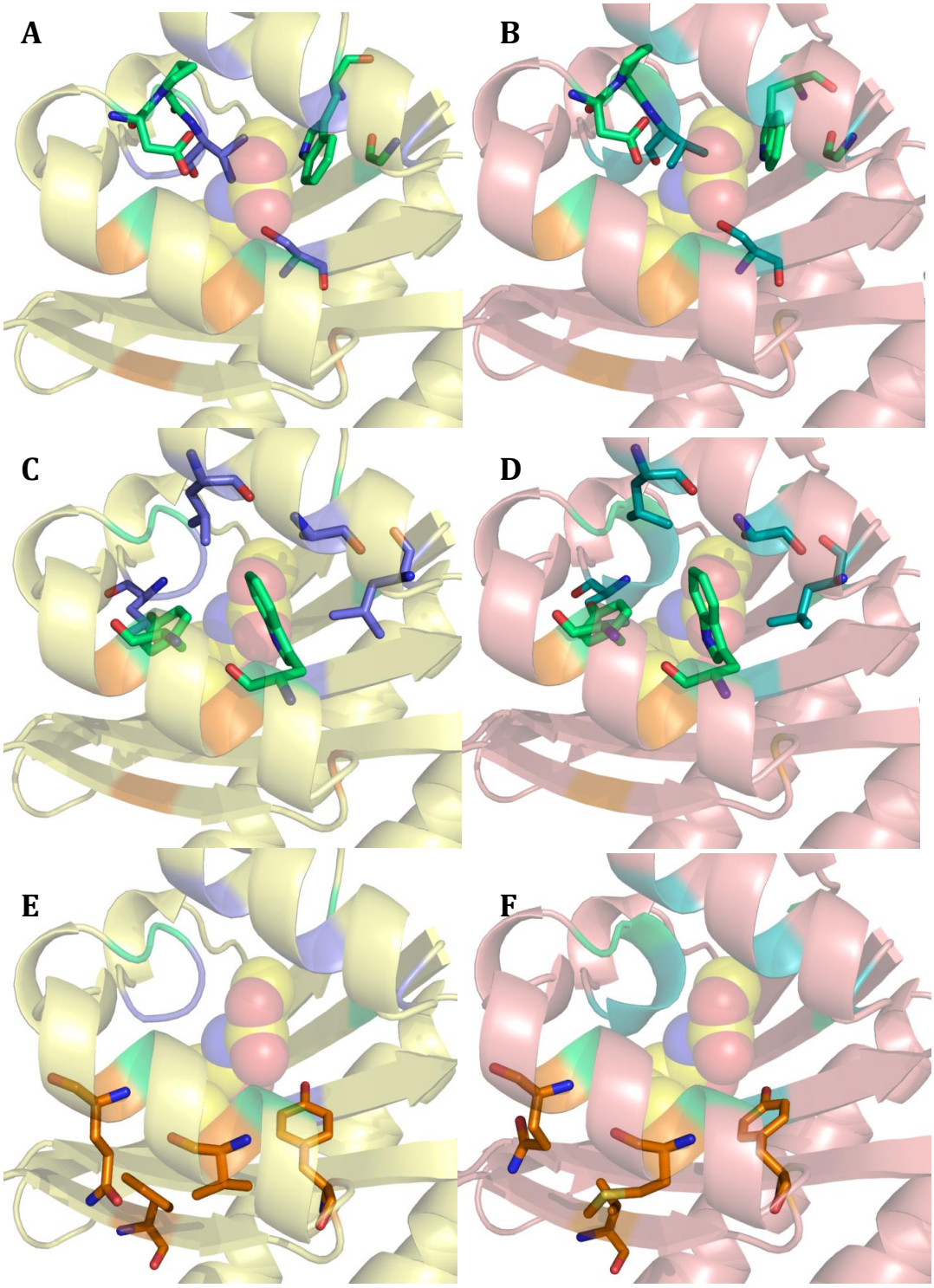


Figure 4.3. Comparison of the ligand-binding sites of LoxR with the QS LuxR solo SdiA.

Mapping the protein residues defining the three Clusters (colored with the same color code used in Figure 4.2) that delineate the conserved core (**A, B**), the specificity patch (**C,D**) and the variability patch (**E,F**), respectively on the structure of SdiA in complex with 3OC6-HSL (PDB_ID 4Y15_A) (Nguyen et al., 2015) (**A, C, E**) and on the 3D structure-based homology model of the LuxR solo LoxR (**B, D, F**). The carbon, nitrogen and oxygen atoms of the 3OC6-HSL ligand shown in the left column are represented by spheres and are colored in yellow, blue and red respectively. Figures produced by Pymol (Delano 2002).

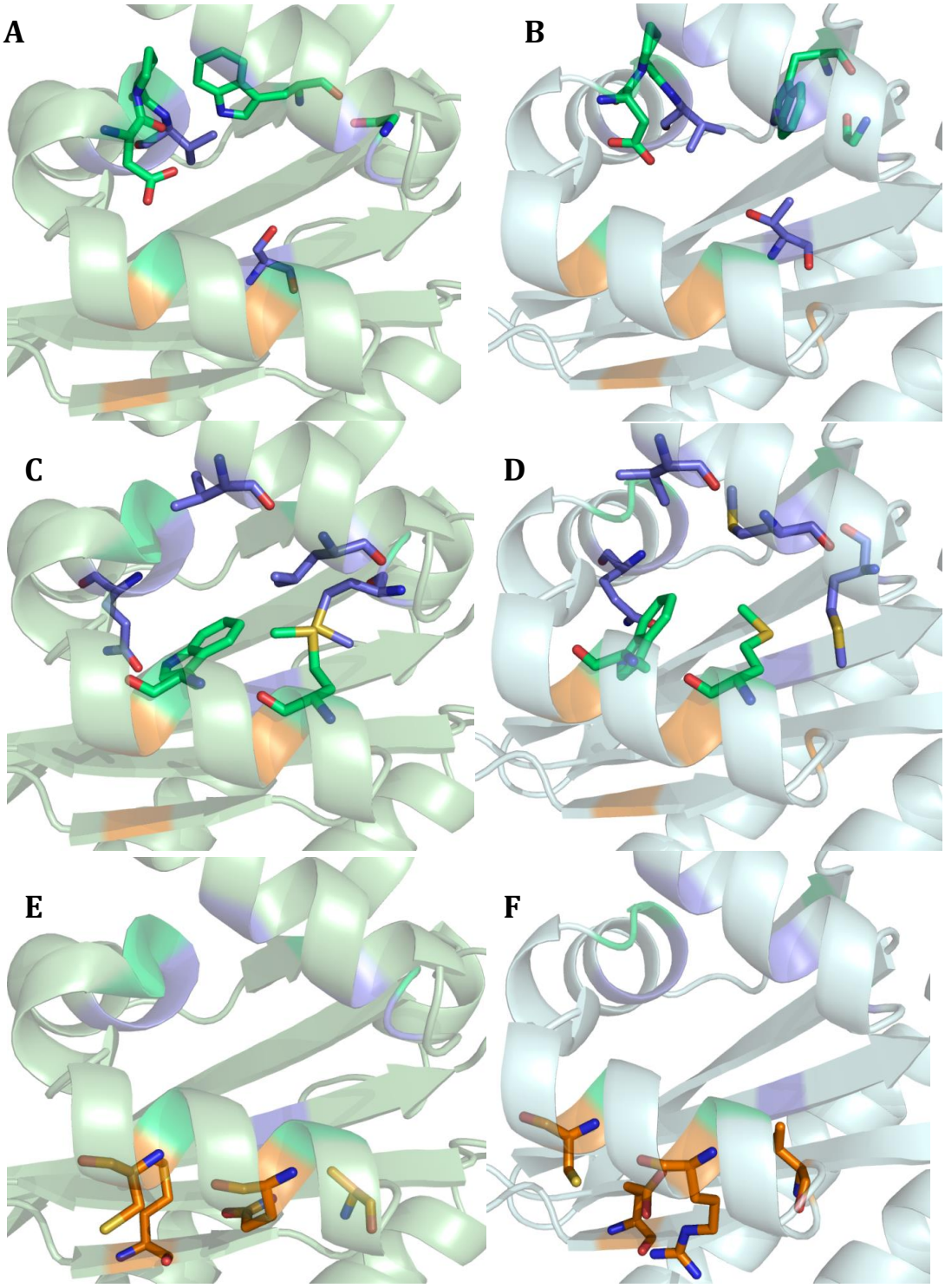


Figure 4.4 Comparison of the ligand-binding sites of PsrR with the prototype of LuxR PAB solo OryR.

Mapping the protein residues defining the three Clusters (colored with the same color code used in Figure 4.2) that delineate the conserved core (**A, B**), the specificity patch (**C,D**) and the variability patch (**E,F**), respectively on 3D structure-based homology models of OryR (Covaceuszach et al., 2013) (**A, C, E**) and of PsrR (**B, D, F**). Figures produced by Pymol (Delano 2002).

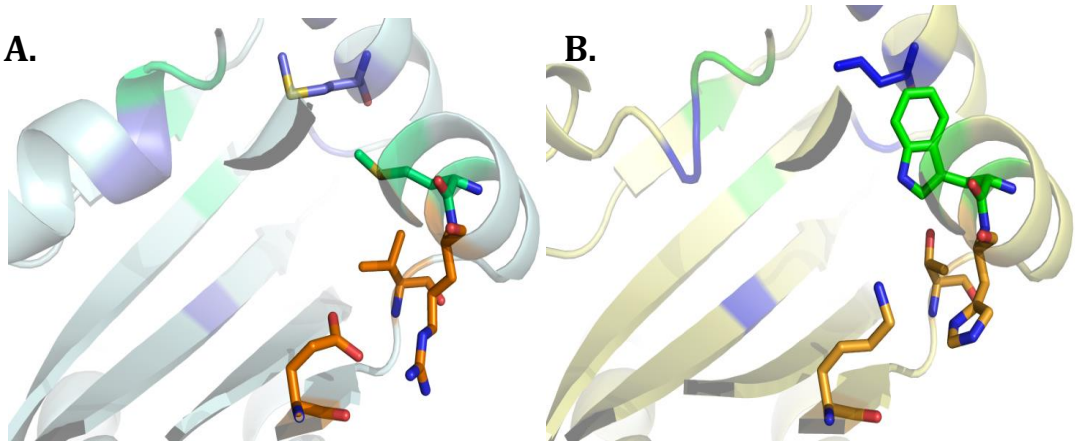


Figure 4.5 Comparison of PsrR and PipR the ligand-binding sites.
Superimposition of the 3D structure-based homology models of (A) PsrR (colored in pale cyan) and (B) PipR (colored in pale yellow), obtained from the orientation in Figure 4.3– 4.4 by 90 degrees rotation around y axis. Among the protein residues defining the three Clusters (colored with the same color code used in Figure 4.2) that delineate the conserved core, the specificity patch and the variability patch, the side chains of ones that are not conserved between PsrR and PipR are displayed. Figures are produced by Pymol (Delano 2002).

In particular, LoxR not only conserved the binding site core (including Clusters 1 and 2 residues and delimiting the binding site floor and the distal wall, *i.e.* 70, 71, 72, 85, 113, 129, according to TraR_*At* numbering, marked by *c* in **Figure 4.2**), but also conserved all its residues of the specificity patch (belonging to Cluster 1 and 2 and mainly delimiting the binding site roof and the nearby proximal and distal walls, *i.e.* 57, 61, 73, 101, 105, 110 according TraR_*At* numbering, marked by *s* in **Figure 4.2**) which differ from the subfamily of PAB LuxR solos and are conserved within the members of the canonical QS LuxRs family, pinpointing that LoxR could have a common specificity towards AHLs. Interestingly residues belonging to both the conserved and the specificity patches in LoxR (**Figure 4.3B** and **4.3D** respectively) are identical to those of the corresponding regions in the canonical QS LuxR solo SdiA (**Figure 4.3A** and **4.3C** respectively), whose crystal structure (PDB_ID 4LGW_A) has been used, as a template for homology modeling, being

68% and 83% their overall primary sequence identity and homology respectively. Moreover some degree of conservation has been identified also in the respective variability patches (**Figure 4.3E - 4.3F**) that comprise Cluster 3 residues (delimiting the proximal wall and the nearby roof and of the floor of the binding site, *i.e.* 49, 53, 58, 62 according TraR_*At* numbering), usually less conserved even within the members of canonical QS LuxR family. Indeed besides two identical residues (*i.e.* Y63 and Q72), a conservative (*i.e.* M68 in LoxR instead of V68 in SdiA) and a semi-conservative substitution (*i.e.* V59 in LoxR instead of F59 in SdiA) have been found. Overall, a close specificity of LoxR for AHLs can be inferred.

On the other hand the molecular determinants of the PsrR binding site (**Figure 4.4**, right column) suggest a different specificity, most likely towards plant compounds that are unrelated to AHLs. Indeed all the residues belonging to the specificity patch are quite conserved with respect to the PAB LuxR solos subfamily (**Figure 4.4**, left column) and differ with respect to the canonical QS LuxRs family. Among the residues of the roof of the binding site, PsrR and OryR W71, which belongs to Cluster 1 and it is highly conserved among all members of the PAB LuxR solos subfamily, differs from Y61, the TraR_*At* corresponding residue, that is conversely highly conserved within the canonical QS LuxRs family. Alike, the two roof residues PsrR and OryR V115 and a conserved hydrophobic/aliphatic residue (L/M) in position 120 are replaced by the quite conserved TraR_*At* F101 and A105 residues respectively. Among the residues of the distal wall of the binding site, PsrR and OryR Q83, which belongs to Cluster 2 and is highly conserved in the PAB LuxR solos subfamily, it is substituted in the canonical QS LuxRs by a conserved hydrophobic/aliphatic residue (V/L), TraR_*At* V73. Nevertheless

important differences can be detected in Cluster 3 residues that are less conserved: the only position in this cluster that PsrR and OryR share is C71. Indeed PsrR is characterized by the presence of two charged residues (E and R, instead of hydrophobic/aliphatic residue M and of a hydrophilic residue Q in positions 58 and 67 respectively) and a V62 that substitute A62 in OryR. These key differences suggest a different specificity towards unrelated plant compounds for PsrR and OryR.

