Isolation of rhizobacteria from salt tolerant plant species and evaluation of their plant growth-promotion

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Publications

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Statement

"I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice

Gießen, May 07, 2015 **Christian Suárez Franco**

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Summary

Bacteria from rhizosphere samples of *Hordeum secalinum* and *Plantago winteri* from a natural salt meadow in Hessen, Germany, a natural undisturbed ecosystem, were isolated with emphasis on diazotrophs (NFB &LG agar), phosphate- and phytate-mobilising bacteria (CP & IHP agar), ACC (1-aminocyclopropane-1-carboxylate, a precursor of ethylene) deaminase-active bacteria (DF agar) as well as IAA (indole-3-acetic-acid)-producing bacteria (LBT agar) as source of potential halotolerant potential growth-promoting rhizospheric bacteria. Twenty-two isolates, out of 100, mostly belonging to diazotrophic, ACC deaminase producer and P-mobilising bacteria, were selected and their potential plant growth-promoting activities determined in pure culture by evaluating their abilities to grow on specific media, measuring specific metabolic functions. The effect of these 22 isolates on barley plants (*Hordeum vulgare* L.) was evaluated in non-sterile soil under salt stress in greenhouse conditions. Results surprisingly showed that the best performing isolates in pure culture were not those ones displaying the best plant growth-promoting activity in plant growth assays. The partial 16S rRNA gene sequence of the bacteria showed that they belong to the *Proteobacteria*, *Actinobacteria,* and *Firmicutes.* Among the isolates two new species belonging to genus *Rheinheimera, R. hassiensis* E48^T and *R. muenzenbergensis* E49^T, a new species of genus *Cellvibrio*, *C. diazotrophicus* E50^T, and new genus and species *Hartmannibacter diazotrophicus* E19^T were proposed and accepted. Strain E19^T is able to grow on agar containing not water-soluble phosphate sources ($Ca_3(PO_4)_2$, $AlPO_4$ $FePO_4$ and Phytate), DF media agar supplemented with ACC, nitrogen free media, reduce acetylene, and it has phosphatase and ACC-deaminase activities. In addition, reduction of ethylene emission measured on barley plantlets under salt stress showed positive stress relieving effect of E19^T due to its ACC deaminase activity.

Plant growth-promoting activity of the new taxonomically proposed strains were first tested under gnotobiotic conditions in a newly designed liquid plant growth system, and in Mitscherlich pots using subsoil under salt stress with unsuccessful results under such experimental conditions. However, experiments using non-sterile soil under salt stress allowed to evidence the plant growth-promotion capability of *H. diazotrophicus* E19^T on barley plants (*Hordeum vulgare* L.) by significantly increasing root and shoot dry weights, water content in the root system, root-to-shoot ratio and decreasing root Na^+ concentration and root surface sodium uptake. The capability of strain E19^T to colonize barley roots under salt stress conditions was revealed with a specifically designed fluorescence *in situ* hybridization (FISH) probe.

Draft genome sequence of *H. diazotrophicus* E19^T is used at the moment in order to identify the presence of genes contributing directly or indirectly to enable PGPR effects on plants aiming to better understand the mode of interaction of the bacterium and plants, and to furthermore study the phylogenetic relationship with closest genome sequenced related organisms.

Chapter 1

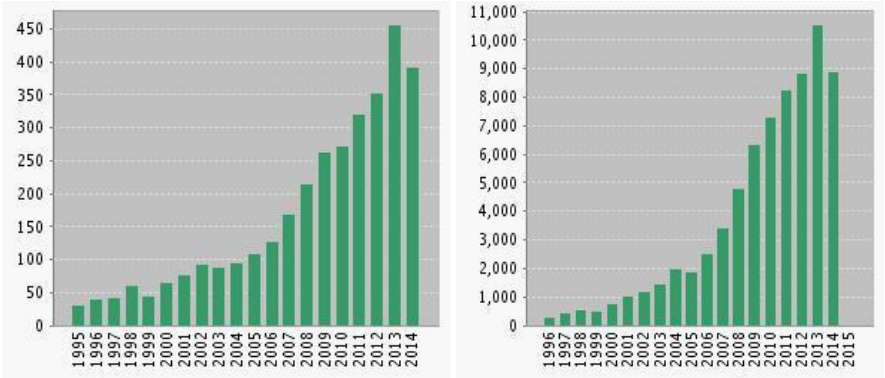
Introduction

1. Introduction

The rhizosphere is defined as a region or volume of soil influenced by the plant root system activity. Plants rhizosphere usually extends a few mm from the root system (Bringhurst *et al.*, 2001), represents a versatile and dynamic ecological environment of plant-microbe interactions influencing plant nutrition (Mayak *et al.*, 2004). Interaction of rhizobacteria and growing plants can be neutral, negative or positive. Neutral interactions are related to commensals bacteria exhibiting no visible effect on growth or physiology of the host (Beattie, 2006). Negative interactions are related to phytopathogenic rhizobacteria and their metabolic products while positive interactions are related to enhance plant growth (Zahir *et al.*, 2003).

1.1 Plant Growth-Promoting Rhizobacteria

The term Plant Growth-Promoting Rhizobacteria (PGPR) is used to define bacteria that colonize the rhizosphere and stimulate plant growth. Since Kloepper & Schroth (1981) introduced the termed PGPR up to day there are more than 4400 search results related to this term in public databases available at web of science v 5.15 (https://webofknowledge.com). 2060 of these results have been published in the last 5 years revealing the increasing interest of the scientific community in this research area (Fig 1).



A: Published papers per year **B: Citations per year**

Fig 1. Number of articles per year in Web of Science (v 5.15) for key words 'plant-growth promoting bacteria', 'plant-growth promoting bacterium', ' PGPR', 'PGPA' and 'PGPRs' obtained from web of science date: A) Published items in each year, B) Citation in each year.

PGPR can/are be considered as an indispensable part of rhizosphere biota that when grown in association with the host plants can stimulate the growth of the host. PGPR strains may have at least three of the following criteria: aggressive colonization, plant growth stimulation or biocontrol properties (Weller et al., 2002; Vessey, 2003). PGPR have also been classified based on their degree of association with plant root cells and their beneficial roles. Classification based on their degree of association with plant root cells divides PGPR in extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR). ePGPR are found as part of the rhizosphere, rhizoplane or endophytic bacteria located at the spaces between root cortex cells. iPGPR are found as intra cellular endophytic bacteria that are located inside specialized nodular structure of plant root cells (Gray & Smith, 2005). PGPR enhance plant growth by direct mechanisms such as nitrogen fixation, nutrient solubilization and production of plant growth regulators and indirect mechanisms such as competitive exclusion of pathogens and stimulation of other beneficial organisms for the plant (Zahir *et al.*, 2003), moreover they have been classified based on the beneficial roles either as biofertilizers, phytostimulators or biopesticides (Martínez-Viveros *et al.*, 2010).

2. PGPR traits

It is widely reported that the application of PGPR influences plant growth by different mechanisms such as fixation of atmospheric nitrogen, solubilization and mobilization of phosphorus, sequestration of iron by siderophores, production of phytohormones, 1-aminocyclo-propane-1-carboxylate (ACC) deaminase, antibiotics, hydrogen cyanide, organic molecules such as vitamins, amino acids and volatile compounds and synthesis of hydrolytic enzymes (Babalola, 2010).

2.1 Nitrogen fixation

Nitrogen fixation by diazotrophic bacteria is the process of conversion of atmospheric N_2 to NH_3 to be assimilated by plants to synthesize nitrogenous biomolecules. Diazotrophic bacteria carry out nitrogen fixation by a highly conserved enzyme complex called nitrogenase. This enzyme complex consists of two components, Fe protein (dinitrogenase reductase) and FeMo protein (dinitrogenase) and is produced and regulated by the *nif* genes (Drummond *et al.*, 1996) or alternative nitrogenases that replace Mo with V or Mo

with Fe regulated by the *vnfH* and *anfH* gene respectively (Zehr *et al.*, 2003). Among these genes the *nifH* gene, that encodes for the dinitrogenase reductase, is a useful molecule in order to detect bacterial diazotrophic diversity (Raymond *et al.*, 2004).

Diazotrophic bacteria in plant microbe interaction could be grouped as symbiotic fixers, rhizosphere-associated or endophytic nitrogen fixers according to their biological nitrogen fixation (BNF) systems. As symbiotic nitrogen fixers, occurring in nodules, utilize organic acids biosynthesized by plants as energy source to fix nitrogen. Rhizosphere-associated bacteria use carbon compounds from exudates for N_2-fixation, and endophytic nitrogen fixers use carbon sources available within the plant tissues and/or from degradation of plant compounds (Terakado-Tonooka *et al.*, 2013) (Sessitsch *et al.*, 2012).

2.2 Mineral solubilization

Solubilization of unavailable forms of minerals, used by plants as nutrients, it is another desirable trait of PGPR because they influence the maintenance of soil fertility by increasing its availability. Inoculation of rhizobacteria increased uptake of nutrient elements like Ca, K, Fe, Cu, Mn and Zn by plants by decreasing the soil pH-value in the rhizosphere, enzymes and/or siderophores (Richardson *et al.*, 2009).

Phosphorus (P) is an essential growth limiting compound for plant growth involved in several important metabolic processes (Vance, 2001). P is generally present at levels of 0.04-0.12 % as organic and inorganic insoluble forms, and its soluble form normally is present in very low levels of 0.1 % or less (Zou *et al.*, 1992). Soluble P concentration in soil is a growth-limiting factor due to the fact that plants absorb P form as mono basic ($H_2PO_4^-$) and di basic (HPO_4^{2-}) ions (Glass, 1989). Phosphate solubilization is an important trait for PGPR and strains holding this activity are considered as phosphate-solubilizing bacteria (PSBs). PSBs act by converting insoluble inorganic forms of P such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite and rock phosphate (Goldstein, 1986; Rodríguez et al., 2006) to soluble forms by producing organic anions or protons (Nahas, 1996). Organically bound phosphorus content in upper layer of arable soils can range considerably between 20 to 80 % total P content, and approximately 40 % corresponds to phytate fraction (Dalal, 1977). Mineralization of most organic phosphorus is mediated

enzymatically by nonspecific phosphatases, phytases, phosphonatases and C–P lyases produced from plants or microorganisms (Tarafdar & Claassen, 1988).

Siderophores are low molecular weight molecules that solubilize and sequester iron(III) from the soil to make it available for microorganisms and plant cells (Neilands, 1995). Although iron(III) oxides and hydroxyls are present in sufficient amounts in arable soils it is due to their extreme low solubility in water that plants and microorganisms suffer from iron limitation. Siderophores secreted by microorganisms can be classified in four main types: catecholates, phenolates, hydroxamates and carboxylates (Saghir *et al.*, 2009).

2.3 Plant growth regulators

Plant growth regulators or phytohormones are organic compounds produced by plants or microorganisms at low concentration able to regulate multiple physiological processes including root initiation, elongation architecture and root hair formation which all influence physiological processes in plants. Plant hormones are grouped in abscisic acid (ABA), auxins, cytokinins (CKs), ethylene, gibberellins (GAs), jasmonic acid (JA) and salicylic acid (SA) compounds. Although, the activity of each plant regulator is known they interact in complex networks involving feedback and cross-talk regulation, being a subject of present study (Woodward and Bartel, 2005; Kochar et al., 2013). PGPR are able to produce and affect plant growth regulator levels in plants benefiting the plant growth by affecting root and shoot hormone concentrations, mediate shoot hormone status or alter root-to-shoot long-distance signaling (Dodd, 2005); nevertheless it is important to take in account that not all PGPR strains able to produce plant growth hormones in vitro alter plant growth *in vivo*.

2.3.1 Ethylene and ACC deaminase

Ethylene (ET) is a gaseous hormone that regulates processes in plants such as seedling emergence, leaf and flower senescence, ripening, organ abscission, and also upregulates plant response to biotic and abiotic stresses such as pathogen attack, heat and cold stress, water logging, drought, excess of heavy metals, high soil salinity, and soil compaction (Morgan and Drew, 1997; Dodd et al., 2010). ET synthesis is described to be produced in a biphasic model presenting two peaks of production in plants (Van Loon et al. 2006; Pierik et al., 2006; Glick et al., 2007). First ET peak is typically smaller that the second peak and

it is believed to be responsible for the transcription of genes involved in plant defensive/protective proteins (Robison *et al.*, 2001). Second ET peak occurs as response to stress, normally several days after the initial ethylene peak, and it is considered to cause detrimental effects by initiate processes such as senescence, chlorosis and leaf abscission in plants. Therefore, the lowering action of ET in the second peak will benefit plant health (Glick, 2014). A sustained high level of ethylene, due to stress, would inhibit root elongation leading to an abnormal root growth, which would affect plant growth and development (Babalola, 2010).

The enzyme 1-aminocyclo-propane-1-carboxylate (ACC) deaminase cleaves ACC, an immediate precursor of ethylene in plants, to form ammonia and α-ketobutyrate (Fig. 2). ACC is produced and exuded by plant tissues and can be metabolized by ACC deaminase-containing microbe (Glick *et al.*, 1998). When stress occurs, ACC is produced and exuded from seeds or roots providing then conditions for ACC deaminase-containing PGPB in the rhizosphere to degrade it. Since ACC is used by bacteria more ACC is exuded from the plant tissue in order to keep an internal-external equilibrium resulting in a reduction of the ACC amount inside the plant cells for ET synthesis (Fig.2). Therefore, the inhibitory effect of ET on plants is reduced by bacterial influence allowing plants to grow and develop longer roots and shoots by ACC deaminase bacterial activity that can reduce by 50 to 90% the second ethylene peak in plants (Glick *et al.*, 1998).

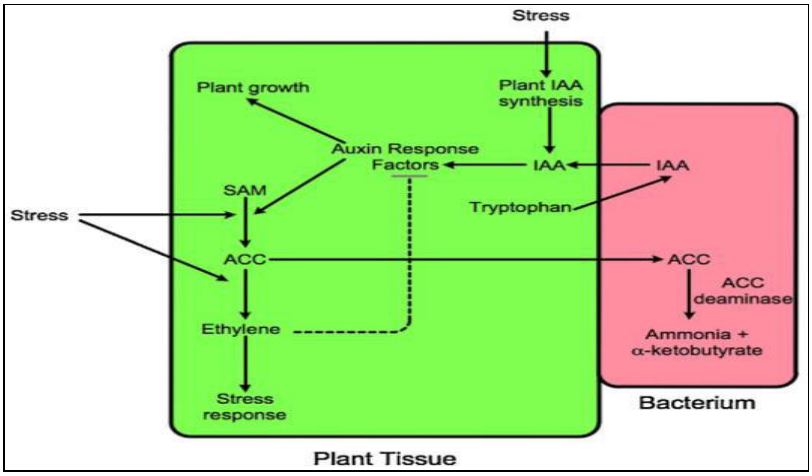


Fig.2: Model of how PGPB can produce ACC deaminase and synthesize IAA facilitating plant growth. ACC; 1-aminocyclo-propane-1-carboxylate; SAM, S-adenosyl methionine; IAA, Indol acetic acid (Glick, 2014).

2.4. Other PGPR traits

PGPR have been reported also to promote plant growth by producing volatile organic compounds (VOCs) able to elicite plant growth by signal molecules mediating plant-microbe interaction when produced at sufficient concentrations (Ryu et al., 2005; Santoro et al., 2011). Some PGPR are able to produce water-soluble B group vitamins niacin, pantothenic acid, thiamine, riboflavine and biotin that can influence plant cells and also beneficial rhizospheric bacteria (Revillas et al., 2000; Lugtenberg et al., 2001). PGPR strains are known to influence the reduction of phytopathogens and deleterious rhizobacteria by their production of antibiotics, hydrogen cyanides and hydrolytic enzymes (Bhattacharyya & Jha, 2012):

3. Salinity

Salinity is one of the most severe environmental stresses on plants (Tester and Davenport, 2003; Munns and Tester, 2008). It affects about 20 % of all irrigated agricultural fields and over 7 % of the world land surface (Szabolcs, 1994) and causes economical global annual costs by loss in crop production in the order of US$ 27.3 billion (Qadir *et al.*, 2014). Moreover, climate change scenarios showed the increasing risk of salinization at different latitudes, and therefore a special effort will be required for maintaining crop production under salt stress (Turral *et al.*, 2011).

3.1 Soil salinization

The soil salinization is primarily influenced by natural causes such as salt water intrusion, deposition of salt by oceanic salt carried in wind and rain and mineral weathering (Rozema & Flowers, 2008). Irrigation of crops with salt waters, inorganic fertilizers and soil amendments are anthropogenic factors that also lead to soil salinization (Ghassemi et al., 1995; Kotuby-Amacher et al., 2000). Soil salinity refers to the accumulation of water soluble mineral salts in soil including cations (Na^+, Ca^{2+}, Mg^{2+}, and K^+), and anions (Cl^-, SO_4^{2-}, HCO_3^-, CO_3^{2-}, and NO_3^-). It is usually reported in mg l^{-1} (ppm), mmol l^{-1} or mmol charge l^{-1} (meq l^{-1}) in solution extracted from a soil saturated with water (Tanji, 2002).

Salinity is measured as electrical conductivity (EC) which is the measure of the amount of electrical current that a material can carry. EC is used to express the magnitude of the total

dissolved electrolytes in soils (Abrol *et al.*, 1998), generally reported as deci Siemens m^{-1} (dS m^{-1}, equivalent to mmhos cm^{-1}) or its transformed units milli Siemens cm^{-1} (mS cm^{-1}) (Tanji, 2002). Soils are classified according to its EC value in classes in non-saline 0-2, slightly saline 2-4, moderately saline 4-8, strongly saline 8-16, very strongly saline > 16 conductivity of the saturation extract (dS m^{-1}) (Abrol *et al.*, 1998).

3.2 Salinity causes different effects in plants

Salinity causes nutritional imbalance in plant growth, development and yield mainly because salt affects nutrient availability, competitive uptake and mineral transport inducing nutritional disorders (Grattan & Grieve, 1999). Salinity reduces N uptake/accumulation (Feigin, 1985)**,** reduction of phosphate uptake/accumulation by reducing phosphate availability (Sharpley *et al.*, 1992)**,** reducing K^+ net uptake and its translocation by lowering K^+ content in shoot and increasing K^+ in root (Botella *et al.*, 1997).

Limitation of plant growth by salinity is primarily due to reduction of water uptake from soil by osmotic effects. Damage is mainly caused by excess of Na^+ and Cl^- ions and nutrient deficiencies caused by Na^+ competition with other ions (K^+, NO_3^- and $H_2PO_4^-$), needed for plant nutrition (Tester & Davenport, 2003). Toxicity by Na^+ affects plants more than toxicity caused by Cl^- because Na^+ causes cell swelling and several disorders at enzyme activation and protein synthesis processes resulting in reduced energy production and other physiological changes (Tester and Davenport, 2003; Larcher, 1980). Excess of Cl^- in plants accumulates in shoots inhibiting photosynthesis mainly by inhibition of nitrate reductase activity (Xu et al., 2000; Flowers, 1988). Effects of salinity on plants lead to anatomical and morphological changes, leaf discoloration, inhibition of seed germination, seedling growth, flowering and fruit set (Tester and Davenport, 2003; Sairam and Tyagi, 2004). In order to maintain water homeostasis and normal physiological functions produced by salinity plants overproduce compatible organic solutes such as proline and glycine betaine (Serraj & Sinclair, 2005). Proline maintains higher leaf water potential and protects plants against oxidative stress by adjusting osmotic pressure and stabilizing membranes, constitutive proteins and enzymes, scavenging free radicals, and buffering cellular redox potential during salt stress (Ashraf and Foolad, 2007; Peng et al., 2008; Kohler et al., 2009). The time frame of salt effects on plants have been described by Munns and Sharp (1993) and it is proposed as a two-phase growth response concept. First

phase or osmotic phase is of short duration and reduce growth by the water stress due to the root surrounding salt. The second or ion-specific phase takes time to develop and it is caused by the excessive levels of salt accumulation in cell vacuoles of transpiring leaves leading to the reduction of growth of younger leaves by the lack of carbohydrates supply to growing cells (Munns, 2002).

Plants have different mechanisms to handle salinity tolerance that are grouped in three different categories. As a primarily mechanisms in order to reduce osmotic stress plants decrease leaf area and stomatal conductance that benefits the plants only if there is sufficient soil water available. The second mechanism consists of Na^+ exclusion by roots in order to avoid its accumulation to toxic concentration in leaves. The third mechanism is the tissue tolerance that consists in accumulation of Na^+, or in some species such as barley also Cl^-, by compartmentalization of these ions at cellular and intracellular level in order to avoid toxic concentration at cytoplasmatic level. This process occurs especially in leaves mesophylic cells and leads to toxic levels of Na^+ with time (Munns & Tester, 2008).

3.3 Crops and salt stress

Most of the cultivated plants declined yields even at values that are lower than the defined value for salinity (EC= 4 dS m^{-1}) (Maas, 1990). Tolerance of some crops to saline conditions is resumed in Table.1. Salt-sensitive plants when exposure for few days to salt will reduce the plant growth rate with no many visible changes. Extended exposure effects of few weeks will become evident by the yellowing or death of older leaves and a more evident reduction of growth. On the other hand under moderate salinity salt-tolerant plants are able to grow for several months, although flowering or decreased production of florets may result (Munns, 2002).

Table.1. Tolerance threshold values of some crops to saline conditions. Salinity expressed as electrical conductivity of the saturation extract (Brady & Weil, 2008)

Sensitive (0-4 dS m^{-1})	**Moderately tolerance** (4-6 dS m^{-1})	**Tolerant** (6-8 dS m^{-1})	**Highly tolerance** (8-12 dS m^{-1})
Almond	Corn	Fig	Barley
Bean	Grain Sorghum	Oats	Cotton
Clover	Lettuce	Pomegranate	Olive
Onion	Soyben	Sunflower	Rye
Potato	Tomato	Wheat	Wheatgrass

Barley (*Hordeum vulgare* L.) is one of the world´s most extensively cultivated crops and being, according to FAO, the European Union its highest producer (Table.2). Barley is the most salt tolerant cereal, reported to die only after extended periods at salt concentrations higher than 250 mM NaCl (equivalent to 50 % seawater) (Munns *et al.*, 2006). Due to its salt tolerance barley crops may be suitable to be used in salt remediation of salt impacted soils (Chang *et al.*, 2014).

Table.2. Last year barley crop production in millions of tons data from FAOSTAT (FAOSTAT, http://faostat.fao.org/):

Countries	2009	2010	2011	2012	2013
European Union + (Total)	62.2	53.3	51.9	55	59.8
Russian Federation	17.8	8.3	16.9	13.9	15.3
Northern America + (Total)	14.4	11.5	11.1	12.8	14.9
Germany	12.2	10.4	8.7	10.4	10.3
France	12.8	10.1	8.7	11.3	10.3
Canada	9.5	7.6	7.7	8.0	10.2
Spain	7.3	8.1	8.3	5.9	10
Australia & New Zealand + (Total)	8.3	7.6	8.3	8.6	7.8

4. PGPR under salt stress

4.1 Bacterial osmotolerance

Microorganisms have developed different adaptations to counteract the outflow of water which enables them to also grow in high osmolarity environments that cause a rapid lost of cell water along the osmotic gradient causing reduction in turgor and dehydration of the cytoplasm. When bacterial cells are exposed to high osmolarity the cytoplasm is exposed to high ionic strength, in order to maintain osmotic equilibrium accumulation of K^+ could serve as a second messenger activating additional osmotic responses. As response, cells upregulate genes involved in adaptive, protective, metabolic, and amino acids transport processes and production of organic compatible solutes in order to equilibrate the intracellular potassium concentration. (Miller and Wood, 1996; Shabala, 2009). Osmolytes produced by bacteria as organic compatible solutes can be sugars and derivates, polyols, α- and β-amino acids and their derivatives, betaines and/or ectoines (Paul and Nair, 2008; Lamosa et al., 1998; Roesser and Müller, 2001). Compatible solutes function as osmoprotectants and also supporting protein stability, folding and function *in vitro* and *in vivo* (Street *et al.*, 2006). Other mechanisms to survive under salt stress is the production of

exopolysaccharides to enhance water retention to protect cells from osmotic stress and fluctuations in water potential (Sandhya *et al.*, 2010) changes in the fatty acid composition of the bacterial membrane (Klein *et al.*, 1999) and/or shortening peptidoglycan inter-peptide bridges (Piuri *et al.*, 2005).

4.2 Plant promotion of PGPR in crops

The use of PGPR is a promising agricultural practice to help less salt tolerant horticultural crops to maintain an acceptable level of productivity under higher salt concentrations (Nadeem et al., 2012; Singh et al., 2011). PGPR have been related to influence plant health under salt stress on several parameters such as increasing biomass, root system surface, improving germination rate, enhancement of chlorophyll content and resistance to diseases. Among PGPR mechanisms reported to influence plant growth under salt stress are enhancement of plant nutrient uptake, production of ACC deaminase, production of phytohormones, increase K^+ ion concentration, induce systemic tolerance, ion homeostasis mediation, induced antioxidative enzymes, contributing to osmolyte accumulation and production of bacterial extracellular polymeric substance (Paul and Lade, 2014; Ryu et al., 2005; Nadeem et al., 2012; Yang et al., 2009).

4.2.1 Enhancement of plant nutrient uptake

PGPR improve nitrogen and phosphorus uptake, solubilizing inorganic phosphate and mineralizing organic phosphate (Diby et al., 2005; Ogut et al., 2010, Upadhyay et al., 2011). PGPR inoculation influencing positively plant biomass, increase of N, P, K^+, and Ca^{2+} absorption and decrease of Na^+ absortion have been reported in cotton by *Klebsiella oxytoca* Rs-5 and *Pseudomonas putida* Rs-198 under salt stress (Yue et al., 2007; Yao et al., 2010).

4.2.2 Plant growth regulators

Beside the effect of PGPR lowering the ethylene concentration and thereby stress signal for the plant Glick (2014) suggested a cross-talk between IAA and ACC deaminase where by lowering plant ethylene levels, ACC deaminase facilitates the stimulation of plant growth by IAA (Fig. 2). There are several reports of ethylene emission reduction by inoculation ACC deaminase producing bacteria e.g. *Achromobacter piechaudii* on tomato

plants (Mayak *et al.*, 2004), *Achromobacter xylosoxidans* on Madagascar periwinkle (*Catharanthus roseus*) (Karthikeyan *et al.*, 2012) and *Bacillus licheniformis*, *Brevibacterium iodinum* and *Zhihengliuella alba* on red pepper seedlings (Siddikee *et al.*, 2011). Also a *Streptomyces* strain reported to promote growth in wheat under salt stress by production of indole acetic acid and auxin, phosphate solubilization and siderophore production even though no ACC deaminase is evaluated (Sadeghi *et al.*, 2012). Bacterially-mediated plant tolerance to salt stress has been reviewed and includes diverse functional and taxonomical groups of bacteria (Dimkpa *et al.*, 2009). Diversity of rhizobacteria mediated plant tolerance to salinity stress involving ACC deaminase activity in different plant species is reviewed in Table.3. ACC deaminase production has been reported in strains belonging to *Proteobacteria*, *Actinobacteria*, *Firmicutes* and '*Bacteroidetes*' (Glick, 2014; Nadeem et al., 2010).

4.2.3 Induced systemic tolerance

Yang et al. (2009) proposed the term induced systemic tolerance (IST) to the effect of VOCs, produced by PGPR, that induce physical and chemical changes in plants enhancing tolerance to abiotic stresses, including salt stress (Farag *et al.*, 2013). Zhang et al., (2008) reported that plant growth promotion triggered by VOCs from *Bacillus subtilis* GB03 confers salt tolerance in *Arabidopsis thaliana* reducing Na^+ levels and recirculation of Na^+ in the whole plant under salt condition by accumulation of tissue specific high affinity potassium transporter HKT1, that mediate Na^+ transport, expression down regulated in roots and upregulated in shoots. Furthermore, PGPR inoculation increased iron uptake, redistributed whole-plant auxin, increased leaf cell expansion, and influenced root branching (Zhang et al., 2007; Zhang et al., 2008). Similar effects have been also studied in white clover and wheat (Han et al., 2014; Zhang et al., 2014).

4.2.4 Ion homeostasis mediation

As an effect of salinity the availability, transport and mobility of Ca^{2+} and K^+ are affected in growing parts of plants. Potassium can act as a cationic solute responsible for stomatal movements as a response to changes in water status on bulk leaf (Caravaca *et al.*, 2004) and Ca^{2+} regulates early signaling processes at the onset of salt stress. PGPR can influence in host physiology and in the foliar reduction of Na^+ and Cl^- ions accumulation by

increasing K^+ and Ca^{2+}. Wheat plants separately inoculated with *Pseudomonas putida*, *Enterobacter cloacae*, *Serratia ficaria* and *Pseudomonas fluorescens* have been reported to increase the K^+/Na^+ ratio by increasing K^+ effectively influencing salinity tolerance (Nadeem *et al.*, 2013).

Table.3. Rhizobacteria reported in literature as PGPR under salt stress involving ACC deaminase activity in different plant species.

Rhizobacteria	Plant	Reference
Achromobacter xylosoxidans	*Catharanthus roseus*	Karthikeyan et al. (2012)
Achromobacter piechaudii	Tomato (*Lycopersicon esculentum*)	Mayak et al., (2004)
Acinetobacter sp.	Barley (*Hordeum vulgare*), Oats (*Avena sativa*)	Chang et al. (2014)
Alcaligenes faecalis	Rice (*Oryza sativa*)	Bal et al. (2013)
Azospirillum sp.	Durum wheat (*Triticum durum*)	Nabti et al. (2010)
Bacillus pumilus	Rice (*O. sativa*)	Jha et al. (2011)
Bacillus pumilus	Rice (*O. sativa*)	Bal et al. (2013)
Bacillus sp.	Avocado (*Persea gratissima*)	Nadeem et al. (2012)
Bacillus licheniformis	Red pepper seedlings (*Capsicum annuum*)	Siddikee et al. (2011)
Bacillus aryabhattai	Canola *(Brassica campestris)*	Siddikee et al. (2010)
Brevibacterium casei	Pea nut (*Arachis. hypogaea*)	Shukla et al. (2011)
Brachybacterium saurashtrense	Pea nut (*A. hypogaea*)	Shukla et al. (2011)
Brevibacterium iodinum	Red pepper seedlings (*Capsicum annuum*)	Siddikee et al. (2011)
Brevibacterium epidermidis	Canola *(Br. campestris)*	Siddikee et al. (2010)
Burkholderia sp.	Tomato (*L. esculentum*)	Onofre-Lemus et al. (2009)
Burkholderia caryophylli	Wheat (*T. aestivum*)	Shaharoona et al. (2007)
Haererohalobacter sp.	Pea nut (*A. hypogaea*)	Shukla et al. (2011)
Hartmanibacter diazotrophicus (from this study)	Barley (*H. vulgare*)	Suarez et al. (2015)
Klebsiella oxytoca	Cotton (*G. hirsutum*)	Yue et al. (2007)
Enterobacter sp.	Tomato (*L. esculentum*)	Kim et al. (2014)
Enterobacter cloacae	Wheat (*T. aestivum*)	Nadeem et al. (2013)
Enterobacter aerogenes	Maize (*Z. mays*)	Nadeem et al. (2007)
Methylobacterium fujisawaense	Canola *(Br. campestris)*	Madhaiyan et al. (2006)
Micrococcus yunnanensis	Canola *(Br. campestris)*	Siddikee et al. (2010)
Ochrobactrum sp.	Rice (*O. sativa*)	Bal et al. (2013)
Pseudomonas sp.	Barley (*H. vulgare*), Oats (*Avena sativa*)	Chang et al. (2014)
Pseudomonas putida, *P. fluorescens*	Wheat (*Triticum aestivum*)	Nadeem et al. (2013)
Pseudomonas sp.	Mung bean (*Vigna radiata* L.)	Ahmad et al. (2013)
Pseudomonas sp.	Avocado (*Persea gratissima*)	Nadeem et al. (2012)
Pseudomonas pseudoalcaligenes	Rice (*O. sativa*)	Jha et al. (2011)
Pseudomonas fluorescens, P. aeruginosa, P. stutzeri	Tomato (*L. esculentum*)	Tank and Saraf, (2010)
Pseudomonas putida, P. fluorescens	Wheat (*T. aestivum*)	Nadeem et al. (2010)
Pseudomonas spp.	Wheat (*T. aestivum*)	Shaharoona et al. (2007)
Pseudomonas syringae, P. fluorescens	Maize (*Z. mays*)	Nadeem et al. (2007)
Pseudomonas fluorescens	Groundnut (*Arachis hypogaea*)	Saravanakumar and Samiyappan, (2007)
Pseudomonas putida	Canola *(Brassica campestris)*	Cheng et al. (2007)
Rhizobium sp.	Mung bean (*Vigna radiata* L.)	Ahmad et al. (2013)
Serratia ficaria	Wheat (*T. aestivum*)	Nadeem et al. (2013)
Serratia sp.	Wheat (*T. aestivum*)	Zahir et al. (2009)
Streptomyces sp.	Tomato (*L. esculentum*)	Palaniyandi et al. (2014)
Variovorax sp.	Avocado (*Persea gratissima*)	Nadeem et al. (2012)
Zhihengliuela alba	Red pepper seedlings (*Capsicum annuum*)	Siddikee et al. (2011)

Inoculation with *Pseudomonas* sp. on eggplant (*Solanum melongena* L.) significantly increased K^+ and Ca^{2+}, and decreased Na^+ shoot concentrations under saline conditions but not under non stress conditions (Fu *et al.*, 2010). Similar results in cotton by inoculation of *Pseudomonas putida* Rs-198 increased K^+ and Ca^{2+}, and decreased Na^+ in leaves and roots (Yao *et al.*, 2010).