It was of interest to obtain the structure-based homology model of the full-length PipR, a PAB LuxR solo from *Pseudomonas sp.* GM79 that responds to an specific plant compound N-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide (HEHEAA) (Coutinho et al. 2018) and to compare its homology modeling to the one of PsrR to unveil the structural determinants responsible for a possible ligand specificity. **Figure 4.5** compares the ligand-binding sites of PsrR and PipR, highlighting the side chains of residues that are not conserved between PsrR and PipR. These residues define mainly the roof and part of the floor and of the proximal wall of the two binding pockets that are significantly different in terms of electrostatic potentials (E58 and R67 in PsrR instead of K96 and H105 in PipR) and hydrogen-bond donor capabilities (V62 and M66 in PsrR instead of T100 and W104 in PipR) that may indicate a different specificity of PsrR and PipR towards unrelated plant compounds.

4.3.3 LoxR binds AHLs

It was therefore of interest to determine if LoxR was binding AHLs due to its high homology to the SdiA 3D-structure and due to the conserved AHL binding docking residues (see above). His-tagged LoxR was expressed and purified in *E. coli* in the

presence of different AHLs (C6, OHC6, OC6, OC8 and C10; **Figure 4.6**). QS LuxR-family proteins are soluble when bind their cognate AHL, and insoluble in their absence (Zhu and Winans 2001) (**Figure 4.6)**. After optimization by adding metal ions, the best purification was obtained when LoxR was expressed in the presence of C6 or OC12 (**Figure 4.7 - 4.8**). For confirmation of the binding of LoxR to AHLs; LC-MS/MS was performed to the corresponding protein eluting fractions corresponding to the putative LoxR-OC12 complex. Here is was established that the OC12 and LoxR proteins were present confirming the binding of LoxR to OC12 (**Figure 4.9 - 4.10**). The same analysis for the LoxR-C6 has not yet been performed.

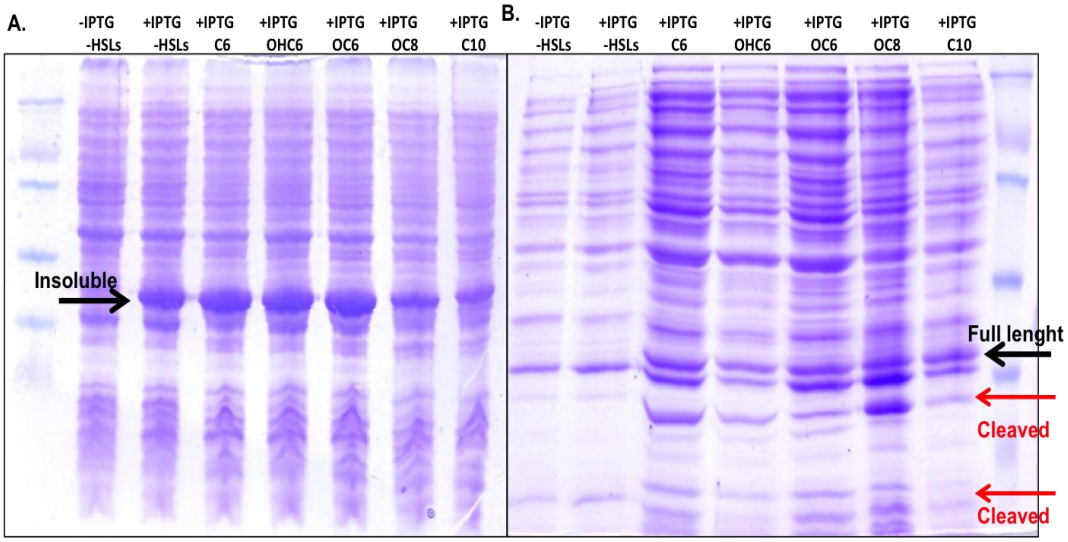


Figure 4.6. LoxR protein is soluble only in presence of AHLs.
LoxR was expressed in *E. coli* BL21, induction was performed using of 2mM IPTG in presence of different AHLs (10uM) at 37°C. LoxR is expressed in a soluble form only in presence of AHLs.

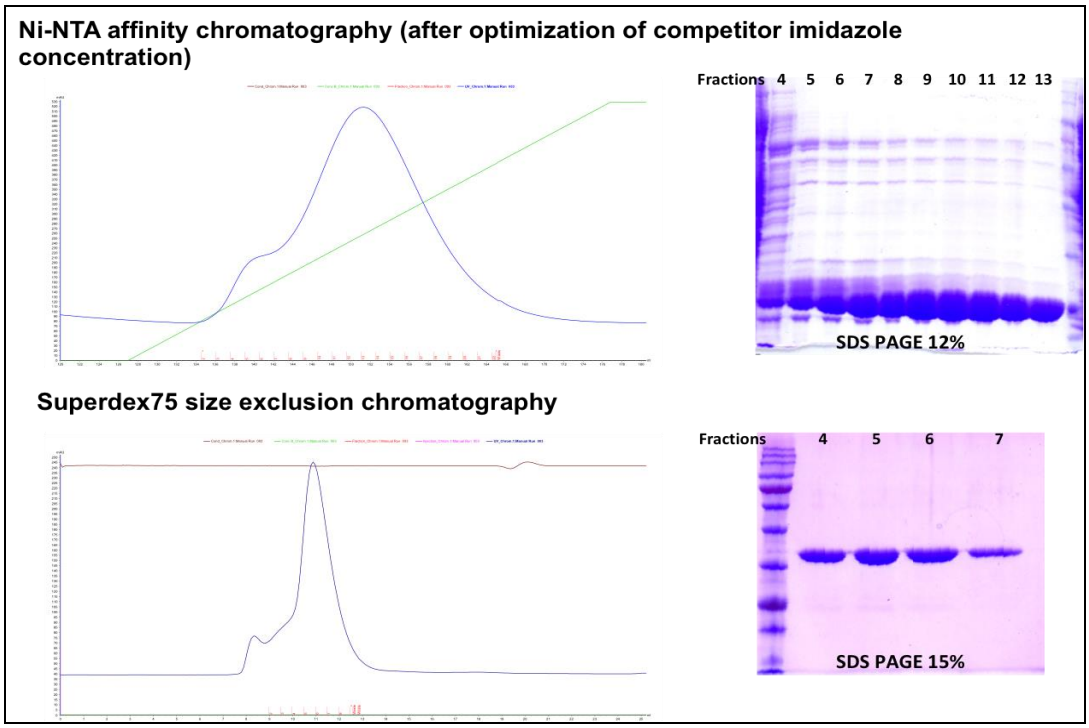


Figure 4.7 Purification of the complex LoxR-C6.
LoxR was autoinduced in *E. coli* BL21 in presence of 10uM of C6, expression tests were performed at 16°C. Purification was performed by Ni-NTA chromatography in presence of metal ions followed by a size exclusion cromatography.

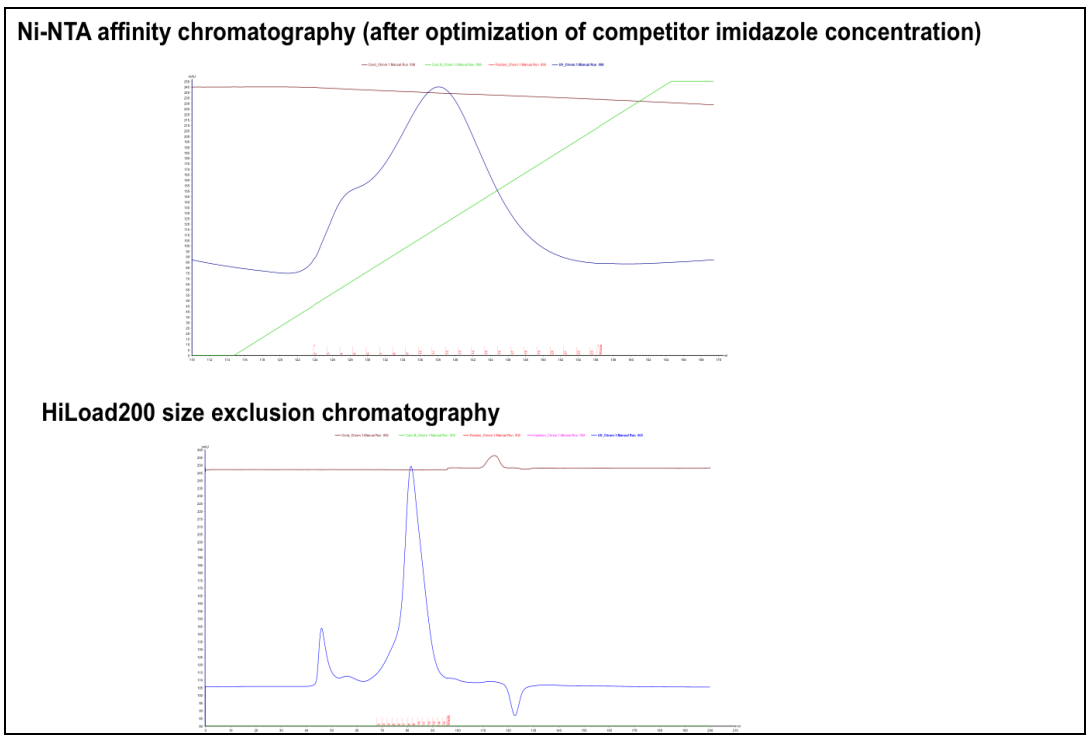


Figure 4.8 Purification of the complex LoxR-OC12.
LoxR was autoinduced in *E. coli* BL21 in presence of 10uM OC12, expression tests were performed at 16°C. Purification was performed by Ni-NTA chromatography in presence of metal ions followed by a size exclusion chromatography.

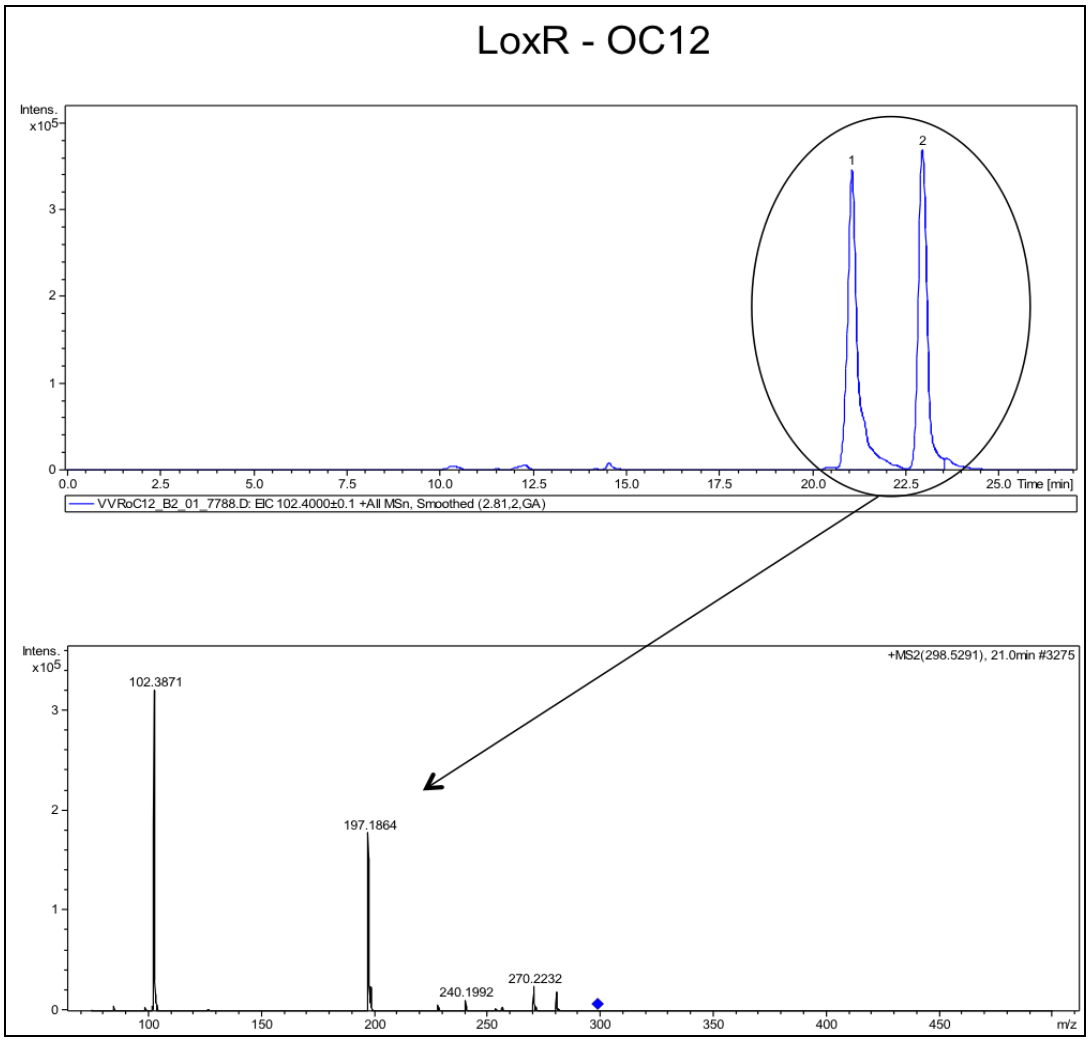


Figure 4.9 Mass spectra of the LoxR-OC12 complex.
OC12 presents two peaks (in the circle) identified during the OC12 standarization method, the same peaks were found in the MS spectra of the LoxR-OC12 complex.

rank	log(e)	log(l)	%/%	#	total	Mr	Accession
1	-221.4	9.13	59/69	22	79	28.0	fig|6666666.110358.peg.240: gpmDB | psyt | snap homo (0/1) protein peptide no protein information available
2	-127.7	8.56	25/31	11	26	50.0	gi|16128765| gpmDB | psyt | snap protein peptide rhlE:p, ATP-dependent RNA helicase [Escherichia coli str. K-12 substr. MG1655]. DEADc, DEAD-box helicases. A diverse family of proteins involved... PRK10590, ATP-dependent RNA helicase RhlE HELICc, Helicase superfamily c-terminal domain
3	-62.1	8.33	36/37	6	21	20.2	gi|90111568| gpmDB | psyt | snap protein peptide bacterial transferase hexapeptide domain protein [Escherichia coli str. K-12 substr. MG1655]. PaaY, Carbonic anhydrase or acetyltransferase, isoleucine patch ... LbH_gamma_CA_like, Gamma carbonic anhydrase-like: This family is ...
4	-61.3	7.95	9.2/15	6	12½	65.8	sp|K2C1_HUMAN| gpmDB | psyt | snap homo (0/1) protein peptide no protein information available
5	-57.6	7.67	18/56	5	13	20.8	gi|16131228| gpmDB | psyt | snap protein peptide FKBP-type peptidyl prolyl cis-trans isomerase (rotamase) [Escherichia coli str. K-12 substr. MG1655]. SlpA, FKBP-type peptidyl-prolyl cis-trans isomerase 2 [Posttrans... PRK10737, FKBP-type peptidyl-prolyl cis-trans isomerase
6	-49.9	8.08	25/29	5½	17	23.6	gi|16131236| gpmDB | psyt | snap protein peptide cAMP-activated global transcription factor, mediator of catabolite repression [Escherichia coli str. K-12 substr. MG1655]. CAP_ED, effector domain of the CAP family of transcription facto... HTH_CRP, helix_turn_helix, cAMP Regulatory protein C-terminus PRK11753, DNA-binding transcriptional dual regulator Crp

LoxR (*Kosakonia* KO348)

Figure 4.10 LoxR identification by The Global Protein Machine Organization library. Lox R was identified after the LC/MS-MS of the purifiec complex LoxR-OC12.

4.3.4 LoxR and PsrR target gene regulation

It was of interest to test whether some loci known to be regulated by LoxR or PsrR homologs in other bacteria, were regulated by the two LuxR solos in *Kosakonia*. In the case of LoxR, the transcription driven by five gene promoters, which are regulated by SdiA (Sabag-Daigle et al. 2015), were studied by comparing the expression driven by the promoters of a transcriptional fusion with *lacZ* reporter gene in a plasmid construct in *Kosakonia* KO348 and *loxR* mutant KO348*loxR*. The five gene promoters studied were controlling the expression of the virulence associated *srgE* gene and four typeVI secretion system (T6SS)-related genes (one hypothetical and three different vgrGs). The T6SS has been shown to be regulated by SdiA in *Enterobacter cloacae* (Sabag-Daigle et al. 2015) and *srgE* in *Salmonella* ser. Typhimurium (Smith and Ahmer 2003). It was established that *srgE,* the hypothetical T6SS locus and *vgrG2* were not regulated by LoxR in presence or absence of AHLs (**Figure 4.11**). Interestingly, the promoters of *vgrG1* and *vgrG3* displayed differential expression in the presence and absence of AHLs in the KO348loxR mutant in comparison to the WT (**Figure 4.11**). Gene promoter activity *vgrG1* and *vgrG3* was restored to the wild-type levels when the *loxR* mutant was complemented with a *loxR* gene in a plasmid (**Figure 4.11**). These results implicated LoxR in the regulation of two T6SS loci in *Kosakonia*.

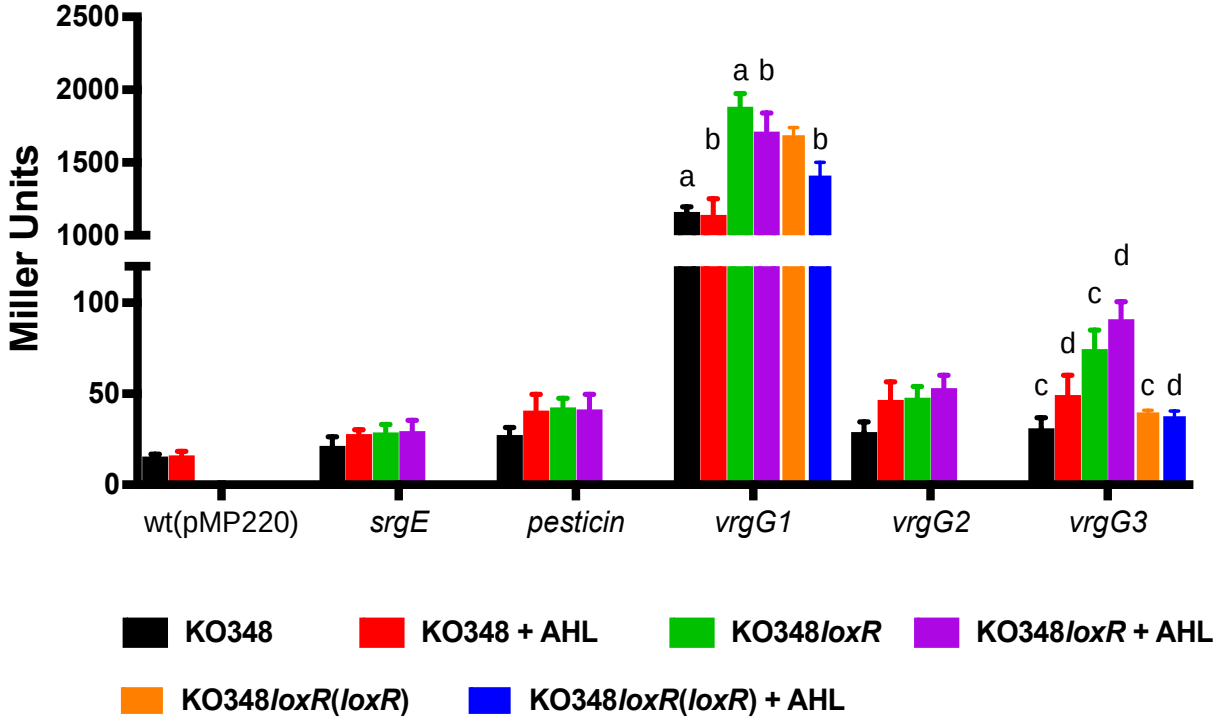
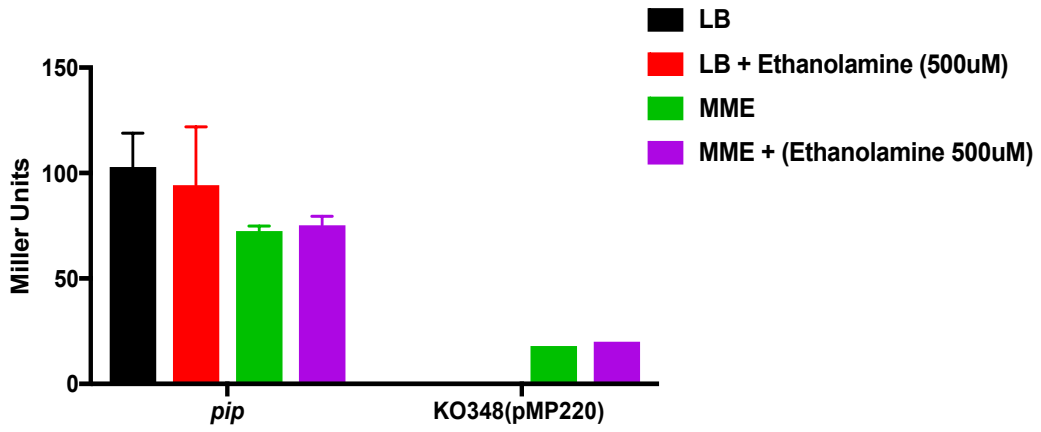


Figure 4.11 Gene promoter activities in the presence or absence of AHLs in *Kosakonia* KO348 and KO348loxR.
β-galactosidase levels (Miller Units) of five gene promoter transcriptional fusions (*srgE*, type VI cluster hypothetical protein, vgrG1, vgrG2 and vgrG3 were tested) by comparing the expression levels between WT (KO348), *loxR* mutant (KO348loxR) and when necessary complemented mutant KO348*loxR*(pBBR*loxR*) in presence or absence of AHLs. The WT strain with empty plasmid wt(pMP220) was used as control. All experiments were performed in triplicate. Statistical analysis were calculated using Student's t-test (P = 0.05) and letters (a–d) indicate statistically different values.

In the case of PsrR, since it belongs to the subfamily of PAB solos responding to plant low molecular weight molecules (Coutinho et al. 2018; González and Venturi 2013), it was of interest to study the expression of the adjacent *pip* gene encoding for a proline imino peptidase. All members of the subfamily of PAB LuxR solos have a *pip* gene located adjacently which is regulated by the LuxR solo (González and Venturi 2013). The *pip* promoter activity was tested in presence of (i) rice root macerate, (ii) ethanolamine and (iii) ethanolamine derivative HEHEAA. The latter two compounds were recently reported to activate *pip* expression and require a PAB LuxR solo in a *Pseudomonas* endophyte (Coutinho et al., 2018). No PsrR dependent *pip* promoter was detected and no induction of *pip* was observed in *Kosakonia* in any of the conditions tested (**Figure 4.12A, 4.12B** and **Figure 4.13**).

a.-



b.-

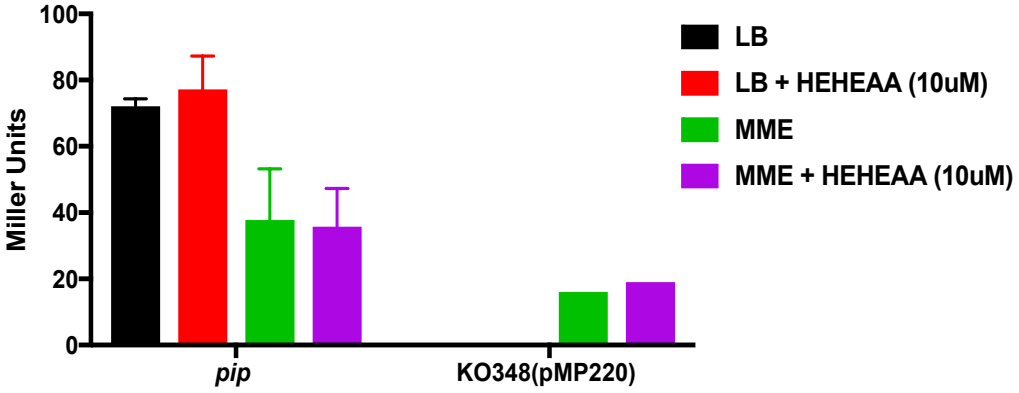


Figure 4.12. *Kosakonia* KO348 *pip* promoter activity is not influenced by ethanolamine

(A) or its derivative HEHEAA (B) β-galactosidase levels (Miller Units) for the *pip* promoter transcriptional fusion (*pip*) and KO348 WT containing the empty plasmid KO348(pMP220) in LB or MME media. All experiments were performed in triplicate.

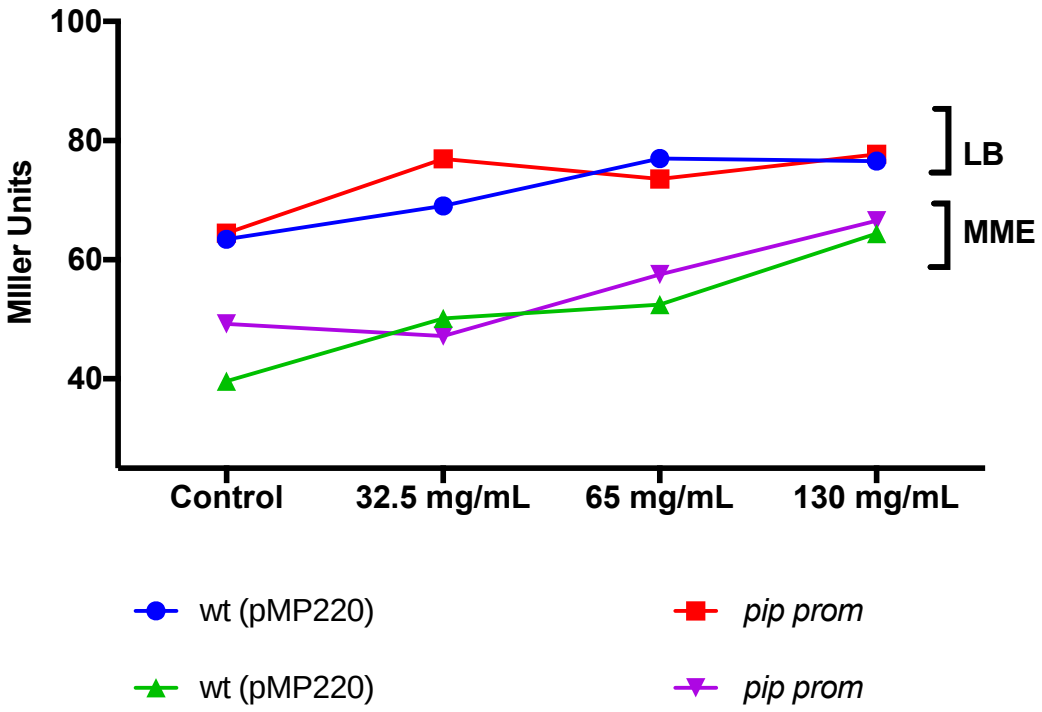


Figure 4.13 .*Kosakonia* KO348 *pip* promoter activity in presence of different concentrations of rice root extract

β-galactosidase levels (Miller Units) for the *pip* promoter transcriptional fusion (*pip*) and KO348 WT containing the empty plasmid KO348(pMP220) grown in LB or MME media supplemented with rice root extract.

4.3.5 Role of LoxR and PsrR *in planta*

Kosakonia strain KO348 is a very efficient rice root colonizer thus it was of interest to determine the role LoxR and PsrR LuxR solos, on colonization ability of the rhizoplane and root endosphere. The knock-out mutants *loxR* and *psrR* were generated and their rice rhizoplane and endosphere colonizing abilities determined. No statistically significant differences in colonization between KO348 wild type and KO348*loxR* were observed in the presence or absence of AHLs both in the rhizoplane and endosphere (**Figure 4.14)**. Similarly, no statistically significant difference in colonization of the rhizoplane between KO348 WT and KO348*psrR* was observed. In the root endosphere however, the wild type was a significantly

better colonizer than mutant KO348*psrR* (**Figure 4.15)** indicating the involvement of PsrR in the rice endosphere colonization of *Kosakonia* KO348.