4.2.5 Induced antioxidant enzymes

Salinity induces in plants the formation of reactive oxygen species (ROS) due to a salt shock that brings about damage to lipids, protein and nucleic acids and eventually death (del Rio *et al.*, 2003). As a response to salt stress antioxidant enzymes such as catalase, guaicol peroxidase and superoxide dismutase are increased in plants to improve salt tolerance (Mittova *et al.*, 2002). Gururani et al. (2013) reported improvement in plant tolerance to salt stress by two ACC deaminase producers and phosphate solubilizers, *Bacillus pumilus and B. firmus* strains, inoculated on potato (*Solanum tuberosum*) by positively influencing photosynthetic activity, higher proline content in tubers, enhancing of mRNA expression, and specific activities of ROS scavenging enzymes. Similarly, Kim et al. (2014) reported increases in fresh weight, dry weight, plant height of tomato, and enhancement of reactive oxygen species scavenging enzyme activities in aerial plant tissue (Nakano and Asada, 1981) under salt stress by an ACC deaminase and IAA producer *Enterobacte*r sp. strain inoculation.

4.2.6 Contribution to osmolyte accumulation

Improvement of plant growth parameters on wheat plants under different salt stress conditions by the inoculation of *Azospirillum* strains has shown an increase in proline accumulation (Zarea *et al.*, 2012). Moreover, plant growth promotion and increasing of proline and also total soluble sugar content accumulation in wheat plants inoculated with single bacteria and coinoculation of *Bacillus subtilis* and *Arthrobacter* sp have been reported to influence the osmotolerance under salt stress (Upadhyay *et al.*, 2012).

4.2.7 Production of bacterial extracellular polymeric substance

PGPR have been related to increase water holding and fertilization by influencing soil particles aggregation and enhance volume of macropores helping plants to manage salt

stress by producing extracellular polymeric substances (Roberson & Firestone, 1992). Furthermore, extracellular polymeric substances are able to bind to cations decreasing their availability for plant uptake and therefore helping to alleviate plants stress conditions (Upadhyay *et al.*, 2011). Plant growth-promotion by exopolysaccharides producing bacteria efficiently reduced plant cation uptake under salt stress as reported by Siddikee et al. (2011) on red pepper seedlings by *Bacillus licheniformis*, *Brevibacterium iodinum* and *Zhihengliuella alba,* and by Upadhyay et al. (2011) on wheat by co-inoculations of *Bacillus* species and *Enterobacter* species.

5. Bacterial root colonization

The study of the interaction of PGPR with rhizosphere, roots and their natural environment is essential in order to elucidate the successful inoculation, colonization, behavior, functioning and successful application (Jones et al., 2004; Bloemberg, 2007). In order to evaluate root colonization and interaction the gnotobiotic system may be used to accomplish this aim. One example for a gnotobiotic system was described by Lugtenberg et al. (2001) as a sterile system that allows sterile seedling germination in interaction with high bacterial suspension in long quartz sand column moisturized with a plant nutrient solution (PNS) without added carbon source (Lugtenberg *et al.*, 2001). Microscopic techniques have been used to observe localization of soil microorganisms on soil and plants systems in controlled or field environments by light microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and fluorescence microscopy.

Fluorescence microscopy has been used to detect nucleic acids, proteins and polysaccharides of microorganisms in soil (Li *et al.*, 2004). Fluorochromes such as 4,6 diamidino-phenylindole (DAPI), allow staining of bacteria by binding to the AT-rich regions of double stranded DNA of vital and dead cells and have been used for bacterial enumeration in soil. However, DAPI binds to DNA but no differentiation of bacterial species can be achieved by this technique (Hannig *et al.*, 2010). Fluorescence *in situ* hybridization (FISH) allows to specifically stain bacteria with fluorescence-labeled molecular probes that bind specifically to rRNA (Macnaughton *et al.*, 1996). FISH allows to determine structure and dynamics of microbial communities from environmental samples (Amann et al., 1995; Daims et al., 2005), target microorganisms on different

phylogenetic levels (Amann *et al.*, 1990), as well to localize, quantify and identify rhizospheric microorganisms on soil particles, rhizosphere biofilm, root cells, axial grooves between epidermal cells, cap cells, or root hairs (Watt *et al.*, 2006) using confocal laser scanning microscopy (CLSM) and epifluorescence microscopy techniques (Hannig *et al.*, 2010).

6. Salt environments as potential new sources of microbial diversity

Among all microorganisms observable in nature it was estimated that more that 99 % cannot be cultivated by standard techniques (Hugenholtz *et al.*, 1998). Among cultivated bacteria the phylogenetic class α-proteobacteria and phyla *Actinobacteria, Acidobacteria* and *Verrucomicrobia* are ubiquitous to almost all soil types. The phyla *Proteobacteria, Cytophagales, Actinobacteria* and *Firmicutes* are the most represented among all cultivated bacteria and the ones that presented constantly taxa rearrangement and new descriptions (Zhang & Xu, 2008).

Meta-analysis of publicly available 16S rRNA gene sequences suggested that in saline soil habitats less than 25 % of bacteria have been sampled and that among these sequences *Proteobacteria* and *Actinobacteria* represented the most common taxon (Ma & Gong, 2013). Pyrosequencing analysis of 16S rRNA-based datasets from saline soil correlates salinity as the strongest factor influencing significant differences in bacterial community composition and diversity. Furthermore, phyla have been found that cannot be classified as salinity related and also other phyla which are typically related to this environment (Canfora *et al.*, 2014).

7. New taxa description

The species concept for prokaryotes has been long debated, however one of the most considered and accepted concept is the so-called phylo-phenetic species concept. This concept defines species as a monophyletic and genomically coherent cluster of individual organisms (strains) that show a high degree of overall similarity in many independent characteristics and is diagnosable by one or more discriminative phenotypic properties (Rosselló-Mora & Amann, 2001). Then this species concept is based on a polyphasic approach, that includes the description of phenotypic analysis combined with genomic data (Vandamme *et al.*, 1996).

7.1 Phenotypic traits

Phenotypic traits are the observable characteristics that result in the overall gene expression of the organism (Moore *et al.*, 2010), that can be influenced largely by environmental conditions such as temperature, pH-value, oxygen tension and others. Individually phenotypic characteristics, that also include chemotaxonomic characteristics, are insufficient to delineate a species, but together they provide sufficient descriptive information. The more characteristics that are included in the descriptions, the more robust and stable classification will be. Among the different characteristics some have resolving power at species level while others are valuable to discriminate genera, families and orders (Rainey & Oren, 2011)

Phenotypical characteristics analysis includes cell morphology and physiology, metabolism and enzymatic activities. Chemotaxonomical characteristics comprise analysis of composition of prokaryote cells including components of cell wall, outer cell membrane or cytoplasm membrane such as fatty acids, polar lipids, quinones, polyamines, the presence or absence of mycolic and teichoic acids, pigments and/or certain proteins (Fig. 3) (Moore et al., 2010; Tindall et al., 2010). Most of bacterial descriptions have been analyzed by classical standard microbial methods although new phenotyping systems such as the matrix-assisted-laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) or the high field ion cyclotron fourier transform mass spectroscopy (ICP-FTMS) techniques have been introduced into systematic (Welker & Moore, 2011).

7.2 Genotypic traits

Genotypic traits of an organism are those within its genetic material and its analysis for characterization purposes include several techniques (Fig. 3) such as DNA G + C content, DNA-DNA hybridization and analysis of 16S rRNA gene sequence have been of great value in bacterial taxonomy (Tindall *et al.*, 2010). The 16S rRNA gene is widely used in microbiology as a phylogenetic marker because it is functionally stable, ubiquitous, highly conserved and poorly subject to horizontal gene transfer. All prokaryotes are classified on the basis of the 16S rRNA gene sequence into the ‘Bacteria’ or ‘Archaea’ domains, and subdivided following a hierarchical manner into the non-overlapping ranks ‘phylum’, ‘class’, ‘order’, ‘family’, ‘genus’, ‘species’ (Brenner *et al.*, 2001). As a general statement

values below the mean (96.4 % ± 0.2) or minimum identity (94.9 % ± 0.4) to the type species may lead to a new genus circumscription (Yarza *et al.*, 2008). Cut-off values of the 16S rRNA gene sequence identity for novel taxa at the species level have been considered 97 % (Tindall *et al.*, 2010), although Stackebrandt and Ebers (2006) suggested increasing value up to 98.7 %. However, cut-off values of the 16S rRNA gene sequence identity does not apply for all genera as is illustrated in *Streptomyces* genus where >99 % sequences similarity is shared within 30 type species strains, and contrasts with < 94 % sequence similarity within 44 of the 47 described species contained in genus *Deinococcus* (Rainey, 2011). Furthermore, nucleotide variations among multiple rRNA operons in a single genome (Rainey et al., 1996; Acinas et al., 2004) and rare but occurring horizontal gene transfer (HGT) distorting relationships between taxa in phylogenetic analysis (Jain *et al.*, 1999) have been also disadvantages discussed on the use of 16S rRNA gene as a molecular marker.

The DNA G+C content of prokaryotes has been often used to grossly classify prokaryotes and its intra-genomic variability between >5 and >10 % between strains make it useful to classify within distinct in some cases species or genera, respectively (Goodfellow *et al.*, 1997). Among genomic DNA methods, DNA–DNA hybridization (DDH) (Johnson & Ordal, 1968) evaluate the relatedness degree of highly related organisms and is still recognized as a 'gold standard' for delineation of prokaryotic species (Tindall *et al.*, 2010). As a general statement DNA-DNA relatedness threshold for the definition of a species is 70 % (Stackebrandt & Goebel, 1994).

Multi locus sequence analysis (MLSA) (Maiden *et al.*, 1998), consists of the phylogenetic analysis of 16S rRNA gene together with protein-encoding marker gene sequences of housekeeping genes (*dnaJ*, *dnaK*, *gyrB*, *hsp60*, *recA*, *rpoB*) alignments. MLSA analysis increase the species phylogenetic resolution of 16S rRNA gene up to subspecies delineation and is useful to eliminate phylogenetic inconsistence such as lateral gene transfer (Kämpfer & Glaeser, 2011). Some authors consider this technique to represent the novel standard in microbial molecular systematics that improves phylogenetic resolution at the species level and that could replace DDH studies (Gevers et al., 2005; Konstantinidis and Tiedje, 2007).

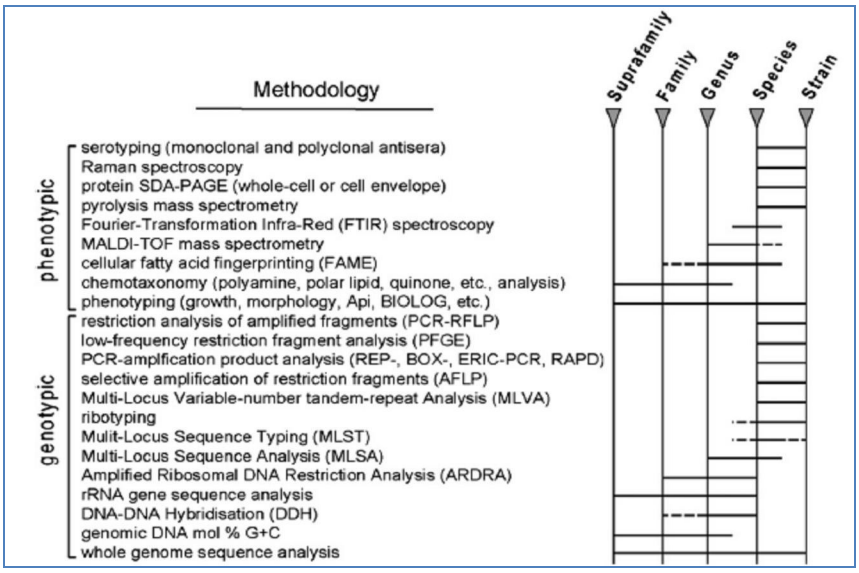


Fig.3. Different methodologies used for the characterization of prokaryotes and their approximate taxonomic resolution levels (Moore *et al.*, 2010).

The lowering costs and high throughput of next-generation sequencing methods have enabled fast increase of sequenced genomes (Soon *et al.*, 2013). Up to date more than 29000 prokaryotic genome sequences are available in public databases (http://www.ncbi.nlm.nih.gov/genome/browse/) facilitating genomes sequence comparison (Ramasamy *et al.*, 2014). Genome sequence can be used in taxonomy by using several indices obtained from pairwise genomes comparison such as the average nucleotide identity (ANI) (Konstantinidis & Tiedje, 2005) and maximal unique matches (MUM) (Deloger *et al.*, 2008) that is suggested as a valid alternative to DDH (Goris *et al.*, 2007). It is also proposed that an ANI value ≥ 95 % between genomes corresponded to a DDH value of ≥ 70 % (Goris *et al.*, 2007). However, analysis of genome sequence analysis provides a high taxonomic resolution (Fig.3) and it should be include among taxonomic criteria as an additional parameters to the phenotypic and chemotaxonomic parameters and not as a replacement of them (Ramasamy *et al.*, 2014).

8. Genome sequence comparison

Gene-based genome comparison allows genotypic characterization of prokaryotes based on their similarities or differences of gene contents. In order to perform comparison a genome is compare to a selected group of related organisms in order to describe its core genome,

singleton genes and pan-genome. Core genome references to the set of genes that is shared by an analyzed group organisms, meaning that all genomes of the studied group possess an orthologous gene in any other strain of the genome group. Singleton genes describes genes that are unique within the studied group, it means that no orthologous genes are identified in any other strain of the comparison set. Pan-genome refers to all independent genes within a group of analyzed organisms, it means that comprises all singleton genes and all genes that can be founded in more than one but not in all compared genomes (Borriss *et al.*, 2011). In terms of bacterial species description it could be said that a bacterial species can be described by its pan-genome, that is the sum of a 'core genome' and a 'dispensable genome' (Tettelin et al., 2005, Blom et al., 2009).

Comparison of multiple genomes of related species have become of great interest and could be done through databases such as the Comprehensive Microbial Resource (CMR) (Peterson *et al.*, 2001) or the Microbial Genome Database (MBGD) (Uchiyama, 2003). Such databases allow the use of different parameters to define a homology cutoff for genome comparison depending on the user expertise and in order to avoid different parameters analysis an automatic estimation of an adequate homology criterion software such as the Efficient Database framework for comparative Genome Analyses using BLAST score Ratios (EDGAR) has been developed (Blom *et al.*, 2009). EDGAR uses a generic orthology criterion adjusted to the set of compared genomes based on BLAST score ratios and provides several analysis and visualization features. EDGAR also provides a precalculated public databases for 116 genera with 1008 genomes, but it also allows to create projects to user-defined sets of genomes with publish and un-publish data (Borriss *et al.*, 2011).

Comparative whole genome sequencing could be used for several proposals such as phylogenetic, epidemiological, and ecological studies, and is increasingly being used in place of PCR-based sequencing or typing methods (Edwards & Holt, 2013). The use of comparative genomic analysis in PGPB will help on the understanding of genes on metabolism, potentially involved in plant growth promotion, compound and metal resistance, rhizosphere colonization, lifestyle, ecological adaptation and on physiological role in their interaction with plants (Bruto et al., 2014; Shen et al., 2013; Duan et al., 2013).

9. Aims of this study

As soil salinization is a major concern of modern agriculture and an expected threat in climate change scenarios, special effort will be required for maintaining crop production under salt stress (Turral et al., 2011). The use of plant growth-promoting rhizobacteria (PGPR) is a promising agricultural practice to help less salt tolerant crops to maintain an acceptable level of productivity under higher salt concentrations (Nadeem et al., 2012; Singh et al., 2011). A lot of research has been conducted in order to understand plant stress effects of salt and the mechanisms involved in alleviation and promotion of plant growth in different kinds of crops by bacteria (Sections 3 and 4). Many studies have focused on isolation of effective halotolerant PGPR from salt affected soils (Siddikee et al., 2011), and from the rhizosphere of natural salt tolerant and halophytes plant species (Ruppel et al., 2013; Paul and Lade, 2014). Even so, there are no studies exploring both, the microbial and functional diversity, of halotolerant rhizobacteria with plant growth-promoting abilities (PGPA) isolated from the rhizosphere of natural salt tolerant plant species growing in natural protected areas.

In the following study, the aims were to investigate the microbial diversity of PGPR from the rhizosphere of natural salt tolerant plant species, and the selection of isolates that effectively promote the growth of *Hordeum vulgare* L. under salt stress. Furthermore, special attention is intended on isolates of taxonomical interest and not belonging to bacterial genera reported as PGPR. The study involved the evaluation of qualitative and quantitative PGPA of the isolates in pure culture, plant growth (*ad planta*) experiments using a gnotobiotic liquid system and non-sterile soil under salt stress, and root colonization experiments using fluorescence *in situ* hybridization (FISH). Additionally, it involved polyphasic approaches to describe new bacterial taxa and detection of genes involved in plant growth promotion. The main aims of this study were:

1. Analysis of the halotolerant microbial diversity of plant growth-promoting rhizobacteria from the rhizosphere of *Hordeum secalinum* and *Plantago winteri* using selective enrichment media.

2. Among the plant growth-promoting rhizobacteria isolated it should be selected isolates of taxonomical interest with plant growth-promoting abilities able to promote *Hordeum vulgare* L. growth under salt stress.
3. The localization and effective plant growth promotion abilities of the isolates *ad planta* should be determined and correlated with their respective gene content.

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Chapter 2

Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress

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Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (*Hordeum vulgare* L.) under salt stress

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ABSTRACT

Soil salination is a severe problem in agricultural plant production and it is expected to become more relevant in the next decades. Plant growth promoting rhizobacteria (PGPR) isolated from salty habitats can contribute to sustain growth and alleviate salt stress of crop plants. In this work, 100 potential PGPR were isolated on enrichment media for relevant functions (ACC-deaminase activity, Auxin synthesis, P-solubilization and N-fixation), from the rhizosphere of *Hordeum secalinum* and *Plantago winteri* grown in a natural salt meadow. Twenty-two isolates, including representatives of each function and depicting a high taxonomical diversity, were tested for their promotion effect on barley under salt stress and non-sterile substrate. The two best promoters were tested in a second plant assay. In parallel to the plant assay, the complete spectrum of PGP-activities of our isolates was assessed by pure culture assays. Results surprisingly showed that the best promoters did not display a promising PGP-spectrum., whereas isolates showing multiple PGP-activities in pure culture, eventually did not promote barley growth. The most effective isolate (E108, identified as *Curtobacterium flaccumfaciens*, which increased barley growth up to 300%) would have never been recognized as best candidate based on its PGP-activities in pure culture. Its mechanisms of action involved amelioration of Na^+ tissue tolerance and reduction of cation root surface uptake. Our results highlight how screening based on pure culture assays may not be suitable for recognition of best PGP candidates, and could preclude the detection of both new PGPR and new plant growth promotion mechanisms.

Keywords

salt stress; salt meadow; *Curtobacterium*; PGPR, plant microbe interaction

Highlights

High phylogenetic diversity of PGPR in a natural salt meadow

PGP activities assessed in pure culture do not allow good prediction in plant assays

Curtobacterium flaccumfaciens isolate was most effective PGPR on barley under salt stress

Amelioration of Na^+ tissue tolerance

Reduction of cation root surface uptake

INTRODUCTION

Soil salination is a serious threat for agriculture. It is expected that more than 50 % of all agricultural soils will be affected by salinity increase by the year 2050 (Ashraf 1994; Vinocur & Altman 2005). It is therefore imperative, in the context of a responsible soil management for sustainable agriculture, to discover beneficial microorganisms able to alleviate the salinity stress and improve plant growth (Grover et al. 2011; Singh et al. 2011) thus allow sufficient yields.

The results obtained so far lead to the general conclusion that bacteria producing ACC-deaminase are most suitable candidates, as they are able to remove the precursor of ethylene, the inducer of the plant response to several abiotic stresses including salt-stress (Dimkpa et al. 2009; Yang et al. 2009). Numerous rhizosphere-inhabiting pseudomonads possess this function and, since they are often efficient root colonizers, they have been regarded as optimal candidates for salt-stress alleviation. Promising results were obtained with cucumber (Egamberdieva et al. 2011), lettuce (Kohler et al. 2009), mung bean (Ahmad et al. 2011), paddy rice (Jha et al. 2011), sunflowers (Shilev et al. 2012), tomato (Tank and Saraf 2010) and wheat (Zahir et al. 2009; Nadeem et al. 2010). Further ACC-deaminase producers, such as *Achromobacter* sp. (Mayak et al. 2004; Karthikeyan et al. 2012), *Burkholderia* (Shaharoona et al. 2007), *Bacillus* sp. and *Brevibacterium* sp. (Siddikee et al. 2010 and 2011) did also significantly alleviate salt stress. However, additional rhizocompetent bacteria with different PGP traits (Pii et al. 2015) can also play a major role in supporting the plant growth under stress conditions, as demonstrated, for example, for the exopolysaccharide (EPS)-producing *Bacillus*, *Serratia* and *Aeromonas* (Ashraf et al. 2004; Han and Lee 2005).

Barley (*Hordeum vulgare* L.) is a relatively salt tolerant crop and the most salt tolerant cereal (Nevo et al. 1993), domesticated the first time about 10,000 years ago in the Fertile Crescent (Badr et al. 2000) and a second time, more recently, in an undefined region located between Turkmenistan and Pakistan (Morrell and Clegg 2006). It is the fourth most cultivated cereal in the world after corn, rice and wheat (source: Encyclopedia of Life, http://eol.org).

In this work we exploited the rhizosphere bacteria associated with monocotyledon wild barley (*Hordeum secalinum*) and the dicotyledonous greater plantain (*Plantago winteri*),

isolated from a salt meadow in Germany, as bioinoculant for mitigating salt stress of the crop plant *Hordeum vulgare*. We selected bacteria with various potential PGP activities and broad taxonomic affiliations, and tested their potential on *Hordeum vulgare* under salt stress conditions, using non-sterile soil, in order to select competitive strains with higher probability of being effective in future field applications. Our hypothesis was that typical plate assays for PGP activities might not detect the actual plant growth promoters, which could explain the typical low efficacy of such laboratory assays as screening method for selecting promising bacterial isolates to be tested *ad planta*.

MATERIALS AND METHODS

Sampling campaigns and bacterial isolation on plate.

Plants were taken in a natural salt meadow near Münzenberg, Hessen, Germany (50° 279´ 360´´ N 8° 449´ 350´´ E) where salty water welled from a subsurface salt deposit to the surface. Chloride (Cl^-), sulfate (SO_4^-) and nitrate (NO_3^-) concentrations were measured with a ion chromatograph (Bak et al. 1991) from the surface water of nearby ditches in June 2004. Chloride concentrations were 133 ± 1.4 mM, sulfate concentrations were 0.039 ± 0.04 mM, nitrate was not detectable. The pH-values (7.66 ± 0.22) were measured directly in the water of the ditches with a mobile pH meter (WTW, Weinheim, Germany). All values are means of duplicates ± the range of the values). *Hordeum secalinum* and *Plantago winteri* plants *were dug at 3 time points of the year* (May 2007, July 2006, October 2006) and *Plantago winteri* plants in addition also in December 2005. In the laboratory, the root systems were carefully separated from the roots of other plants with sterile forceps. Loose soil was separated from the root by hand shaking. The roots with attached rhizospheric soil were weighed in sterile glass flasks and sodium pyrophosphate (0,18 %) was added to detach the bacteria and soil from the root material.

Detaching was done by vigorous shaking of the flasks by hand for 5 min. From each resulting soil suspension, three independent serial dilutions with 0.9 % NaCl were prepared and from the dilutions, different media were inoculated. The media used for the isolation were chosen among those allowing the enrichment of specific functional groups: potential nitrogen fixing bacteria (LG medium, Turner and Gibson 1980; NFB medium, Kirchhof et al. 1997); potential ACC-deaminase producers (DF medium, Penrose and Glick 2003); potential organic phytate-mobilizing bacteria (IHP medium, Unno et al. 2005); potential inorganic phosphate-mobilizers (CP medium, Suarez et al. 2014c); potential indol-acetic-

acid (IAA) producers (LBT medium with L-tryptophan, followed by confirmation on nitrocellulose membrane with the Salkowski reagent Bric et al. 1991). Total load of heterotrophic bacteria was assessed with liquid and solid K7 medium (per liter water: 1 g glucose, 1 g yeast extract, 1 g peptone and 15 g agar). Cycloheximide [per 1 l media: 40 ml steril filtrated solution (4 mg cycloheximide ml^{-1} deionised water)] was added to the K7-media, the CP-media and the DF-media to suppress fungal growth. All isolation plates and liquid cultures were incubated at 25 °C and cell numbers were checked regular until no change occurred any more. For the liquid (DF) and semisolid (NFB, IHP) media, the MPN-method (Man 1983) was used for the calculation of the cell numbers per gram dry weight soil. Cell numbers on the solid media were calculated as colony forming units (CFUs) per gram dry weight soil. After visible growth, recurrent colonies with different morphology were chosen and strains were purified by using standard streaking technique on the corresponding medium and then stored in 20 % glycerin at -80 °C.

Taxonomical identification of the isolates

For the sequencing of the partial 16S rRNA gene one part of a colony was picked with a sterile toothpick and directly put in the PCR reaction cup. PCR reaction was performed with the primer pair EUB9f (9-27) (Lane 1991) and EUB 1492r (Weisburg et al. 1991) as described by Kampmann et al. (2012). For sequencing 10-15 ng of the purified PCR product was amplified with 10 pmol of the forward primer 616V (Johnson 1994) and partial with 10 pmol of the reverse primer EUB 1492r (Weisburg et al. 1991) to receive a more complete sequence Sequencing was performed by the sequencing facility of the IFZ (Research Centre for Bio Systems, Land Use and Nutrition, Giessen, Germany) with either the Abi Prism 310 Genetic Analyser (Applied Biosystems, Weiterstadt, Germany) and the Abi Prism BigDyeTMPrimer Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany), according to the manufacturer instructions or DNA was sent for sequencing to the company LGC (Berlin, Germany). Chimera were detected according to Kampmann et al. (2012). Pairwise similarities values of next the relatives were retrieved from the EzTaxon server (Kim et al. 2012). The 16S sequences obtained in this work were submitted to ... under the accession numbers … to … .

Plant growth promoting assay with Hordeum vulgare

Plant assay 1: Twenty-two isolates were selected based on their taxonomic and functional diversity, and were tested for their efficiency in alleviating salt stress on *Hordeum vulgare*

(cultivar Propino) *ad planta*, in greenhouse. Bacterial isolates were grown in liquid AC medium (Sigma-Aldrich Chemie, Steinheim, Germany), centrifuged at 8000 g and resuspended with 30 mM $MgSO_4$ to reach a concentration of 10^7 to 10^8 CFUs ml^{-1}, depending on the isolate. Barley seeds were sterilized with 2.5 % bleach, washed five times with sterile water and then incubated for one hour in the respective bacterial suspensions under gentle shaking. Immediately after, the seed were planted into non-sterile Classic Tonsubstrat ED 73 substrate (Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co.KG, Sinntal – Altengronau, Germany), into squared plastic pots (13 cm x 13 cm) containing ~750 ml (~270 g dry weight) of substrate. Physico-chemical properties of the substrate are given in Supplement 1. The water capacity (WC) was estimated to be 233 ml, and each pot was irrigated with 180 ml rainwater (~75 % WC). This amount of water allowed the whole substrate in the pot to be moistened, yet avoiding percolation. Eight seeds were placed on each pot and covered with a one cm-layer of the same substrate. Treatments were named after the isolate name. The mixture of all isolates was also tested (treatment "MIX"). Barley seeds incubated in sterile 0.03M $MgSO_4$ solution served as negative control (treatment "S+ B-"). Additional uninoculated seeds were placed into pots that did not receive NaCl, to assess the normal growth of barley in absence of salt stress and bacterial inoculants (treatment "S- B-"). *Pseudomonas fluorescens* PCL1751 (Kamilova et al. 2005; Egamberdieva et al. 2011), effective in promoting the growth of different plants under salt stress, was used here as an additional treatment to have a comparison for the effect of our isolates. Five pots per bacterial treatment/uninoculated control were prepared and arranged in a greenhouse with a randomized complete block design (RCBD; Clewer and Scarisbrick 2001) to account for possible gradient effects in the greenhouse. Plants were grown for 5 weeks with daylight from 06:00 to 22:00 (artificial light switched off when natural light exceeded 10 Klx), and temperature of 20 °C and 16 °C (day and night, respectively).

Four days after first seedling emergence, the germination was considered complete and each pot was rarified to five plants. Depending on the number of germinated seeds, the smallest, the biggest and then the smallest seedling were removed.

Beginning with the third day after germination (DAG), pots treated with bacteria and those of the treatment "S+ B-" were irrigated with increasing NaCl solutions in rainwater (50 mM, 100 mM, 150 mM, 200 mM and finally 250 mM NaCl, with three days interval each;

Supplement 2) to impose the salt stress, until reaching a salinity of 4.8 % (g NaCl soil DW^{-1}) 27 DAG. Treatment "S- B-" received the same amount of rainwater without NaCl.

Five weeks after sowing, from each pot, the shoots and the leaves of the 5 plants were separately collected in paper bags, and their fresh weight was recorded (gram pot^{-1}). Leaves and shoots were then dried at 80 °C for 48 h before assessing the dry weight.
Salt stress resistance (barley FW in presence of salt / barley FW without salt * 100) of inoculated and uninoculated plants was compared by using the fresh weight of the five plants per pot (N=5). To evaluate the effect of the inoculation on the plant fitness, the percentage of relative increase (Crane-Droesch et al. 2013) was calculated on both the accumulated dry biomass and the water content (calculated as the difference between fresh- and dry weight). Effect on both germination and roots was not assessed in this first plant assay.

Plant assay 2:

The two best performing isolates (E108 and E110, see results) were tested in a second experiment to assess their effect on barley germination and growth when salt stress occurred already at seeding to have conditions that are more similar to the field. The two selected isolates were inoculated alone (treatments "E108" and "E110", respectively) and in combination (treatment "E108+E110"), and were compared to both uninoculated barley (treatment "S+B-") and to barley inoculated with dead bacteria produced by autoclaving an aliquot of the E108+E110 mixture (treatment "S+ D"). Seed inoculation was performed in the same way as plant assay 1. E108 and E110 bacterial suspensions were applied at a concentration of 10^7 CFUs ml^{-1}. Squared pots and the non-sterile Classic Tonsubstrat ED 73 substrate soil as in plant assay 1 were used, but the salt stress was applied already at the seeding stage (Supplement 2) to assess the effect on the germination under salt stress, and then increased until a final NaCl concentration of 4.4 % (reached 15 DAG). At this NaCl concentration, the electrical conductivity (EC 1:5, Guang-Ming *et al.* 2006) was 22.40 ± 0.7 mS cm^{-1}. Treatment S- B- received the same amount of rainwater without NaCl.
The experimental design was the same of plant assay 1 (5 pots with 5 plants each, RCBD randomized) but fifteen seeds, instead of eight, were placed into each pot, to account for the reduced germination rate as affected by salt. Germination was monitored until eight days after sowing, and then the pot were rarified to five plants. Plants were grown under

the same greenhouse conditions of plant assay 1, and were harvested 42 DAG. Fresh weight of leaves, stems and roots was recorded and plant material was left to dry for 48 h at 80 °C before assessing the dry weight. As in the plant assay 1, salt stress resistance was calculated on fresh weight, while the relative increase of both dry biomass and water content on dry weight (leaves, stems and roots separately).

Characterization of PGP activities of the selected isolates in pure culture experiments (plate assay)

From the twenty-three isolates evaluated in the plant assay 1, fifteen strains were selected for the determination of their PGP activities in pure culture assays. The selected isolates included all those showing largest effects on plant growth (both positive and negative) as well as neutral isolates, and in addition also strain PCL1751. Every isolate was inoculated on plates of different selective media for 3-5 days at 28 °C to determine their PGP activities qualitatively: SRSM1 medium (Sundara Rao and Sinha 1963) and IHP medium (Unno et al. 2005) were used to test the ability to grow in presence of calcium phosphate and phytate, respectively. Additionally, for confirmation of phytate mobilization activity, the isolates grew on IHP solid medium were then inoculated into 5 ml of IHP liquid medium at 28 °C in a rotary shaker for 15 days. NFB medium (Kirchhof *et al.* 1997) prepared with washed agar and supplemented with 1 % of saccharose was used to check the ability of growing on nitrogen free media. Growth tests were evaluated at least twice on the respective medium and compared with the growth on Tryptic Soy Agar medium. DF medium with addition of ACC (Penrose and Glick 2003) and Lauria-Bertani broth supplemented with L-tryptophan (LBT) medium (Bric et al. 1991) were used to test the potential ACC deaminase activity and IAA production, respectively; ACC deaminase activity and IAA production of positive isolates were then quantitatively measured according to Penrose and Glick (2003) and Glickmann and Dessaux (1995), respectively.