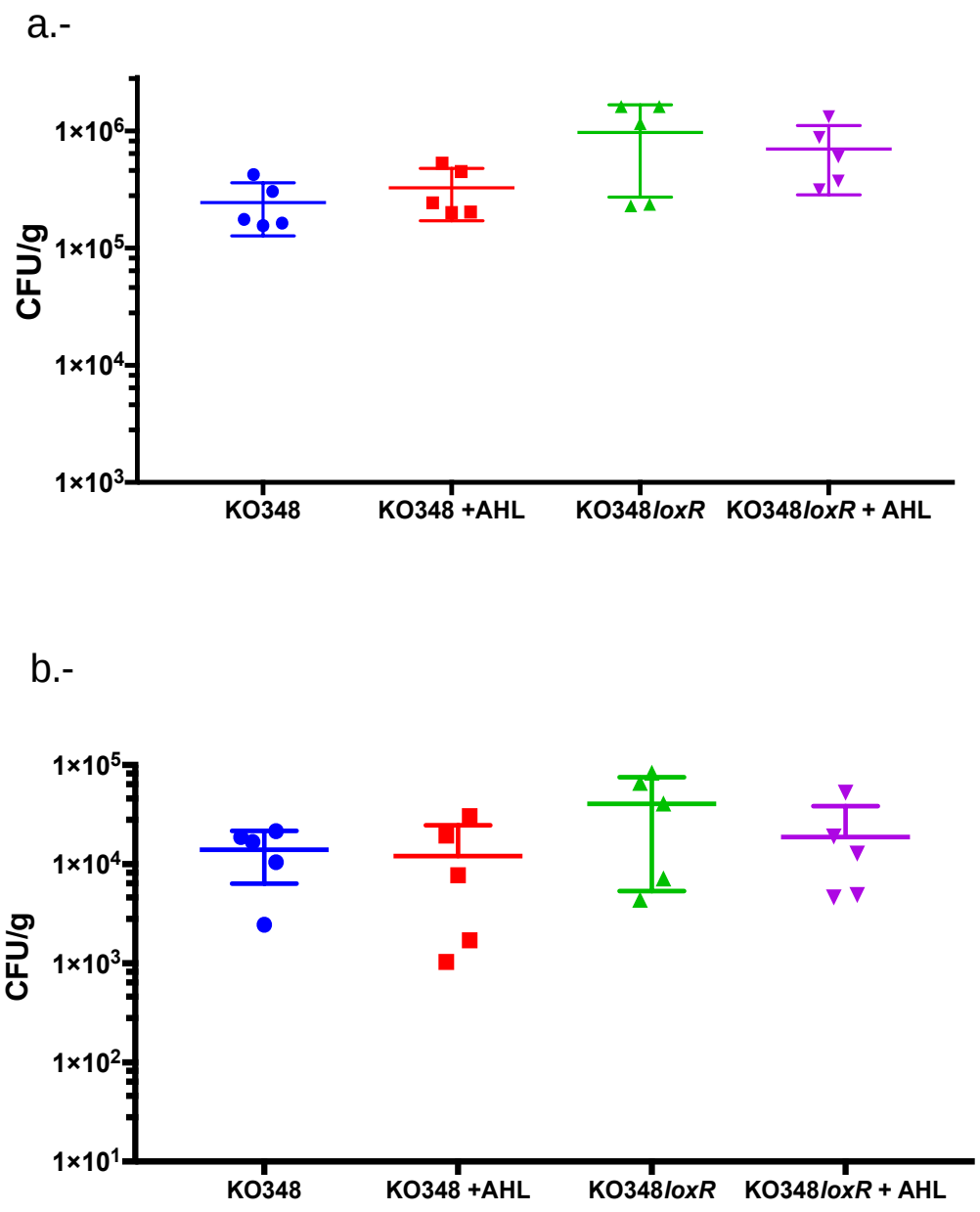


Figure 4.14 Role of LoxR solo of *Kosakonia* KO348 in rhizoplane and endosphere rice root colonization.
The effect of LoxR was tested by comparing CFU/g of KO348 WT vs. KO348*loxR* mutant in the rhizoplane (**A**) and in the endosphere (**B**) of rice root plants at 14 dpi in presence or absence of *N*-acyl-homoserine lactones (AHL).

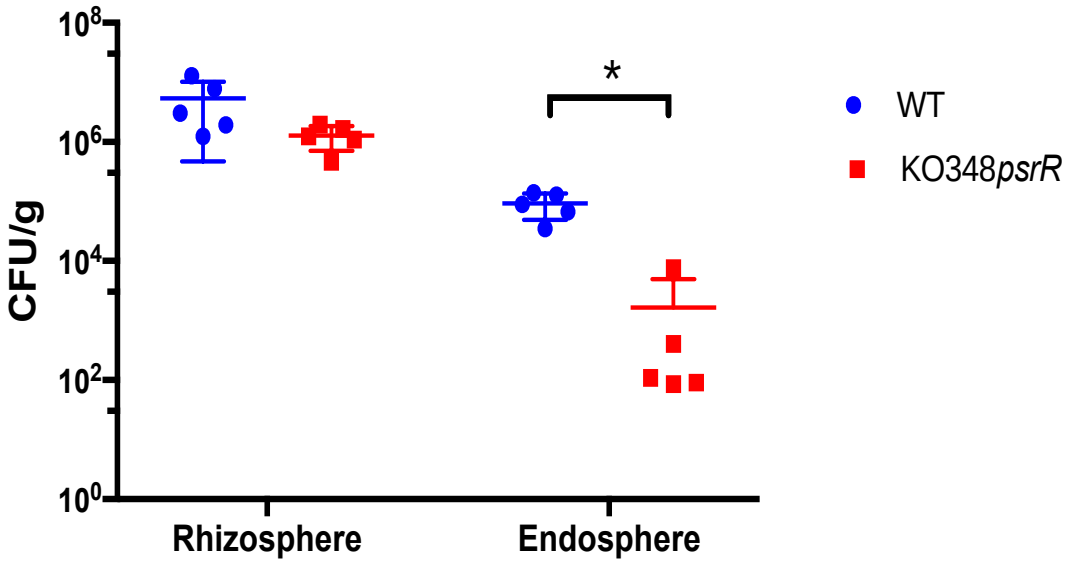


Figure 4.15 Role of PsrR solo of *Kosakonia* KO348 in rhizoplane and endosphere rice root colonization.
The effect of PsrR was tested by comparing CFU/g of KO348 WT vs. KO348*psrR* mutant at rhizoplane and at endosphere of rice root plants at 14 dpi.

4.4 Discussion

LuxR solos are very widespread in proteobacteria (Subramoni et al. 2015; Case et al. 2008), however only few solos have been thoroughly studied. In this chapter two LuxR solos in the recently described genus *Kosakonia* are reported*;* LoxR is an SdiA homolog and is able to bind AHLs whereas PsrR belongs to PAB-subfamily of LuxR solos.

Modeling analysis of LoxR showed high conservation of amino acid residues forming the binding pocket just like *E. coli* SdiA (PDB ID_4LGW), indicating that it was likely to bind AHLs. The LoxR has conserved the amino acids residues, Y63, W67, Y71, D80 and S134, according to *SdiA* numbering (Figure 3) which are important for docking different types of AHLs (Almeida et al. 2016). Evidence for LoxR binding to AHLs included protein solubilization following expression in *E. coli* only in presence of AHLs, which is likely necessary for proper folding and homomultimerization (Zhu and Winans 2001), and the detection of LoxR and AHLs after the fraction (complex AHL-LoxR) purification. These results suggest that the role of LoxR in *Kosakonia* is to detect AHLs produced by neighbouring bacteria for possible interspecies/community interactions, as is the case of SdiA from *Salmonella* and *E. coli* (Michael et al. 2001; Smith and Ahmer 2003; Sperandio 2010).

An SdiA target in *S. enterica* serovar Typhimurium is the *srgE* gene encoding for a type III effector protein (Smith and Ahmer 2003; Habyarimana et al. 2014). The *srgE* gene is not regulated by LoxR indicating that regardless that LoxR is highly identical in structure and probably an ortholog of SdiA, in *Kosakonia* it has evolved to regulate a different set of target genes. In murine isolated *E. cloacae*, SdiA

regulates a hypothetical protein from the T6SS cluster (Sabag-Daigle et al. 2015). In *Kosakonia,* two *vgrG* loci, involved in the assembly of the tip of the T6SS tube, are negatively regulated by LoxR. In endophytes, T6SS has been implicated in two different roles, firstly in host interaction delivering effectors into host cells and secondly in plant defense by antagonizing other microbes (Frank 2011). Interestingly, in *Kosakonia* KO348, T6SS plays a role in rhizoplane and rice root endosphere colonization (see Chapter 2), thus LoxR can possibly play a role *in planta* in response to AHLs produced by neighboring bacteria. Interestingly, in *P. aeruginosa,* AHL quorum sensing (QS) regulates T6SS (Lesic et al. 2009), moreover transcriptomic analysis of a steady co-culture of *P. aeruginosa* and *Klebsiella pneumonia w*here *P. aeruginosa* remained dominant over time, showed an inverse correlation between QS and T6SS expression (Zhao et al. 2018). SdiA is well conserved among members of the Enterobacteriaceae family; SdiA orthologs are present in *Escherichia, Kosakonia, Salmonella, Enterobacter, Citrobacter, Cronobacter, Klebsiella, Pantoea,* and *Erwinia* (Sabag-Daigle and Ahmer 2012). SdiA and T6SS are commonly found in endophytic bacterial genomes, however they are more prevalent among the genomes of phytopathogens (Hardoim and Hardoim 2017). More studies are needed to understand the role and regulation of T6SS by SdiA in plant beneficial bacteria.

I*n planta* studies showed no involvement of LoxR in rhizoplane nor endosphere colonization under the tested conditions. In a PGP *E. cloacae* strain, an SdiA mutant displayed a 4-fold more efficient rhizoplane colonization with respect to the wild type (Shankar et al. 2013). The *in planta* experiment described in this chapter has some limitations since it lacks soil which contains a rich and native microbial community most probably undergoing cell-cell communication including via

AHLs. Therefore, the role of SdiA in root colonization under wild/field conditions may not be inferred from results presented in this chapter.

Homology modeling for the PAB LuxR solo PsrR was analyzed using as models two described PAB LuxR- solo proteins, OryR from rice pathogen *Xanthomonas oryzae* pv. oryzae (Ferluga et al. 2007) and PipR from endophytic *Pseudomonas* strain GM79 (Schaefer et al. 2016). Some key differences between PipR and PsrR by homology modeling were found; PsrR has two important amino acids M and W (at positions W57 and Y61 according to TraR) as they are part of the auto-inducer binding domain and which are conserved among the PAB LuxR-solo subfamily (Patel et al. 2014), and shared between PsrR and OryR; however, in the same positions, PipR has a W and W (at positions W57 and Y61 according to TraR) (Subramoni et al. 2011). This indicates a key difference in the binding-site residues conformation between PsrR and PipR probably resulting in a different response/specificity to plant compounds. PipR activates *pip* gene expression in response to ethanolamine and a derivative (Coutinho et al. 2018); such response did not occur in *Kosakonia* KO348 which confirms that these PAB LuxR solos respond to different (probably related) plant compounds. Rice root extract could not activate *pip* expression in *Kosakonia*; several *pip* genes can be activated by plant extract via PAB LuxR solos (e.g. OryR, PipR and XccR; (Ferluga and Venturi 2009; Schaefer et al. 2016; Zhang et al. 2007) whereas some could not (e.g. PsaR2, Patel et al. 2014). The reason for this is currently unknown, it could be that the signal molecule is present in very low concentrations or only in specific plant part and/or growth stage(s).

In planta studies indicated that PsrR was involved in root endosphere colonization since the mutant displayed significantly less colonization. This phenotype could not

be complemented when providing the wild-type gene in a plasmid; it is believed that this is due to the high-percentage of plasmid loss (see Chapter 2). Other PAB LuxR solos have been implicated in plant colonization, for example PsoR of *P. fluorescens;* XocR of *X. oryzae,* PsaR2 of *P. syringae* pv. *actinidiae* and XagR of *X. axonopodis* pv. *glycines* (Patel et al. 2014; Chatnaparat et al. 2012).

This chapter represents the first report of LuxR solos in *Kosakonia*, here it is described that LoxR binds AHLs and its involvement in the regulation of the T6SS. PsrR has a different binding-pocket conformation than PipR and its unresponsiveness to the PipR signal and plays a role in root endosphere colonization. Further studies in these solos and their target genes, *in vitro* and in planta, will help in the understanding of their role in the endophytic lifestyle of *Kosakonia.*

Chapter V.

Summarizing Discussion

5.1 Scope of the thesis

This thesis was aimed to provide novel insights into the lifestyle of *Kosakonia* rice root endophytes. These studies included comparative genomics, secretome profiling, *in-planta* tests, a field release trial and type VI secretion system involvement in the rhizosplane and endosphere colonization. Additionally, an enrichment strategy was presented for the identification of *Kosakonia* bacterial co-inhabitants for rice root endosphere colonization. Lastly, cell-cell signaling studies involving two LuxR solos and their possible involvement in interspecies and interkingdom signaling was also investigated.

5.2 *Kosakonia* is an efficient rice rhizoplane and root endophytic colonizer

5.2.1 Genomic features

Both *Kosakonia* strains studied in this thesis shared genomic features related to their ability to enter and colonize the endosphere. For example, they have high copy numbers of flagella-related genes and have complete clusters of type VI secretion system. Both flagella and T6SS were evidenced in the secretome profile and were amongst the most abundant proteins. T6SS domains are present among all *Kosakonia* available genomes and in addition, a recent comparative genomic study based in the endophytic model *Kosakonia radincincitans* DSM 16656 described

multiple flagellar and secretion systems contributing to high motility and high competitiveness thus increasing bacterial fitness (Becker et al. 2018). Other genomic features include several loci which are also involved in enzyme activities, signaling, metabolism and uptake of nutrients; however validation studies need to be performed in order to link them to endophytic lifestyle.

5.2.2 *In-planta* colonization studies

Kosakonia strains were able to colonize the rice rhizosphere and endosphere very efficiently and when using two different strains they did not out-compete each other possibly indicating a cooperative behavior or a compatible and non-interfering co-existence. Localization studies by fluorescence microscopy showed the ability to attach and colonize the rhizoplane and root endosphere of rice plants as communities were detectable up to 50 dpi.

5.2.3 Type VI secretion system is involved in endosphere colonization

It has been determined that a T6SS mutant of *Kosakonia* sp. KO348 displayed a significant decrease in rice rhizosphere and root endosphere colonization studies thus suggesting a role in the host-bacteria colonization/interaction. T6SS in endophytes could be involved in host interaction and/or antagonizing other microbes in the endosphere (Frank 2011). As mentioned above, all *Kosakonia* possess a T6SS and some possess more than one system (Becker et al. 2018), future studies will need to determine whether T6SS plays a role in plant life in other members of this genus .