For the siderophore production test, the isolates were inoculated in liquid King's B medium (Schaad et al. 2001). After 7 days of bacterial growth, 1 ml was centrifuged for 5 min at 2600 g at room temperature. One-hundred microliters of supernatant were placed in a micro-plate and 100 µl of 2 mM chrome azurol S (CAS) solution (Alexander and Zuberer 1991) were added. After 30 minutes, a colour change to yellow or orange indicated the production of siderophores of the type hydroxamate.

Chemical analysis of plant material

The concentration of the cations Na^+, Ca^{2+}, Mg^{2+} and K^+ in the leaves, stems and roots of barley plants inoculated with most effective isolate E108 and uninoculated controls was measured under a full-factorial design 2X3 (two inoculation treatment: inoculated/not inoculated; 3 plant habitats: leaves/stems/roots). Plant material was dry ashed at 550 °C, dissolved in 5 M HNO_3, and finally measured by atomic absorption spectroscopy (Steffens and Schubert 2011). Total intake was calculated as the product of the cation concentration and the dry weight of the corresponding plant habitat. Root surface uptake was calculated as the total intake of each cation in the whole plant (in mg) divided by the root dry weight (Schubert et al. 2009). Root-to-shoot translocation was calculated as the total cation content of the shoots (leaf + stems) divided by the total cation content of the roots, and was further normalized to the biomass partition (shoots DW/roots DW), to eliminate the bias due to the different effects on the growth of shoots and roots (Saqib et al. 2005). Therefore, root-to-shoot translocation has no units.

Statistical analysis

Statistical differences of germination, plant growth parameters, cations concentration/intake and MPN values between treatments were assessed by ANOVA (either one-way, multivariate or factorial, depending on the tested dataset), followed by LSD Post-hoc test at $p<0.05$, using the software Statistica (Statsoft Inc., Tulsa, USA). Effect size (partial-η^2) and analysis power (1-β) of each statistical test are also reported. Student's T-test was used to compare root surface uptake and root-to-shoot translocation between E108-inoculated plants and uninoculated control plants.

RESULTS

Bacterial enumeration form rhizospheric soil of Plantago winteri and Hordeum secalinum, strain isolation and identification.

Bacterial cell numbers determined in the rhizopheric soil of *P. winteri* and *H. secalinum* sampled at 4 time points are shown in Supplement 3. The cell numbers of the rhizospheric soil were as expected higher as the bulk soil. Between the different PGPA no statistical differences could be found. The cell numbers trended to be lower in the sampling of December and October (Supplement 3). Out of the dilutions of the counting a total of 100 pure bacterial isolates were obtained from all media, including members of 39 genera (Fig. 1), 22 families, 15 orders and 8 classes (Alphaproteobacteria 33 %, Actinobacteria 27 %,

Gammaproteobacteria 18 %, Bacilli 15 %, Betaproteobacteria 2 %, Cytophaga 2 %, Sphingobacteria 2 % and Flavobacteria 1 %).

Among such isolates, three new species and one new genus were already described (Suarez et al. 2014a, b and c). Additionally, 22 more isolates showed pairwise similarities lower than 98.7% to next described relatives, potentially indicating further new species/genera (Stackebrandt and Ebers 2006) (Supplement 4).

The highest number of the isolates (40 %) was obtained on the enrichment media for potential nitrogen fixers, while potential auxine producers were the smallest functional category retrieved (6%). Phosphate mobilizers, phytate mobilizers and ACC-deaminase producers accounted for 26, 15 and 13% of the isolates, respectively.

Twenty-two isolates were selected based on their functional and taxonomical diversity for the *ad planta* test on barley.

Effect of bacterial inoculation on barley under salt stress

Plant assay 1: Inoculation with bacteria caused significant changes in the salt stress resistance of barley (ANOVA, $F_{24, 99} = 2.66$, $P< 0.001$; partial-$\eta^2 = 0.39$; 1-$\beta = 0.99$; Fig. 2). LSD post-hoc test ($P< 0.05$) revealed that 20 out of 24 tested bacterial inoculations (including the reference strain *Pseudomonas fluorescens* PCL1751 and the mixture of all isolates) had no significant effect on barley growth; one isolate (E136B, identified as *Bacillus* sp.) and the mixture of all isolates had a significant negative effect. The isolate E108, identified as *Curtobacterium flaccumfaciens*, significantly increased the salt-stress resistance of barley (Fig. 2), followed by isolate E110 identified as *Ensifer garamanticus*. Bacterial inoculation affected significantly the relative increase (RI) of both dry biomass and water content (MANOVA, $F_{96, 4382.82} = 2.35$, $P< 0.001$; partial-$\eta^2 = 0.37$; 1-$\beta = 1$; Fig. 3). In particular, in comparison with the uninoculated barley, more water was accumulated in both leaves and stems of E108 inoculated plants (Fig. 3 C and D) and in the stem of E110 inoculated plants (Fig. 3 D); the accumulated biomass also was increased in the stems by inoculation with isolates E108 and E110, (Fig. 3 A and B), suggesting a general positive effect of these two bacteria on barley fitness. Isolate E19 slightly increased the water content of the leaves (Fig. 3C). Isolate E108 showed the strongest effect on both biomass accumulation and water content in both leaves and stems, thus resulting the best performing isolate among the tested ones, followed by strain E110. These two strains were therefore selected for the second plant assay, performed under stronger salt stress.

Plant assay 2: Isolates E108 and E110 were tested in a second plant assay, with salt stress occurring already at the seeding stage (1.75 % NaCl) and then further increased (Supplement 2). Germination rate of barley was significantly increased especially by strain E108 (ANOVA Repeated measures, $F_{5, 24} = 33.40$, $P < 0.001$; partial-$\eta^2 = 0.87$; 1-$\beta = 1.0$). The germination rate in presence of strain E108 was comparable to that of seeds without salt (Fig. 4), although delayed of about 4 days. Germination rates of E110 and E108+E110 were higher than uninoculated control, and also higher (but not significantly different) from the seeds inoculated with dead bacteria (Fig. 4).

Salt stress resistance of leaves, stems and roots (based on separate fresh weights) was significantly increased by inoculation with E108, while only root salt resistance was increased by inoculation with E110 (MANOVA, $F_{12, 47.92} = 2.66$, $P = 0.0080$; partial-$\eta^2 = 0.40$; 1-$\beta = 0.95$) (Fig. 5). The mixture of the two isolates was not effective.
Biomass accumulation of leaves, stems and roots were significantly increased by inoculation with E108 (MANOVA, $F_{24, 53.54} = 6.21$, $P < 0.001$; partial-$\eta^2 = 0.74$; 1-$\beta = 1$), with respect to both uninoculated plants (treatment "S+B-") and plants inoculated with dead bacteria (S+D, Fig. 6). The water content was significantly higher than the uninoculated plants only, while not significantly different from treatment "S+D" (Fig. 6). Only in roots, E110-inoculated barley plants accumulated more water than the control plants (Fig. 6). The positive effect of E108 on biomass accumulation overcame that on water content and was highest in the roots (+319.1 %), followed by stems (+152,2 %) and leaves (+105,7 %) (Fig. 6). The mixture E108+E110 was less effective than E108 alone and more than E110 alone, although water content of leaves and stems was even lower than that of uninoculated control (at harvesting, the E108+E110 inoculated plants appeared very dry and partially withered already). Plant of this treatment accumulated significantly more root biomass (Fig. 6). Plants inoculated with the dead bacteria accumulated more water and more biomass than uninoculated plants, but the difference was not statistically significant (Fig.6).

Characterization of PGP activities of the isolates in pure culture

From the twenty-two isolates evaluated in the plant assay 1, fifteen were selected to analyze their PGP activities (Table 1). Results showed an unexpected high rate of positive results. The isolates E134 and E38 presented most of the activities: E134 was positive for

indol-acetic-acid production (IAA), nitrogen fixation (NF) phosphate mobilization (PHO),phytate mobilization PHY and siderophore production (SID), whereas E38 was positive for ACC, IAA, NF, PHO and PHY. Two isolates, PCL 1751 and E31 presented activity for ACC, IAA, NF and SID. Isolate E110 was positive for IAA production (the highest among the tested isolates), NF, PHO and PHY. Isolate E8 showed a very high ACC deaminase activity (8,545 nmol h^{-1}, followed by E136A with 292 nmol h^{-1}), as well as the second highest IAA production, NF and SID. Surprisingly the isolates with highest PGP score in pure culture (including the ACC-deaminase producer pseudomonads) were not the best performing *ad-planta* under salt stress condition, while the best *ad-planta* performers (E108 and E110) showed low PGP potential in pure culture.

Cation analysis of the plant tissues

The contents of the four cations mainly involved in plant sensitivity and adaptation to high salinity (Na^+, Ca^{2+}, Mg^{2+} and K^+) were measured in the plants inoculated with isolate E108, the most effective promoter, and compared to those of uninoculated plants (treatment "S+B-"). Cation concentrations were significantly affected by inoculation (Factorial ANOVA, $F_{4,20} = 12.71$; $P<0{,}001$; partial-$\eta^2 = 0.72$; 1-$\beta = 1$) and by plant habitat (Factorial ANOVA, $F_{8,40} = 46.09$; $P<0{,}001$; partial-$\eta^2 = 0.90$; 1-$\beta = 1$), but not by the interaction Inoculation*Habitat (Factorial ANOVA, $p=0.069$). Post hoc test indicated that only the concentration of K^+ was significantly reduced in both leaves and stems by inoculation with isolate E108, while concentrations of all other cations (Ca^{2+}, MG^{2+} and Na^+) were not significantly different (Supplement 5). However, since the E108 inoculated plants grew more than the uninoculated control plants, the total intake of all cations was significantly higher (Factorial ANOVA, $F_{4,20} = 25.03$; $P<0.001$; partial-$\eta^2 = 0.83$; 1-$\beta = 1$; Supplement 5). Plant habitat and interaction Inoculation*Habitat were also significant ($p<0.001$ and $p=0.017$, respectively). Differences between total intakes of E108-inoculated plants and uninoculated controls were significant in both stems and leaves for all cations, whereas only Na^+ and Mg^{2+} were significantly different in the root (Supplement 5). In order to shed light on the possible mechanism of action of isolate E108, root surface uptake and root-to-shoots translocation were calculated, two parameters that indicate how the plant manages the cations under high salinity conditions. Root surface uptake of all four cations was significantly reduced by inoculation of E108, while normalized root-to-shoot translocation was not significantly changed (Tab. 3).

DISCUSSION

A strong effort is currently devoted to seek for new bioresources, due to the need of discovering new organisms and substances with biotechnological potential. Natural salt environments seem to be a promising source of bacteria able to alleviate salt stress in important crops, such as cereals (Tiwari et al. 2011; Nabti et al. 2014). Therefore, isolation campaigns at different seasons and with rhizosphere soil of different plants were performed to find new effective PGPR from a natural salt meadow. The chloride concentration at June of the water in the meadow was with ~ 133 mM in the range where plants not belonging to the groups of the extreme halophytes and halophytes already show reduced substantially growth (Greenway and Munns, 1980). At this concentration, the growth of *Triticum trugidum* ssp. *durum* (durum wheat), *Triticum aestivum* (bread wheat) and *Hordeum vulgare* (barley) is already decreased by around 70 %, 60 % and 51 %, respectively (Colmer et al. 2005). The natural occurring monocotyledon *Hordeum secalinum* (meadow barley) and the dicotyledon *Plantago winteri* (great plantain) are adapted to this relatively harsh conditions by plant physiological adaptions and likely also by the help of naturally occurring PGPR. We supposed that in contrast to crop which often have been used as inoculum for isolation of new PGPR, these plants and the associated PGPR coevolved in the salty environment over a much longer period, whereas the modern crop plants are bred in the last 9000 years and cultivated mostly under no salt stress. Therefore, it was expected to find PGPR with a high efficiency to promote growth under salt stress. Moreover choosing the close relative plant species *Hordeum secalinum* to the crop plant *Hordeum vulgare* may increase the chances that the new PGPR also interact with the crop plant. Altogether around 50 pure cultures could be isolated from each plant, by choosing six different already PGPR-selective media instead of the often used universal media and therefrom a high percentage of 22 % were potential new not yet described species. The diversity was high since the isolates belonged to 40 different genera and 8 different classes. As other cultivation-dependent studies the major part of the isolates belonged to the phyla *Proteobacteria*. Whereas other studies (Aranda et al., 2011, Marasco et al., 2013, Bafana, 2013) found *Firmicutes* as the next most abundant phylum, in this study *Actinobacteria* was the second most abundant. This is coherent with the meta-analysis performed by Ma and Gong (2013), which indicated *Proteobacteria* and *Actinobacteria* as the most frequent phyla among submitted 16S rRNA gene sequences obtained from saline rhizospheric soils and also by Turner et al. (2013) in a metatranscriptomics study looking at the active

bacteria in the rhizospheric soils of wheat and pea. However, in a cultivation-independent analysis of the rhizosphere of wild and domesticated barley, the bacterial families *Commandadaceae*, *Flavobacteriaceae*, and *Rhizobiocea* were found dominant (Bulgarelli et al. 2015). Other phyla also found dominant in cultivation independent analyses of the rhizospheric microbiomes are *Bacteroidetes*, *Acidobacteria*, *Saccharibacteria* (formerly TM7), *Chloroflexi*, *Gematimonadetes* and *Verrucomicrobia* (Buée et al. 2009). Between the plants and the seasons investigated in our study, no difference of cell numbers was found (Supplement 3). Concerning the taxonomic affiliation of the isolates, neither *Plantago* nor *Hordeum* specifically favoured any taxon, with the exception of the genus *Rhizobium* that was isolated more often from the *Plantago* rhizosphere.

Among the 22 isolates selected based on their taxonomical and functional diversity, and tested *ad planta* on *Hordeum vulgare*, isolate E108 (identified as *Curtobacterium flaccumfaciens*) was the most effective growth promoter. The second best promoter was isolate E110 (identified as *Ensifer* (*Sinorhizobium*) *garamanticus*. Both species are well known as typical plant endophytes. While *Ensifer*, as well as other rhizobia, can promote the growth and reduce the salt stress in different hosts (including non-legumes; Galleguillos et al. 2000), *Curtobacterium flaccumfaciens* is shown here for the first time as PGPR and stress alleviator. *Curtobacterium flaccumfaciens* was already shown to be able to reduce the symptoms of both *Xylella fastidiosa* (in *Catharanthus roseus*; Lacava et al. 2007) and angular leaf spot (in cucumber, by inducing systemic resistance; Raupach and Kloepper 2000). Interestingly, *Curtobacterium flaccumfaciens* is a well-known pathogen of different plant species (Collins and Jones 1983), including common bean, soybean, sugar beet, poinsettia and tulip. To the best of our knowledge, *Curtobacterium flaccumfaciens* represents the only case of a phytopathogenic bacterium acting as PGPR on a different host, and this work is the first report of its plant growth promoting effect not mediated by biocontrol activity. The mechanisms at the basis of the *Curtobacterium flaccumfaciens* pathogenicity are not known, but its endophytic lifestyle is well-recognized (Bent and Chanway 1998; Zinniel et al. 2002; Gagne-Bourgue et al. 2013), as well as its transmission by seed (Diatloff et al. 1992; Maringoni et al. 2006). Possibly, the same colonization strategy may result either in a negative or in a positive net-output on different hosts (Partida-Martínez and Heil 2011). The fact that our results with *Hordeum vulgare* (barley) were obtained under non-sterile conditions in greenhouse, adds robustness and significance to the PGP effect of isolate E108.

In the second plant assay, the effect of the inoculation was much stronger than in the first one. This likely occurred because the stress was present from the beginning (at the sowing stage already), thus determining a larger benefit of the inoculant (Partida-Martínez and Heil 2011). Indeed, this is coherent with the results of Rolli et al. (2015), who showed that beneficial effects of bacterial inoculants are stress-dependent, in the case of drought stress. Single inoculation of isolate E108 lead to the best performance, including a strong increase of both biomass and water content in all plant compartments. Therefore our isolate E108 acts a "fitness enhancing" bacterium (FEB) on *Hordeum vulgare* under salt stress. Co-inoculation of isolates E108 and E110 did not result in a more efficient synergy but in a sort of "averaging" of the effects of both inoculants. This is coherent with a few studies showing that the beneficial effects of singularly inoculated PGPR disappeared or were reduced in co-inoculation, probably due to competition (Probanza et al. 2002; Elkoca et al. 2010; Golding et al. 2012). There are far more examples of increased beneficial effect of co-inoculations (especially in the case of rhizobia + PGPR in legumes), but this likely represents a bias due to the common practice to not publish negative results. Moreover, the non-sterile conditions used in our plant assay obviously resulted in a complex microbe-microbe interaction network in the *Hordeum vulgare* rhizosphere, a more realistic situation compared to the gnotobiotic conditions of most published results. The changes in the individual contributions after co-inoculations, as well as the net output in such a system is therefore more difficult to predict and challenging to explain. In this case, further plant assays under gnotobiotic conditions will be required to unravel the mechanism of action and interaction of the inoculated PGPRs.

Results of *ad planta* tests showed that assays for PGP activities of pure cultures might not be the appropriate screening method for selecting the best performing strains *ad planta*, under certain conditions such as salinity stress. ACC-deaminase activity, in particular, was expected to be the best indicator of the most effective PGPR under soil salinity, yet our results showed that the best performers (E108, E110 and E19) shared the phosphate mobilization activity instead (Tab. 1). Of these three isolates, only E19 (*Hartmannibacter diazotrophicus*, Suarez et al. 2014c) possessed the ACC-deaminase activity additionally. Although the positive effect of isolate E110 could be explained by both the multi-activity and the abundant production of auxine, the best performing isolate (E108) could not have been predicted from its assay for PGB activity (Tab. 1). However, it cannot be excluded that other untested PGP activities may have also played a role under the non-gnotobiotic conditions of our plant assays, or that the beneficial effects of the inoculated strains may

have been indirectly mediated by other *Hordeum vulgare*-associated microbes. Our results suggest a possible explanation for the typical low efficiency of the traditionally PGPR laboratory screenings, which detect "promising" strains, performing then inconsistently in the field. Our study shows in fact that the best promoters would have been discarded because not performing in pure culture assays, while the supposed "best candidates" eventually did not perform *ad planta*: as scientists devotedly seeking for environmental friendly solutions for sustainable agriculture, are we systematically discarding the best PGPRs, while wasting our energies on hopeless strains?

Cation analysis showed that E108 affected both concentrations and intake of all four analyzed cations in the plant tissues (Tab. 2). There are three recognized strategies of plant resistance to salt stress: osmotic stress tolerance, Na^+ exclusion and tissue tolerance to accumulated Na^+

(Carillo et al. 2011; Munns and Tester 2008). We could not test osmotic stress tolerance, since the salt was already present in the substrate of the second plant assay at the sowing stage. The results of the chemical analysis suggested that E108 leads to the increase of tissue tolerance to Na^+ accumulation: in fact, despite to a similar concentration of Na^+, inoculated plants were able to grow more (thus accumulating more cations). This suggests that intracellular mechanisms, such as compartmentalization of cations or organic solutes accumulation, might take place in barley leaf cells. Moreover, isolate E108 reduced the root surface uptake, so improving the Na^+ exclusion in an untypical manner, since the effect was not specific for the Na^+ as expected (Carillo et al. 2011) but generalized over all analyzed cations.

Since only phosphate solubilization was identified as PGP activity of E108 in pure culture assays, we cannot hypothesize additional growth promoting mechanisms. Further plant assays under gnotobiotic systems would be required in this case to unravel the possible PGP mechanisms of this strain on *Hordeum vulgare*, as resulting from the specific host-microbe interaction. This was not done here, because the primary scope of this work was to identify efficient PGPR under more natural (non-sterile) conditions.

CONCLUSIONS

The use of salt-stress alleviating PGPR can be an environmental friendly remedy to contrast the emerging problem of salinity in agriculture. Our direct screening approach to identify isolates of interest, revealed new taxa as fitness-enhancing bacteria (FEB) of *Hordeum vulgare* under salt stress and semi-natural conditions. The highest effect was

obtained when the salt stress was applied already at the sowing stage, which represents a more realistic situation compared to the imposition of salt stress gradually after seed germination. The best FEB was the isolate E108, belonging to the species *Curtobacterium flaccumfaciens*, an otherwise typical phytopathogen. E108 is therefore a suitable and highly interesting candidate for comparative genomics and for the investigation of the host-microbe interaction mechanisms in the differently affected host species. The discordance between pure culture assays and *ad planta* results suggests that the current PGP-screening methods commonly used may need to be re-evaluated, in order to detect promising plant growth promoters, thus increasing the efficiency of the PGPR investigations.

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Table 1 PGP activities of some bacterial isolates tested in pure cultures. Values indicate quantitative analysis (means ± SD), while +/- indicate qualitative results of plate/liquid assay (see material and methods for details).

Isolate	Next relative	ACC* (nmol h^{-1})	IAA (µg mL^{-1})	NF	PHO	PHY	SID
E38	*Microbacterium natoriense*	56.77 ± 34.16	15.85 ± 0.23	+	+	+	-
E134	*Streptomyces* sp.	-	11.39 ± 1.05	+	+	+	+
E8	*Pseudomonas brassicacearum*	8545.4 ± 2515.4	30.96 ± 2.52	+	-	-	+
E31	*Pseudomonas kilonensis*	114.15 ± 106.76	13.72 ± 0.26	+	-	-	+
PCL1751	*Pseudomonas fluorescens*	96.03 ± 4.27	12.82 ± 0.23	+	-	-	+
E110	*Ensifer garamanticus*	-	38.03 ± 0.98	+	+	+	-
E136A	*Bacillus subtilis*	292.31 ± 42.71	10.36 ± 0.22	+	-	-	-
E19	*Hartmannibacter diazotrophicus*	74.40 ± 24.45	-	+	+	-	-
E136B	*Brevibacterium frigoritolerans*	44.69 ± 42.71	-	+	-	+	-
E65	*Streptomyces bacillaris*	38.65 ± 0.00	-	+	-	+	-
E105	*Sphingopyxis taejonensis*	-	11.65 ± 0.59	+	-	-	-
E108	*Curtobacterium flaccumfaciens*	-	7.49 ± 0.16	-	+	-	-
E47	*Mycobacterium aurum*	83.95 ± 64.05	-	-	-	-	-
E48	*Rheinheimera aquimaris*	-	19.61 ± 1.28	-	-	-	-
E50	*Cellvibrio diazotrophicus*	-	-	+	-	-	-

*ACC=ACC-Deaminase production; IAA = Indol-Acetic-Acid production; PHO=Phosphate mobilization; PHY=Phytate mobilization; NF = Nitrogen fixation; SID = Siderophore production. Details in materials and methods.

Table 2 Cation Root surface uptake and normalized root-to-shoot cation translocation of plants inoculated with isolate E108 and uninoculated controls (S+B-). Values are mean ± SE. Different letters indicate significantly different means (Student's T-test, $p<0.05$) between treatments, within the same cation (N=5).

Cation	Treatment	Root surface uptake (mg g^{-1} root DW)	Normalized root-to-shoot translocation
Na^{+}	E108	121.28±6.26 A	1.62±0.19 a
	S+B-	157.39±11.21 B	1.67±0.32 a
Ca^{++}	E108	42.60±3.33 A	1.84±0.24 a
	S+B-	73.60±8.61 B	1.85±0.71 a
K^{+}	E108	138.68±3.28 A	2.07±0.17 a
	S+B-	262.02±30.97 B	2.86±0.81 a
Mg^{++}	E108	11.39±0.48 A	1.35±0.28 a
	S+B-	18.03±1.37 B	2.02±0.95 a

Figure Legends

Fig 1 (a) Bacterial genera isolated from the meadows "Münzenberg" (Germany; 50° 279´ 360´´ N; 8° 449´ 350´´ E), in the four sampling campaigns (b) Relative abundance of the five functional groups as resulted from bacterial isolation on different function-specific enrichment media (ACC= potential ACC-Deaminase producers; IAA = potential Indol-Acetic-Acid producers; PHO= potential phosphate mobilizers; PHY= potential phytate mobilizers; NF = potential nitrogen fixators).

Fig 2 Salt stress resistance of *Hordeum vulgare* (barley), calculated as percentage ratio between the fresh weight in presence of salt and without salt (boxes and whiskers represent mean ± SE, respectively). Effect of inoculation with single bacterial isolates or with the mix of all isolates (MIX) is compared with the uninoculated *H. vulgare* (S+ B-). Asterisks indicate means significantly different from control treatment "S+B-" (LSD Post-hoc test, $p< 0.05$)

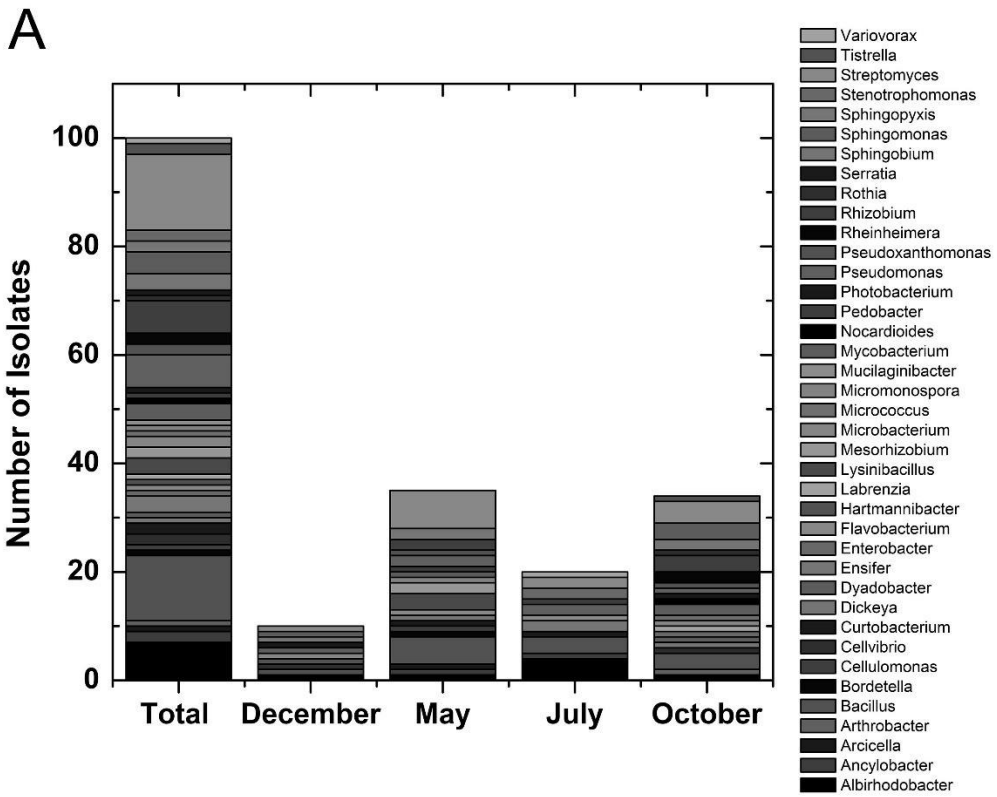
Fig 3 Percentage of relative increase (bars indicate mean ± SE) of both biomass and water content, separately shown for leaves and stems. Effect of inoculation with single bacterial isolates or with the mix of all isolates (MIX), compared with the uninoculated *H. vulgare* (treatment "S+ B-"). Asterisks indicate means significantly different from control treatment "S+B-" (LSD Post-hoc test, $p< 0.05$)

Fig 4 Germination rate of barley calculated on five pots (15 seeds per pot) eight days after sowing. Box and whiskers represent mean ± SE, respectively. The effect of the inoculation with single bacterial isolates (E108 or E110) and with the bacterial mixture (E108+E110) under salt stress is compared with uninoculated *H. vulgare* without salt stress (treatment "S-B-"), with *H. vulgare* inoculated with dead bacteria (treatment "S+D") and with uninoculated *H. vulgare* (treatment "S+B-"). Different letters indicate significantly different means at $p<0.05$ (LSD Post-hoc test)

Fig 5 Salt stress resistance of *H. vulgare*, calculated as percentage ratio between the fresh weight in presence of salt and without salt (boxes and whiskers represent mean ± SE, respectively). The effect of the inoculation with single bacterial isolates (E108 or E110) and with the bacterial mixture (E108+E110) under salt stress is compared with

uninoculated H. vulgare without salt stress (treatment "S-B-"), with H. vulgare inoculated with dead bacteria (treatment "S+D"). Different letters indicate significantly different means at $p<0.05$ (LSD Post-hoc test)

Fig 6 Percentage of relative increase (RI; mean ± SE) of both biomass and water content, separately calculated for leaves, stems and roots. The effect of the inoculation with single bacterial isolates (E108 or E110) and with the bacterial mixture (E108+E110) under salt stress is compared with uninoculated H. vulgare without salt stress (treatment "S-B-"), with H. vulgare inoculated with dead bacteria (treatment "S+D"). Different letters indicate significantly different means at $p<0.05$ (LSD Post-hoc test)


A
Number of Isolates
0
20
40
60
80
100
Total
December
May
July
October
Variovorax
Tistrella
Streptomyces
Stenotrophomonas
Sphingopyxis
Sphingomonas
Sphingobium
Serratia
Rothia
Rhizobium
Rheinheimera
Pseudoxanthomonas
Pseudomonas
Photobacterium
Pedobacter
Nocardioides
Mycobacterium
Mucilaginibacter
Micromonospora
Micrococcus
Microbacterium
Mesorhizobium
Lysinibacillus
Labrenzia
Hartmannibacter
Flavobacterium
Enterobacter
Ensifer
Dyadobacter
Dickeya
Curtobacterium
Cellvibrio
Cellulomonas
Bordetella
Bacillus
Arthrobacter
Arcicella
Ancylobacter
Albirhodobacter

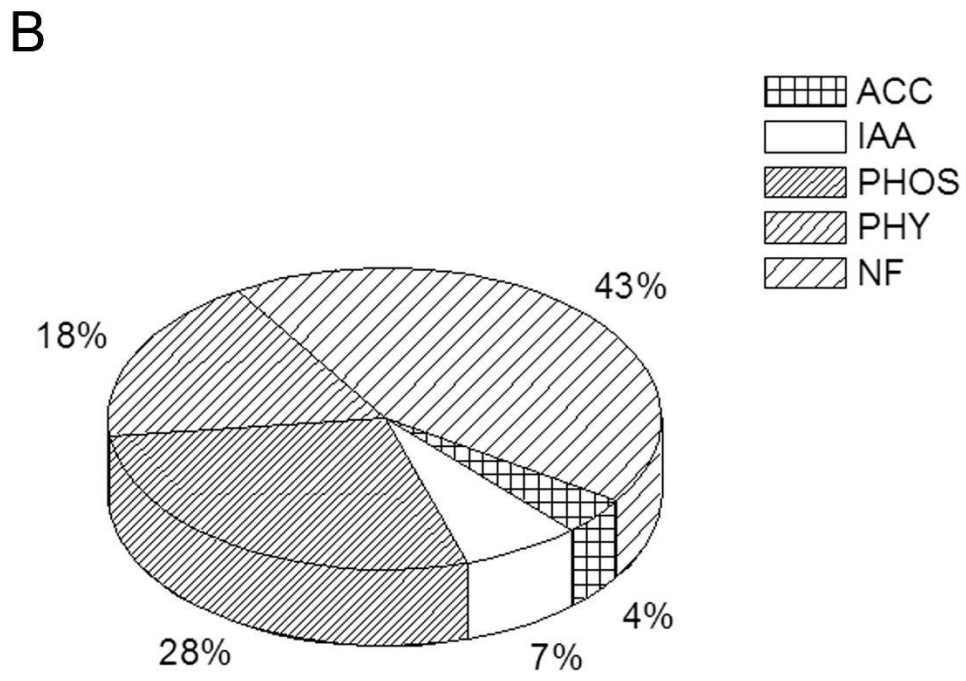

B
ACC
IAA
PHOS
PHY
NF
43%
18%
4%
28%
7%

Fig. 1

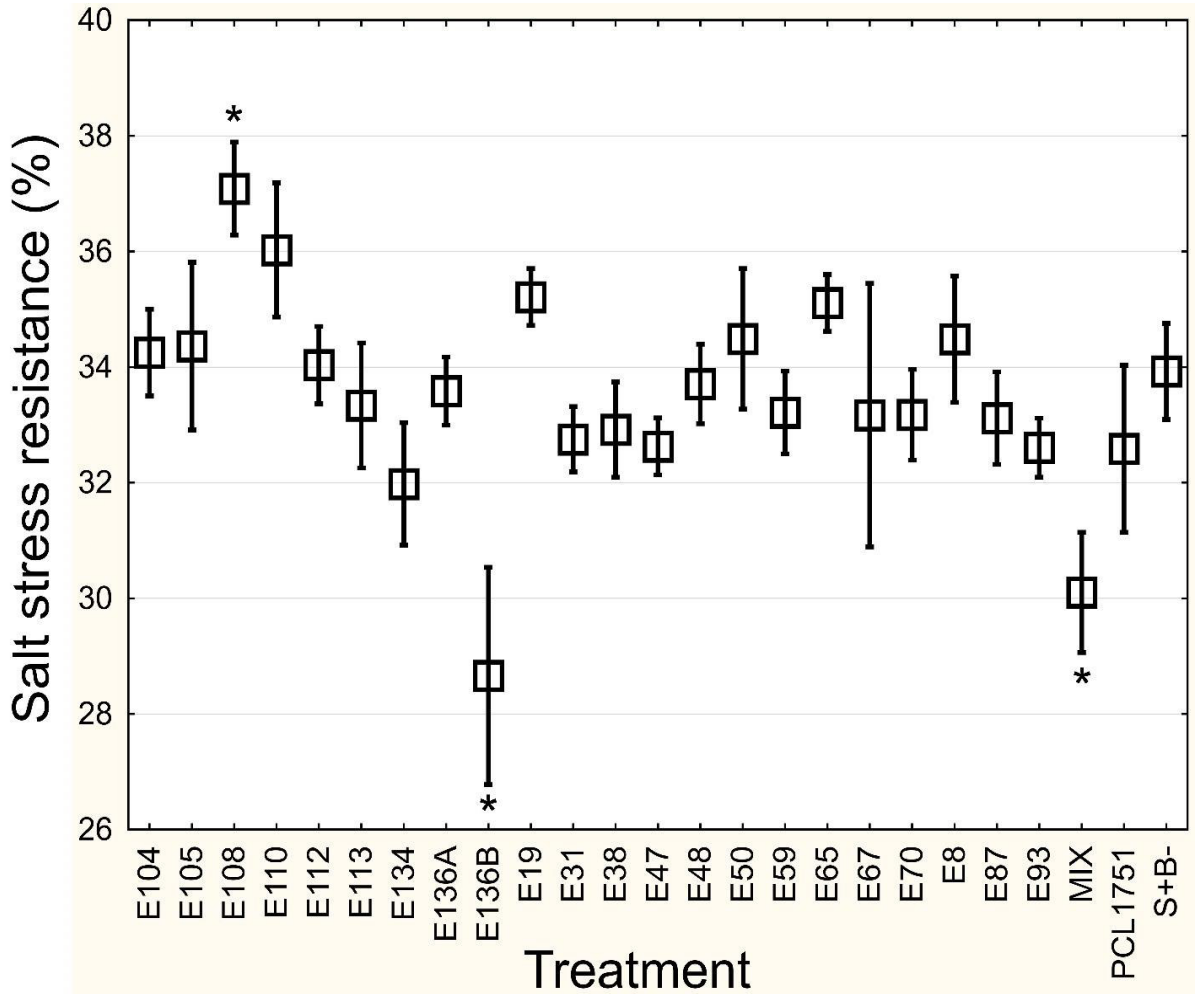

Salt stress resistance (%)
40
38
36
34
32
30
28
26
E104
E105
E108
E110
E112
E113
E134
E136A
E136B
E19
E31
E38
E47
E48
E50
E59
E65
E67
E70
E8
E87
E93
MIX
PCL1751
S+B-
Treatment