5.2.4 *Kosakonia* LuxR solos are involved in interspecies and interkingdom signaling

Kosakonia KO348 possesses two LuxR solo proteins; these are known to be involved in cell-cell signaling in bacteria. PsrR is a LuxR solo from *Kosakonia* KO348, which belongs to the sub-family of plant-associated solos that responds to plant signals (González and Venturi 2013; Venturi and Fuqua 2013), and plays a role in rice root endosphere colonization since the knock-out mutant displayed significantly less colonization ability. Other PAB LuxR solos have been implicated in plant colonization, for example PsoR of *P. fluorescens;* XocR of *X. oryzae,* PsaR2 of *P. syringae* pv. *actinidiae* and XagR of *X. axonopodis* pv. *glycines* (Patel et al. 2014; Chatnaparat et al. 2012; González and Venturi 2013).

LoxR is a LuxR solo from KO348 which binds AHLs is involved in the regulation of the T6SS as it negatively regulates two *vgrG* loci, involved in the assembly of the tip of the T6SS tube. LoxR is therefore likely to play a role *in planta* in response to AHLs produced by neighboring bacteria. However, i*n planta* studies showed no involvement of LoxR in rhizoplane nor endosphere colonization; since soil was not used in these studies, the absence of a rich microbial community most probably affected this experiment as LoxR responds to AHL signals produced by neighboring bacteria. In a PGP *E. cloacae* strain, an SdiA (LoxR solo ortholog) mutant displayed a 4-fold more efficient rhizoplane colonization with respect to the wild type (Shankar et al. 2013); this indicates that interspecies signaling is occurring *in planta* via LuxR solos.

5.3 *Kosakonia* as a member of the rice root microbiome community

5.3.1 Microbiome studies of a rice field trial release study of a *Kosakonia* strain

The root endomicrobiome composition from a field *Kosakonia* release trial of rice infected seeds showed that under the tested conditions, *Kosakonia* sp. KO774 was able to colonize the rice root only in the first time point analyzed (30 days post-sowing), being rather inefficient over a longer period of time. In addition, the presence of *Kosakonia* strain was not significantly changing the microbiome composition. This release field trial was performed under conditions of nitrogen stress with the aim that diazotrophic endophytic *Kosakonia* would supplement for the lack of nitrogen. Unfortunately, seed inoculation with *Kosaknoia* did not help the plant in the recovery of insufficient nitrogen. More experimentation is necessary on order to optimize the inoculation of rice under different growth conditions before it can be concluded whether *Kosakonia* can be used as plant probiotics.

5.3.2 Identification of *Kosakonia* rice root endomicrobiome cooperators/co-inhabitants

An enrichment strategy was devised in order to identify *Kosakonia* bacterial co-inhabitants/cooperators for endophytic colonization. Strains belonging to *Pseudomonas* and *Sphingomonas* were significantly enriched through the

endophyte inoculation passages in the presence of *Kosakonia*. If the enrichment experiment was performed under salinity stress, strains of *Pantoea* and *Stenotrophomonas* were significantly enriched. Several bacterial genera were enriched in the rice endophyte enrichment strategy and these merit further study as they could represent the most efficient rice endosphere colonizers. Interestingly, under salinity stress, these experiments have clearly evidenced that *Achromobacter* is significantly enriched*;* this genera has been previously connected to salinity stress tolerance (Mayak et al. 2004).

5.4 Future directions

The work of this thesis involved a series of studies in order to begin to understand endophytism by *Kosakonia* sp. therefore it opens several avenues form many future investigations/experiments. Since it has been established these are efficient colonizers, throughput studies like RNAseq and Tn-seq will provide a global picture of the loci that are mostly expressed and most important for bacterial life in the endopshere. In addition, identifying bacterial co-inhabitants/cooperators of *Kosakonia* sp. will be fundamental for interspecies studies and for devising multistrain inocula for rice PGP and/or abiotic stress tolerance. Finally, the PsrR LuxR solo could lead the way in unraveling the first plant-bacterial signaling system in the endopshere.

Chapter VI.

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Chapter VII.

Appendix

Appendix 1. Type VI secretion system annotated proteins present in KO348

>2652308089 Ga0077644_106131 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MINKAWREELQELKRDGSACQFHDAEWGVIRLKLLYRGEFLFFQLNERAL
ICEVSARYSTLDKTSLKRWDDGSVIGADEREALAKIIARYYRLCWKDDLR
IN

>2652308090 Ga0077644_106132 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MKIDKLIKKRVMLHDGVETRFFNQPVELICPRCQKPIEPDLYQSGDFARL
PEDIQAAVAARIKVITFNPETFTRYSAPEGSLLCSAHSCEGGQGKTLVIF
SYKEQQPARYIATLYALLVVSDDQ

>2652308091 Ga0077644_106133 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MRRIFPVFALLFAFSAQAAQTPLTENDFTVEINKQAITLGQDWDRNLLTV
LGKQTREDFVGEVPFGEENYKYYRHIFAGFDIYSANIDWQQRGKSVDSYV
IGQITLHAPTLHTARGVAPSDAKQRVIEHYGAGETDNSDGEEWIMYSLAH
KNIAFDVTGGKVRAINISTGND

>2652308092 Ga0077644_106134 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MHAIHSTLQRLYVVVASFLLSCCACAASATDFSGQWHGSESNESTLTLKL
SQQNNKLTGSYCFITQRGNRIDCPDEQQDNLRGEVKNDTAIVTFDSSFGG
KNGKATLVINGDKLAWHLTQPPEHGDYYAPENYALVKETVHAGATTKIIR
TDNFMLAIRNNCGAFTTPCDDLLYTGARNRDSQQISLHGKTRQDNENRVI
GAEFRNGDVLYLVDYNPPKLVVTQAGKTLVNQAGSWLK

>2652308093 Ga0077644_106135 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MDLLTREEGEALLLKFLSRALKNPSDIETLMTMAREHPSTIPMKGIIYQY
DRMEKNTLSKAELDDLSTLMFFYGP

>2652308094 Ga0077644_106136 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MRNLFTGNVRLSILFICLFSFFSFDAAAVIDINHYDTLETVENITVDKQV
QASLKNVLGADYAAFAGNFDVYGEPRHTADGGLFVEGWLKDLYLENASAF
VIYPDGRLSAAWVVPAASVAHYKSNTGEKRIPDALQQWVSRFQDVSFNTP
AITQTAETFVDFFETPKFKIKVVTVCGNGAHCDEATYYGVRKNDRAEVNL
HGFAVRKSCEQLICPVITYTFKNGTTTYLLSKIDNSLTVIQNNKILLDEK
GIWKAHE

>2652308095 Ga0077644_106137 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MAEVNVIHKEKGDFFILQSDDKYLICMIWPYNSMWDVQKCFILDHAELSR
YSDFHEMVSLAKDMRDNYDKYKHREVPVPEFKVR

>2652308096 Ga0077644_106138 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MSNALCDHIPFLNVNHYTIIPSFIFLLNIASRTAIVQISAWSFN

>2652308097 Ga0077644_106139 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
VFSFCPIHSVARDATVSKKIHEALERNIIVDDIVELTIVQPDENMAKQHV
LVEQQLLNELGVNNVIVLYDRDRVNKKMNDRVVINKLSSHLCNVCPICLS
QKLGLKISK

>2652308098 Ga0077644_106140 RHS repeat-associated core domain-containing protein [Kosakonia oryzae KO348 : Ga0077644_106]
MSDKHAARQGDEIIHSSVFADITSIVAEGVAYAAIGSAVAFAAATAAPLL
GAGAAAASVAAIGSSCLLSGIVGGILANVAGITDDISNMANSLGDALFPP
SPAGKIVTGSANVLTNNKLAARAAGTLTPADTPPAEPQSPASFADYAGML
LAGAKHFGSEMWQPTVGSADAGTSPLEQDKVACKKHSGPQYLAQGSKSVF
INGQPAVRAKDKTTCEGTVSDNVSPNVIIGGETLTVRDIKSGKLPGLAVA
MIALSLIRGRPGKILKNMPCALASAGGGMLADMAVNAIFGSPHPVHAATG
VKVLNDEQELDFSLPGRFPLRLQRSYNSLTSRAGLFGAGWSTVFDSYLVL
TGDEACWFDETGRELRFTLPSVDQAMYSISEGVIIRRNDNGDVAIADDDG
AVWRLFKPTRANPAILRLASLSDEYGNALETGWDEHGRLVRLHDAPCAID
VTFAYDDARFSQRVTSASHFDGEHHWPLMRWHYDARGQLATVTDASGIVT
REYRYNDDGLMVWHRAAGGLESEYRWAMFDHWRVIENRTNTGDGCRFAYD
LDAGLTTVTHYDGQTRQHYWNTQGLIVRFVDERGENWRFEWNDNEQLTRR
IDPLGNAMTFVYDEMGNRVQEIDADGNERATQWLENRALPAVITEPGGST
TRFFYDPHFGLARTVDALGQSTVWHRDEFGQVIEEVDAAGNSRRMEYNDA
GQVIRETDCSGHLTRYHYHPLGWLVAVQTADGEETRYHYDAAGRPVQLER
AEGWLETLRWNEQGLPTEHEAADGSRSAFRYDNTGRLVATRNHLGEEIRR
SWDSRGRLVALHNENGEAYQFRWGADSLLLEEQGLDGVVSRYTYDACGRT
LSRTFAAGHPEAITHRFSWSAAGQLLARSTPEGQTRWRWSAAGFPERISL
HPALDENSWSAEAEQELNFTFDALGRVIGEQGENGTLGWGYDALGNRTSL
QLPDGRELKQFYYGSGHLLSIALDNLPISDFGRDTLHREISRTQGLLTAR
SDYDRLGRLHRRDVFSGNAQRPAPRRWSRRWDYDHRNNLVREERDDNPFN
WYRWKYDDAGRLLTQDGTLPGQEQWRWDAASNPVDTTVQHAVRHNRVTQL
HGIRWQYDIHGRTVEKDNGQTRWRYRYDGEHRLTEVISQPRDRNKPQVQV
SFRYDPLGRRISKTRRQMRAGQPVGQSVTTHFVWDGFRLLQEIHDDVPLT
YVYSEQGSFDPLARIDGITDPDVYWFHNQPNGTPERLTDAEGELRWEGQN
SAWGKLLHETPLRAPEYAQNLRMQGQYLDRETGLHYNLFRYYDPDCGRFT
QQDPIGLAGGLNLYQYAPNAQGWVDPWGLSNRKCSNTNSKQGHTSSAIRN
QSGTAVIHWHDNRSTTNRFGHYSVEIKLNGTSLHTHQAGAPGEHTMVTTR
GFEWLPPAAKKAEVPLPKADEAIKYLGGKLEKDGPLYDLKTQSCVTHVCD
VLRSGGVDVPTEPGAQMKYLLKLLR

>2652308099 Ga0077644_106141 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MQYTLQEGSFSLFPAGWQDTSMTMLRDEESGLSLIVSRGPIPDGSDFEKE
FYRQWDVLRTQMGDIAQSEFARILVGRDNKTRAVEVETVFTRNGQQIWQK
QFAVQAPGAAVVMIFTLSALRAFTDEDGERWDAIKHSLTLHE

>2652308100 Ga0077644_106142 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MTICKVRHLTKIIVMTFALFSSFYSCAEENNMKVQSLWQFATQLKNTIGQ
DVEELDAIIPGRFVRENPNIAERLKAEPFTIDGGIEIRNMEVRLDLHNPG

KVYIISYDVANADILLEDVRKTYPQLKLIDVPRGRSKDETFSWITPLDEK
GNGIGFGFPYAQPSYLKNMTLRNFQD

>2652308101 Ga0077644_106143 type VI secretion system secreted protein VgrG [Kosakonia oryzae KO348 : Ga0077644_106]
MANRITATLPVGDLLFWKLSGREALSESFTLALTVLGTDARADRSKLLGQ
SATINIPTQGTGTRYINGKITRVAVSAVELSGTRYAVYQLTVEPDLWPMK
RDRNLRIFQGQTAPQIVKTLLGEYQVNVEDKLTGSYRTWDYCVQYQESSL
DFISRLMELEGIAYHFRHEADRHVLVLTDAATEHQPFSGYETIPYHQTPS
GGSTDEEGISQWALEDSVTPGIYSLDDYDFRKPNAWLFQARQNPASPQPG
SIDVYDWPGRFVEHGHGEYYARIRQERWQVEHQQIQGTATAVGVAPGNTF
ALYNAPFFSDNGEYLTTEANYFFEENRYASGSDGETVHRIDFTVIPSSVV
FRPAAVTAWPKTYGPQTAKVVGPQGESIWTDKYGRVKVKFHWDRLAKGDD
TSSCWVRVSSAWAGQGFGGVQIPRVGDEVVIDFINGDPDRPIVTGRVYNE
ASMPPWALPAAATQMGFLSRSKDGSVDNANALRFEDKAGEEQVWIQAERN
MDVHVKNDASRSIGSNHSHYVRKNELYRVETNQTQAVKGQTEILTGKGKL
DAVVEQFILASGTQLRLVSGHSAIELNANGKINLIGKSFNFFVEEDGHIT
TGGKLHLNAPGTKAPTTAPGADHKGNINSAVQAKFSPQGNVQHAAPVAAA
PAGAAKPVTKYKAPPPLKGDYVFSNEKSKSQFMPFSDGVVKKINSSPKMQ
SDLKKLMDDQWNISPNVPGGGSWTDTKNKVMVLDPESMADDNEAVMTLAH
EVGHATSPYKNDFSSKSNFVNGMLKDEGQATLNEIQVRREIYHNSGIDIG
SMTDSSNEMKYIQAFKDMDSGKITRDEASKAIGEIYRRGEVASGSTTNEV
YEDHYGNMYDDYMQGKAPH

>2652308102 Ga0077644_106144 protein of unknown function (DUF4150) [Kosakonia oryzae KO348 : Ga0077644_106]
MFANCQLMGVDLAFPDVCLTPMPAPTPIPYPDIALGPTAIPNALNILFMG
MPAHNMATITPLTNGDNPGVATGVASGTVMGPSRHLTGAFTVLLKGTPAT
RLTSVSLQNSTNAIGMRIVPSQFKVLMLAP

>2652308103 Ga0077644_106145 Protein of unknown function (DUF3540) [Kosakonia oryzae KO348 : Ga0077644_106]
MNNLNQPLTLATLPGGQFSARVTHCFDDGSLMVECDGRGWHCRRAVSCVI
APQAGDTVLISAVDNQMWLLAVLERGNEDATELSVPGDLRITSQGALILS
SDALNVSAAKGDCHISEMNYSGDKISAWVTLSRIVGKRAESVWQTVTQMS
QHLFRTTRQTEHVRAGQLDMKAEDYLRMHAQNTVITSKAITKVDSEQIHM
G