Fig 2

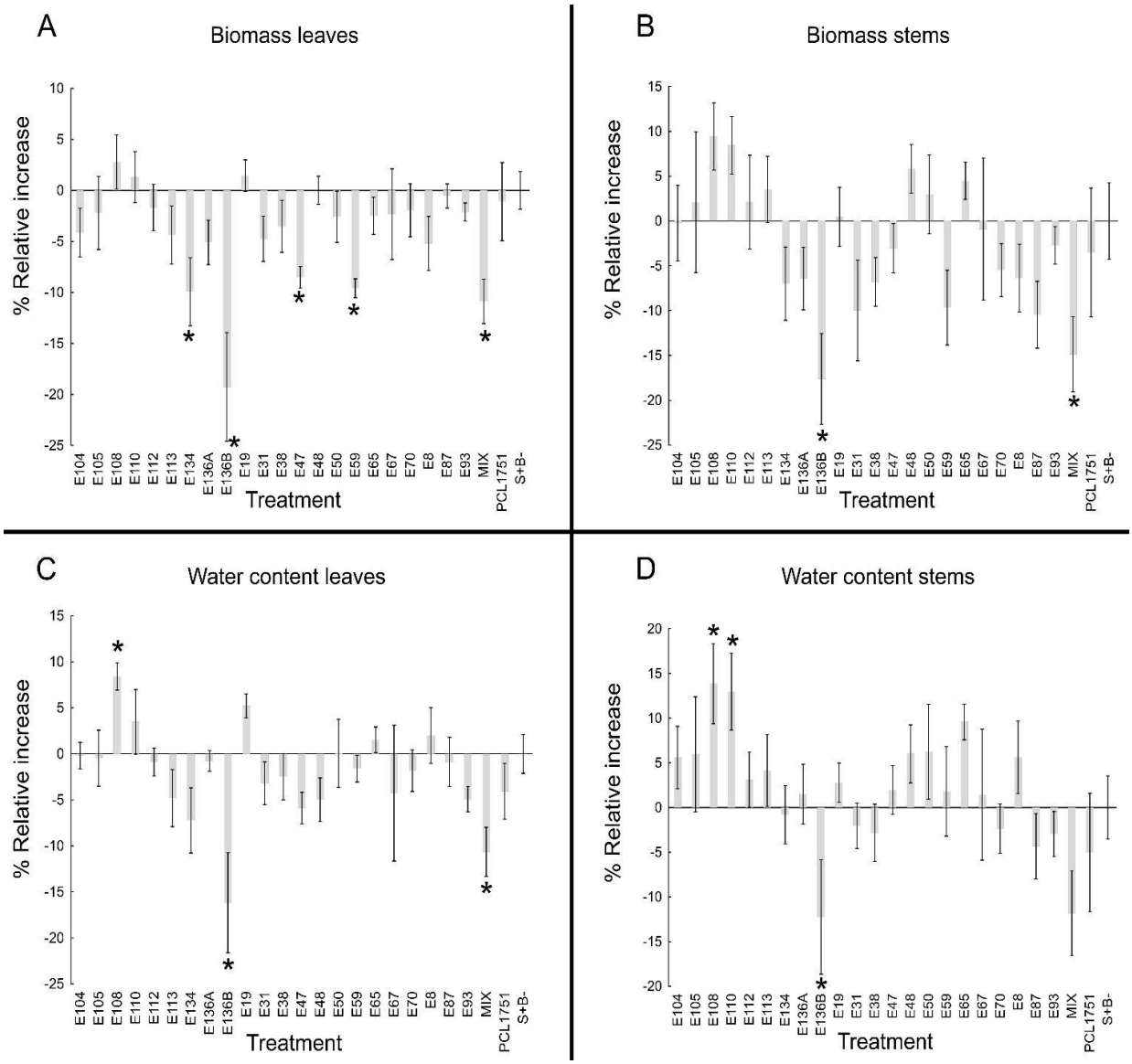

A
Biomass leaves
% Relative increase
Treatment
B
Biomass stems
% Relative increase
Treatment
C
Water content leaves
% Relative increase
Treatment
D
Water content stems
% Relative increase
Treatment
E104 E105 E108 E110 E112 E113 E134 E136A E136B E19 E31 E38 E47 E48 E50 E59 E65 E67 E70 E8 E87 E93 MIX PCL1751 S+B-

Fig 3

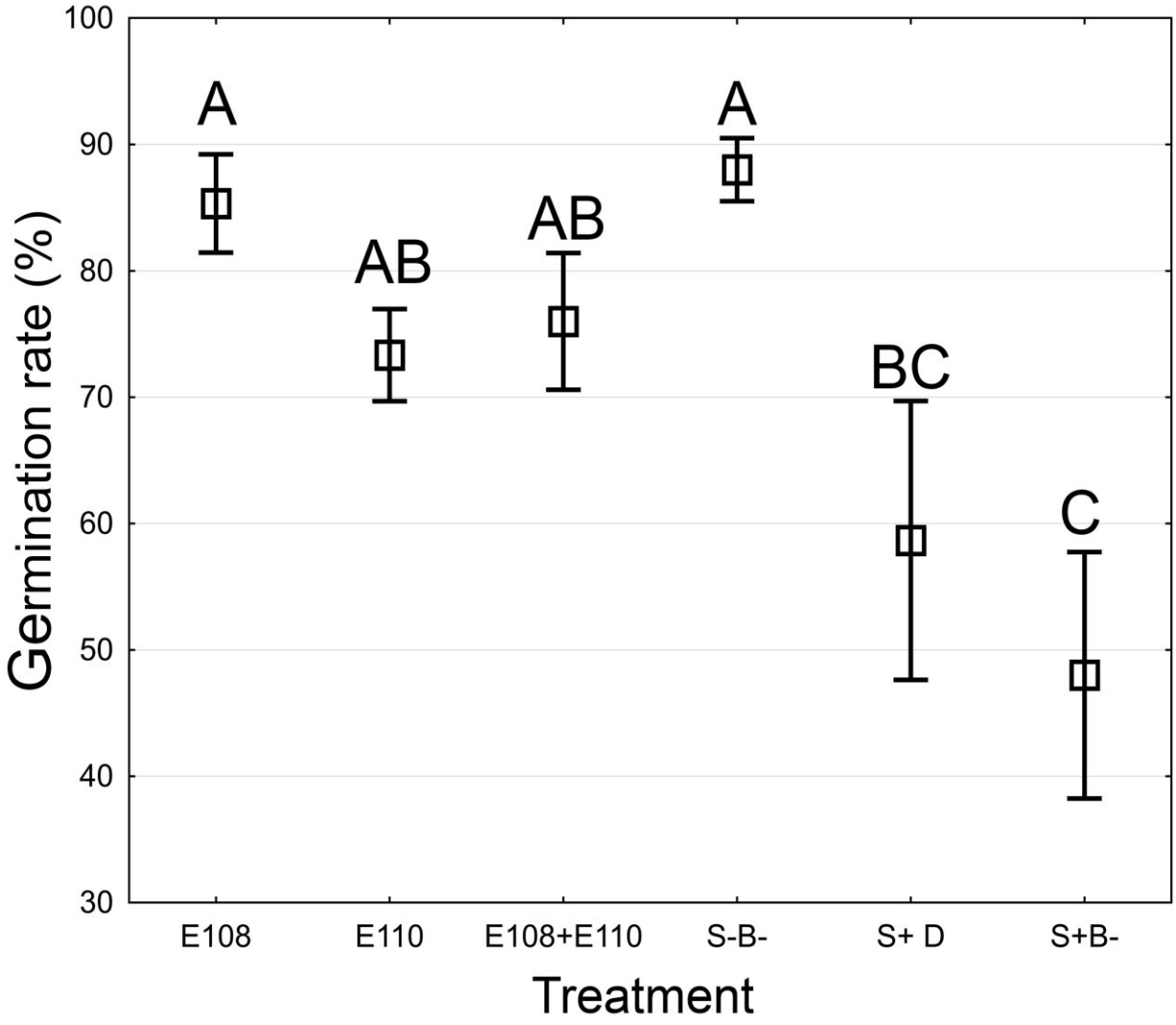

100
90
80
70
60
50
40
30
Germination rate (%)
A
AB
AB
A
BC
C
E108
E110
E108+E110
S-B-
S+ D
S+B-
Treatment

Fig 4

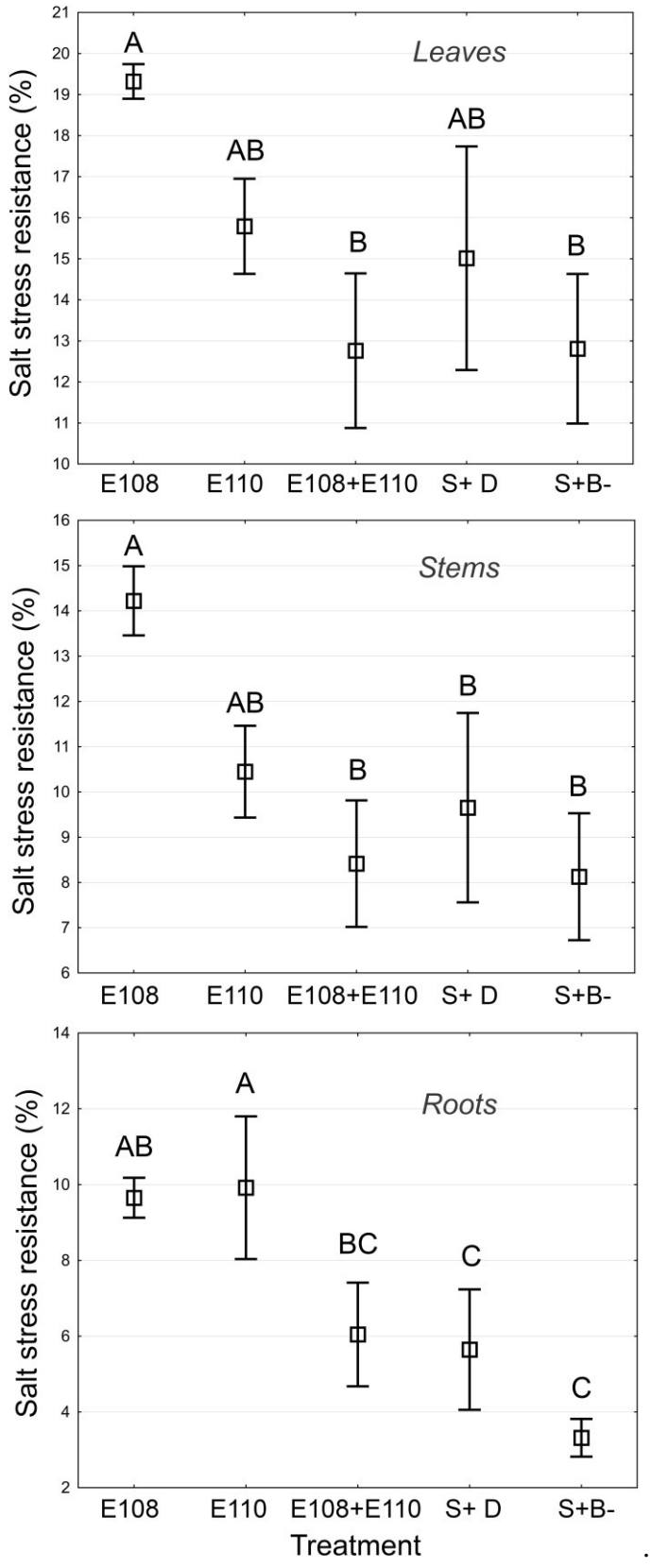

Leaves
Stems
Roots
Salt stress resistance (%)
A
AB
B
AB
B
A
AB
B
B
AB
A
BC
C
C
E108
E110
E108+E110
S+ D
S+B-
Treatment

Fig 5

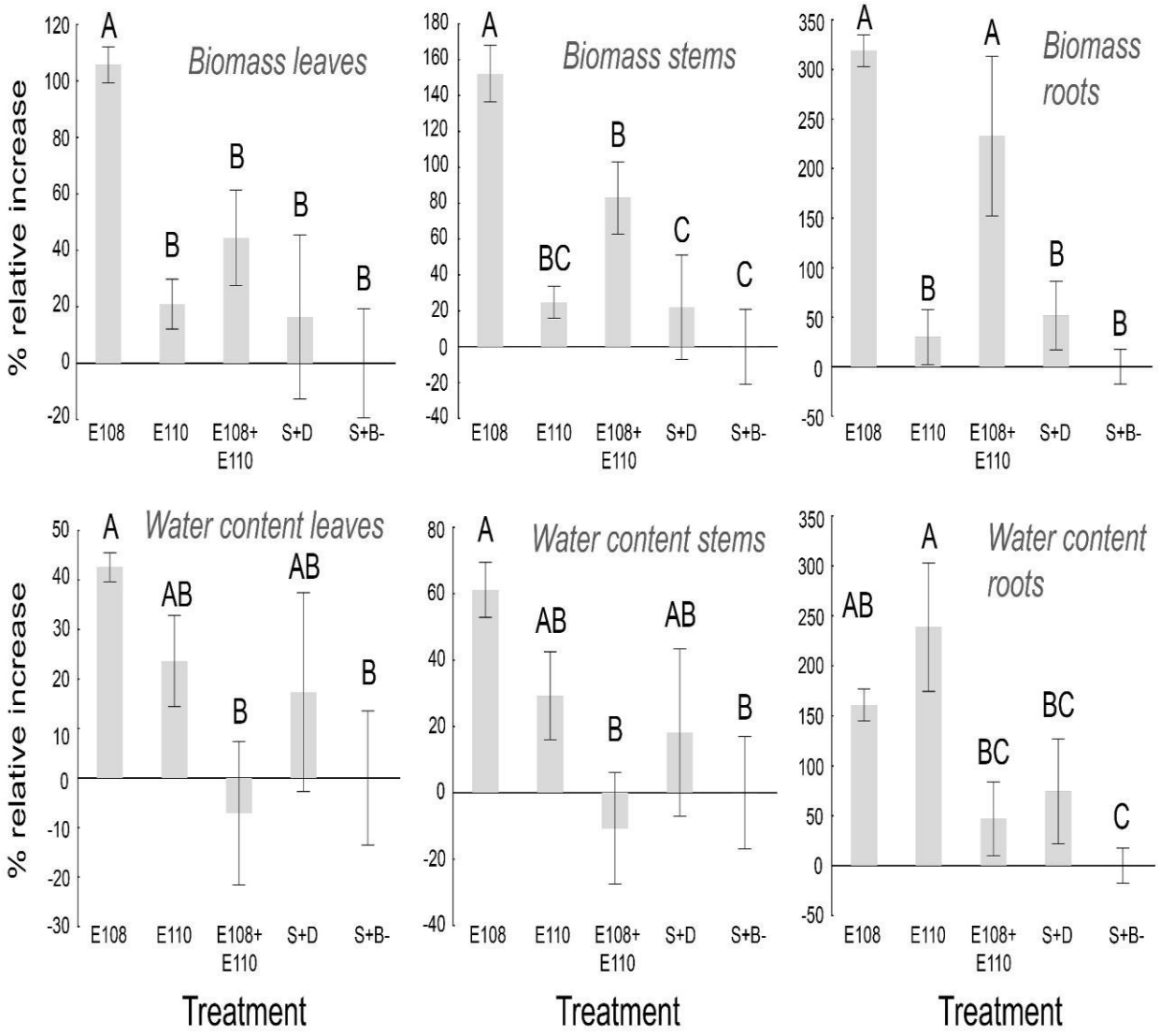
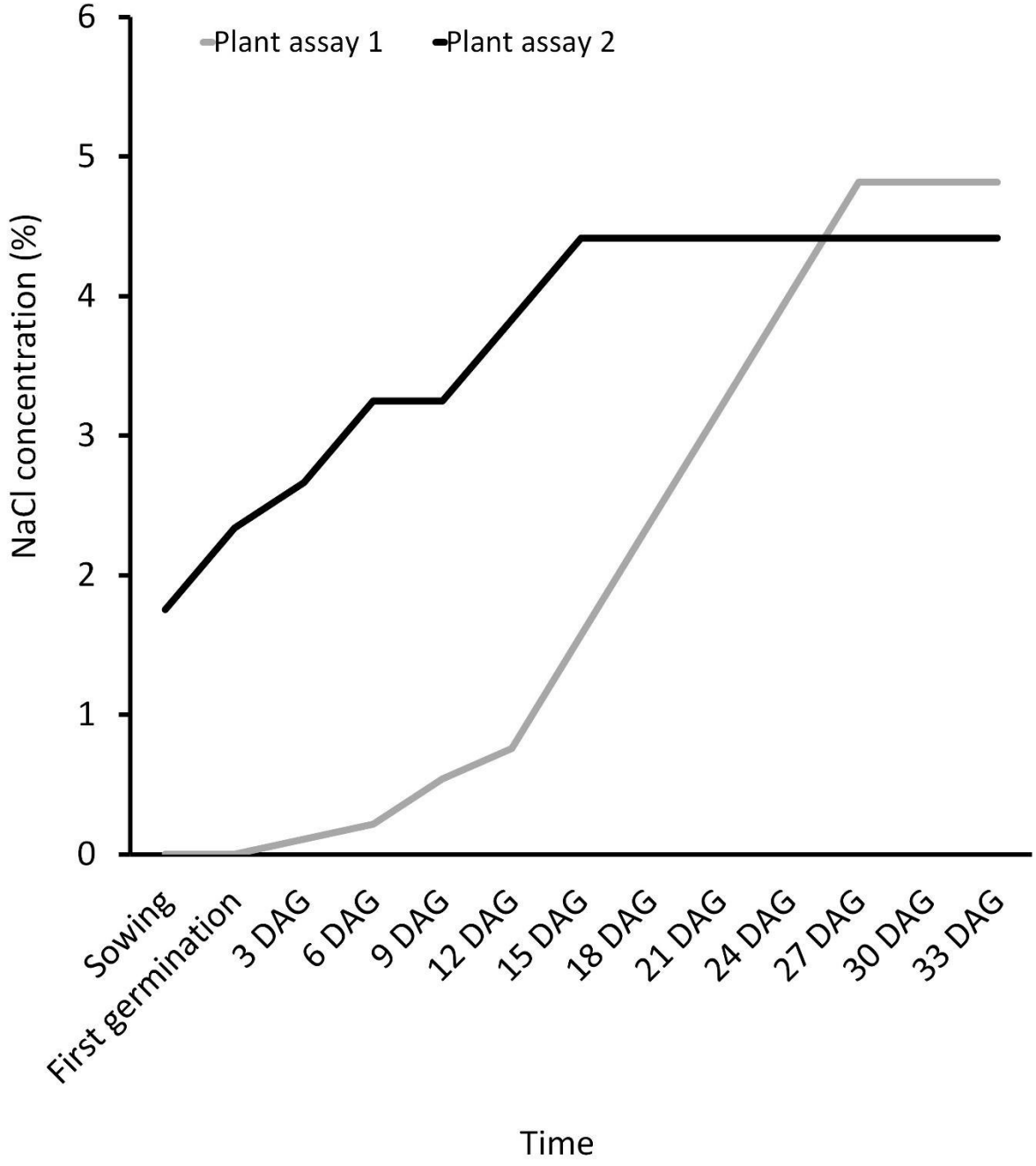

Biomass leaves
Biomass stems
Biomass roots
Water content leaves
Water content stems
Water content roots
% relative increase
% relative increase
E108
E110
E108+ E110
S+D
S+B-
Treatment
Treatment
Treatment

Fig 6

Supplement 1 Physico-chemical properties of the Classic Tonsubstrat ED 73 substrate (Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co.KG, Sinntal – Altengronau, Germany), the substrate used for the plant assays.

Physico-chemical parameter	**Value**
pH ($CaCl_2$)	5.8
KCl	2.5 g l^{-1}
EC	0.3–0.9 mS cm^{-1}
Nitrogen ($CaCl_2$)	250 mg l^{-1}
Phosphate (CAL)	300 mg l^{-1}
Kalium (CAL)	400 mg l^{-1}
Sulphur (fresh mass)	200 mg l^{-1}
Magnesium (fresh mass)	700 mg l^{-1}



Supplement 2 Scheme of the salt stress application to *H. vulgare* (barley) for the two plant growth promotion assays in greenhouse. Salt stress was induced by watering the pots with NaCl solutions to increasing NaCl concentration in the substrate. DAG: Days after germination.

Supplement 3 CFUs (a) and MPN (b) cell numbers of plant growth-promoting bacteria determined for four sampling times. The numbers are expressed as per g of dry rhizospheric soil.

Plant rhizosphere	Medium*	1st Sampling (December 2005)	2nd Sampling (July 2006)	3rd Sampling (October 2006)	4th sampling (May 2007)
Aerobic heterotrophic Bacteria					
P. winteri	K7 (a)	3.0×10^9	1.28×10^{11}	$2.9\ 10^9$	$7.3\ 10^{10}$
	(b)	$6.\ 910^{10}$	$4.5\ 10^{10}$	$5.3\ 10^8$	$2.3\ 10^{10}$
H. secalinum	K7 (a)	nd **	$1.5\ 10^{10}$	$1.8\ 10^9$	$1.2\ 10^{11}$
	(b)	nd	$5.4\ 10^9$	$6.2\ 10^8$	$2.5\ 10^{10}$
Nitrogen-fixing Bacteria					
P. winteri	LG (a)	$3.3\ 10^9$	$1.7\ 10^{11}$	8.8×10^9	$1.3\ 10^9$
	NFb (b)	$> 2.0\ 10^7$ (#)	$5.3\ 10^{10}$	1.010^9	$3.8\ 10^{10}$
H. secalinum	LG (b)	nd	1.510^9	1.110^{10}	$2.9\ 10^{10}$
	NFb (b)	nd	$5.4\ 10^9$	1.310^9	$2.5\ 10^{10}$
Phosphate-mobilising & Phytate-mobilising Bacteria					
P. winteri	CP (a)	$1.1\ 10^8$	$9.4\ 10^9$	$2.5\ 10^8$	$1.3\ 10^9$
	IHP (b)	$> 2.0\ 10^7$ (#)	$5.3\ 10^{10}$	$2.1\ 10^8$	$1.1\ 10^9$
H. secalinum	CP (a)	nd	$4.3\ 10^8$	$5.2\ 10^8$	$7.1\ 10^9$
	IHP (a)	nd	$3.4\ 10^8$	$1.3\ 10^9$	$6.2\ 10^9$
ACC-Deaminase-producing Bacteria					
P. winteri	DF (b)	$2.0\ 10^7$ (#)	$5.3\ 10^{10}$	$1.0\ 10^9$	$1.1\ 10^{10}$
H. secalinum	DF (b)	nd	4.0×10^{10}	6.810^9	$2.5\ 10^9$
IAA-producing Bacteria					
P. winteri	LBT (a)	nd	$2.2\ 10^{11}$	$8.8\ 10^8$	$1.4\ 10^{10}$
H. secalinum	LBT (a)	nd	$1.7\ 10^9$	$1.7\ 10^9$	$6.7\ 10^9$

* Selective media used for enrichment of different functional groups: K7 = complex medium, LG and NFb (N-free media) = nitrogen fixers; DF + ACC as only N-source: ACC-deaminase producers; CP (mineral medium with calcium phosphate): phosphate solubilizers; IHP (phytate containing mineral medium): Phytate solubilizers; LB + tryptophan: IAA producers.

** nd= not determined

= All dilution steps were overgrown; therefore the actual MPN cell number is expected to be higher.

Supplement 4 Bacterial isolates obtained by enrichment on specific culture media. Sampling campaign, 16S rDNA-based taxonomical identification, isolation source and isolation medium (with corresponding functional activity) are shown.

Sampling date	**Isolate name**	**Result of Identify Analysis (EZTaxon)**	**Sequence length (bp)**	**Similarity (%)**	**Isolation source**	**Isolation medium**	**Activity**
Dec 05	E1	*Sphingobium xanthum* (KF437579)	534	99.25	*Plantago*	NFB	NF
Dec 05	E4	*'Albirhodobacter marinus'* (FR827899) *Paracoccus caeni* (GQ250442)	556	98.92 95.55	*Plantago*	IHP	PHY
Dec 05	E5	*Serratia plymuthica* (AJ233433)	531	99.24	*Plantago*	IHP	PHY
Dec 05	E8	*Pseudomonas brassicacearum* (AF100321)	605	99.67	*Plantago*	DF-ACC	ACC
Dec 05	E9	*Sphingomonas sanxanigenens* (DQ789172)	568	98.24	*Plantago*	LG	NF
Dec 05	E10	*Streptomyces bacillaris* (AB184439)	419	100	*Plantago*	LG	NF
Dec 05	E14	*Bacillus aryabhattai* (EF114313)	550	100	*Plantago*	CP	PHOS
Dec 05	E15	*Micrococcus luteus* (CP001628)	484	99.79	*Plantago*	CP	PHOS
Dec 05	E19	*Hartmannibacter diazotrophicus* (KC567245)	1426	100	*Plantago*	CP	PHOS
Dec 05	E20	*Cellvibrio diazotrophicus* (JQ922426)	1474	98.08	*Plantago*	LG	NF
Jul 06	E21	*Streptomyces coeruleorubidus* (AJ306622)	452	98.64	*Hordeum*	LG	NF
Jul 06	E22	*Mucilaginibacter sabulilitoris* (JQ739458)	1429	99.30	*Hordeum*	LG	NF
Jul 06	E23	*'Albirhodobacter marinus'* (FR827899) *Paracoccus caeni* (GQ250442)	589	98.95 94.90	*Plantago*	LG	NF
Jul 06	E24	*Ensifer garamanticus* (AY500255)	931	96.99	*Plantago*	LG	NF
Jul 06	E25	*Pseudomonas corrugata* (D84012)	679	94.15	*Hordeum*	NFB	NF

Jul 06	E26	*Rhizobium selenitireducens (*EF440185*)*	560	98.04	*Plantago*	NFB	NF
Jul 06	E27	*Ensifer garamanticus (*AY500255*)*	880	97.95	*Plantago*	NFB	NF
Jul 06	E28	*Bacillus safensis (*AF234854*)*	524	99.81	*Hordeum*	CP	PHOS
Jul 06	E29	*Streptomyces rishiriensis (*AB184383*)*	503	100	*Hordeum*	CP	PHOS
Jul 06	E30	*Bacillus safensis (*AF234854*)*	594	99.81	*Hordeum*	CP	PHOS
Jul 06	E31	*Pseudomonas kilonensis (*AJ292426*)*	574	99.82	*Hordeum*	CP	PHOS
Jul 06	E32	*Ancylobacter dichloromethanicus (*EU589386*)*	576	99.82	*Plantago*	CP	PHOS
Jul 06	E33	*Bacillus safensis (*AF234854*)*	640	99.02	*Plantago*	CP	PHOS
Jul 06	E34	*Curtobacterium pusillum (*AJ784400*)*	632	99.20	*Plantago*	CP	PHOS
Jul 06	E37	*Variovorax paradoxus (*D88006*)*	593	99.65	*Hordeum*	IHP	PHY
Jul 06	E37II	*Stenotrophomonas rhizophila (*AJ293463*)*	603	98.77	*Hordeum*	IHP	PHY
Jul 06	E38	*Microbacterium natoriense (*AY566291*)*	518	98.61	*Hordeum*	IHP	PHY
Jul 06	E39	*Stenotrophomonas rhizophila (*AJ293463*)*	591	99.15	*Hordeum*	IHP	PHY
Jul 06	E40	*'Albirhodobacter marinus' (*FR827899*) Gemmobacter tilapiae (*HQ111526*)*	559	99.45 95.21	*Plantago*	IHP	PHY
Jul 06	E41	*'Albirhodobacter marinus' (*FR827899*) Paracoccus caeni (*GQ250442*)*	537	99.04 95.58	*Plantago*	IHP	PHY
Jul 06	E42	*'Albirhodobacter marinus' (*FR827899*) Paracoccus caeni (*GQ250442*)*	510	99.20 96.55	*Plantago*	LBT	IAA
Oct 06	E47	*Mycobacterium aurum (*X55595*)*	688	99.47	*Hordeum*	LG	NF
Oct 06	E48	*Rheinheimera hassiensis (JQ922423)*	1412	100	*Hordeum*	LG	NF
Oct 06	E49	*Rheinheimera muenzenbergensis (JQ922424)*	1416	100	*Hordeum*	LG	NF
Oct 06	E50	*Cellvibrio diazotrophicus (*JQ922426*)*	1409	100	*Hordeum*	LG	NF
Oct 06	E51	*Rhizobium alkalisoli (*EU074168*)*	621	98.82	*Plantago*	LG	NF
Oct 06	E54	*Mycobacterium aurum (*X55595*)*	679	99.15	*Plantago*	LG	NF
Oct 06	E55	*Streptomyces goshikiensis (*AB184204*)*	646	98.91	*Plantago*	LG	NF
Oct 06	E57	*Rothia amarae (*AY043359*)*	646	99.64	*Plantago*	LG	NF
Oct 06	E59	*Sphingomonas ginsenosidimutans (*HM204925*)*	551	100	*Hordeum*	NFB	NF

Oct 06	E60	*Pseudoxanthomonas japonensis (*AB008507*)*	626	99.47	*Hordeum*	NFB	NF
Oct 06	E61	*Sphingomonas ginsenosidimutans (*HM204925*)*	555	100	*Hordeum*	NFB	NF
Oct 06	E62	*Sphingobium limneticum (*JN591313*)*	612	97.85	*Plantago*	NFB	NF
Oct 06	E63	*Arthrobacter russicus (*AB071950*)*	459	100	*Plantago*	NFB	NF
Oct 06	E64I	*Dyadobacter ginsengisoli (*AB245369*)*	598	98.6	*Plantago*	NFB	NF
Oct 06	E64II	*Sphingobium limneticum (*JN591313*)*	649	96.11	*Plantago*	NFB	NF
Oct 06	E65	*Streptomyces bacillaris (*AB184439*)*	585	100	*Hordeum*	CP	PHOS
Oct 06	E66	*Tistrella mobilis (*AB071665*)*	1453	100	*Hordeum*	CP	PHOS
Oct 06	E67	*Bacillus safensis (*AF234854*)*	616	99.63	*Hordeum*	CP	PHOS
Oct 06	E69	*Streptomyces sanyensis (*FJ261968*)*	583	100	*Hordeum*	CP	PHOS
Oct 06	E70	*Rhizobium leguminosarum (*JH719379*)*	602	99.00	*Hordeum*	CP	PHOS
Oct 06	E71	*'Enterobacter oryzendophyticus' (*JF795011*)* *Enterobacter ludwigii (*AJ853891*)*	612	100 99.67	*Plantago*	CP	PHOS
Oct 06	E74	*Sphingomonas sanxanigenens (*DQ789172*)*	1409	98.36	*Plantago*	CP	PHOS
Oct 06	E75	*Bacillus safensis(*AF234854*)*	577	99.81	*Plantago*	CP	PHOS
Oct 06	E78	*Streptomyces malachitospinus (AB249954)*	624	99.20	*Plantago*	CP	PHOS
Oct 06	E80	*Nocardioides hankookensis (EF555584)*	628	98.54	*Plantago*	CP	PHOS
Oct 06	E83	*Bacillus simplex (*AB363738*)*	580	99	*Plantago*	CP	PHOS
Oct 06	E85	*Labrenzia suaedae (*GU322907*).*	644	97.66	*Plantago*	CP	PHOS
Oct 06	E87	*'Albirhodobacter marinus' (*FR827899*)* *Pseudorhodobacter antarcticus (*FJ196030*)*	581	99.11 95.05	*Hordeum*	IHP	PHY
Oct 06	E88	*Pseudomonas geniculata (*AB021404*)*	564	99.24	*Hordeum*	IHP	PHY
Oct 06	E89	*Photobacterium halotolerans (*AY551089*)*	1546	100	*Plantago*	IHP	PHY
Oct 06	E90	*Rhizobium mesoamericanum (*JF424606*)*	621	98.43	*Plantago*	IHP	PHY
Oct 06	E92	*Dickeya dadantii (*AOOE01000052*)*	628	96.72	*Plantago*	LBT	IAA
Oct 06	E93	*Microbacterium oxydans (*Y17227*)*	678	100	*Plantago*	LBT	IAA

May 07	E101	*Lysinibacillus xylanilyticus* (FJ477040)	698	100	*Hordeum*	LBT	IAA
May 07	E102	*Lysinibacillus xylanilyticus* (FJ477040)	652	97.88	*Hordeum*	LBT	IAA
May 07	E103	*Lysinibacillus xylanilyticus* (FJ477040)	701	100	*Hordeum*	LBT	IAA
May 07	E104	*Tistrella bauzanensis* (GQ240228)	622	99.66	*Plantago*	LBT	IAA
May 07	E105	*Sphingopyxis taejonensis* (AF131297)	581	100	*Hordeum*	IHP	PHY
May 07	E106	*Pseudomonas corrugata* (D84012)	637	99.83	*Hordeum*	IHP	PHY
May 07	E107	*Sphingopyxis taejonensis* (AF131297)	602	100	*Hordeum*	IHP	PHY
May 07	E108	*Curtobacterium flaccumfaciens* (AJ312209)	576	99.82	*Plantago*	IHP	PHY
May 07	E109	*'Albirhodobacter marinus'* (FR827899) *Paracoccus caeni* (GQ250442)	624	99.00 95.46	*Plantago*	IHP	PHY
May 07	E110	*Ensifer garamanticus* (AY500255)	614	97.87	*Plantago*	IHP	PHY
May 07	E111	*Mesorhizobium gobiense* (EF035064)	578	99.64	*Hordeum*	NFB	NF
May 07	E112	*Pedobacter koreensis* (DQ092871)	598	97.65	*Hordeum*	NFB	NF
May 07	E113	*Arcicella rosea* (AM948969)	543	100	*Hordeum*	NFB	NF
May 07	E114	*Rhizobium giardinii* (ARBG01000149)	662	95.91	*Plantago*	NFB	NF
May 07	E115	*Streptomyces flavovirens* (AB184834)	627	99.84	*Plantago*	NFB	NF
May 07	E117	*Pseudoxanthomonas spadix* (AM418384)	532	98.85	*Plantago*	NFB	NF
May 07	E118	*Streptomyces pratensis* (JQ806215)	636	100	*Hordeum*	LG	NF
May 07	E119	*Streptomyces zaomyceticus* (AB184346)	636	99	*Hordeum*	LG	NF
May 07	E122	*Pseudomonas corrugata* (D84012)	622	99.83	*Hordeum*	LG	NF
May 07	E123	*Mesorhizobium metallidurans* (CAUM01000060)	534	100	*Hordeum*	LG	NF
May 07	E124	*Bordetella trematum* (AJ277798)	613	99.67	*Hordeum*	LG	NF
May 07	E125	*Mycobacterium hodleri* (X93184)	510	98.62	*Hordeum*	LG	NF
May 07	E128	*Streptomyces pratensis* (JQ806215)	526	100	*Plantago*	LG	NF
May 07	E129	*Rhizobium sphaerophysae* (FJ154088)	602	99.65	*Plantago*	LG	NF
May 07	E130	*Ancylobacter rudongensis* (AY056830)	543	97.76	*Plantago*	LG	NF
May 07	E131	*Cellulomonas humilata* (X82449)	601	99.49	*Plantago*	LG	NF
May 07	E132	*Flavobacterium resistens* (EF575563)	649	96.04	*Plantago*	LG	NF
May 07	E134	*'Streptomyces siamensis'* (AB773848) *Streptomyces spiroverticillatus* (AB249921)	605	99.30 98.60	*Hordeum*	DF-ACC	ACC

May 07	136A	*Bacillus simplex*	1393	100	*Hordeum*	DF-ACC	ACC
May 07	136B	*Bacillus simplex*	1401	100	*Hordeum*	DF-ACC	ACC
May 07	E146	*Streptomyces canus (*AY999775*)*	621	100	*Hordeum*	CP	PHOS
May 07	E147	*Bacillus subtilis (*AMXN01000021*)*	617	99.03	*Hordeum*	CP	PHOS
May 07	E148	*Bacillus safensis (*AF234854*)*	599	99.83	*Plantago*	CP	PHOS
May 07	E151	*Micromonospora fulviviridis (*X92620*)*	578	99.47	*Plantago*	CP	PHOS
May 07	E155	*Streptomyces arenae (*AB249977*)*	392	99.49	*Hordeum*	CP	PHOS
May 07	E157	*Bacillus subtilis (*AMXN01000021*)*	556	99.82	*Plantago*	CP	PHOS

ACC = ACC-Deaminase production; PHOS = Phosphate mobilization; PHY = Phytate mobilization; NF = Nitrogen fixation; IAA = Indol-Acetic-Acid production

Supplement 5 Cation content of *Hordeum vulgare* plants. Values indicate means ± SE (N=5), while different letters indicate significantly different means within the same cation (Tukey HSD test, $P<0.05$).

Cation	Habitat	Treatment	Concentration (mg g^{-1} DW)	Total intake (mg pot^{-1})
Na^{+}	Leaves	E108	25.61±1.19 ab	18.53±1.15 a
		S+B-	21.69±2.35 bc	7.01±0.99 b
	Stems	E108	30.16±2.30 a	17.44±1.32 a
		S+B-	27.40±3.31 ab	5.85±1.01 b
	Roots	E108	17.25±1.20 c	6.00±0.54 b
		S+B-	15.13±1.41 c	1.31±0.14 c
Ca^{++}	Leaves	E108	11.93±0.96 a	8.64±0.83 a
		S+B-	12.46±0.72 a	4.47±0.82 b
	Stems	E108	7.25±0.34 bc	4.22±0.32 b
		S+B-	8.72±0.09 b	2.00±0.36 c
	Roots	E108	5.38±0.32 c	1.86±0.14 c
		S+B-	7.66±2.31 bc	0.58±0.05 c
K^{+}	Leaves	E108	33.50±0.77 b	24.17±0.71 a
		S+B-	41.87±0.44 a	14.67±2.50 b
	Stems	E108	31.51±0.33 b	18.31±0.97 b
		S+B-	39.75±1.55 a	9.41±1.89 c
	Roots	E108	18.81±0.54 c	5.46±0.22 cd
		S+B-	15.74±2.28 c	1.35±0.18 d
Mg^{++}	Leaves	E108	2.55±0.16 ab	1.85±0.15 a
		S+B-	2.66±0.11 a	0.90±0.14 c
	Stems	E108	2.49±0.13 ab	1.44±0.09 b
		S+B-	2.84±0.09 a	0.65±0.12 c
	Roots	E108	1.91±0.09 bc	0.66±0.03 c
		S+B-	1.78±0.50 c	0.14±0.03 d

Chapter 3

Gnotobiotic plant growth liquid system and subsoil experiments

Summary

The newly designed plant liquid growth system allows evaluation of NaCl salt stress effect on barley plants but in order to evaluate a bacterium plant growth-promoting effect using this system it is needed the use of germ-free plantlets or seeds. Analysis for bacteria vertically transfer in seeds is remarkably important for plant microbe interaction studies under gnotobiotic conditions. The subsoil sand mixture in Mitscherlich pots allows to evaluate barley plants growth under nitrogen nutritional deficiencies, different P fertilization sources and salt stress effect in growth chamber conditions.

1. Material and methods

1.1 Seeds surface sterilization and bacterial inoculum

Barley (*Hordeum vulgare* L. cultivar Morex or cultivar Propino) seeds were surface sterilized using a solution with final 3 % bleach concentration (Hurek et al., 1994) and washed seven times under gentle agitation for 10 min with sterile water. Growth curves of the strains (Appendix 1) were measured in TSB (strains $E48^T$ and $E50^T$) and/or marine broth half concentration (strain $E19^T$) under optimal conditions to determinate cell concentrations needed in liquid culture for the inoculation process in bacteria-plant interaction experiments. The parameters of absorbance and cells per ml were obtained by measuring the OD with a spectrophotometer (Genesys 10UV, Thermo Spectronic) at 600 nm. The OD was calibrated using a Thomma counting chamber (Labor Optik, Germany). With these values the regression curve from the log phase for each strain was calculated. In all cases results of cell ml^{-1} were confirmed by counting colony forming unit per ml (CFU ml^{-1}). In order to produce bacterial inoculums for plant experiment, selected strains were grown in their respective media under best growth conditions (Suarez et al., 2014 a,b,c), centrifuged at 3345 g and resuspended in 30 mM $MgSO_4$ solution to reach a 10^7– 10^8 CFU ml^{-1} concentration in exponential growth phase.

1.2 Gnotobiotic system

Surface sterilized barley (*Hordeum vulgare* L. cv. Morex) seeds were placed in petri dishes containing sterile filter paper, moisturized with 3 ml of sterile distillated water, and

incubated for 48 h at 25 °C in darkness. Plantlets were placed in sterile 50 ml tube with 25 ml of grown culture of strain *Rheinheimera hassiensis* strain E48^T (Suarez et al., 2014b) and incubated for 1 h at 28 °C in darkness under slow agitation. Inoculated plantlets were transfer to glass tubes (4 cm width X 14 cm length) filled with 100 g autoclaved quartz sand and 10 ml of sterile plant nutrient solution 5 mM $Ca(NO_3)_2$, 1 mM K_2SO_4, 0.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.5 mM $CaCl_2$, 10 µM H_3BO_3, 2 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.01 µM$(NH_4)_6Mo7O_{24}$, 0.2 µM Fe-EDTA (Hatzig et al., 2009). Greenhouse conditions for plant growth were 1000 lux, 20 °C during day and 14 °C during night for 1 week. For sampling shoots (leaf blades + sheaths) and roots of the 5 inoculated and uninoculated plants were separated and collected in separated paper bags, and their fresh weights were recorded. Dry biomass accumulation and water content were assessed after 3 days drying at 80 °C.

1.3 Plant growth liquid system

Surface sterilized barley (*Hordeum vulgare* L. cv. Morex) seeds were peeled under sterile conditions using forceps and placed individually in SG agar (yeast extract, peptone, glucose, and agar) for 72 h at 25 °C in darkness in order to detect bacterial growth and provide conditions for seed germination (Fig. 1A). Seedlings that presented none bacterial growth on the agar SG where transfer with forceps under sterile conditions to 50 ml sterile centrifugation tubes (Falcon; Becton Dickinson, Paramus, N.J.). Bacterial inoculum of *Cellvibrio diazotrophicus* E50^T (Suarez et al., 2014c) was added to the tube containing the seedlings and incubated for 2 h at 28 °C in darkness under slow agitation (Fig.1B). Inoculated plantlets were transfer to sterile rubber plugs (1.7 cm, 2 cm length), with a central hole of approximately 0.5 cm, placing the root system at the lower part of rubber plug to protect it from light influence, and immediately fixed with sterile agar 1.5 % (50 °C). Four rubber plugs with the fixed plantlets were transfer to round grey plastic racks (4.5 cm diameter x 3 cm depth) and placed in sterile transparent plastic boxes (9.5 x 8.0 x 12 cm) filled with 175 ml $CaSO_4$ 1 mM solution. (Fig.1C). The boxes were placed under controlled conditions for 72 h daylight from 06:00 to 22:00 (artificial light switched off when natural light exceeded 10 Klx), and temperature of 20 °C and 16 °C (day and night, respectively).

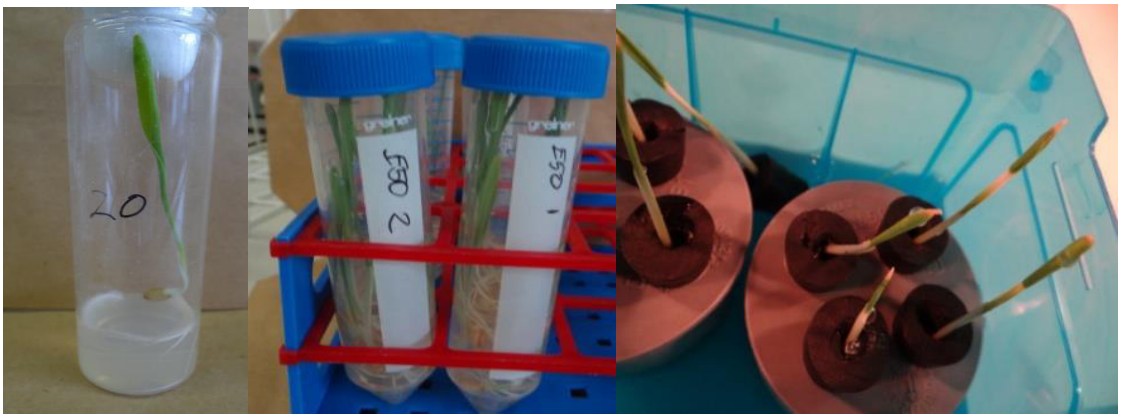


Fig.1. Surface sterilized barley (*H. vulgare* L. cv. Morex) seeds plantlets. A) 72 h plantlets on SG agar tubes; B) Plantlets incubated with inoculums of *C. diazotrophicus* $E50^T$; C) transparent plastic boxes containing plastic racks with inoculated plantlets fixed in the rubber plugs.

Plantlets fixed in the rubber plugs were transfer to a root sterile liquid system with 2 l capacity (Fig 2A). Plant nutrient solution was supplied to the system laterally at ~160 ml min^{-1} under sterile conditions by transferring the corresponding sterile solution from a 2 l glass flask (Schott, Mainz, Germany), connected through a hose to the system, by air flow strength generating an overpressure in the Schott flask with an air pump (Marina model 200, Hagen Germany) injecting filtrated air through a 0.2 µm pore membrane (Sarstedt Nümbrecht, Germany) (Fig. 2B). Liquid was removed from the system at around 250 ml min^{-1} by gravity and slight overpressure through a fluid outlet at the bottom of the vessel. To maintain plant root and bacterial growth under oxygen conditions filtered sterile air was supplied the liquid medium of the system through a hose entering the system from the top and ending at the lateral bottom of the vessel using an air pump (Marina model 200, Hagen Germany) at a 90 l h^{-1} flow. An air outlet was located on the top of the system covered by a 0.2 µm pore membrane filter.

Plant nutrient solution (PNS) containing 5 mM $Ca(NO_3)_2$, 1 mM K_2SO_4, 0.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.5 mM $CaCl_2$, 10 µM H_3BO_3, 2 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 0.2 µM Fe-EDTA (Hatzig et al., 2009). Conductivity was measured for all solution with conductivity meter (Model 3110) with measuring cell (TetraCon 325) (WTW, Weilheim Germany).

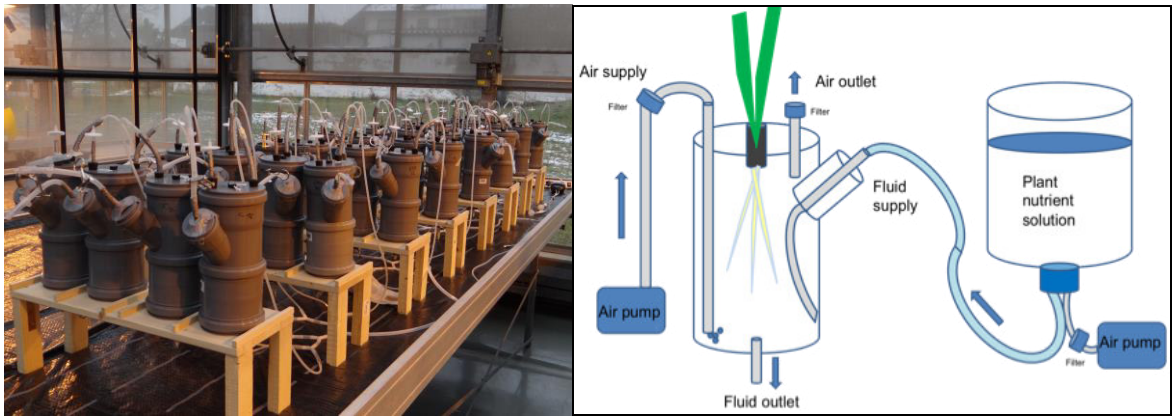


Fig.2. Root sterile liquid systems A) Liquid sterile system disposition; B) Liquid sterile system functioning diagram.

Experimental design was set to obtain five homogeneous plants inoculated with strain E50^T and five un-inoculated homogeneous plants grown for 45 days in plant nutrient solution at 0 mM, 100 mM and 150 mM NaCl concentration respectively. Solutions were changed every 5 days in order to maintain equal conditions through the experiment and to increase the salt concentration stepwise as described in Table 1, and pH was set to 6.5.

Table.1. Barley (*H. vulgare* L. cv. Morex) plant growth conditions though experimentation describing the day plan of the plant nutrient solution concentration and its NaCl concentration stepwise increase with their respective electrical conductivity in dS m^{-1}.

NaCl plant nutrient solution concentration	Day	Plant nutrient solution concentration	Day	dS m^{-1}
SG agar	0	-	0	-
Sterile rubber in plastic boxes	3	¼	3	0.34
0 mM	7	½	7	0.65
25 mM	12	½	12	3.31
50 mM	17	½	17	6.18
75 mM	22	½	22	8.71
100 mM	27	½	27	11.15
125 mM	32	½	32	13.39
150 mM	37	½	37	15.87
150 mM	42	½	42	15.87

Plant sampling for this assay was done after 45 days of growth from shoots and roots of the 5 plants of each treatment measuring their length. Plant material was collected in separated paper bags and their fresh weights recorded. Dry biomass accumulation and water content were assessed after 3 days drying at 80 °C. In order to determine rhizospheric CFU ml^{-1} approximately 0.2-0.5 grams from different parts of the root system were cut, placed in a 50 ml centrifugation tube (Falcon), weighted and 9 ml sodium pyrophosphate 0.18% was added. After 30 min gentle shaking 0.1 ml of the dilutions 10^5-10^7, from decimal serial dilution in NaCl 0.9 %, were inoculated in TSA and incubated for 48 h 28 °C. For endorhizospheric the sampled roots used previously for rhizospheric analysis were immersed for 10 min in a 1 % sterile chloramine solution followed by 5 times washes with PBS 1X and shortly place and moved over TSA agar using forceps. Subsequently, the root samples were transfer to a Stomacher bag containing 9 ml 1X PBS sterile solution, shaken 120 s in high speed and pressed on a mortar using a pestle. Obtained suspension (1 ml) was used to make a decimal serial dilution in NaCl 0.9 % and 0.1 ml from dilutions 10^1-10^3 was inoculated on TSA and incubated for 48h 28 °C. CFU ml^{-1} of each corresponded treatment were counted and colonies with different morphological characteristics were used for PCR colony amplification of their 16S rRNA gene using PCR protocol using EUB9f (9-27) and EUB 1492r primer pair (Lane, 1991) as described by Kampmann et al. (2012). The PCR products were cleaned using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and sequenced by the company LGC genomics (Berlin, Germany). The obtained 16S rRNA gene sequences of the isolates were compared with relatives retrieved from the GenBank database using the MEGA software version 5.0 (Tamura *et al.*, 2011).

1.4 Sub soil plant growth promotion experiment using Mitscherlich pots.

1.4.1 Plant growth promotion experiment under nitrogen deficiency and insoluble phosphate source

Twenty surface sterilized seeds of *Hordeum vulgare* L. cv. Propino seeds (Syngenta, Bad Salzuflen Germany) inoculated with *Hartmannibacter diazotrophicus* strain $E19^T$ were seeded evenly with tweezers in Mitscherlich pots containing a mixture of 3.25 kg of subsoil (Appendix 3) and 3.25 kg quartz sand. The soil in the pots were treated, in order to avoid deficiencies, with 160 mg N (NH_4NO_3), 100 mg P ($Ca(H_2PO_4)_2*H_2O$), 400 mg K (K_2SO_4), 50 mg Mg ($MgSO_4$ x 7 H_2O), 0.46 mg B (H_3BO_3), 0.11 mg Mo ($(NH_4)_6MoO_{24}$ x

$4H_2O$), 40 mg Mn ($MnSO_4$ x H_2O), 10 mg Zn ($ZnSO_4*7H_2O$) and 5 mg Cu ($CuSO_4*5H_2O$) mg (kg soil)$^{-1}$ respectively and similarly as Steffens et al. (2010). In order to set treatment for nitrogen deficiency the nitrogen source was reduced to 16 mg N NH_4NO_3 (kg soil)$^{-1}$ and treatment with water insoluble phosphate source was 100 mg P $Ca_3(PO_4)_2$ mg (kg soil)-^{1} instead of the water soluble source $Ca(H_2PO_4)_2$ H_2O. Twenty days after sowing, barley plants were fertilized with 83.3 mg N (NH_4NO_3) (kg soil)$^{-1}$ excluding those settled under nitrogen deficiency. Water-holding capacity was kept at 60 % with tap water throughout all experimentation.

Seven days after sowing 10 homogeneous seedlings were left for experimentation removing non-homogeneous and extra seedlings. For all treatments, Mitscherlich pots with un-inoculated seeds were set in same growth conditions to evaluate plant growth promotion by the inoculated bacteria. After 8 days of the first inoculation, a re-inoculation of 200 ml of bacterial suspension of strain E19^T was applied to all pots belonging to this treatment and 200 ml of 30 mM $MgSO_4$ solution to un-inoculated treatments. Plant growth conditions were 16 h at 22 °C with light and 8 h at 18 °C with darkness at 60 % of relative humidity in growth chamber (Weiss-Technik, Umwelttechnik GmbH, Reiskirchen).

Plant sampling for this assay was done after 35 days of growth after sowing. For sampling shoots (leaf blades + sheaths) were measured and cut with scissors 1 cm over subsoil mixture surface. Shoots from each pot were collected in separated paper bags, and their fresh weights recorded. Dry biomass accumulation and water content were assessed after 3 days drying at 80 °C. CFU from rhizosphere and endorhizosphere from pots inoculated plants with strain E19^T where analyzed in order obtain predominant colonies to determine the presence of the strain. For this 0.2-0.5 grams from different parts of the root were cut, placed in a 50 ml centrifugation tube, weighted and 9 ml sodium pyrophosphate 0.18 % added. After 30 minutes gentle shaking 0.1 ml of the dilutions 10^4-10^7, from decimal serial dilution in NaCl 0.9 %, were inoculated on calcium phosphate agar (CP) containing 1 % saccharose, 0.01 % NaCl, 0.05 % $MgSO_4.7H_2O$, 0.02 % yeast extract, 0.05 % NH_4Cl, 0.01 % $MnSO_4.H_2O$, $Ca_3O_8P_2$ 0.5 %, 0.5 % (v/v) bromocresol green (0.5 % in 0.2M KOH) and 1.5 % agar, and incubated for 48 h 28 °C. For assessment of endorhizospheric bacteria the sampled roots used previously for rhizospheric analysis were immersed for 10 min in a 1 % sterile chloramine solution followed by 5 times washing with PBS 1X and shortly place and moved over TSA and half marine agar using forceps. Subsequently, the root

samples were transfer to a Stomacher bag containing 9 ml 1X PBS sterile solution, shaken 120 sec in high speed and pressed on a mortar using a pestle. 1 ml of obtained suspension was used to make a decimal serial dilution in NaCl 0.9% and 0.1 ml from dilutions 10^1-10^3 was inoculated on CP agar and incubated for 48h 28°C. Several colonies with similar growth characteristics of strain E19^T on CP agar were sub-cultured on CP agar and their colony morphology, catalase activity and microscopic characteristics compared with strain E19^T characteristics. Selected colonies were used for PCR colony amplification of their 16S rRNA gene using PCR protocol using EUB9f (9-27) and EUB 1492r primer pair (Lane, 1991) as described by Kampmann et al. (2012). The PCR products were cleaned using the QIA quick PCR purification kit (Qiagen) and randomly restricted using endonucleases *HpaII* and *Hin6I.* Restriction products were separated by electrophoresis in 2.75 % TBE-agarose gel. As control it was use E19^T genomic DNA restricted under same conditions in order to compare ARDRA pattern of the16S rRNA gene PCR products for the selected isolates.

1.4.2 Plant growth promotion experiment using different phosphate sources under salt stress

H. diazotrophicus E19^T was isolated as phosphate-mobilizing bacterium (Suarez et al. 2014C) and therefore its ability to support plant growth under limited phosphate conditions was tested. Twenty surface sterilized seeds of *Hordeum vulgare* L. (cultivar Propino) seeds (Syngenta, Bad Salzuflen Germany) inoculated with *H. diazotrophicus* E19^T were seeded evenly with tweezers in Mitscherlich pots containing a mixture of 3.25 kg of subsoil (Appendix 1) and 3.25 kg quartz sand. The soil in the pots was treated with 160 mg N (NH_4NO_3), 400 mg K (K_2SO_4), 50 mg Mg ($MgSO_4$ x 7 H_2O), 0.46 mg B (H_3BO_3), 0.11 mg Mo (($NH_4)_6MoO_{24}$ x 4H_2O), 40 mg Mn ($MnSO_4$ x H_2O), 10 mg Zn ($ZnSO_4$*7H_2O) and 5 mg Cu ($CuSO_4$*5H_2O) mg (kg soil)$^{-1}$ respectively and similarly as Steffens et al. (2010).

Pots where adjusted with NaCl to generate salt stress conditions at 15 ds m^{-1} and with different P fertilization at 100 mg P kg soil^{-1} for either $Ca(H_2PO4)_2$, GAFSA, or organic P as phytate dodeca-sodium salt (Na-hexaphytate, $C_6H_6O_{24}P_6Na_{12}$) (Sigma- Aldrich, USA). In order to observed P fertilization effect and salt stress effect, pots without P fertilization under salt stress and pots under non salt stress fertilized with highly soluble phosphate form $Ca(H_2PO4)_2$ were settled. Twenty days after sowing, barley plants were fertilized

with 83.3 mg N (NH_4NO_3) kg $soil^{-1}$. Water-holding capacity was kept at 60 % with tap water throughout experimentation.

Seven days after sowing 10 homogeneous seedlings were left for experimentation removing non-homogeneous and extra seedlings. For all treatments, Mitscherlich pots with un-inoculated seeds were set in same growth conditions to evaluate plant growth promotion. After 8 days of the first inoculation, a re-inoculation of 200 ml of bacterial suspension of $E19^T$ was applied to all pots belonging to this treatment and 200 ml of 30 mM $MgSO_4$ solution to un-inoculated treatments. Plant growth conditions were 16 h at 22 °C with light and 8 h at 18 °C with darkness at 60 % of relative humidity in growth chamber (Weiss-Technik, Umwelttechnik GmbH, Reiskirchen).

Plant sampling for this assay was done after 35 days of growth after sowing. For sampling shoots (leaf blades + sheaths) were cut with scissors 1 cm over subsoil mixture surface. Shoots from each pot were collected in separated paper bags, and their fresh weights recorded. Dry biomass accumulation and water content were assessed after 3 days drying at 80 °C.

1.5 Statistical analysis

Statistical differences of the different experiments were performed using Shapiro-Wilk test to check the normal distribution of the data. The analysis of variances was performed using ANOVA and either Tukey HSD or Fisher LSD post-hoc test at $p<0.05$ were assessed using software Statistica (Statsoft Inc., Tulsa, USA) version 12.

2. Results

2.1 Gnotobiotic system

A gnotobiotic system was used in order to analysis plant growth interaction of *R. hassiensis* strain $E48^T$ and surface sterilized barley (*H. vulgare* L. cv. Morex) seeds. However, it was not possible to obtained homogeneous growth parameters on the fresh and dry weight due to the strong attachment of sand particles to the whole root surface altering these values (Fig.3).

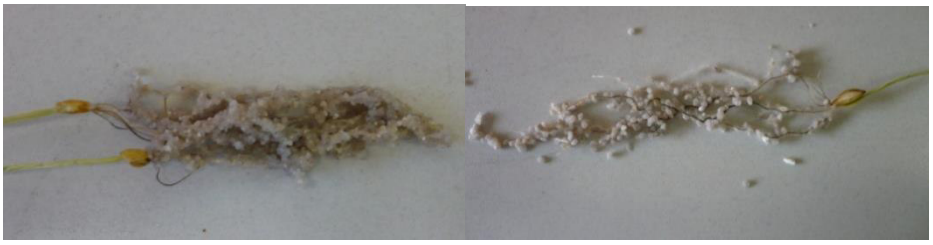


Fig.3. Attachment of sand to the root system on *H. vulgare* L. (cultivar Morex) inoculated with strain E48^T.

2.2 Plant growth liquid system

Barley (*H. vulgare* L. cv. Morex) plants grown in PNS with no NaCl added to liquid medium presented significant differences in shoot length, shoot and root fresh and dry weights values compared with plants grown in PNS settled at 100 and 150 mM NaCl concentration. Fresh and dry weight values for shoots and roots, and shoot length results showed that there were no significant differences between the plants inoculated with *C. diazotrophicus* strain E50^T and uninoculated plants neither in PNS settled at 100 and 150 mM NaCl concentration nor PNS with no NaCl added (Table 2).

Table.2. Effects of *C. diazotrophicus* strain E50^T on barley (*H. vulgare* L. cv. Morex) plants grown in PNS with no NaCl added and settled at 100 and 150 mM NaCl concentration, after 45 days growth. Average ± standard error from 5 replications. Different letters indicate significantly different means (Tukey HSD, p<0.05). DW, dry weight; FW, Fresh weight; gms, grams.

Inoculation	PNS NaCl (mM)	Root DW (gms)	Shoot DW (gms)	Root FW (gms)	Shoot FW (gms)	Root length (cm)	Shoot length (cm)
Ctrl	0	0,43 ± 0.10 a	1,85 ± 0.18 a	8,69 ± 2.14 a	15,38 ± 2.76 a	56,00 ± 10.61 a	65,00 ± 3.48 a
E50^T	0	0,47 ± 0.10 a	2,31 ±0.53 a	8,86 ± 2.04 a	18,45 ± 3.68 a	54,33 ± 6.53 ab	72,82 ± 4.55 a
Ctrl	100	0,20 ± 0.05 b	0,77 ±0.19 b	2,96 ± 0.27 b	5,57 ± 1.76 b	28,90 ± 6.07 b	51,00 ± 5.88 b
E50^T	100	0,24 ±0.07 b	0,75 ±0.26 b	3,03 ± 0.89 b	5,60 ± 1.67 b	33,00 ± 10.56 ab	50,20 ± 6.21 b
Ctrl	150	0,24 ± 0.04 b	1,03 ± 0.26 b	3,45 ± 0.37 b	7,02 ± 1.62 b	40,10 ± 21.77 ab	53,20 ± 5.30 b
E50^T	150	0,26 ± 0.09 b	0,97 ± 0.30 b	3,20 ± 0.55 b	6,36 ± 1.65 b	42,60 ± 16.46 ab	53,78 ± 6.04 b

Root length values showed higher significant differences between un-inoculated plants grown in PNS with no NaCl added compared with those inoculated with strain E50^T under same conditions. No significant root length difference was observed between inoculated and un-inoculated plants grown in PNS settled to 150 mM NaCl, whereas this difference was significant in plants grown in nutrient solution settled at 100 mM NaCl (Table 2). A two-factor ANOVA of these data showed a significant effect of NaCl adjusted PNS ($F_{14, 36}$ = 5.89, p <0.001), a no significant effect for the inoculation of strain E50^T ($F_{7, 18}$ = 1.67,

p=NS) and a non significant interaction between them in the growth of barley (*H. vulgare* L. cv. Morex) plants ($F_{14,36} = 0.88$, p=NS) (appendix 2.1.1).

Table.3. Results for CFU ml^{-1} of rhizosphere and endorhizosphere of *H. vulgare* L. cv. Morex plants grown in PNS non NaCl added and adjusted to 100 and 150 mM NaCl concentration inoculated and uninoculated with strain $E50^T$. Average ± standard error from 4 replications. Different letters indicate significantly different means (Tukey HSD, $p<0.05$).