>2652308104 Ga0077644_106146 Uncharacterized protein YjbI, contains pentapeptide repeats [Kosakonia oryzae KO348 : Ga0077644_106]
MSQLSAAELQQKVKSGEAIMELNLDGCDLRGCDLSGGIFQEVSFEGASLQ
GCNLQESVFTECQLAGAVLTAAHLEETVFNQCDVAAANFSNTSLLRCVFN
ECTLNGCDFTQSAFDSTQFMRSPLNKSLFTGARLERSTLFECPLDGAKLN
HCHNLLTTYYGIDLRDTDLSGSQFERAVFFNCDQRGKNYAQHQFTGCQFT
DNQLDGADFSGAQLTQCNFKGASLKQARLNNVNATQALFMQADLSGAHAH
GSLFDQAIFVGATLQQASFKQSRFFQSILQHVAARQVDFTLCDFTYADFS
GAEVCEADFRGAIFSRSRFHRARQEGARFADRKGILEYDEELLAAEAWTA
ERHSRIYGDLQ

>2652308105 Ga0077644_106147 Uncharacterized protein YjbI, contains pentapeptide repeats [Kosakonia oryzae KO348 : Ga0077644_106]
MKIIKPLRLSVLNRPFRLQGQNHLGVSVLALLDMSAQPKLRPEVELWQLA
ASELQTSGGVLDMAMPKARAEFLAIGFAYSHHHQDKTACAVRIEIGELSK
TLAVTGDRYWAGSRPTAAKPFEQMRLDWSRAFGGEGFEENPHGIGAVEEN
HNGTRFRRLPNIELLNQRVTSPRAKPEPASFGPLDLLWPRRFRRMGKNYD
ARWLQHDFPGFAPDIDWRVFNAASPDQWWEERDALPPQAAWRIWNMHPEQ
HLQEGTLPPWQARCFMQRQRGDEILFEEIALRATTVWFFPHLEQMVLIWQ
GNQRINEDDAADVLQLMPALEKIGAPRSVNHYRKVLHQRLDKEKGALFAF
REKDLIPEEVIGPWIDSEVQENHSPMRDNQQNRAMQLREQHRARLEAQGA
DTADLLQEMEEPALPKLEDLPEFIEEMERKARQMQAQAETRKAEMEARFP
QMNAQENQPRGPESMMRMQDLLERNADSMSEKKLKQSREALHKLYLMSAA
EQPPAIKLTGDIALIIRQRAERTMAQGGDFSGLDLTGADFSGMDLRGANF
RNALLECANLSQCQLDGADFSNAMLARTDLQGASLCECNFTEASLALAQC
HQTNFTGAQFTESEMTDALFDACDFSHARLEKLLLRKTGFSQCVFRHATL
DNCVFMELTLPQPDFSAATMNKSSFIQCDLQSASFAGAHLEGCSWVESRL
ERALFRDATLITCAVASGGTLCGADFSGAQLKQSNLRQAVLTDARFVRAK
LDNSDLSEAQCEGADFSGATLTGSLFMRTDFRRVRFTDANLMGAMMQKSR
LEGADLSFSNLFRADLSQSIADSTTKFEGAYNKRVKTLPKRDGEVI

>2652308106 Ga0077644_106148 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MDYFKYLAYLFPSLVIVVFIKIAIRDIKNNRIKKRIASDPVHVNARITQA
VAGTPAPNGIVNVTLDYEFNDHTGKVFTQQNVVTVVKTMEMLNYKAGETV
PVIYLRSDPSLNKVNLPRVF

>2652308107 Ga0077644_106149 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MTDLIAKNLTLFSWSIMAIFIYLFIKGLREPTDDAKLIQYRATLRDWYGS
EKEGVLETWAPLKSTPKGEYFLFHFIIVIDRKTQKKSSGFN

>2652308108 Ga0077644_106150 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MMGILLAIGIIAVVVIMCFISVNKTEKIIREGRPIMAVIENIRPVSTDDS
GNTTVSYVLNVEGRKIEGREKIDTFYAPQMQPGMHIKIMYVDDKHFVFIF
EK

>2652308109 Ga0077644_106151 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MFTGDNVGLILSIVAPCFLIYLVFHTGMVHDDFKKNGIRTVAKINNIKQI
STSGTGSPKCVFTLSFTTQDGHDISLEKTQVVTVLDMMPLERERKVDIYY
KKENPKKIWLILESESRIK

>2652308110 Ga0077644_106152 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
VNLYCHHTIHESKFLLLVQFFIYITVNMSVFTETPLIIGVIVSILGIILA
FSTLKPNKDEVTTLRDWSSREKWSGQIIETSLESWHQTDTKYGNDFLYDF
TFTATINDTRKKYVAKGLVRPNEIHKIQKGLTLIIKCNADNPPRIAVMAI
NYK

>2652308111 Ga0077644_106153 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]

MDYLKVLHIILVGLVAGLIIYLICRDIVWGVMKKIILRNPVYTHATISAV
MPGTPSSNGLVNLTIDYEFKDLTGANYSRKNVLTIIKTINLVEYQIGSAV
PVVYLRTNPEKHFLNEGKDGVLIR

>2652308112 Ga0077644_106154 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MSYLSYIMPKLAWFIPLCILLFVGYVVYKNRQRDAQDAYIKEHGVSLDAE
ISDVVYDKIQRINNYFVVVASVKYNYSDKIFVSKRGFSFLITEKEKIIPG
QIIKIRVNRNMPEQFYYEDYQNY

>2652308113 Ga0077644_106155 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MDYLLLIAMAAVFLIIIYKGTARHDRLMRDGRPVMATIENVTPVSSDDAG
NTTIVYTLNIEGRHVKGKEKIDTFFSPQFQPGMQIKIMYINDEDYMFVFK
K

>2652308114 Ga0077644_106156 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MAISYFVTYPLLVVFFGMFVFFVYNIIMAGRGAVKIDPTDAGQAQGEIVN
IRSSSGENSAFINVIIQVRFITADHKIVNTEGKAVIDVVKIPEYQKGVKV
PVTYSKKEPENIKINIPSPLDK

>2652308115 Ga0077644_106157 Protein of unknown function (DUF3592) [Kosakonia oryzae KO348 : Ga0077644_106]
MAAIVFDSPVFIISLIFIVCIGILLYCYYTGVVHDKFKKEGVRTEAKVLS
KEKIGASGTGNTRFRMVVEFTTKNETVTVTTKRYFTPEDLIKIMRNNTVV
LYYLPQDPQQVLLMPGEMK

>2652308116 Ga0077644_106158 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MSDSTMWSIVIVVLIIAWLIYGIMKAGYDDDRFRAKGIKVEAKILDKKNI
GVSGTGNVKFKVKVEFETKDGVVRAQAKRYFTPEDLIKVMRKNTVQLFYL
PENPQQIYLVPQDME

>2652308117 Ga0077644_106159 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MGIEYYSKYFLYGGIAFSLLLSVVFYFMFRGDKTDVAGMKNYLSSSQWQG
EFIDSRIESWQQTNARYGNDFFYDISFRLNDEKLYTAQTLVRPSQMHLMK
EGLKIKVKKGSKNRLAVVEVNFEDN

>2652308118 Ga0077644_106160 type VI secretion system secreted protein VgrG [Kosakonia oryzae KO348 : Ga0077644_106]
MQNRITATLPVGGLLFWKLAGREALSESFTLALTVLGTDARADRSKLLGQ
SATINIPTQGTGTRYINGKITRVAVSAVELSGTRYAVYQLTVEPDLWPMK
RDRNLRIFQGQTAPQIVKTLLGEYQVNVEDKLTGSYRTWDYCVQYQESSL
DFISRLMELEGIAYHFRHEADRHVLVLTDAATEHQPFSGYETIPYHQTPS
GGSTDEEGISQWALEDSVTPGIYSLDDYDFRKPNAWLFQARQNPASPQPG
SIDVYDWPGRFVEHGHGEYYARIRQERWQVEHQQIQGTATAVGVAPGNTF
ALYNAPFFSDNGEYLTTEANYFFEENRYASGSDGETVHRIDFTVIPSSVV
FRPAAVTAWPKTYGPQTAKVVGPQGESIWTDKYGRVKVKFHWDRLAKGDD
TSSCWVRVSSAWAGQGFGGVQIPRVGDEVVIDFINGDPDRPIVTGRVYNE
SSMPPWTLPDDSTRMGFMTRSKDGSKDNASYLFFEDRAGSEAVELHSEKD

MKVSVENDKTVNIDGNRTTTILKEQKDDVTGDASFYYRAKRTTTVDEAET
TTFNNSQTETIKNGRTLNITSGGDVVTVKEGRITEVEGTESHHVTGLVTE
KFDSGQMTTIEGGLTVDVNSGNWTQNVNGGTITISSPNMIRISSKEQIVM
DAPEAVFKPKFHTLSVTAFSESFVGHSASGTGMTSSATGIALSANGMSIG
FKLKDLSKTLFKKEANGVVINYEVTNISNRTLNITNNALYIFT

>2652308119 Ga0077644_106161 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MNLLTHSNQGGGEFTFVVSRSNAKADDKVHALAARKTRELEMTLIDFHLE
SSEMLEVDGYPAVELFYQFKNDNHVIFQRQTLILLDDPAGKKMVSYVGTC
PGEFSEEHQKQYQQIIQSIKFHRAK

>2652308120 Ga0077644_106162 Serine/threonine protein kinase [Kosakonia oryzae KO348 : Ga0077644_106]
MTDHDNNRSVPNALPPGYRFNEFEILEVIGGGGFGIVYRAKDHQLERTIA
IKEFMPSSLAVRNDDMTLVLRSERFSKAFTAGLNSFIQEARLLARFNHPN
LLHVLRFWVQNDTAYMGTVFYSGTTLSRLREKNPTLINEAWIRRMLPMLL
GAIKTIHDEGYLHRDISLDNIQIQDNGLPVLLDFGSARRSIGSVSDETET
MLRPGYAPIEQYTDDNESEQGPWTDIYALGAVLHTLIIGSPPPVSVVRSI
QDTYVPLTQRGLPGYSHTLLQAVDRALSLKMEDRPQTVDEFAALIEMPVA
GIDDVMSVKQPGTMLVPVEETTEKTDALSSVKRYKVPGLVAAGVIVGVIA
GAVLFGGNSSSDTAATQNASNSDQQQTTQQAAEPPRQSEQETTTAPAATP
SQTTQTQAQSQTTAPAVQEPVAQIFVRMNEGEKLTLNGEAQSVSPATNGF
ASLKLQPGRYNLVLQGNGQTRSQTITIGQPGTWLINPQAQ

>2652308121 Ga0077644_106163 type VI secretion system protein VasG [Kosakonia oryzae KO348 : Ga0077644_106]
MSEISRAVLFGKLDTLLFTSLESATAFCKLRGNPYVELVHWLHQLMQQTD
GDLQQVIRHFSLDEEALTRDIVAALDKLPRGASSVSDLSEHIDTAVERAW
VYGSLKYGVTRIRGGHLLAGILKTWSLANVLKGISPQFERISADALLDNF
DAIFANSKESQQVTVALNDDAGAGVPQQQGTLAQYGQDLTARARDGKIDP
VVGRDEEIRQMVDILMRRRQNNPLLTGEAGVGKTAVVEGLALRIAAGDVP
EPLVDVQLWLLDIGMLQAGAGMKGEFEARLQSLINEVQSSPTPIILFIDE
IHTLIGAGGQQGTGDAANLLKPALARGQLRTIGATTWAEYKKYIEKDPAL
TRRFQTVQVAEPDEEKAVLMLRSTVSALEKHHRVLLLDEAVVAAVKLSHR
YIPARQLPDKAVALLDTACARVAVSQSSPPPQLEDCLHRIAALDVEVEIA
NREAKMATGESDRVEKLQVELEKLAQQRDELTARWEQEQALVDAIIALRA
QLHTSPEEAQADIRATLTQQQAELRALQGDAPLLFTSVDANVVAAVVSDW
TGIPLGRMVKNEIDAVLKLADTLNERVIGQRHGLELIAKRVQTSRARLDD
PNKPVGVFMLCGPSGVGKTETALALAESLYGGEQNVITINMSEFQEAHTV
STLKGAPPGYVGYGEGGVLTEAVRRRPYSVVLLDEIEKAHPDVHEIFFQV
FDKGWMEDGEGRHIDFRNTIIILTSNVGTELITGMCADPELMPEPDALRD
ALRPPLLQVFPPALLGRLLVVPYYPLSDEMLAMIVRLQLKRIQRRLADNH
GIVSEVDDSVVEQIVARCTEVESGGRMVDAILTNTLLPLMSQLLLDASAR
DEQYKRLRVTFEQGEFHCQFAA

>2652308122 Ga0077644_106164 type VI secretion system protein ImpH [Kosakonia oryzae KO348 : Ga0077644_106]
MTETLSSAPVITRASALPEAFWQNVMATPWRYDLFTLLRRVDARGGERYP

LGRAPLPRFEPLRIGQTPSLGFAPSTLSSVRQRENSSLYDVSILSFGLFG
PNGPLPVHLTEYASERIAHHQDDSLSAFADLFHHRLALLFYRAWADAQPT
VSLDRQDNKRFEQYIASLIGMGQPGQLEKGSLSPHARFALAGHLTRNGRD
PEGLAKILRSYFNVPVTIVENVPQWMPLSERERARLQGGRHAPRLGQSAF
LGEAVRDVCHKFRIEIGPLNVDTYRRFMPGEKWVTALRDWVRQYLGIEYQ
WAVKVILRSEDVAGATLGGAGRLGYSAWLGTQPRPQARGDLVFSPEG

>2652308123 Ga0077644_106165 type VI secretion system protein ImpG [Kosakonia oryzae KO348 : Ga0077644_106]
MDSKLLEYYNRELAWLREMGQEFAARYPKVAGRLGMRGMDVSDPYVERLM
EGFAFLTSRVQLKMDAEFPRFSQRLLEMVAPNYLAPTPSMAIAELQPDSA
KGDLSNGFVVPRGTMMDSQVMKKNGVTCSYTTAHDVTLLPLKISQVELGG
VPADLPLAQVGLSQRGAQSALRIRLSCDGPVNLSHLDFDRLEFFLSGPDM
QALKLLELVMEHQVGILCQANGQKTPPLVLADDALRQEGFSADQALLPDD
LRNFDGYRLLQEYFAFPSRFLFISLHGLRTMLAQSGEAKSFDIIILLDKA
DAQLERVVDKSHLALHCTPVINLFPKVAERQKLSDSLHEYHLVVDNIRPL
DYEIYAVTKIHASIDGQRDEETFRPFWSSWSQDEGNYGAYFSLRREQRAL
SEHAQRYGTRTGYIGSEVFASLVDEQHAPWREELRYITAEVLCTSRDLPL
MLQQEIGQFVLPDSLPVKTLQLRKGPTPPRPALAEGLSTWRLISQLQMNY
LSLMDGEDGEGAAALRQLLGLYTRLAEAPVARQIEGVRHCVLEPVHRRVP
EPGPIVFARGIGITLTVDEQAFSGFSPYLFGSVLERVFARLVGMNSFTEF
TLKSQQRGEVGYWPPRMGKRALI

>2652308124 Ga0077644_106166 type VI secretion system protein ImpF [Kosakonia oryzae KO348 : Ga0077644_106]
MSNSAHDEESDLLRSGWRSRRGKDTVGARDKMQPSLLDRLTDDAPDKVQE
PVNNNLVSHSALRRHVLRDLQWLFNTINNEAQQDLSGFDQVRRSVVNFGV
SPLAGKRMSDIEWQDIQRKLTDAILHFEPRILPQGLQVRCISDTKSLDLH
NVLSIEIKGRLWCVPYPLEFLFRTDVDLENGHFELKDAG

>2652308125 Ga0077644_106167 type VI secretion system protein ImpE [Kosakonia oryzae KO348 : Ga0077644_106]
MNTLFQQLAGESLRESLAQLESRIRTQPGDADLRAAFAQLLCLDGNWSRA
LAQLKSWQALKPQAQPTVTLLEQAIEGERQRADVMAGRARPVTPDQQWPW
LASMVSALEPEAANASADREAALEMADANPGQLTTQDGQTLNFDWLMDGD
CRFGPVCEAIVNGRYFWLPFSAISAMQFQPPASVTDLVWRHTLVRLQDGS
EQVCQIPARYPLDANADDRFKLCRVTEWQPLPGDAPHYIGQGQKVWLNDS
AEYSLLDLATVSFNVEAADE

>2652308126 Ga0077644_106168 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MKLWLPGLALLAVCGSVQAENYRIVQSPSQKLDVWIDNVADNTPKSWCAK
TLPLRIVASGDKKPSVLNSFLPRLGALLENQCGTLTQVRWKLTDPQGTTL
AEGTADKAKEWDPVVTSTGATTAPAATPSAGLVAPTGRAEDLSPPASRAP
WQEFTLQDGCHLRTFWQGGAGTPALFIPAKEDGKCEKGGWLNGRSVVTQL
SNGVEKKITMTFVHGFPVSGLNASVDADRLLITTVNNERMVVSEGTLAQS
WMILPYIDSLNGWQANGTVAVEISRDVANDPARLQARIEEVRKAWMPWFE
PGTHLNILLIDSLHPQLRNPAVGTYKTVN

>2652308127 Ga0077644_106169 Serine/threonine protein phosphatase PrpC [Kosakonia oryzae KO348 : Ga0077644_106]
MNISTASISRQGERASNQDQTGETIGERAACFVVCDGIAGLPGGDVAAKL
ARNAIITRFDGDEHLNAQYIRQYVNGANRAIREEQKAVQDYHRMGTTMVS
LFIDRDYQLAYWAHAGDSRLYLFRRGWLYHVTTDHSLVQQMKDAGHQTEG
INGNLLYFALGLGEDEREPSYSDVVPIEDGDAFLLCTDGFWHGVTEEQMQ
QSLHMVNTPDEWLTLMNQILLKNGVGAQTQQDNYSAVAVWMGTPQETTLL
HTLSEAAQFFPLRD

>2652308128 Ga0077644_106170 FHA domain protein [Kosakonia oryzae KO348 : Ga0077644_106]
MRFTIITSKPGHQPPQSSCDFYPPGGTIGRGTDNNLVLPDNDRSISRLQA
IVHIAGNGECRVTNRGNVTRVVLNDIPLERGRQVELQDGDILGIDEYRIE
VTDLIQDTRPVTRMAEEMYPKPAQPQVAKPAPAPAQKNAPEGAPASVPTE
IWDSLMQEFSISDSISSSRAKPQEAQNLNPFAAPKGPERNPEDPLSLLNN
NEPLVTPKSLASDQLFNDEQLFKNDSIFNDSTPSALVPPVEPTQKSHAPD
SDELDPLALFGGSGSTKQTRSDDPLGLLSGAVPLAHADELAAQKPAEPQP
IITDLNTPEPPPTAQPIITELRPEDLSSTPLFADDAPLSAPEEVQEQQDY
AGITLPTPQAVQRSAAQTPKGRLRIDPVQSNGTKSTPSVSTGDSGDVLKG
ELLDALLEGMGLSDMQPVPQFDKENMRQFGQMLSMFSQGTVALLSSRSIL
KRGVKADMTMVLDDANNPFKLLPSGKTVLMQMFGTRMPGFMPPKKSVRDA
LIDLQAHQLGMISGIRAIIAAMLQQFNPEQLEDDAKRDGATARLGVLSNR
KAALWDYYVRTYAQTAGEIDDDFHTLFGEAFLHAYDMEVNQYKDSQSGSE
E

>2652308129 Ga0077644_106171 Protein of unknown function (DUF1311) [Kosakonia oryzae KO348 : Ga0077644_106]
MTGSIIRFTPARVKCVALAALVSLSHCAASAAEAPASISGPWRIIANSTD
TQATISFYTKVNDPSYVGRVVRFDNHAVSGDAALNIDCQQPAYLQQSPMT
LNEAIVKTSGERRFPPKIPVAEDFGLNGQGMQKITPIVLQCQQGHLGPDG
ESIGNWVALLSADKLLMYGNDNSYFVLKRVTADEKITPRFSCNAKLSATE
QAICGDNELAAWDRSVTDAYTIQLQQQQEIDPADKATLAGMKAAQRDWLK
KRNQCQTDAACLTKSMQERTFELVSKIQ

>2652308130 Ga0077644_106172 Protein of unknown function (DUF1311) [Kosakonia oryzae KO348 : Ga0077644_106]
MTTHTKQIVAATMTCLALFSSAVQAGNSPVQGEWQVEKAFINTETERTLN
YQFNDDRLVGRFLSVTPQGISTTLPGGSNCQSPAMKESSSTLDAWVAATQ
SIPEKDAAKTYELGLDGSAKTQVENITCASGHFANGDAGSDASLAFVNQR
LLLNWTDGTILLLKPVDKNSKPQASFDCAKAASAPEKAICGDRELAALDN
SVARSYKAFRKEAASLGNNDLENKLQSQQKAWLSQRNSCNGDVQCLKKSM
NDRLETLAHSLDGV

>2652308131 Ga0077644_106173 Phage lysozyme [Kosakonia oryzae KO348 : Ga0077644_106]
MSVMRKGDRGTNVKELQQLLNKSGAKVGEDGIFGLKTEMAVKQFQAKQNL
HVDGIVGRRTLAALGKPVTTRAPQPPISGSAGRQAVGAMDISASGMTFIF
HREAWANKSCYLHWPGGASGVTLGPGYDMKERSEQSIKAKMIEIGIDAIT
AEKISKAARLHDDQASQFVADNAKLVRLTADQETNLLRATVPPYVNAVRN
GIFVPLKQYEFDALVSFAYNPGGRLNNVFGFINRGQISDAMTEIKRANTS

KHKVMKGLINRRNFEVNLFLNGDYGTN

>2652308132 Ga0077644_106174 type VI secretion system secreted protein Hcp [Kosakonia oryzae KO348 : Ga0077644_106]
MAIDMFLKVDGVTGESKDSNHTGWTDITSFSWGASQPGNMSVGGGGGAGK
VNFNDLHVNALIDKSTTAILKHCASGKHLTKVELSVCKAGGQQVEYARIT
LEDVLVTSVQYTGADNGDTVGVTYAFQAAKVKQQYWEQSSSGGKGAETTA
GWNIKENKEA

>2652308133 Ga0077644_106175 Type VI secretion system (T6SS), amidase effector protein 4 [Kosakonia oryzae KO348 : Ga0077644_106]
MRPLYEQLKRYHRSSLKYQPGFLSPEELFKEIGYDYQALKASNPNYENTC
GVRMSLALLKNNIDFTGRFIIKDGPCKGKKIEPGAKLLADQLYKDSVFGK
AEVYTDIHEAGRKLRNRKGVVYFHRIAGYGGGHIDLLEPLSNNMFQCNSG
CHTDSKEVWFWELK

>2652308134 Ga0077644_106176 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_106]
MKMRIGLAGFLVMFSTMISAETKTYSYPQASPLPEDSVLNLMRDPALLNG
FDVNMSAQRFADAWFSKTNERERIKADMYLLGVLDTTEGKTWCGYNRLLP
SSIHENLYSYFENLTAEKGKLRASKVISDAMTELMPCKKGNNK

>2652308135 Ga0077644_106177 type VI secretion system protein ImpC [Kosakonia oryzae KO348 : Ga0077644_106]
MSNPSQQQELQQGAQAFSQDEFSALLSKEFRPKTDQARSAVESAVKTLAQ
QALENTVTFSSDTYRTIQNLIAGIDEKLSQQINQIIHHEDFQKLESAWRG
LSHLVNNTETDEMLKIRFMSISKQELGRNLKRYKGVGWDQSPLFKKIYEE
EYGQFGGEPFGCLVGDYYFDHSPQDVELLGEMARIGAAAHCPFITGTAPS
VMQMESWQELANPRDLTKIFQNTEYAAWRSLRESEDARYLGLVMPRFLSR
LPYGIRTNPVDSFDFEEETDGANHNNYSWANAAYAMATNINRSFKEYGWC
TSIRGVESGGAVENLPCHTFPSDDGGVDMKCPTEIAISDRREAELAKNGF
MPLIHRKNSDFAAFIGAQSLQKPMEYHDADATANARLASRLPYLFACCRF
AHYLKCIVRDKIGSFRERDEMERWLNDWVMNYVDGDPANSSQETKARKPL
AAAEVQVQEIEDNPGYYAAKFFLRPHYQLEGLTVSLRLVSKLPSLKTKEA

>2652308136 Ga0077644_106178 type VI secretion system protein ImpB [Kosakonia oryzae KO348 : Ga0077644_106]
MAISNSGQKFIARNRAPRVQIEYDVEVYGAERKIQLPFVMGVMADLVGKP
VENLPSIEDRKFLEIDVDNFDERMKALKPRVAFNVDNTLTGEGRLNVDLT
FDSMDDFLPDAVARKVEPLNKLLEARTQLSNLLTYMDGKNGAEELIAKVL
QDPTLLKSLSQLPNSEDSAQGKEE

>2652308137 Ga0077644_106179 type VI secretion system protein ImpA [Kosakonia oryzae KO348 : Ga0077644_106]
MTIESLLAPVSPEQPCGENLEYDADFQAMEQASLGKAEQQFGSTIIPAEP
ADWTRVEKLATGLLARTKDIRVMMALTHAWTRRRGLEGYADGLMLLGQAL
ALYWDQLWPSLTDGGEFDPFYRINALAGLSDKSSLTTTLRQSTLLRSNGD
ELNVRDAQALLDGSKTECAGYPGGRVRLIDELTRGGQPGIEAICQIEGRL
QTIRTWLLEQLGESGVPEMEQLLKTVGLIAGVSRANRTEEQQATEQTAPA
DSAPQPVVATPLAAHTDWRTAQVTTRADAQLMLEKVKQYFTQHEPSHPAP

LMIDRVQRLIELDFMEIIRDLAPDGVNQLQNIFGRQD

>2652308138 Ga0077644_106180 type VI secretion system protein ImpM [Kosakonia oryzae KO348 : Ga0077644_106]
MTTTSAISWYGKLPSAGDFLQRRFPDALQRQWSHWFQVGLMNWQKEEQRT
NDRQFSNAPIWNFVVPPMLGGQQVQMGCLLPGRDSVGRQYPLCALMAINP
LEWSPRHLAKAGDWYEQLGRTMLHAVRNGFSPEQLDQALLSIPPVQLVEP
ETRSEILDVIGYDGDGESTIGWRQAAECFDPLRQISFWWTNRSDGYPLYT
HVHSGNFTGQLFTLLFEPAGGARPGRHGLYPPMFEE

>2652308139 Ga0077644_106181 type VI secretion system protein ImpL [Kosakonia oryzae KO348 : Ga0077644_106]
MLLSLLTNRILWGFLGVTGLAAVIWMIGPLLSVVDSRPLESEQNRMITIA
VMYLIWVHSHIVPRLYNAWLNRKLMDNLKSEEAKDPVERKRLNNEDQVLN
ERFEEAAQVLKKAHFNQPGQRGQWAQRFSTQYLYQLPWYVIIGAPGSGKT
TALVNSGLQFPLADRFGKTALRGIGGTRNCDWWFTNEAVLLDTAGRYTTQ
ESEQVQDASEWLKFLGLLQKYRRRQPINGVIVTVSIADLLTQSAEASREQ
ALNLRQRLTELHEQLGIRFPVYVLVTKADLLKGFRAYFAGFDKAQRDQIW
GFTFPWEKAKLADFDLQGSFIQEFALLQQRLDAALPDTLLRESDAQARAE
CFLFPQEFAALRPLLADYLNTIFARSNFETEFSPRGIYFASGTQEGLPFD
RVMGELNRALSLPQGKSGDNWDSVSKEEPVPGGKGQSFFIKHLLQNVIFQ
EAGIAGQNRWWELRSRAVIWSGYAALLALLIIISALWFTSYGNNRDYLDE
VQTKVPALDQQIKALRNRQQGDLFALLPLLNGLSALPESEKFDVNNPPIT
RRMGLYRGNDVADASQTLYQKALQQMLLPEVAMRITTWLRNDNGSDVEYS
YEALKAYQMLYQPKHYDGKFLHSWVMLNLQRNLPQNVTKAQLSQLEWHLT
QLLEPQIQSSPYAKDEALISREQALINQQPLSTRVYGRLKRLLERDENLK
QVSLASLGGPQSELVFSRKSGKPVGEGLPGLYTPDGYWNSFNAQIAPVTA
SLHEDDVWVLGSTTQAEDKQQTDNAVRQLYARDFIAIWDNFLSDIQLNNS
ADLNQRINTARLLSGNNSPLRRLVINLSQQLKLSRDDVADGKEKAPESSN
RGTQMLETLFSNHDGASATANAAASQTPEQRVTEHFAPIIELAQPLEKGG
KTIVFDDFLKQVDELYRYLTAVQDAANSGMPPPGGEAISRLQASAGRLPG
GLQTMFSNMAVGASSDTQRRDMENVRKRISVEVGSFCRQAIAGRYPLVRS
ASSEVTPDDLARMFAPGTGLMDVFFRDNLTNKVDTTQATWRFMPGIDGKT
LPGSEGVLVPFQQAQSIRDAFFANGSATPSFRTTVRTVRMDNTILNMTLD
VDGQILRYSHGPQAVQIVSWPGSGGTNQVRMQLGLANGTTATLVTNGAWA
LNRFFDKARVSPGSSSLSRQATFTVDGHQVTLEFAPNSIRNPFQLPRFAC
P