PNS	Rhizophere (CFUml^{-1})		Endorhizosphere (CFU ml^{-1})	
NaCl [ ]	Ctrl	$E50^T$	Ctrl	$E50^T$
0 mM	$1.5\pm 1.5\ 10^9$ a	$2.1 \pm 0.3\ 10^8$ a	$5.2 \pm 3.4\ 10^3$ x	$1.9 \pm 0.8\ 10^4$ x
100 mM	$2.0 \pm 3.7\ 10^9$ a	$2.2 \pm 0.7\ 10^8$ a	$6.1 \pm 4.7\ 10^5$ x	$5.6 \pm 1.5\ 10^5$ x
150 mM	$6.2 \pm 1.6\ 10^8$ a	$2.2 \pm 1.1\ 10^8$ a	$5.1 \pm 5.5\ 10^5$ x	$3.8 \pm 3.1\ 10^5$ x

Results for CFU ml^{-1} of rhizosphere of *H. vulgare* L. cv. Morex plants grown in PNS non NaCl added and adjusted to different NaCl concentration inoculated and uninoculated with strain $E50^T$ (Table 3), presented no significant differences ($F_{2,18} = 0.63$, p=NS) (Appendix 2.1.2). Similarly, endorhizospheric CFU ml^{-1} values of barley plants grown in PNS non NaCl added and adjusted to different NaCl concentration inoculated and uninoculated with strain $E50^T$ presented no significant differences (Table 3). There was no significant interaction between inoculation of strain $E50^T$ and the different NaCl adjusted PNS on the endorhizospheric CFU ml^{-1} barley (*H. vulgare* L. cv. Morex) plants ($F_{2,18} = 0.10$, p=NS) (appendix 2.1.3). From the TSA agar plates used for CFU ml^{-1} analysis from rhizosphere and endorhizosphere predominant characteristic colonies were identified by their partial 16S rRNA gene sequence (Table 4).

Fig.4. SG tube agar used to confirm seed surface sterilization A) 2 days seedlings *H. vulgare* L. cv. Morex growth on SG agar, B) Same SG agar tube after 15 days incubation at 28 °C.

After surface sterilization of barley (*H. vulgare* L. cv. Morex) seeds and incubated for 72 h no presence of bacteria was observed (Fig. 4A). But after 15 days incubation of the

respective SG agar tubes from where the plantlets used for this study come from bacterial growth was observed. Colonies predominant in this step from this experiment and previous similar experiments from the same seed batch were isolated and identified by their partial 16S rRNA gene sequence (Table 4).

Table.4. Blast similarity of the partial 16S rRNA gene sequences from isolated colonies from rhizosphere and endorhizosphere CFU $m^{-1}l$ analysis, and colonies isolated from SG agar after seed sterilization over incubation.

Source of isolation	**Isolate**	**Length**	**ID Blast**	**No. access**	**ID %**
Plant rhizosphere inoculated with strain $E50^T$	IA1	1061	*Pseudomonas* sp. BBI9	GQ868355	100
Plant rhizosphere inoculated with strain $E50^T$	IA2	1009	*Cellvibrio diazotrophicus* $E50^T$	JQ922446	99
Plant rhizosphere uninoculated.	IA3	946	*Curtobacterium flaccumfaciens* A4-16	JF496347	99
SG agar tube (after seed surface sterilization)	C5EA	989	*Curtobacterium flaccumfaciens* A4-16	JF496347	99
Previous experiment (after seed surface sterilization)	SB1	1052	*Microbacterium phyllosphaerae* KUDC1780	KC355287	99
Previous experiment (after seed surface sterilization)	C13EA	1059	*Pseudomonas* sp. AW4	HQ911371	100

2.3 Plant growth promotion under nitrogen deficiency and insoluble phosphate source

Since *H. diazotrophicus* strain $E19^T$ was isolated as phosphate-mobilizing bacterium and able to fix nitrogen, its ability to promote growth of summer barley (*H. vulgare* L. cv. Propino) under nitrogen deficiency and a water insoluble phosphate source was tested. Experiments were done in the growth chambers with Mitscherlich pots containing a mixture of 3.25 kg subsoil (appendix 3) and 3.25 kg quartz sand.

After 35 days growth no significant differences in shoot fresh or dry weight were obtained within plants inoculated with strain $E19^T$ and strain $E19^T$ dead biomass under nitrogen deficiency (Fig. 5).

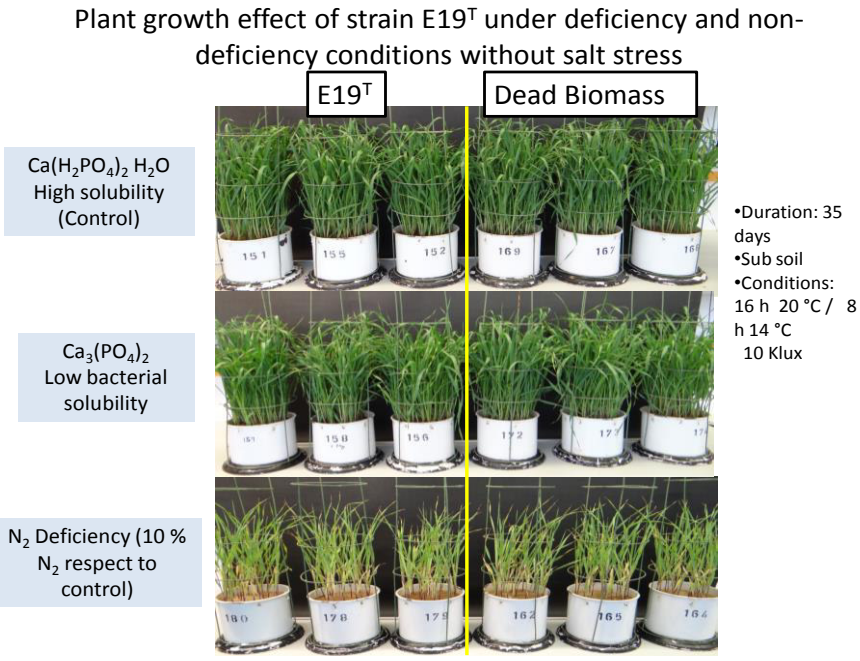


Fig.5. Mitscherlich pot experiment evaluating plant growth effect of *H. diazotrophicus* strain E19^T in summer barley (*H. vulgare* L. cv. Propino) under nitrogen deficiency and different phosphate sources without salt stress after 35 days growth.

Results from summer barley plants fertilized with water soluble phosphate source $Ca(H_2PO_4)_2$ H_2O and water insoluble phosphate $Ca_3(PO_4)_2$ mg (kg soil)$^{-1}$ inoculated with strain E19^T and inoculated with strain E19^T dead biomass showed no significant difference in shoot fresh weight, but presented statistically significant differences on shoots dry weight.

Also, neither in shoots fresh or dry weights there were significant differences within the summer barley plants inoculated with strain E19^T and inoculated with E19^T dead biomass in treatment with nitrogen deficiency (Fig. 6).

A two-factor ANOVA of these data showed a significant effect of nitrogen deficiency, or water soluble or insoluble phosphate sources fertilization ($F_{4,\ 46}$ = 181.5, p <0.001), a significant effect for the inoculation of strain E19^T ($F_{2,\ 23}$ = 15.6, p <0.001) but no significant interaction between them in the growth of summer barley (*H. vulgare* L. cv. Propino) plants ($F_{4,46}$ = 2.3, p=NS)(appendix 2.2.1).

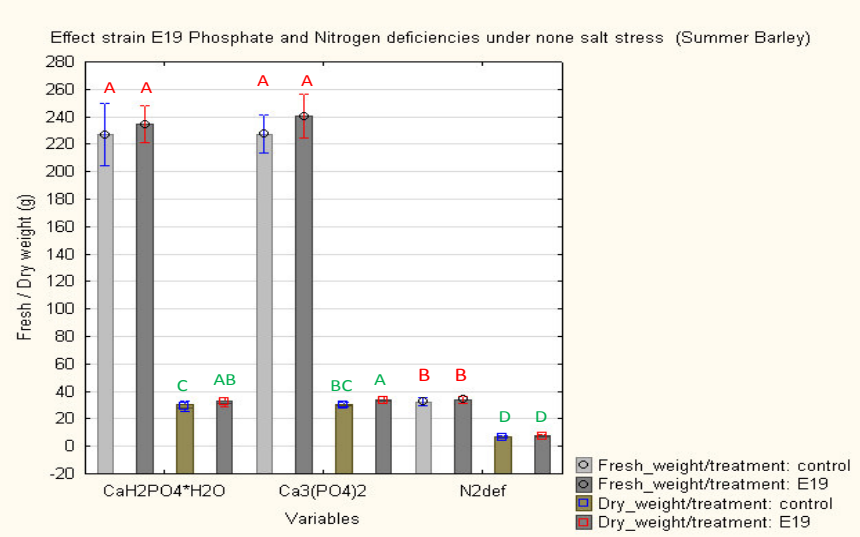


Fig 6. Effects of *H. diazotrophicus* strain E19^T on summer barley (*H. vulgare* L. cv. Propino) plants grown under non salt stress fertilized with different phosphate sources or deficiency in nitrogen. Different letters indicate significantly different means (Tukey HSD, p<0.05). Ctrl, treatments inoculated with strain E19^T dead biomass.

Results from CFU ml^{-1} analysis from rhizosphere and endorhizosphere summer barley plants inoculated with strain E19^T fertilized with different P sources and under N deficiency on agar CP are shown in Table 5.

Results from rhizosphere summer barley plants fertilized with nitrogen deficiency and fertilized with water insoluble phosphate $Ca_3(PO_4)_2$ inoculated with strain E19^T and inoculated with strain E19^T dead biomass showed no significant difference in CFU ml^{-1}, whereas rhizosphere CFU ml^{-1} on the treatment fertilized with water soluble phosphate source $Ca(H_2PO_4)_2$ H_2O inoculated with strain E19^T and inoculated with strain E19^T dead biomass presented a significant differences. CFU ml^{-1} endorhizosphere showed no significant differences neither by different fertilization nor inoculation with strain E19^T and inoculated with strain E19^T dead biomass.

Statistical analysis of these data showed no significant effect of nitrogen deficiency or water soluble or insoluble phosphate sources fertilization treatments ($F_{4,10}$ = 9.3, p=NS), no significant effect for the inoculation of strain E19^T ($F_{2,5}$ = 12.8, p=NS) and no significant

interaction between them on the CFU ml^{-1} at the rhizosphere and endorhizosphere ($F_{4,10}$ = 8.4, p=NS)(appendix 2.2.2)

Table.5. Rhizosphere and endorhizosphere CFU ml^{-1} on CP agar from summer barley plants inoculated with strain $E19^T$ fertilized with different P sources or under N deficiency with non salt stress. Average ± standard error from 2 replications. Different letters indicate significantly different means (Tukey, HSD $p<0.05$).

Fertilization	Inoculation	Rhizosphere (CFU ml^{-1})	Endorhizosphere (CFU ml^{-1})
$Ca(H_2PO_4)_2$ H_2O	$E19^T$	5.25 ± 1.06 10^8 a	4.40 ± 2.26 10^3 x
$Ca(H_2PO_4)_2$ H_2O	Dead Biomass $E19^T$	8.70 ± 10.3 10^7 b	9.05 ± 12.7 10^3 x
$Ca_3(PO_4)_2$	$E19^T$	9.75 ± 8.84 10^6 b	7.50 ± 3.54 10^2 x
$Ca_3(PO_4)_2$	Dead Biomass $E19^T$	3.80 ± 3.11 10^7 b	1.58 ± 0.10 10^3 x
Nitrogen deficiency	$E19^T$	3.6 ± 4.8 10^7 b	1.0 ± 0.0 10^4 x
Nitrogen deficiency	Dead Biomass $E19^T$	3.80 ± 0.14 10^7 b	2.33 ± 3.07 10^3 x

Fifty colonies isolated from the agar CP plates used for rhizosphere and endorhizosphere CFU ml^{-1} were sub-cultured in agar CP and their colony morphology, catalase activity and microscopic characteristics compared with strain $E19^T$ growth on CP under same conditions. A PCR amplification product of the 16S rRNA gene of 6 possible isolates with similar characteristics to $E19^T$ and their 16S rRNA gene were restricted with enzymes *HpaII* and *Hin6I* using as control a PCR amplification product of the 16S rRNA gene of strain $E19^T$ from a genomic DNA extraction (Fig. 7). For all isolates analyzed none of them corresponded to the 16S rRNA gene restriction pattern of the strain $E19^T$ strain profiles and some of them have the same restriction pattern between them.

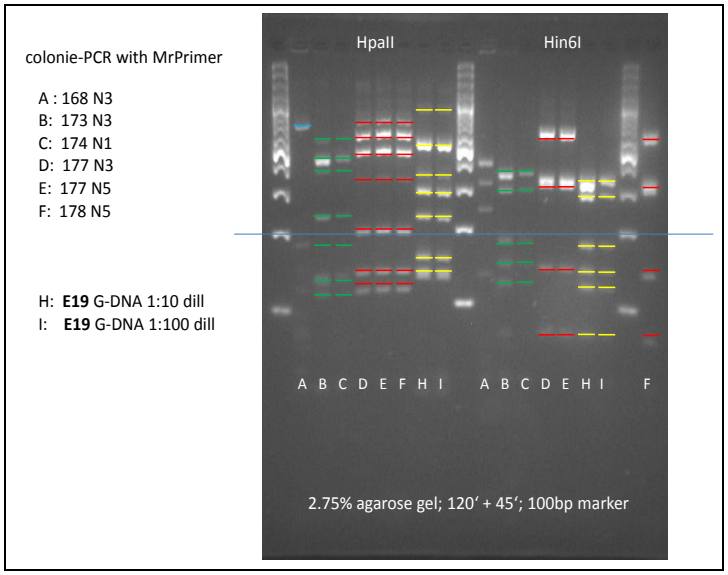


Fig.7. ARDRA pattern of the16S rRNA gene PCR products, using the endonucleases *HpaII* and *Hin6I* for the selected isolates with similar colony morphology, catalase activity and microscopic characteristics to strain

$E19^T$ isolated from the rhizosphere and/or endorhizosphere of summer barley plants grown in Mitscherlich pots under nitrogen deficiency or different phosphate sources without salt stress and inoculated with strain $E19^T$.

2.4 Plant growth promotion using different phosphate sources under salt stress

H. diazotrophicus strain $E19^T$ was tested *in vivo* for the ability to promote growth of summer barley (*H. vulgare* L. cv. Propino) under salt stress and different phosphate sources. Experiments were done with Mitscherlich pots containing a mixture of 3.25 kg of subsoil (Appendix 3) and 3.25 kg quartz sand fertilized with 100 mg P (kg $soil^{)-1}$ of water soluble phosphate $Ca(H_2PO_4)_2H_2O$, GAFSA, organic P and a treatment without P application, respectively in growth chamber conditions. Pots under non salt stress fertilized with highly soluble phosphate form $Ca(H_2PO_4)_2H_2O$ were settled in order to evaluated salt stress effect.

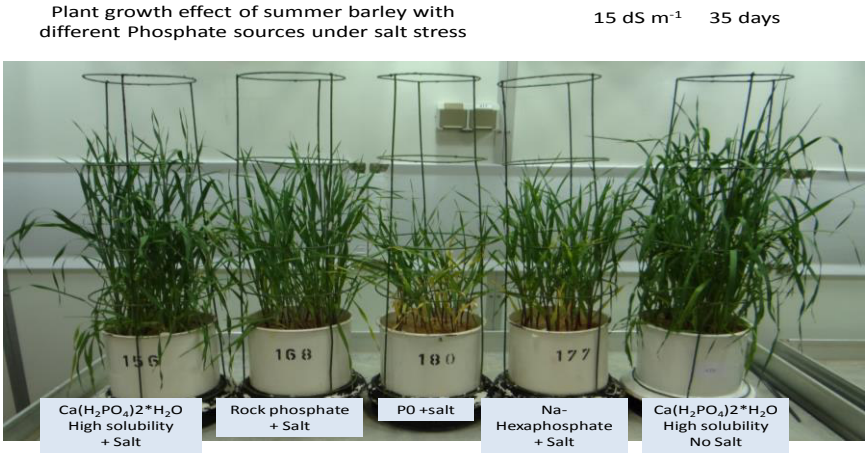


Fig.8. Mitscherlich pot experiment evaluating plant growth effect of *H. diazotrophicus* strain $E19^T$ in summer barley (*H. vulgare* L. cv. Propino) fertilized with different phosphate sources under salt stress after 35 days growth.

After 35 days growth significant differences were obtained within all different phosphate sources variables under salt stress (Fig. 8), although no significant differences were observed in shoot fresh and dry weights within summer barley plants inoculated with strain $E19^T$ and those inoculated with $E19^T$ dead biomass among all different phosphate sources tested under salt stress (Fig. 9). Shoot fresh and dry weights of summer barley plants grown in pots containing water soluble phosphate $Ca(H_2PO4)_2$ without salt stress presented

the best growth but as well as in the other P sources pots under salt stress there were no significant differences within plants inoculation with strain E19^T or E19^T dead biomass (Fig. 9). A two-factor ANOVA of these data showed a significant effect of different phosphate sources fertilization under salt stress ($F_{8, 58}$= 82.5, p <0.001), a no significant effect for the inoculation of strain E19^T ($F_{2, 29}$ = 2.59, p=NS), and no significant interaction between them in the growth of summer barley (*H. vulgare* L. cv. Propino) plants ($F_{8,58}$ =1.78, p=NS)(appendix 2.2.1)

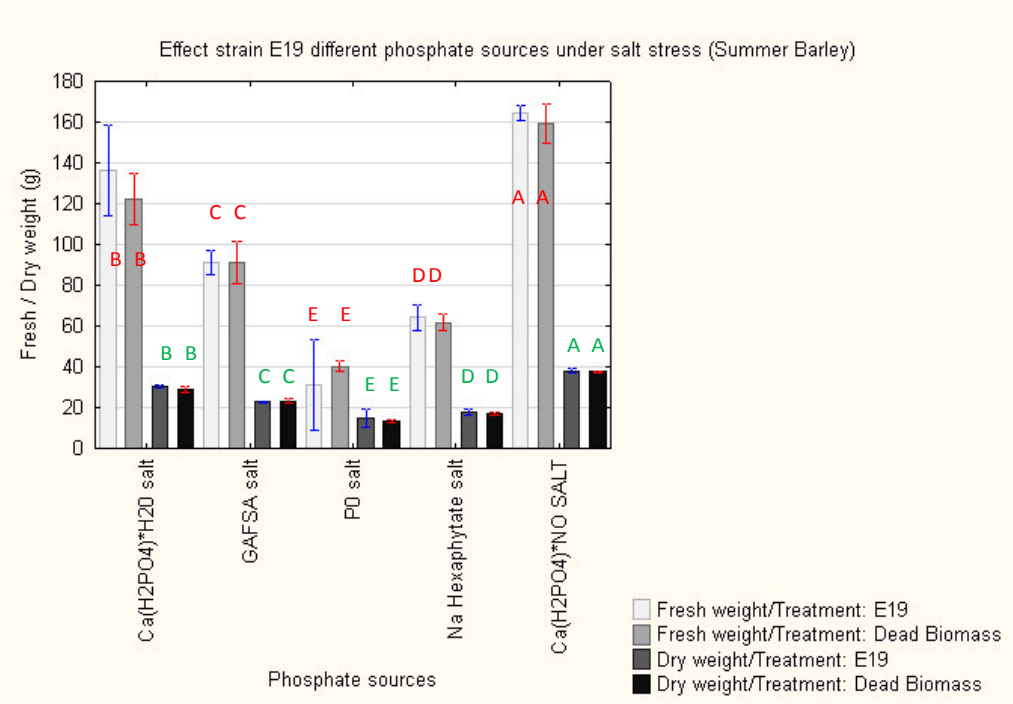


Fig.9. Mitscherlich pot experiment evaluating plant growth effect of *H. diazotrophicus* strain E19^T in summer barley (*H. vulgare* L. cv. Propino) fertilized with different phosphate sources under salt stress after 35 days growth. Different letters indicate significantly different means (Tukey HSD, p<0.05).

3. Discussion

The evaluation of the plant growth effect *R. hassiensis* strain E48^T and surface sterilized barley (*H. vulgare* L. cv. Morex) seeds in the gnotobiotic system presented difficulties due to the strong attachment of sand particles to the whole root surface which did not allow to determine root biomass and to localize the bacteria on the root. The gnotobiotic system is widely used to evaluate bacterial root colonization and bacterial cell concentration. Some of the advantages of a gnotobiotic system are better reproducibility, allow to test bacterium on the root in absences of indigenous bacterial competitor as occurs in soil and it makes

simple to test individually the interaction effect with other bacteria, fungi or protozoae Simons et al. (1996).

The used gnotobiotic system was not suitable for sterile root experiments allowing growth over a time period of weeks thus in order to evaluate root colonization, bacterial cell numbers, plant growth parameters and increase stepwise NaCl concentration in PNS the plant growth liquid system (numeral 1.3) was designed. The plant growth liquid system allowed the correct stepwise increase of PNS adjusted to different NaCl concentration and their corresponding electrical conductivity as shown in Table 1. The induction of salt stress was confirmed statistically and by the negative effect on all plant growth parameters of the barley plants grown at 100 and 150 mM NaCl adjusted concentration in PNS (Table.2).

Even though, there was no significant effect by the inoculation of $E50^T$ on barley plants, the presence of strain $E50^T$ through experimentation was confirmed by its isolation (isolate IA2) from the CFU plates colonies sampled from $E50^T$ inoculated barley plants rhizosphere samples at the end of the experiment (Table.4). Furthermore inoculation of strain $E50^T$ at barley seedling stage with an active inoculum at 10^8 cells ml^{-1}concentration can be assume as enough to colonize root surface barley seedling. In accordance, Simons et al. (1996) reported 80 to 90 % root tip surface colonization of 7 days old tomato grown seedlings inoculated with *Pseudomonas fluorescens* WCS365 at a concentration of 10^7 - 10^8 CFU ml^{-1} in a gnotobiotic system.

One of the objectives of the plant growth liquid system was to keep gnotobiotic conditions, in which all microorganisms are either known or excluded, during all experimentation to evaluate the interaction of strain $E50^T$ with barley (*H. vulgare* L. cv. Morex) seeds. Different kind of colonies where isolated from CFU ml^{-1} from rhizosphere and endorhizosphere (Table 4), confirming that neither the plant liquid growth system was able to keep sterile conditions nor the seed surface sterilization process was not successful although it was optimized in respect of disinfectant concentration and time.

Several colonies with isolate IA3 characteristics were isolated from inoculated and uninoculated endorhizosphere barley plant samples. Furthermore, isolate C5EA, with same colony characteristic of isolate IA3, grow on SG agar tube after 72 h incubation probably because it was not present on the seed surface but in the spermoplasm and thus seed

surface sterilization process was not effective to eliminate its growth. Blast similarity of 16S rRNA gene identification of isolates C5EA and IA3 showed both isolates sequences similarity with *Curtobacterium flaccumfaciens* A4-16 (JF496347).

Member of the genus *Curtobacterium* have been frequently reported as bacterial seed endophytes (Truyens et al., 2014) as PGPR, *Curtobacterium flaccumfaciens* ME1 (Raupach and Kloepper, 1998), and as phytopathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (EPPO/CABI, 1997). *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* is transmitted both within and on the seed, it is very resistant to drying and it can remain viable for long periods in stored seeds (EPPO/CABI, 1997; Camara et al., 2009). For instance, presence of several isolates with similar characteristics as isolates C5EA and IA3 in endorhizosphere samples and after surface seeds sterilization lead to hypothesize that all corresponded to a same strain of *Curtobacterium flaccumfaciens* and it may be vertically transmitted in the barley (*H. vulgare* L. cv. Morex) seeds used for this experiment. Although, several attempts to improve sterilization protocol and different batch of seeds from cv Morex to eliminate the presence of *Curtobacterium flaccumfaciens* colonies could not be achieved.

For further experimentation the commercial available summer barley (*H. vulgare* L. cv. Propino) was selected in order to replace the use of barley (*H. vulgare* L. cv. Morex). For evaluation of plant growth promotion by *H. diazotrophicus* strain E19^{T} on summer barley (*H. vulgare* L. cv Propino) mixtures of subsoil, sand and mineral salts were used to set plant growth conditions with nutritional deficiencies, different P fertilization sources fertilization and salt stress adjustment in Mitscherlich pots under growth chamber conditions. Similarly, Steffens et al. (2010) used sub soil to determined the bioavailability of phytate soil P for various plant species due to the fact that it contains low organic-matter, restricts microbial activity, and provides low nutritional conditions that can be adjusted by mineral salt or fertilizers supplementation.

In both experiments, the effect on summer barley plant by nitrogen deficiency, water soluble and insoluble phosphate sources fertilization under no salt stress, and by different phosphate sources fertilization under salt stress in Mitscherlich pots were statistically significant confirming that the nutritional effects set for the experiments were reached. However, plant growth promotion by PGPR has been reported to have a prominent

beneficial effect on limited resources in poor soils (Ramos Solano et al., 2006), no significant interaction between the nutritional effects evaluated, in both Mitscherlich pots experiments, and inoculation of $E19^T$ were observed. Although, post hoc analysis of the dry weight of shoots of plants fertilized with water soluble phosphate $Ca(H_2PO4)_2$ and water insoluble phosphate $Ca_3(PO_4)_2$ sources under no salt stress showed an effect of strain $E19^T$. It is important to remark that the parameters evaluated corresponded to the plant shoot and not to the complete plant biomass and that the effect of the inoculation could be determined in a biomass increase of the whole plant including the roots and not only the shoots.

An attempt to isolate and to confirm presence of strain $E19^T$ at rhizosphere or endorhizosphere of inoculated summer barley plants cv Propino grown in Mitscherlich pots was unsuccessful. Isolates were compared based on colony morphology, catalase activity and microscopic characteristics similarities, and for those with similar characteristics a comparison of their 16S rRNA gene restriction pattern showed that none corresponded to strain $E19^T$. It was expected to isolate strain $E19^T$ colonies from rhizosphere or endorhizosphere samples due to its high inoculated concentration on the barley seeds and the expected low microbial activity in the used mixture of sand and subsoil. Agar CP was used for CFU ml^{-1} counting and in for isolation due that it is the originally agar media used for its isolation (Suarez et al., 2014a) and because it selectively allow the growth of PSBs. CP agar allowed great variety of colonies including colonies with similar evaluated characteristics. Also, allowed rapid growth colony bacteria development that hampered to confirm $E19^T$ strain presence in the samples by this method.

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Appendix

1. Strains E48^T, E50^T and E19^T growth conditions used in for bacterial inoculums production for plant growth promoting experiments.

Strain	Media	Temperature (°C)	Time (h)	Agitation (rpm)
E19^T	Marine bouillon half concentration	28	36	150
E48^T	TSB	28	24	150
E50^T	TSB	28	24	150

2. Statistical Analysis

2.1 Statistical results of the effects of *C. diazotrophicus* strain E50^T on barley (*H. vulgare* L. cv. Morex) plants grown in PNS

2.1.1 ANOVA analysis of effects of *C. diazotrophicus* strain E50^T on barley (*H. vulgare* L. cv. Morex) plants grown in PNS with no NaCl added and settled at 100 and 150 mM NaCl concentration, after 45 days growth.

Effekt	Multivariate Signifikanztests PNS SALT strain E50 (Tabelle42) Sigmabeschränkte Parametrisierung Typ VI Dekomposition (Effektive Hypothese)					
	Test	Wert	F	Effekt FG	Fehler FG	p
Konstante	Wilks	0,001	2644,477	7	18	0,0000
NaCl	Wilks	0,092	5,889	14	36	0,0000
Treatment	Wilks	0,607	1,666	7	18	0,1806
NaCl*Treatment	Wilks	0,557	0,875	14	36	0,5899

Post hoc results

Zelle Nr.	Tukey HSD Test; PNS Variable Root DW (Tabelle42) Homogene Gruppen, Alpha = ,05000 Fehler: MQ(Zwischen) = ,00573, FG = 24,000				
	NaCl	Treatment	Root DW Mittel	1	2
3	100 mM	Control	0,1985	****	
4	100 mM	E50	0,2383	****	
5	150 mM	Control	0,2429	****	
6	150 mM	E50	0,2599	****	
1	0mM	Control	0,4273		****
2	0mM	E50	0,4682		****

Zelle Nr.	Tukey HSD Test;PNS Variable Shoot DW (Tabelle42) Homogene Gruppen, Alpha = ,05000 Fehler: MQ(Zwischen) = ,09664, FG = 24,000				
	NaCl	Treatment	Shoot DW Mittel	1	2
4	100 mM	E50	0,7458	****	
3	100 mM	Control	0,7740	****	
6	150 mM	E50	0,9732	****	
5	150 mM	Control	1,0292	****	
1	0mM	Control	1,8466		****
2	0mM	E50	2,3144		****

Tukey HSD Test; PNS Variable Shoot FW (Tabelle42)
Homogene Gruppen, Alpha = ,05000
Fehler: MQ(Zwischen) = 1,6765, FG = 24,000

Zelle Nr.	NaCl	Treatment	Shoot FW Mittel	1	2
3	100 mM	Control	2,9556	****	
4	100 mM	E50	3,0298	****	
6	150 mM	E50	3,2043	****	
5	150 mM	Control	3,4539	****	
1	0mM	Control	8,6873		****
2	0mM	E50	8,8615		****

Tukey HSD Test; PNS Variable Shoot FW (Tabelle42)
Homogene Gruppen, Alpha = ,05000
Fehler: MQ(Zwischen) = 5,4013, FG = 24,000

Zelle Nr.	NaCl	Treatment	Shoot FW Mittel	1	2
3	100 mM	Control	5,5740	****	
4	100 mM	E50	5,5960	****	
6	150 mM	E50	6,3600	****	
5	150 mM	Control	7,0220	****	
1	0mM	Control	15,3760		****
2	0mM	E50	18,4533		****

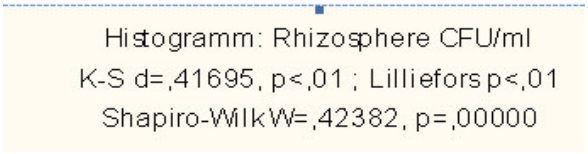
Tukey HSD Test; PNS Variable Root length (Tabelle42
Homogene Gruppen, Alpha = ,05000
Fehler: MQ(Zwischen) = 174,72, FG = 24,000

Zelle Nr.	NaCl	Treatment	Root length Mittel	1	2
3	100 mM	Control	28,9000C	****	
4	100 mM	E50	33,0000C	****	****
5	150 mM	Control	40,1000C	****	****
6	150 mM	E50	42,6000C	****	****
2	0mM	E50	54,3320C	****	****
1	0mM	Control	56,0000C		****

Tukey HSD Test; PNS Variable Shoot length (Tabelle42)
Homogene Gruppen, Alpha = ,05000
Fehler: MQ(Zwischen) = 28,435, FG = 24,000

Zelle Nr.	NaCl	Treatment	Shoot length Mittel	1	2
4	100 mM	E50	50,2000C	****	
3	100 mM	Control	51,0000C	****	
5	150 mM	Control	53,2000C	****	
6	150 mM	E50	53,7800C	****	
1	0mM	Control	65,0000C		****
2	0mM	E50	72,8200C		****

2.1.2 ANOVA analysis and post hoc for results for CFU ml^{-1} of rhizosphere of *H. vulgare* L. cv. Morex plants grown in PNS non added and adjusted to 100 and 150 mM NaCl concentration inoculated and uninoculated with strain E50^{T}



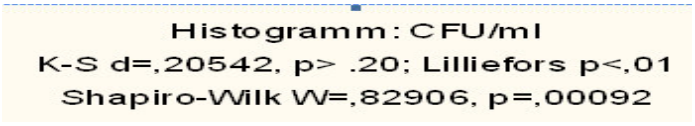
Univariate Tests für Signifikanz, Effektgröße und Teststärke für Rhizosphere CFU/ml
Sigmabeschränkte Parametrisierung
Typ VI Dekomposition (Effektive Hypothese)

Effekt	SQ	FG	MQ	F	p	Partielles Eta-quadr.	Nicht-Zentralität	Teststärke (alpha=0,05)
Konstante	1,265224E+19	1	1,27E+19	4,6750	0,044	0,2062	4,675	0,534
NaCl cencentration	3,440049E+18	2	1,72E+18	0,6355	0,541	0,0660	1,271	0,140
Treatment	6,193197E+18	1	6,19E+18	2,2884	0,148	0,1128	2,288	0,299
NaCl cencentration*Treatment	3,437058E+18	2	1,72E+18	0,6350	0,541	0,0659	1,270	0,140
Fehler	4,871495E+19	18	2,71E+18					

Tukey HSD Test; Variable Rhizosphere CFU/ml (Tabelle rhizosphere 06-12-2014)
Homogene Gruppen, Alpha = ,05000
Fehler: MQ(Zwischen) = 271E16, FG = 18,000

Zelle Nr.	NaCl cencentration	Treatment	Rhizosphere CFU/ml Mittel	1
5	150 mM	Control	1,985000E+08	****
2	0 mM	E50	2,100000E+08	****
6	150 mM	E50	2,200000E+08	****
4	100 mM	E50	2,242500E+08	****
1	0 mM	Control	1,516667E+09	****
3	100 mM	Control	1,987000E+09	****

2.1.3 ANOVA analysis and post hoc for results for CFU ml^{-1} endorhizosphere *H. vulgare* L. cv. Morex plants grown in PNS non added and adjusted to 100 and 150 mM NaCl concentration inoculated and uninoculated with strain E50^{T}



Univariate Signifikanztests für CFU/ml (Tabelle24)
Sigmabeschränkte Parametrisierung
Typ VI Dekomposition (Effektive Hypothese)

Effekt	SQ	FG	MQ	F	p
Konstante	2,8901E+12	1	2,890E+12	27,23687	0,000058
NaCl	1,4268E+12	2	7,134E+11	6,72301	0,006597
treatment	2,0134E+10	1	2,013E+10	0,18974	0,668310
NaCl*treatment	2,2904E+10	2	1,145E+10	0,10792	0,898272
Fehler	1,9100E+12	18	1,061E+11		

Tukey HSD Test; Variable CFU/ml (Tabelle endorhizosphere 06-12-2014)
Homogene Gruppen, Alpha = ,05000
Fehler: MQ(Zwischen) = 1061E8, FG = 18,000

Zelle Nr.	treatment	NaCl	CFU/ml Mittel	1
1	Control	0 mM	5200,0	****
4	E50	0 mM	18666,7	****
6	E50	150 mM	375500,0	****
3	Control	150 mM	512750,0	****
5	E50	100 mM	560000,0	****
2	Control	100 mM	610000,0	****

2.2 Statistical results of effects of *H. diazotrophicus* strain E19^T on summer barley (*H. vulgare* L. cv. Propino) plants grown under non salt stress fertilized with different phosphate sources or deficiency in nitrogen.

2.2.1 ANOVA and post hoc analysis for Mitscherlich pot experiment evaluating plant growth effect of *H. diazotrophicus* strain E19^T in summer barley (*H. vulgare* L. cv Propino) under nitrogen deficiency and different phosphate sources without salt stress

Histogramm: Fresh_weight	Histogramm: Dry_weight
K-S d=,34803, p<,01 ; Lilliefors p<,01	K-S d=,29103, p<,01 ; Lilliefors p<,01
Shapiro-Wilk W=,66373, p=,00000	Shapiro-Wilk W=,72495, p=,00000

	Multivariate Tests für Signifikanz,Pots E19 Testgröße und -stärke (data pot 08 14) Sigmabeschränkte Parametrisierung Typ VI Dekomposition (Effektive Hypothese)								
Effekt	Test	Wert	F	Effekt FG	Fehler FG	p	Partielles Eta-quadr.	Nicht-Zentralität	Teststärke (alpha=0,05)
Konstante	Wilks	0,0014	8208,252	2	23	0,0000	0,998601	16416,50	1,000000
variable	Wilks	0,0035	181,520	4	46	0,0000	0,940421	726,08	1,000000
treatment	Wilks	0,4234	15,663	2	23	0,0001	0,576629	31,33	0,998218
variable*treatment	Wilks	0,6935	2,309	4	46	0,0720	0,167226	9,24	0,625463

	Tukey HSD Test; Variable Fresh_weight (data pot 08 14) Homogene Gruppen, Alpha = ,05000 Fehler: MQ(Zwischen) = 48,351, FG = 24,000				
Zelle Nr.	variable	treatment	Fresh_weight Mittel	1	2
5	N2def	control	32,2820		****
6	N2def	E19	33,9420		****
1	CaH2PO4*H2O	control	227,0500	****	
3	Ca3(PO4)2	control	227,3900	****	
2	CaH2PO4*H2O	E19	234,5580	****	
4	Ca3(PO4)2	E19	240,4860	****	

	Tukey HSD Test; Variable Dry_weight (data pot 08 14) Homogene Gruppen, Alpha = ,05000 Fehler: MQ(Zwischen) = 1,4508, FG = 24,000						
Zelle Nr.	variable	treatment	Dry_weight Mittel	1	2	3	4
5	N2def	control	6,8260	****			
6	N2def	E19	7,3920	****			
1	CaH2PO4*H2O	control	29,1960		****		
3	Ca3(PO4)2	control	30,0880		****	****	
2	CaH2PO4*H2O	E19	32,1940			****	****
4	Ca3(PO4)2	E19	33,6060				****

2.2.2 ANOVA and post hoc analysis of rhizosphere and endorhizosphere CFU ml^{-1} on CP agar from summer barley plants inoculated with strain E19^T fertilized with different P sources or under N deficiency with non salt stress

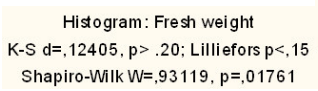
Histogramm: Rhizosphere	Histogramm: Endorhizosphere
K-S d=,34276, p<,10 ; Lilliefors p<,01	K-S d=,21770, p> .20; Lilliefors p<,15
Shapiro-Wilk W=,64057, p=,00024	Shapiro-Wilk W=,81306, p=,01324

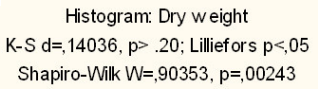
	Multivariate Tests für Signifikanz, Testgröße und -stärke (Tabelle2) Sigmabeschränkte Parametrisierung Typ VI Dekomposition (Effektive Hypothese)								
Effekt	Test	Wert	F	Effekt FG	Fehler FG	p	Partielles Eta-quadr.	Nicht-Zentralität	Teststärke (alpha=0,05)
Konstante	Wilks	0,119814	18,36569	2	5	0,0050	0,8802	36,73137	0,974626
Fertilization	Wilks	0,044729	9,32071	4	10	0,0021	0,7885	37,28286	0,981575
Inoculation	Wilks	0,163400	12,79990	2	5	0,0108	0,8366	25,59980	0,906167
Fertilization*Inoculation	Wilks	0,052354	8,42609	4	10	0,0030	0,7712	33,70437	0,969444

	Tukey HSD Test; Variable Endorhizosphere (Tabelle2) Homogene Gruppen, Alpha = ,05000 Fehler: MQ(Zwischen) = 2915E4, FG = 6,0000			
Zelle Nr.	Fertilization	Inoculation	Endorhizosphere Mittel	1
3	Ca3(PO4)2	E19	750,00	****
4	Ca3(PO4)2	DB E19	1575,00	****
6	N defic	DB E19	2330,00	****
1	Ca(H2PO4)2*H2C	E19	4400,00	****
2	Ca(H2PO4)2*H2C	DB E19	9050,00	****
5	N defic	E19	10000,00	****

	Tukey HSD Test; Variable Rhizosphere (Tabelle2) Homogene Gruppen, Alpha = ,05000 Fehler: MQ(Zwischen) = 421E13, FG = 6,0000				
Zelle Nr.	Fertilization	Inoculation	Rhizosphere Mittel	1	2
6	N defic	DB E19	4000000	****	
3	Ca3(PO4)2	E19	9750000	****	
5	N defic	E19	35950000	****	
4	Ca3(PO4)2	DB E19	38000000	****	
2	Ca(H2PO4)2*H2C	DB E19	87000000	****	
1	Ca(H2PO4)2*H2C	E19	525000000		****

2.3 ANOVA and post hoc analysis for Mitscherlich pot experiment evaluating plant growth effect of *H. diazotrophicus* strain E19^T in summer barley (*H. vulgare* L. cv Propino) fertilized with different phosphate sources under salt stress.





Effect	Multivariate Tests of Significance (Spreadsheet1_MC) Sigma-restricted parameterization Effective hypothesis decomposition Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0,001313	11029,66	2	29	0,000000
Variable	Wilks	0,006525	82,50	8	58	0,000000
Treatment	Wilks	0,848591	2,59	2	29	0,092496
Variable*Treatment	Wilks	0,644246	1,78	8	58	0,099148

Tukey HSD test; variable Fresh weight (Spreadsheet1_MC)
Homogenous Groups, alpha = ,05000
Error: Between MS = 58,624, df = 30,000

Cell No.	Variable	Treatment	Fresh weight Mean	1	2	3	4	5
5	P0 salt	E19	31,1975	****				
6	P0 salt	Dead Biomass	40,3900	****				
8	Na Hexaphytate salt	Dead Biomass	61,7400		****			
7	Na Hexaphytate salt	E19	64,1600		****			
3	GAFSA salt	E19	91,0375			****		
4	GAFSA salt	Dead Biomass	91,2375			****		
2	Ca(H2PO4)*H20 salt	Dead Biomass	122,4100				****	
1	Ca(H2PO4)*H20 salt	E19	136,2775				****	
10	Ca(H2PO4)*NO SALT	Dead Biomass	159,0500					****
9	Ca(H2PO4)*NO SALT	E19	164,5325					****

Tukey HSD test; variable Fresh weight (Spreadsheet1_MC)
Homogenous Groups, alpha = ,05000
Error: Between MS = 58,624, df = 30,000

Cell No.	Variable	Treatment	Fresh weight Mean	1	2	3	4	5
5	P0 salt	E19	31,1975	****				
6	P0 salt	Dead Biomass	40,3900	****				
8	Na Hexaphytate salt	Dead Biomass	61,7400		****			
7	Na Hexaphytate salt	E19	64,1600		****			
3	GAFSA salt	E19	91,0375			****		
4	GAFSA salt	Dead Biomass	91,2375			****		
2	Ca(H2PO4)*H20 salt	Dead Biomass	122,4100				****	
1	Ca(H2PO4)*H20 salt	E19	136,2775				****	
10	Ca(H2PO4)*NO SALT	Dead Biomass	159,0500					****
9	Ca(H2PO4)*NO SALT	E19	164,5325					****

3. Sub Soil physicochemical characteristics

Texture

Texture	Percentage (%)
Sand	44.92
Silt	34.71
Clay	20.38

pH-value (0.01 M CaCl2) = 6.4

M after Schachtschabel 287.8 mg Mg kg^{-1} Soil

	Percentage (%)
Ct	0.169
Nt	0.031
St	0.027

CAL-K 23.35 mg K kg^{-1} soil

CAL-P 6.30 mg P kg^{-1} soil

DTPA extractable micronutrients	mg kg^{-1} soil
Cu	0.57
Mn	14.40
Fe	30.85
Zn	0.63

NO_3-N (0.01 M $CaCl_2$)	5.17 mg N kg-$^{-1}$ soil
NH_4-N (0.01 M $CaCl_2$)	0.43 mg N kg^{-1} soil
Norg-N (0.01 M $CaCl_2$)	0.34 mg N kg^{-1} soil
DOC (0.01 M $CaCl_2$)	14.75 mg C kg^{-1} soil
NO_3-N1. + 2.EUF-Fraction	5.22 mg N kg^{-1} soil

Chapter 4

Cellvibrio diazotrophicus sp. nov., a nitrogen-fixing bacteria isolated from the rhizosphere of salt meadow plants and emended description of the genus *Cellvibrio*

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Cellvibrio diazotrophicus sp. nov., a nitrogen-fixing bacteria isolated from the rhizosphere of salt meadow plants and emended description of the genus *Cellvibrio*

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Two Gram-reaction-negative, aerobic, nitrogen-fixing, rod-shaped bacteria, designated strains E20 and E50^T, were isolated from the rhizosphere of salt meadow plants *Plantago winteri* and *Hordeum secalinum*, respectively, near Münzenberg, Germany. Based on the 16S rRNA gene sequence analysis both strains E20 and E50^T are affiliated with the genus *Cellvibrio*, sharing the highest similarity with *Cellvibrio gandavensis* LMG 18551^T (96.4 %) and (97.1 %), respectively. Strains E20 and E50^T were oxidase and catalase-positive, grew at a temperature range between 16 and 37 °C and in the presence of 0–5 % NaCl (w/v). The DNA G+C contents were 52.1 mol% (E20) and 51.6 mol% (E50^T). Major fatty acids of strains E20 and E50^T were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$, $C_{18:1}\omega7c$, $C_{12:0}$, $C_{18:0}$ and $C_{12:0}$ 3-OH. The DNA–DNA relatedness of the strains to *Cellvibrio gandavensis* LMG 18551^T was 39 % for strain E20 and 58 % for strain E50^T. The nitrogen fixation capability of strains E20 and E50^T was confirmed by the acetylene reduction assay. On the basis of our polyphasic taxonomic study, strains E20 and E50^T represent a novel species of the genus *Cellvibrio*, for which the name *Cellvibrio diazotrophicus* is proposed. The type strain of *Cellvibrio diazotrophicus* is E50^T (=LMG 27267^T=KACC 17069^T). An emended description of the genus *Cellvibrio* is proposed based on the capability of fixing nitrogen and growth in presence of up to 5 % NaCl (w/v).

The genus *Cellvibrio* was originally proposed by Winogradsky (1929) and comprises Gram-reaction-negative, aerobic, cellulolytic and flagellated rod-shaped bacteria. Originally the two species '*Cellvibrio ochraceus*' (as the type species of the genus) and '*Cellvibrio flavescens*' were included. With loss of the type species culture the genus was excluded from the Approved List of Bacterial Names (Skerman *et al.*, 1980). The members of the genus *Cellvibrio* were relegated to species *incertae sedis* within the genus *Pseudomonas* (Doudoroff & Palleroni, 1974) until the revival proposal for the genus *Cellvibrio*, with a new type species *Cellvibrio mixtus* subsp. *mixtus* ACM 2601^T (Blackall *et al.*, 1985).

At the time of writing, the genus contains seven recognized species and two subspecies, *C. mixtus* subsp. *mixtus* ACM 2601^T and *Cellvibrio mixtus* subsp. *dextranolyticus* (Blackall *et al.*, 1985), *Cellvibrio japonicus* NCIMB 10462^T (Humphry *et al.*, 2003), *Cellvibrio vulgaris* NCIMB 8633^T (Humphry *et al.*, 2003), *Cellvibrio fulvus* NCIMB 8634^T (Humphry *et al.*, 2003), *Cellvibrio ostraviensis* LMG 19434^T (Mergaert *et al.*, 2003), *Cellvibrio fibrivorans* LMG 18561^T (Mergaert *et al.*, 2003) and *Cellvibrio gandavensis* LMG 18551^T (Mergaert *et al.*, 2003).

Besides *C. vulgaris* and *C. fulvus*, isolates from beech leaf matter, all recognized species of the genus *Cellvibrio* have been isolated from soil environments, nevertheless, strains belonging to the genus have been found also in aquatic environments (Rhee *et al.*, 2010). The genus *Cellvibrio*, especially *C. japonicus*, has been recognized and studied for its industrial enzymic potential to degrade a variety of polysaccharides, including those present in the plant cell wall (DeBoy *et al.*, 2008). Using a pyrosequencing analysis of rhizospheric soil Anderson & Habiger (2012) found that the abundance of an operational taxonomic unit related to the genus *Cellvibrio* was positively associated with biomass productivity in wheat. In a soil metagenomic library Voget *et al.* (2006) found various cellulose genes, one of which showed 77 % identity to a cellulose gene of *C. mixtus*. The recombinant cellulose protein expressed in *Escherichia coli* was highly temperature stable, salt- and pH-tolerant. These findings may indicate that members of the genus *Cellvibrio* might have a positive effect in the rhizosphere of plants, possibly by close interaction with root cells enabled by a robust cellulose activity.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains E20 and E50^T are JQ922425 and JQ922426, respectively.

Strains E20 and E50^T were isolated from a natural salt meadow in a nature protection area near Münzenberg, Hessen, Germany (50° 27′ 36″ N 8° 44′ 35″ E), as part of an investigation on rhizospheric bacteria from salt-tolerant plant species and evaluation of their plant-growth-promoting abilities. In this study we demonstrate the presence of a previously unknown metabolic characteristic among the members of the genus. Strains E20 and E50^T have been isolated as nitrogen-fixing bacteria from the rhizosphere of salt-tolerant plants. The sequence of the complete chromosome genome of *C. japonicus* NCIMB 10462 (=Ueda107) (DeBoy *et al.*, 2008) is available. In that genome no homologous gene for a nitrogenase could be found (http://www.genome.jp/kegg/kegg2.html)

Plantago winteri and *Hordeum secalinum* plants were sampled during all four seasons of the year. The root systems of the plants were washed off thoroughly by vigorous shaking in sterile distilled water. Subsequently, the suspensions were diluted with sterile saline using standard dilution methods and then incubated on nitrogen-free LG medium (Turner & Gibson, 1980) at 28 °C for 2–3 days. Strain E20 was isolated from the rhizospheric soil of *Plantago winteri* during winter sampling and strain E50^T was isolated from the rhizospheric soil of *Hordeum secalinum* during autumn sampling using dilution streaking technique and then stored in glycerine stocks at −20 °C. All phenotypic and genotypic tests were done in parallel with the type strain *C. gandavensis* LMG 18551^T obtained from the Belgian coordinated collections of micro-organisms (BCCM) and used for reference in this study.

The colonies of strains E20 and E50^T were subcultured in trypticase soy agar (Oxoid) for handling and analysis purposes. Cell morphology of strains E20 and E50^T were observed by scanning electron microscopy (EM 300, Phillips) using cells grown on trypticase soy broth in stationary and exponential phases (Fig. S1, available in IJSEM Online). The Gram reaction was determined as described by Gerhard *et al.* (1994) on cells from 24 and 48 h colonies grown on trypticase soy agar (Oxoid) using a Zeiss microscope with × 1000 magnification. The presence of flagella and pili was evaluated by light microscopy after flagella staining (Heimbrook *et al.*, 1989) (Fig. S2).

Strains E20 and E50^T were tested for growth at different temperatures (4, 10, 16, 20, 28, 37, 45 and 50 °C) on TSA. Anaerobic and microaerophilic growth was checked on TSA using the Anaerocult A and C systems (Merck). Growth with different salt concentrations (1–8 % NaCl, with intervals of 1 %) was evaluated on TSA. Growth with 0 % NaCl was tested on plates containing 1.5 % casein peptone, 0.5 % soy peptone and 1.5 % agar. Catalase activity was determined by bubble formation in 3 % (v/v) H_2O_2 solution and oxidase activity using oxidase test strips (Merck). Assimilation of different carbon sources was evaluated by adding the carbon sources to a basic mineral medium (Pfennig, 1978). Other biochemical and enzymic activities were evaluated using API ZYM strips (bioMérieux) according to the manufacturer's instructions. Hydrolysis of CM-cellulose was determined with Congo red dye following the protocol of Kasana *et al.* (2008). The sensitivities to antibiotics of strain E20, E50^T and *C. gandavensis* LMG 18551^T were tested with the disc diffusion method using a cell suspension (0.5 McFarland) from trypticase soy agar plates grown at 28 °C for 48 h. The cell suspensions were spread on TSA plates and discs (Oxoid) containing the following antibiotics: ampicillin (10 µg), chloramphenicol (10 µg), kanamycin (30 µg), penicillin G (10 µg), erythromycin (15 µg) and tetracycline (30 µg). The diameter of the inhibition zone were measured after 72 h of incubation at 28 °C and strains were considered susceptible when the diameter of the inhibition zone was >13 mm, intermediate for diameters between 10 and 12 mm and resistant for diameters <10 mm as described.

Genomic DNA extractions from cells of the strains E20 and E50^T were obtained according to the method of Moré *et al.* (1994). The G+C content of genomic DNA was measured using fluorimetric methods according to the method of Gonzalez & Saiz-Jimenez (2002). The full-length 16S rRNA gene sequences were obtained by PCR (Kampmann *et al.*, 2012) using the EUB9f (9-27) and EUB 1492r primer pair (Lane, 1991). The PCR products were cleaned using a QIA quick PCR purification kit (Qiagen) and sequenced by LGC genomics (Berlin, Germany). The obtained 16S rRNA gene sequences of strains E20 (1474 bp) and E50^T (1409 bp) were compared with those of relatives retrieved from the GenBank database using MEGA software version 5.0 (Tamura *et al.*, 2011). Sequences were screened for chimeras using Bellerophon software version 3.0 (Huber *et al.*, 2004). The alignment of the 16S rRNA gene sequences of strains E20 and E50^T was performed online with the alignment tool SINA (v1.2.9) (Pruesse *et al.*, 2012). The obtained alignment was merged with the Living Tree Project (LTP) online database (LTPs108, July 2012) (Yarza *et al.*, 2008) using the ARB software version 5.2 (Ludwig *et al.*, 2004). The similarity and the phylogenetic analysis were also performed using the ARB software version 5.2.

Maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) trees were reconstructed using 1000 bootstrap analyses (Felsenstein, 1985) using the termini filter between positions 52 and 1467 (*E. coli* numbering; Brosius *et al.*, 1978) of the 16S rRNA gene sequences.

For the fatty acid methyl ester analysis cell biomass was harvested from cultures of strains E20, E50^T and *C. gandavensis* LMG 18551^T grown 48 h on TSA plates at 28 °C. The fatty acid methyl esters were extracted and analysed according to the method of Kämpfer & Kroppenstedt (1996). Fatty acids were separated by a gas chromatograph (model 5898A, Hewlett Packard). Peaks were automatically integrated and fatty acid names and percentages were determined using the Microbial Identification standard software package MIDI (Sasser, 1990). DNA–DNA hybridization experiments were performed with E20, E50^T and *C. gandavensis* LMG 18551^T using the method described by Ziemke *et al.* (1998).

The marker gene for nitrogen fixation *nifH* was evaluated using the primers described by Poly *et al.* (2001) with both the original protocol and with modification. The nitrogenase activities were detected using a modification of the acetylene reduction assay method (Hardy *et al.*, 1968). Cells grown in TSB for 48 h at 28 °C were washed twice using Nfb medium (Albrecht & Okon, 1980) and resuspended using the same medium to give an OD_{600} of 0.04. Samples (5 ml) of this suspension were suspended in 60 ml bottles containing 5 ml Nfb semisolid medium and were incubated at 28 °C for 10 days. After growth of the cultures the bottles were sealed with butyl rubber stoppers, degassed and the gas phase was replaced by nitrogen. Acetylene and oxygen were added to a final concentration of (10 %, v/v, and 2 %, v/v, respectively). The bottles were incubated at 28 °C and the acetylene-reducing activity was measured by analysing acetylene and ethylene concentrations using a Perkin Elmer Auto system XL chromatograph equipped with a thermal conductivity detector and a Hayesep R column (2 m length, 2 mm diameter) at a helium (quality 5.0) flow of 28 ml min^{-1}. Protein concentrations were quantified using the Bradford assay with BSA as standard protein (Bradford, 1976). All tests for the acetylene-reducing activity and the *nifH* gene PCR were performed in parallel using as a positive control the type strain *Azotobacter vinelandii* DSM 2289^T.

Strains E20 and E50^T were Gram-reaction-negative, aerobic, positive for oxidase, catalase-positive, chemoheterotrophic, non-pigmented, curved rod-shaped bacteria. Cells were motile by the means of single polar flagella (Fig. S2), grew in 0–5 % (w/v) NaCl and at a temperature range between 16 and 37 °C. No growth was observed with 6 % NaCl or at 4 or 45 °C. After 48 h at 28 °C colonies were 2.0–2.5 mm in diameter, circular, slightly convex, non-mucoid, smooth and creamy when grown on TSA. On LG medium at 28 °C the colonies were 2.5–3 mm in diameter, brilliant, non-pigmented, non-mucoid and smooth after 48 h.

Strains E20 and E50^T presented the following phenotypic characteristics in common with all the species of the genus *Cellvibrio* with validly published names. Positive activity for alkaline phosphatase, leucine arylamidase, C4 and C8 esterases and *N*-acetyl-β-glucosaminidase; negative activity for C14 lipase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase and hydrolysis of cellulose, starch and aesculin. A comparison of the morphological, biochemical, physiological and cultural characteristics of strains E20 and E50^T with those of the other species of the genus *Cellvibrio* with validly published names are given in Table 1.

BLAST analysis of the almost complete 16S rRNA gene sequences for the strains E20 (1474 bp) and E50^T (1409 bp) (GenBank accession numbers JQ922425 and JQ922426, respectively) showed that the novel isolates were most closely affiliated to the genus *Cellvibrio*. All 16S rRNA gene sequences of the species of the genus *Cellvibrio* were retrieved from GenBank and used to calculate the 16S rRNA gene similarity values. Strains E20 and E50^T share more than 95 % 16S rRNA gene sequence similarity with the species of the genus *Cellvibrio* with validly published names and therefore both belong to this genus (Ludwig *et al.*, 1998).

The 16S rRNA gene similarity values for the strain E50^T showed the highest similarity with strain E20 (98 %), followed by *C. gandavensis* LMG 18551^T (97.1 %) and both *C. mixtus* subsp. *mixtus* 2601^T (Blackall *et al.*, 1985) and *C. japonicus* NCIMB 10462^T (Humphry *et al.*, 2003) each with 96 % similarity value. The 16S rRNA gene sequences of the other species of the genus *Cellvibrio* with validly published names showed less than 95.9 % similarity. These results provided an indication that the strains E20 and E50^T were strains of the same species and could be distinguished from the other members of the genus *Cellvibrio* due to the principle that 16S rRNA gene sequences with values less than 97 % similarity may provide the first indication that a novel species has been isolated (Tindall *et al.*, 2010).

The DNA G+C contents of the E20 and E50^T strains were 52.1 ±0.6 mol% and 51.6 ±0.4 mol%, respectively, and corresponded with the values of this parameter obtained previously for the members of the genus *Cellvibrio* of between 44 mol% (Mergaert *et al.*, 2003) and 53 mol% (Humphry *et al.*, 2003). The DNA–DNA hybridization results showed that the genomic DNA of strain E20 and E50^T was 39 % (reciprocal value 63%) and 58 % (67%) similar, respectively, to that of *C. gandavensis* LMG 18551^T. Whereas the similarity between strains E20 and E50^T was 86 % (100%) and therefore both belong to the same species.

These results revealed that strain E50^T, proposed as the type strain, represents a novel species considering that the DNA–DNA relatedness threshold for the definition of a species is 70 % (Stackebrandt & Goebel, 1994) and the value of similarity for this strain and the most closely related reference strain was lower that the reference threshold. It was also shown that strains E50^T and E20 are representatives of the same species since the DNA–DNA hybridization value was higher than the mentioned threshold. The phylogenetic trees reconstructed with neighbour-joining and the maximum-likelihood algorithms showed that strains E20, E50^T, *C. gandavensis* LMG 18551^T (AJ289162) and *C. japonicus* NCIMB 10462^T (AF452103) formed a monophyletic group with a bootstrap value of 73 % and 100 % respectively (Fig. 1) The tree topologies generated using neighbour-joining, maximum-likelihood and maximum-parsimony algorithms were very similar. (Fig. S3).

The major fatty acids for strain E20 (>5 %) were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (28.15 %), $C_{16:0}$ (18.33 %), $C_{18:1}\omega7c$ (11.72 %), $C_{12:0}$ (9.78 %), $C_{18:0}$ (8.92 %), $C_{12:0}$ 3-OH (6.22 %) and $C_{10:0}$ 3-OH (5.24 %). Similarly the major fatty acids for strain E50^T (>5 %) were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (30.0 %), $C_{16:0}$ (21.54 %), $C_{18:1}\omega7c$ (11.66 %), $C_{12:0}$ (8.89 %), $C_{18:0}$ (8.59 %) and $C_{12:0}$ 3-OH (5.40 %). Results showed

Table 1. Morphology, growth characteristics and enzyme activities of strains E20 and E50^T in comparison to the closest phylogenetic relatives

Taxa: 1, strain E20; 2, strain E50^T; 3, *C. gandavensis* LMG 18551^T (Mergaert *et al.*, 2003); 4, *C. ostraviensis* LMG 19434^T (Mergaert *et al.*, 2003); 5, *C. fibrivorans* LMG 18561^T(Mergaert *et al.*, 2003); 6, *C. fulvus* NCIMB 8634^T (Humphry *et al.*, 2003); 7, *C. vulgaris* NCIMB 8633^T(Humphry *et al.*, 2003); 8, *C. mixtus* subsp. *mixtus* ACM 2601^T (Humphry *et al.*, 2003); 9, *C. japonicus* NCIMB 10462$_T$ (Humphry *et al.*, 2003).+, positive; −, negative; D, strain-dependent; CR, curved rods; SR, straight rods; ND, not determined; SP, single polar; MF, mixed flagellation; 2P, two polar flagella.

Characteristic	1*	2*	3	4	5	6	7	8	9
Colony diameter (mm)	2–3	2–3	2–4	2–3	2–3	2–3	2–3	1–3	2–3
Cell morphology	CR	CR	SR	SR	SR	CR	CR	ND	ND
Width (μm)	0.5–0.7	0.5–0.7	0.7	0.7	0.7	0.3–0.4	0.3	0.2–0.5	0.5–0.7
Length (μm)	2.4–4.5	2.4–4.5	2–4	2–4	2–4	1.5–3	2.9–4	1–3	1.2–3
Flagella	SP	SP	SP	SP	SP	SP	SP	MF	2P
Growth at 4 °C (14 days)	−	−	+*	+	+	+	D(−)	−	−
Growth at 37 °C	+	+	−*	−	−	−	+	−	+
Growth with NaCl (%)	0–5	0–5	0*	ND	ND	0	0	0	2
Nitrate reduced to nitrite	−	−	−*	+	+	+	+	−	+
Utilization of:									
Maltose	+	+	+*	D(+)	+	+	+	+	ND
Arabinose	+	−	+*	D(+)	+	+	+	+	ND
N-Acetyl-D-glucosamine	+	+	+*	D(−)	+	+	+	D(+)	ND
API ZYM tests:									ND
Valine arylamidase	+	+	−*	+	−	−	−	−	−
Acid phosphatase	−	+	−*	+	+	−	D(−)	−	−
β-Galactosidase	−	−	+*	−	−	−	−	−	−
α-Glucosidase	+	+	−*	+	+	+	+	+	+
β-Glucosidase	−	−	+*	+	D(+)	+	D(−)	+	−
α-Galactosidase	−	−	−*	D(+)	D(+)	+	+	−	+
Naphthol-AS-BI-phosphohydrolase	+	+	−*	+	−	+	+	+	+
Cystine arylamidase	−	−	−*	D(−)	−	−	−	−	−
Mucoid growth on TSA	−	−	+*	−	−	−	−	+	ND
Yellow pigment on TSA	−	−	−*	D(−)	D(+)	−	D(+)	−	ND
Growth on:									
Xylose (5 mM)	+	+	+*	ND	ND	+	+	+	+
Cellulose (0.1 %)	+	+	+*	ND	ND	+	+	+	+
Pectin (0.1 %)	+	+	+*	ND	ND	+	+	+	+
Amylopectin (0.1 %)	+	+	+*	ND	ND	+	+	+	+
Dextran (5 mM)	−	−	−*	ND	ND	−	−	−	−
Ribose (5 mM)	−	−	−*	ND	ND	−	−	−	−
Casein (0.1 %)	−	−	−*	ND	ND	−	−	−	−
Glucose	+	+	+*	ND	ND	+	+	+	+
D-Galactose	+	+	+*	ND	ND	+	+	+	+
DNA G+C content (mol%)	52.1	51.6	44.2–44.6	47.4–48.4	48	44.6	44.9	52.6	53.3

*Results from this study.

that for the two strains, E50^T and E20, the major fatty acid contents correspond with the profiles reported for the other strains of species of the genus *Cellvibrio*. The $C_{12:0}$ 3-OH fatty acid present in strains E20 and E50^T (6.2 %) and (5.4 %) has not been previously detected in other species of the genus (Table S1).

The *nifH* gene amplification for strains E20 and E50^T did not yield any PCR products using the PolF and PolR primers either with the original protocol (Poly *et al.*, 2001) or after changes in the annealing temperature, $MgCl_2$ concentration, primer concentration and/or DNA concentration. DNA extracted from *A. vinelandii* DSM 2289^T and *Rhizobium leguminosarum* DSM 6044 were used as controls and PCR amplifications with the primera PolF and PolR yielded the 360 bp large, expected band when modified and/or original protocol were tested.

The results for the acetylene reduction assays showed that strains E20 and E50^T were able to reduce acetylene to

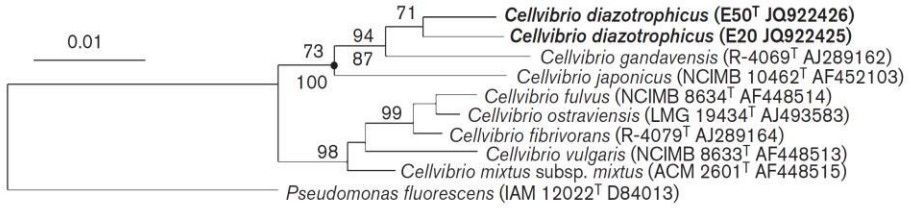

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences for the strains E20 (1474 bp), E50^T (1409 bp) and members of the genus *Cellvibrio* with validly published names. Bootstrap percentage (based on 1000 replicates, Jukes–Cantor) numbers are shown above the lines. Values presented below the lines corresponded to the bootstrap value (1000) of the maximum-likelihood tree. Filled circle indicates that nodes with the same branch topology were found in the neighbour-joining and maximum-likelihood trees. Bootstrap values lower than 70 are not shown. Bar, 0.01 substitutions per nucleotide position.

ethylene at 6.7 μmol (mg protein)$^{-1}$ h^{-1} and 7.1 μmol (mg protein)$^{-1}$ h^{-1}, respectively. The rate of nitrogen fixation was calculated using the theoretical minimum ratio value of acetylene reduction to N_2 reduction 4 : 1 (Schwintzer & Tjepkema, 1994). Strains E20 and E50^T reduce dinitrogen at a rate of 1.68 μmol N_2 (mg protein)$^{-1}$ h^{-1} and 1.78 μmol N_2 (mg protein)$^{-1}$ h^{-1}, respectively. The rate for the positive control *A. vinelandii* DSM 2289^T was 4.5 μmol N_2 (mg protein)$^{-1}$ h^{-1}. Similar results for *A. vinelandii* ATCC 13705 [6.7 μmol N_2 (mg protein)$^{-1}$ h^{-1}], have been previously reported (Linkerhägner & Oelze, 1997).