>2652308140 Ga0077644_106182 type VI secretion system protein ImpK [Kosakonia oryzae KO348 : Ga0077644_106]
MQEQQASGSDAALAGASGNNPLVAAANPLLNAIPQIRYSVSHDDQSGLRQ
HLIDEIRRFEVRCQQSGLAYEVIVGARYCLCTALDEAAALTPWGSRGVWS
GSGLLVTFHNETWGGEKFFQLLARLSQNPREHIALLELINFCLLLGFEGR
YRVMDNGRTQLETIKQRLWQMIRGVRGNYPPPLSPHPEDQPVMRKLWRPV
IPLWACVGLAGFLACLFYIVLNWRLGDSTNPVLAKIYQTQLPEAAIEQPV
QNVQPVLNLRAFLRPEIQAGLVAVRDEADRSVVTLKGDGLFASGSTVARE
SYEPVIDRVAQAMNNVSGKILVVGFSDNVPIRSARFASNYELSLERARSV
QSLLQKHLSQPARVKAEGRGEMNPIAPNNSAENRARNRRVEITLLVSPGN

TAAELNGLPQGN

>2652308141 Ga0077644_106183 type VI secretion system protein ImpJ [Kosakonia oryzae KO348 : Ga0077644_106]
MNKAEKVVWTEGMFLRPHHFQRAESYLQHHIREWGTLQRPYLWGYLDIEL
DDAMLRQGCIALSYASGLLPDGTFFSFHDARQAPTPLAIPDNINNERVVL
ALPARRGGRDEVIFSEEKDSLARYVTWESEVDDDNAMSVGPAAVQFGRLR
LKLMLEKDLSAEWTAIGVAHVVEKRNDNHVRIDSRYIPPMLNAVNNPAIY
AIINDLQSLLMQRSQQIGQRLRQPGRFNTSEMVEFTLLALINHHVGQVSH
LKTLPMIHPEELWRSWLAFATELTTWTASRSPEETLPVYDHDDLAGCFGK
LQLMLRNGLSLVMEEHAIQLPLTERTHGLNIATLPTTTMAREFGFVLAVK
ASVPGEILQTHFPAQMKVAPVTKIRDLVQLQLPGMKLRTMPVAPPQIPWH
AGYNYFELEKGGELWNEMEKSGAFALHLAGEFPGLDMEFWAIRSPTE

>2652308142 Ga0077644_106184 type VI secretion system protein VasD [Kosakonia oryzae KO348 : Ga0077644_106]
MNNNSVVRQISLVFFFVLATLASGCGSSSHSVPTSYNLQFRAHPQINESA
PLKVRVLLLKSDATFMSADFWSLQNNADSVLGANLLNSDEFFLMPGQLSK
TLSGKSAPDARYIGVMAEYQALDGKKWRMSLPLPVQGETHFYEFWKSSSD
ELEANIFLDVNGIRVVSK

>2652309532 Ga0077644_11595 type VI secretion system protein ImpB [Kosakonia oryzae KO348 : Ga0077644_115]
MSDSFQREIPKARINLKLDLHTGGAQKKTELPLKLLVTGDFSNGQETASL
SERVKVNVNKNNFNSVLSDYSPKVNLIVENTLAGNASEENISLTFRDMKD
FTPEEVARQIPQLKAMLAMRNLLRDLKANLLDNQTFRKELEKILLNPSLS
AELRDELSSLAPKQP

>2652309533 Ga0077644_11596 type VI secretion system protein ImpC [Kosakonia oryzae KO348 : Ga0077644_115]
MSVQNESVATGESVVLQGTQAGGVYASLFEKINLNPVTTLSALDIWQDAQ
AMSDATADERLTAGMQVFLECLTKSDSKVEKLDRNLIDHHIAELDYQISR
QLDAVMHHEAFQAVESLWCGLKSLVDKTDFRQNVKIELLDLSKDDLRQDF
EDSPEIIQSGLYKHTYIDEYDTPGGEPIAALISAYEFDASAQDVALLRNI
SKVSAAAHMPFIGSAGPKFFLKDAMADVAAIKDIGNYFDRAEYIKWKSFR
ETDDSRYIGLVMPRVLGRLPYGPDTVPVRSFNYVEEVKGPDHDKYLWTNA
SFAFAANMVRSFINNGWCVQIRGPQAGGAVQDLPIHLYDLGTGNQVKIPS
EVMIPETREFEFANLGFIPLSYYKNRDYACFFSANSTQKPALYDTADATA
NSRINARLPYIFLLSRIAHYLKLIQRENIGTTKDRRLLELELNTWVRSLV
TEMTDPGDELQASHPLRDAKVVVEDIDDNPGFFRVKLYAIPHFQVEGMDV
NLSLVSQMPKAKS

>2652309534 Ga0077644_11597 type VI secretion system protein ImpJ [Kosakonia oryzae KO348 : Ga0077644_115]
MKIYRPLWNEGALLAPQQFQQQSGWESFSRAGLSRLYSPFPWGVERVEFN
EALLASDRVQVQTLRLWLPDGTLVDTQNSDLPPEPREVALPDSGQVESVT
VLIALPVMQPGIVNVQMETVSAERPLRYREEWVAVQDLFGQEEEPMAVAR
FNLAFRFDHESNDAWQTCAVARLLRDGQGGWRQDPDFVPPMAMFSASSLL
RERLVLLNRQLRSRRQRLMAMRRESNDRMADFAVADVSLFWLLNALNTHA

RVLTEFERFPARHPEQVWAELARLAGSLLTFSLDRDLDAIPSYDHHAPEN
TFPPLFELISELLEASLPSRVVAVNMTRLDEQTWKPRRMKQTVAGR

>2652309535 Ga0077644_11598 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_115]
MNQYNYNRLLKQIDSSNDGIYSEQEYLQAVHNPSYRDHLHRIIVMHPSEW
YYGKADIYF

>2652309536 Ga0077644_11599 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_115]
MCNRIYLVYLLLISLLSGIAHSDDDIKETRWVFDKISERNLFGKHNTYTV
NYLNMIYNKSILNIGNKRLIITNDFLENKNICSMEYVKLKKSALSYFMST
NIVNMYSMLFKYENIQFPEDVYEITSLFPGKECPVPYDAIVKAGSNLFVT
DQDYVVFYKQSNVITPQDNALYLNNNWGKYCHNRNVESQFDGTSEYICIF
DNMGIKESYQEVISFDESASGKLSKILPNDNNSYKANGFSVDYKWVDNDR
LKILVAMDSETTSYYFYKNNTGTNLHVLVEAQY

>2652309537 Ga0077644_115100 PAAR motif-containing protein [Kosakonia oryzae KO348 : Ga0077644_115]
MKFGGIGVARKGDKVSCPKEGHGPTTIVEGNPDYLDQGVPVAFHGHKCGC
GCTLISSFSAGKVA

>2652309538 Ga0077644_115101 type VI secretion system secreted protein VgrG [Kosakonia oryzae KO348 : Ga0077644_115]
MVRVIGVSADCRLCAVFFILDWYESGAFYARLHHERELNKSARLHLFSNA
AGLMPGMVLEADGSNLNDLKDGMLMTLITYRAARDTRLHMSVWGMPYSEQ
FCFRPQEEPRPHIHGTLPARIESREQGDIYAWLDNQGRYRVKMDFSREDA
EPGYNYLWIRQAKPCSGDTYGWHTPLTDGTEVGIAYEDGDIDRPYIAHAF
HDSEHQDIVTRDNRSQNILRTAAGNELRMEDLRGQEHIALTTPFGGTQLN
QGHISDEQSKQRGSGFELRTDEYGVIRVAKGLFITADGQQKAVGEVLDME
TALKEIDVCQQQLKALAAAAAEQAQALEADIASQKAMFDQRLKPLNGMIHS
HGPQGVAFTSGEHLQLAAGQNVAVNAGGDFSSGAMGNAAILAGEGVGLFA
RTGSLTLNASEGPVQIQAQNGAMHLSAEQKLSLISASDILFAGKKKVTLI
GGGSYLVIDNGKVEYGTDMTYTRRIKRTYLTAPASLSGMMPSFSQSGICL
SCLLEAAENGAPILIKGE

>2652309539 Ga0077644_115102 protein of unknown function (DUF4123) [Kosakonia oryzae KO348 : Ga0077644_115]
MKHSIVDRLKEIQNESDKRKIYALVDGAQYDRFCTIELFKRNGVMPLFDS
WEDRCLAFAGPWLLALESIDNDLFSILNNLELKYPSVSWILSSSSFDDLF
FHLKNRLEVIMPNNQVAMLRYYDPRVLIFLPEVLTDKQLDVFLMSIISWG
CKYNGEDYFIK

>2652309540 Ga0077644_115103 type VI secretion system secreted protein VgrG [Kosakonia oryzae KO348 : Ga0077644_115]
MGVKVRVQSPSEVARCEDPAISATLPCASCWSKDYAKEIKVNPVKRYSDQ
LNAEGKSYEYNFGARQYKLTIYCKTLKKVSVEIRLKIEPDKGVDETVIEK
SKKSLIKGIKENWDNKFSLKTTDPKCGVKIFPIEFKVEFVASNEHYVFRI
HKQYNREGVTGKFLDVSTDTGSWVYAHEFGHCFGLPDEYGYKAGVQKKDQ
VVYYKPDGKLDAPFSVPYNGGNPAEPSSTIMAAYGNTTILKRHGWLIAIE

ARDLLNEPGLGRKIECDII

>2652309541 Ga0077644_115104 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_115]
MIENNYFIISTNCERQEECVYNNEREIPVVVTIKNISSKGFYIPLKFIEK
TGPGVELIDRRTKKKHALETKYC

>2652309542 Ga0077644_115105 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_115]
MKISTDQLVLLDFYSKNAFANELSEHYKEIFPFIYNELGAKKLKISLAKL
INKAKELNITQRGPLQLYIDMAIVLGYGFDTDPMYSCFSSVNEQYSSGTE
LERSLQIYDKFNGYLKDVMGENNLHILGFKNKLANEKFDGFSNINFSDQI
FDLLNDLYPQKCRYLGCDKVKELICLGLENPDTYYLNLGSRAVFVLVMFV
VGYKFQSDCFHLYEGFFSEETGGDDQFLIYKAKEFLTKYIDAMVT

>2652309543 Ga0077644_115106 type VI secretion system secreted protein VgrG [Kosakonia oryzae KO348 : Ga0077644_115]
MIHFHDPQGVAFTSSEYIQLAAGHNVAVNVCGYFSSGAMGNMAILGGEGV
GLFARTGSLTLNASEGPVQLQAQK

>2652309544 Ga0077644_115107 Protein of unknown function (DUF3304) [Kosakonia oryzae KO348 : Ga0077644_115]
MGLFKLFSKVDNAVNRGYARWGKWIWAALITPFIIYGACIVWASIWGPPV
GPVTLIIHSEIDRPILGFSVNGVGGGNASSYKRNPYSGGGGAATCCGSIS
GKTAEVIWTLDITHEQYLKGMRLEKRRVVMPLPERKWGENDLHVHFLPGD
KVLLGWSDNAWSPYEKRPDIPGKTVKQEHN

>2652309545 Ga0077644_115108 hypothetical protein [Kosakonia oryzae KO348 : Ga0077644_115]
MDSESIIAAANRAQQAEDAGLGNCSRTWHVGFFFDGIHRNIDQDASEQRL
SNVAPSSGYNCKSTRK

>2652309546 Ga0077644_115109 type VI secretion system protein [Kosakonia oryzae KO348 : Ga0077644_115]
MENSSPSLYETLYGNFTGGLDLHQVNEQNQAILSVLDNMQRILNCRAGTL
KHLPDYGLPDMTKILQGMPGTAHQLLQVFSDVLLKYEPRLKKITVVLLEQ
EIPGELRYAIDAELNGIGLVRYGTVFMPEGRVLLRHLKQQQYLDETDKL

>2652309547 Ga0077644_115110 type VI secretion system protein VasL [Kosakonia oryzae KO348 : Ga0077644_115]
MNDISPRKIKTGCDPRTLADYAILRDELSKLTHPARPDVNWRYVEKLCLS
LFEQNGVELQTAAWYTLARTQLAGLFGLNEGLAILEALISHQWGVLWPQP
VHARMEILSNLSQRLQQRMRTLPLNYSDLSQLYRAEQLLTSLGAVLQRLE
LKHLSQLDTLRSMIHNSAVRLENSDGASDSGATIQAGIVLPAPVMNSAGI
LSDALPGIPVTEKREPANTVNWVYVAHPEHQPNVDVLTAMPVQVKKWKPF
AAGMCTMLIISAATVWGWHALHRLDPLQTQLAASLAPLPAILTPAQLDTL
RQHAPIPQTMFTATQQQLVRLGQLPPDWNINYSRQLVEQAQSLWPEQAKP
LAQRWFRQLNAASVPTENLSGWHQGMTKLQQLSNRLSGLDGHKGKYMTVS
ELKSSVFGMMTSFQQTEPTEEQLRQINLLPVASPLRQQLIRQLEQHLRAQ
IYTLGQMKNSAPVTKLLREQAVNPSPLND