Emended description of the genus *Cellvibrio*

The description of the genus *Cellvibrio* is as given by Winogradsky (1929) and in the revival of the genus given by Blackall *et al.* (1985) with the following amendments. Some strains are capable of nitrogen fixation and able to grow in the presence of up to 5 % NaCl.

Description of *Cellvibrio diazotrophicus* sp. nov.

Cellvibrio diazotrophicus (di.a.zo.tro′phi.cus. Gr. prefix *di* two, double; N.L masc. *azotum* nitrogen; Gr. adj. *trophikos* nursing, tending or feeding; N.L. masc. adj. *diazotrophicus* one that feeds on dinitrogen).

Cells are Gram-reaction-negative rods (3.6 μm long and 0.7 μm wide), non-spore-forming and motile by means of single-polar flagella. Colonies on TSA are 2.0–2.5 mm in diameter, circular, slightly convex, non-mucoid, smooth and cream-coloured after 48 h at 28 °C. Colonies grown on LG agar are 2.5–3 mm in diameter, brilliant, non-pigmented, non-mucoid and smooth after 48 h at 28 °C. Growth occurs at a temperature range of between 16 °C and 37 °C, but not at 4 °C or 45 °C. No growth occurs under microaerophilic or anoxic conditions. Nitrate was not reduced to nitrite. Growth occurs with 0–5 % NaCl (w/v). Results are positive in tests for starch and CM-cellulose hydrolysis, oxidase and catalase activities, acetylene reduction and negative for urease. Positive growth results are obtained in basal media with the following carbon sources: fructose, xylose, cellulose, pectin, amylopectin, glucose, D-galactose, sucrose, acid pectin, aesculin, *N*-acetylglucosamine and maltose. No growth occurs with dextran, ribose, pyruvate, casein, adipate, malate, gluconate and citrate. With the API ZYM system positive results are obtained for alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase activities. Negative results are obtained for C14 lipase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase activities. Major fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or iso- $C_{15:0}$ 2-OH), $C_{16:0}$, $C_{18:1}\omega7c$, $C_{12:0}$, $C_{18:0}$ and $C_{12:0}$ 3-OH. Susceptible to kanamycin (30 μg), tetracycline (30 μg) and erythromycin (15 μg) and resistant to ampicillin (10 μg), chloramphenicol (30 μg) and penicillin G (10 μg).

The type strain, E50^T (=LMG 27267^T=KACC 17069^T), was isolated from a rhizosphere of *Hordeum secalinum* in a salt meadow in a nature protection area nearby Münzenberg, Hessen, Germany. Strain E20 (=LMG 27459=KACC 17068) was isolated from a rhizosphere of *Plantago winteri* in the same area and belongs to the species. The DNA G+C content of the type strain is 51.6 mol%.

Acknowledgements

We wish to thank Peter Kämpfer for helpful discussions, Gundula Will and Maria Sowinski for performing the analysis of DNA–DNA hybridizations and the membrane fatty acid analysis, respectively. We also thank Anton Hartmann for providing a culture of *A. vinelandii*, Martin Hardt for help with electron microscopy and Lutz Eckstein for identification of *Hordeum secalinum* and *Plantago winteri* plants in the salt meadows.

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Cellvibrio diazotrophicus* sp. nov., a nitrogen-fixing bacteria isolated from the rhizosphere of salt meadow plants and emended description of the genus *Cellvibrio.

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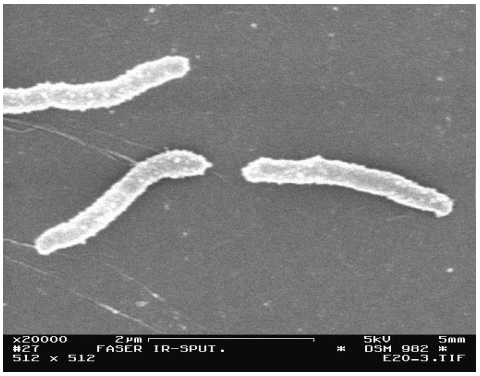
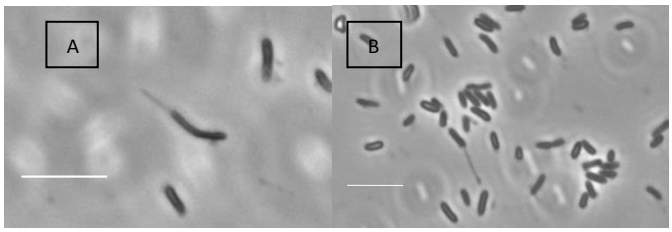
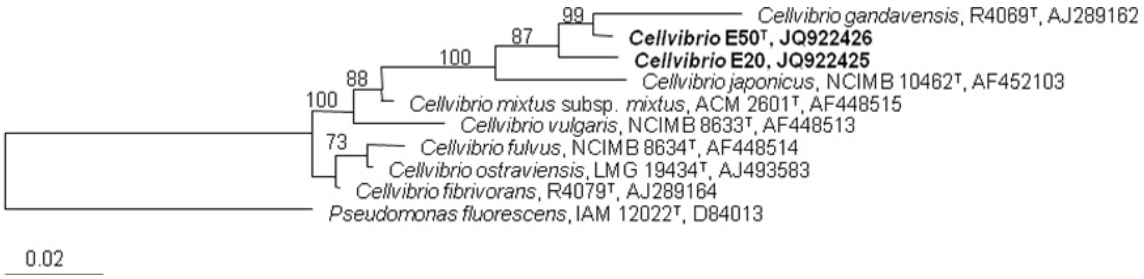
Supplementary materials



Supplementary Fig. S1. Electron micrograph of cells of strain E50^T. Bar, 2 µm



Supplementary Fig. S2. Light microscopy of flagella staining. A. Strain E20, B. Strain E50^T showing both a single polar flagella. (Bars, 5 µm.).



Supplementary Fig. S3. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences for the strains E20, E50^T and members of the recognized *Cellvibrio* genus. Bootstrap percentages higher than 70 (based on 1000 replicates) are showed at nodes. Bar, 0.02 substitutions per nucleotide position.

Table S1. Fatty acids content (in % of total fatty acids) of strains E20, E50^T, and type strains of other *Cellvibrio* species. Strains: 1, E20; 2, E50^T; 3, *C. gandavensis* LMG 18551^T (Mergaert *et al.,* 2003).

Fatty acid	1	2	3	
			This	Mergaert, *et al.* (2003).
$C_{10:0}$	3.2	3,0	1.3	1.6-3.3
$C_{10:0}$ 3-OH	5.2	4.5	2.3	3.0-6.5
$C_{11:0}$ 3-OH	-	-	-	-
$C_{12:0}$	9.8	8.9	4.5	5.0-8.6
$C_{12:0}$ 2-OH	tr	tr		-
$C_{12:1}$ 3-OH	-	-	3.3	3.7-7.4
$C_{12:0}$ 3-OH	6.2	5.4	3	-
$C_{14:0}$	3.5	3.4	-	-
$C_{15:0}$	tr	-	tr	0.0-1.8
$C_{16:0}$	18.3	21.5	25	17.4-25.5
$C_{17:0}$	2.0	1.8	1.7	0.0-1.7
$C_{18:0}$	8.9	8.6	1.5	0.0-1.4
$C_{18:1}{}^{\omega 7c}$	11.7	11.7	17	7.5-11.5
$C_{18:1}{}^{\omega 6c}$	-	-	-	-
Summed feature 3	28.2	30.0	39.6	34.6-47.6
Summed feature 5	-	-	-	0.0-1.1

+, Detected, -, not detected; tr, trace (<1.0 %). Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 3 ($C_{16:1}{}^{\omega 7c}$ and/or iso- $C_{15:0}$ 2-OH), Summed feature 5 ($C_{18:2}{}^{\omega 6,9c}$ and/or $C^{18:0}$ anteiso).

Chapter 5

Rheinheimera hassiensis sp. nov. and *Rheinheimera muenzenbergensis* sp. nov., two new species from the rhizosphere of *Hordeum secalinum*

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Rheinheimera hassiensis sp. nov. and *Rheinheimera muenzenbergensis* sp. nov., two species from the rhizosphere of *Hordeum secalinum*

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Two motile, Gram-staining-negative, aerobic, rod-shaped bacteria designated strains E48^T and E49^T were isolated from the rhizosphere of *Hordeum secalinum* from a natural salt meadow near Münzenberg, Germany. 16S rRNA gene sequence similarity analysis revealed that strains E48^T and E49^T shared similarities of 97.6 % with *Rheinheimera pacifica* KMM 1406^T and 98.5 % with *Rheinheimera nanhaiensis* E407-8^T, respectively. Major fatty acids of strain E48^T were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{17:1}\omega8c$, and of strain E49^T were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{18:1}\omega7c$. The DNA G+C contents were 50.5 mol% (E48^T) and 50.0 mol% (E49^T). Strains E48^T and E49^T grew at 4–37 °C (optimum 28 °C) and with 0–6 % NaCl (optimum 0–3 %) and 0–5 % NaCl (optimum 0–3 %), respectively. The potential for nitrogen fixation by strains E48^T and E49^T was evaluated by molecular techniques and the acetylene reduction assay. The DNA–DNA hybridization, physiological and molecular data demonstrated that strains E48^T and E49^T represent two novel species of the genus *Rheinheimera*, and therefore the names *Rheinheimera hassiensis* sp. nov. (type strain E48^T=LMG 27268^T=KACC 17070^T) and *Rheinheimera muenzenbergensis* sp. nov. (type strain E49^T=LMG 27269^T=KACC 17071^T) are proposed.

Brettar *et al.* (2002) were the first to describe members of the genus *Rheinheimera* as Gram-negative, flagellated, rod-shaped to coccoid, oxidase and catalase-positive bacterial cells that are commonly isolated from marine or estuarine environments. Currently, the genus contains 12 recognized species, *Rheinheimera baltica* OSBAC1^T (Brettar *et al.*, 2002), *Rheinheimera pacifica* KMM 1406^T (Romanenko *et al.*, 2003), *Rheinheimera perlucida* BA131^T (Brettar *et al.*, 2006), *Rheinheimera aquimaris* SW-353^T (Yoon *et al.*, 2007), *Rheinheimera chironomi* K19414^T (Halpern *et al.*, 2007), *Rheinheimera texasensis* A62-14B^T (Merchant *et al.*, 2007), *Rheinheimera soli* BD-d46^T (Ryu *et al.*, 2008), *Rheinheimera tangshanensis* JA3-B52^T (Zhang *et al.*, 2008), *Rheinheimera aquatica* GR5^T (Chen *et al.*, 2010b), *Rheinheimera nanhaiensis* E407-8^T (Li *et al.*, 2011), *Rheinheimera longhuensis* LH2-2^T (Liu *et al.*, 2012) and *Rheinheimera tilapiae* Ruye-90^T (Chen *et al.*, 2013).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains E48^T and E49^T are JQ922423 and JQ922424, respectively. That for the *nifH* gene sequence of strain E48^T is KF430615.

Six supplementary figures and one supplementary table are available with the online version of this paper.

Nine of the 12 recognized species of the genus have been isolated from aquatic environments; nevertheless, the presence of members of the genus *Rheinheimera* in soil environments has been widely reported. The genus *Rheinheimera* has been found associated with the roots of tomato plants (Kim *et al.*, 2006), associated with arbuscular mycorrhizal fungi spores (Roesti *et al.*, 2005), as diazotrophic endophytes of rice (Prakamhang *et al.*, 2009), as a part of a bacterial endophyte community of potato (Manter *et al.*, 2010) and as halotolerant bacteria in non-saline soil (Chen *et al.*, 2010a). Among the recognized species of the genus *Rheinheimera*, only *Rheinheimera soli* BD-d46^T (Ryu *et al.*, 2008) has been isolated from soil and *Rheinheimera tangshanensis* JA3-B52^T (Zhang *et al.*, 2008) from the roots of rice plants.

Strains E48^T and E49^T were isolated from the rhizosphere of salt-tolerant plant species. The salt-tolerant plant species were sampled from a natural salt meadow in a nature protection area near Münzenberg, Germany (50° 27′ 36″ N 8° 44′ 35″ E).

Hordeum secalinum plants were sampled in autumn (2006). The roots were washed off thoroughly by vigorous shaking in sodium pyrophosphate (100 mM). Subsequently, the suspensions were diluted with sterile saline solution using the standard dilution method technique and then incubated on nitrogen-free LG agar plates (Turner & Gibson,

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1980) at 28 °C until colonies were visible. Successive subcultures using streaking techniques were performed to ensure purity of the isolate, which were then stored in glycerol stocks at −20 °C.

The type strain *Rheinheimera pacifica* DSM 17616^T (=KMM 1406^T), obtained from the German collection of microorganisms and cell cultures (DSMZ), was used in comparison in parallel in all phenotypic tests.

For subculture handling and analysis proposes the colonies of strains E48^T and E49^T were subcultured in trypticase soy agar (TSA; Oxoid). Stationary and exponential phase cells grown on trypticase soy broth (TSB; Oxoid) were analysed by scanning electron microscopy (EM 300; Phillips) (Fig. S1, available in the online Supplementary Material). Colonies grown for 24 and 48 h on TSA were used for the Gram staining reaction after Gerhard *et al.* (1994) and visualization was done using a Zeiss microscope with ×1000 magnification. Motility was observed in wet mounts using light microscopy and confirmed by flagella staining (Heimbrook *et al.*, 1989) (Fig. S2).

Growth of E48^T and E49^T at different temperatures (4, 10, 16, 20, 28, 37, 45, 50 °C) was evaluated on TSA. Growth under anaerobic and microaerophilic atmospheres (Anaerocult A and C systems; Merck), and with different NaCl concentrations (1–10 % in intervals of 1 %) were tested on TSA at 28 °C. Growth with 0 % NaCl was tested on plates containing 1.5 % casein peptone, 0.5 % soy peptone and 1.5 % agar. Growth at pH 5.0–12.0 (in intervals of 0.5 pH units) was tested in TSB for 48 h at 28 °C. H_2O_2 solution (3 %, v/v) and oxidase test strips (Merck) were used to determine catalase and oxidase activities.

Biochemical and enzymic activities were evaluated using the API ZYM and API 20NE strips (bioMérieux). Basic mineral media (Pfennig, 1978) with different carbon sources were used to confirm the assimilation results from the API 20NE strip.

Hydrolysis of starch and CM-cellulose were determined with Gram's iodine dye and Congo red dye (Kasana *et al.*, 2008). Hydrolysis activity of Tween 80 was determined using the method of Sierra (1957). The disc diffusion method using a cell suspension (0.5 McFarland standard) from TSA plates grown at 28 °C for 48 h was used to analyse the sensitivity to antibiotics of strains E48^T and E49^T. Ampicillin (10 μg), chloramphenicol (10 μg), kanamycin (30 μg), penicillin G (10 μg), erythromycin (15 μg) and tetracycline (30 μg) discs (Oxoid) were placed on the TSA plates pre inoculated with the cell suspension. The TSA plates were incubated for 72 h at 28 °C and the diameters of the inhibition zones were measured to determine susceptibility, slight susceptibility or resistance according to the protocol of Nokhal & Schlegel (1983).

In order to perform the genomic analysis the genomic DNA from strains E48^T, E49^T, *Rheinheimera nanhaiensis* KACC 14030^T, *Rheinheimera pacifica* DSM 17616^T, *Rheinheimera baltica* LMG 21511^T, *Rheinheimera aquimaris* DSM 22684^T and *Rheinheimera chironomi* DSM 18694^T were extracted according to the method of Moré *et al.* (1994). Genomic DNA G+C contents were determined using a fluorometric method (Gonzalez & Saiz-Jimenez, 2002) and the DNA–DNA hybridization experiments were performed following the method proposed by Ziemke *et al.* (1998).

PCR, using the primers EUB 9f (9-27) and EUB 1492r (Lane, 1991), was used to amplify the 16S rRNA gene according to the method of Kampmann *et al.* (2012). The quick PCR purification kit (Qiagen) was used to clean the PCR products prior to sequencing by LGC genomics (Berlin, Germany). Reverse primers used for sequencing spanned positions (*Escherichia coli* numbering) 785 to 806 and 1099 to 1114, and forward primers spanned positions 7 to 27 and 519 to 536 (Johnson, 1994; Schwieger & Tebbe, 1998).

The closest relatives on the basis of the 16S rRNA gene sequences of strains E48^T (1412 bp) and E49^T (1416 bp) were retrieved from the GenBank database using the BLAST algorithm (Altschul *et al.*, 1990) and aligned using the software MEGA version 5.0 (Tamura *et al.*, 2011). Bellerophon software version 3.0 (Huber *et al.*, 2004) was used to prove that the sequences were not chimeric. The 16S rRNA gene sequences of strains E48^T, E49^T and the closest relatives were aligned online with the alignment tool SINA (v1.2.9) (Pruesse *et al.*, 2012). The ARB software 5.2 (Ludwig *et al.*, 2004) was used to merge the obtained online alignment with the LTP (Living Tree Project) online database (LTPs111, Feb 2013) (Yarza *et al.*, 2008), and to perform the similarity and the phylogenetic analyses of the 16S rRNA gene sequences.

Neighbour-joining (Saitou & Nei, 1987) corrected with the one-parameter model (Jukes & Cantor, 1969), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were reconstructed using nucleotide positions between 104 and 1461 (*E. coli* numbering; Brosius *et al.*, 1978), termini filter and 1000 bootstraps (Felsenstein, 1985).

For the fatty acid methyl ester analysis of strains E48^{T}and E49^T, cell biomass was collected in the exponential growth phase from TSA and marine agar plates Carl Roth grown for 24 h at 28 °C. *R. pacifica* DSM 17616^T cell biomass grown on marine agar was also analysed under the same growth conditions. The fatty acid methyl esters were extracted and analysed according to the methods of Kämpfer & Kroppenstedt (1996) and Kämpfer *et al.* (1997). Fatty acids have been identified based on retention time of reference compounds.

The growth of the type strains *Rheinheimera nanhaiensis* KACC 14030^T, *Rheinheimera pacifica* DSM 17616^T, *Rheinheimera baltica* LMG 21511^T, *Rheinheimera aquimaris* DSM 22684^T and *Rheinheimera chironomi* DSM 18694^T on nitrogen-free LG medium (Turner & Gibson, 1980) was evaluated at their corresponding optimal growth temperatures. Strains E48^T, E49^T and the type strains with positive growth in LG medium were tested to determine their nitrogenase activity using modifications of the acetylene reduction assay proposed by Hardy *et al.* (1968).

The strains to be analysed were grown in TSB or marine broth for 48 h at optimal growth temperature. The cell suspensions (5 ml), double-washed with Nfb-medium (Albrecht & Okon, 1980) and adjusted to give an OD_{600} of 0.5, were placed in 60 ml glass bottles containing 5 ml Nfb semisolid medium. In parallel, a single colony from each strain to be tested was streaked on 10 ml LG sloped agar in the same capacity glass bottles. All bottles were incubated at optimal growth temperature for 3 days and measurement of the acetylene reduction was performed according to the method of Suarez *et al.* (2013).

The presence of the marker gene for nitrogen fixation, *nifH*, was examined using primers and PCR conditions as originally described and with modification of the protocol of Poly *et al.* (2001). The specific PCR product was cleaned with the quick PCR purification kit (Qiagen) and sequenced by LGC genomics. The UCHIME algorithm (Edgar *et al.*, 2011) was used to prove that the sequences were not chimeric. Frameshift errors were detected and corrected and the nucleotide sequence was translated into a protein sequence using the functional gene pipeline and repository (fungene) from the ribosomal database project (Cole *et al.*, 2009). Neighbour-joining phylogenetic trees for the *nifH* gene nucleotide and amino acid sequences corrected with the Kimura two-parameter model (Kimura, 1980) were reconstructed using MEGA version 5.0 (Tamura *et al.*, 2011).

In parallel for all experiments on acetylene-reducing activity and the *nifH* gene amplification, the type strain *Azotobacter vinelandii* DSM 2289^T was used as positive control.

Strain E48^T was aerobic and microaerophilic, chemoheterotrophic, rod-shaped, motile, Gram-staining-negative, positive for oxidase and negative for catalase. Growth occurred within a salt range of 0–6 % (w/v) NaCl, at temperatures between 4 and 37 °C, and between pH 6.5 and 10. Colonies were 2.0 mm in diameter, circular, slightly convex, smooth and greyish yellow in colour when grown on TSA after 24 h. When grown on LG agar, colonies were 1–1.5 mm in diameter, transparent, circular and slightly convex after 48 h at 28 °C.

Strain E49^T was aerobic and microaerophilic, chemoheterotrophic, rod-shaped, motile, Gram-staining-negative, positive for oxidase and negative for catalase. Growth occurred within a salt range of 0–5 % (w/v) NaCl, at temperatures between 4 and 37 °C, and at pH 7.0–9.5. Colonies were 2.0 mm in diameter, circular, slightly convex, smooth and greyish yellow in colour when grown on TSA at 24 h and became slimy with a pale orange colour on TSA after 48 h. Growth on LG agar was slow and transparent, circular, slightly convex colonies of 1–1.5 mm in diameter were formed after 72 h at 28 °C

Positive activities for oxidase, assimilation of *N*-acetylglucosamine and hydrolysis of starch and aesculin were phenotypic characteristics of both new strains and have been described for all recognized species of the genus *Rheinheimera*. Morphological, biochemical, physiological and cultural characteristics of other recognized species of the genus *Rheinheimera* and for strains E48^T and E49^T are given in Table 1.

The 16S rRNA gene sequences of strains E48^T and E49^T showed similarities greater than 95 % with the described species of the genus *Rheinheimera*; the high similarities and the phylogenetic analysis indicated that these species belong to the genus *Rheinheimera* (Ludwig *et al.*, 1998). The highest 16S rRNA gene sequence similarity values for strain E48^T with described species of this genus were (97.6 %) for *Rheinheimera pacifica* KMM 1406^T followed by *Rheinheimera aquimaris* SW-353 (97.5 %), strain E49^T (96.9 %), *Rheinheimera baltica* OSBAC1^T (96.8 %), *Rheinheimera perlucida* BA131^T (96.7 %) and *Rheinheimera nanhaiensis* E407-8^T (96.4 %). Strain E49^T shared the highest similarity with *Rheinheimera nanhaiensis* E407-8^T (98.5 %), followed by *Rheinheimera pacifica* KMM 1406^T (98.2 %), *Rheinheimera aquimaris* SW-353^T (97.9 %), *Rheinheimera baltica* OSBAC1^T (97.8 %), *Rheinheimera chironomi* K19414^T (97.2 %), *Rheinheimera longhuensis* LH2-2^T (97.2 %), strain E48^T (96.9 %) and *Rheinheimera soli* BD-d46^T (96.7 %). The 16S rRNA gene sequences similarity values of the other recognized species of the genus *Rheinheimera* with respect to strain E48^T were less than 96.3 % and for strain E49^T less than 96.9 %.

The DNA–DNA relatedness of strain E48^T with *Rheinheimera pacifica* DSM 17616^T, *Rheinheimera aquimaris* DSM 22684^T, *Rheinheimera baltica* LMG 21511^T and strain E49^T was 38.1 % (39.3 % reciprocal value), 20.7 % (10.1 %), 55.4 % (68.6 %) and 35.2 % (42.8 %), respectively. Relatedness of strain E49^T with *Rheinheimera nanhaiensis* KACC 14030^T, *Rheinheimera pacifica* DSM 17616^T, *Rheinheimera baltica* LMG 21511^T, *Rheinheimera aquimaris* DSM 22684^T, *Rheinheimera chironomi* DSM 18694^T and strain E48^T was 39.2 % (45.5 % reciprocal value), 46.5 % (24.6 %), 33.7 % (20.8 %), 33.4 % (53.2 %), 39.8 % (50.9 %) and 42.8 % (35.2 %), respectively. DNA–DNA hybridization results showed that strains E48^T and E49^T can both be considered as novel species on the basis that neither has a DNA−DNA relatedness value higher than 70 %, considered as the threshold for the definition of a novel species (Stackebrandt & Goebel, 1994).

The neighbour-joining phylogenetic tree (Fig. 1) showed that strains E48^T and E49^T constitute part of a monophyletic cluster formed with all recognized species of the genus *Rheinheimera* confirming that strains E48^T and E49^T belong to the genus *Rheinheimera*. Similar results were obtained with the maximum-likelihood and maximum-parsimony trees (Figs S3, S4).

DNA G+C contents of strains E48^T (50.5 ± 1 mol%) and E49^T (50.0 ± 1 mol%) fell within the range of values for this parameter for the genus *Rheinheimera*, between 47.0 and 51.9 mol%, including the last emendation of the genus considering this issue (Chen *et al.*, 2010b).

The major fatty acids for strain E48^T (>5 %) were $C_{16:0}$ (18.7 %), summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (17.2 %), $C_{17:1}\omega8c$ (15.8 %), $C_{18:1}\omega7c$ (15.0 %) and

Table 1. Morphology, growth characteristics and enzymic activities of strains E48^T and E49^T in comparison with species of the genus *Rheinheimera*

Strains: 1, E48^T; 2, E49^T; 3, *Rheinheimera pacifica* DSM 17616^T (data from Romanenko *et al.*, 2003); 4, *Rheinheimera tangshanensis* JA3-B52^T (Zhang *et al.*, 2008); 5, *Rheinheimera soli* BD-d46^T (Ryu *et al.*, 2008); 6, *Rheinheimera baltica* OSBAC1^T (Brettar *et al.*, 2002); 7, *Rheinheimera perlucida* BA131^T (Brettar *et al.*, 2006); 8, *Rheinheimera aquimaris* JCM 14331^T (Yoon *et al.*, 2007); 9, *Rheinheimera texasensis* A62-14B^T (Merchant *et al.*, 2007); 10, *Rheinheimera chironomi* K19414^T (Halpern *et al.*, 2007); 11, *Rheinheimera aquatica* GR5^T (Chen *et al.*, 2010b); 12, *Rheinheimera nanhaiensis* E407-8^T (Li *et al.*, 2011) 13, *Rheinheimera longhuensis* LH2-2^T (Liu *et al.*, 2012); 14, *Rheinheimera tilapiae* Ruye-90^T (Chen *et al.*, 2013). All strains are Gram-staining-negative and positive for oxidase and hydrolysis of starch and aesculin. +, Positive; −, negative; W, weakly positive; ND, not determined; NA, no data available; R, rods; C, cocci; S, single; P, polar; L, lateral; M, multiple; FA, facultatively aerobic; A, aerobic.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cell morphology	R	R	R	R	SR/CR	R/C	R	R/C	R	R	R	SR	R	R
Cell width (μm)	0.4–0.8	0.5–0.8	0.4–0.8*	1.3–2.5	0.9–1.5	0.5–1.5	0.6–1.2	0.3–0.7	0.7–0.8	0.3–0.7	0.5–1.0	0.3–0.5	0.3–0.5	0.3–0.8
Cell length (μm)	0.7–2.0	1.3–2.4	1.8–2.0*	0.4–1.0	2.0–3.5	0.9–2.5	0.9–2.4	0.3–5.0	1.3–2.5	1.0–2.4	1.5–2.0	1.0–2.0	2.0–4.0	0.9–1.8
Flagella	S, P	S, P	M, P or L	S, P or L	S, P	S, P	S, P	S, P	S, P or M, P, L	S, P	S, P	S, P	S, P	S, P
Catalase	−	−	+*	+	+	+	+	+	+	−	+	+	+	−
O_2 requirement	A	A	A*	A	FA	A	FA	A	FA	FA	A	FA	A	A
Growth temperature														
Range	4–37	4–37	4–37*	10–37	15–35	4–30	4–37	4–43	25–37	4–40	10–45	10–48	0–36	4–30
Optimum (°C)	25–30	25–30	25–30*	30	25–30	20–25	20–30	30–37	30–37	30–37	35	37	26–34	20–30
NaCl for growth (%)														
Range	0–6	0–5	0–8*	0–3	0–4	0–6	0–8	0–8	0–1	0–2	0–2	0–8	0–2	0–2
Optimum	0–1	0–1	0.5–2.5*	1	0–1	0–3	1–3	1–3	0	0.5–1	0.5–1	0.5–2.5	1	0
pH for growth														
Range	6.5–10	7–9.5	5.5–10.5*	6.0–8.5	6.5–8.0	5.7–10	5.7–10	5.5–10.5	6.5–9.6	6.5–10.0	7.0–8.0	5.5–10	6.5–11	7.0–9.0
Optimun	7.0–8.0	7.0–8.0	8–8.5*	7.0	7.0–7.5	7.0	7.0	7.0–8.0	7.5–8.0	7.5–8.0	8.0	7.5–8.5	8.0–8.6	8.0–9.0
Acid production from glucose	–	–	−*	+	+	+	−	+	+	+	+	+	−	−
Assimilation of:														
D-Glucose	−	−	W*	+	+	+	−	+	+	+	+	+	+	−
Maltose	+	−	+*	−	+	+	−	+	−	+	+	+	+	−
Citrate	+	−	+*	−	−	−	−	−	−	−	−	−	−	−
Gelatin hydrolysis	−	+	+*	+	+	+	+	+	+	+	+	+	+	−
Nitrate reduction	−	−	−*	−	+	−	+	+	+	+	+	+	+	+
Growth in nitrogen-free media	+	+	+*	ND	ND	−*	ND	+*	ND	−*	ND	−*	ND	ND
Source of isolation	Barley rhizospheric soil	Barley rhizospheric soil	Pacific deep-sea	Rice roots	Soil	Baltic Sea	Baltic Sea	Seawater	Spring-fed lake	Chironomid egg mass	Freshwater pond	Marine sediments	Lake water	Fresh water culture pond
DNA G+C content (mol%)	50.5	50	49.7*	47.0	49.2	48.9	48.9	50.5	48.2	49.9	51.9	51	47	49

*Results from this study.

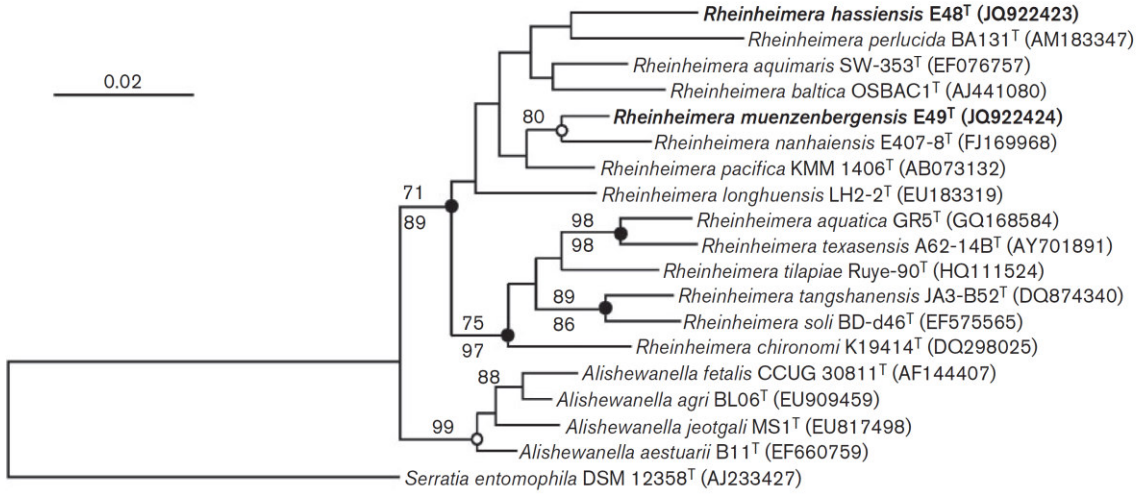

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences for strains E48^T (1412 bp), E49^T (1416 bp), members of the recognized species of the genus *Rheinheimera* and some species of the genus *Alishewanella*. Bootstrap percentages (based on 1000 replicates, Jukes–Cantor) are shown above the nodes. Values presented below the nodes corresponded to the bootstrap values (1000) from the maximum-likelihood tree. Filled circles indicate that the same branch topology was formed using the neighbour-joining, maximum-likelihood and maximum-parsimony methods. Open circles indicate that the same branch topology was formed using the neighbour-joining and maximum-parsimony methods. *Serratia entomophila* DSM 12358^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position. Only bootstrap percentages greater than 70 are shown.

$C_{17:0}$ (8.6 %). The major fatty acids for strain E49^T (>5 %) were $C_{16:0}$ (26.8 %), summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (20.2 %), $C_{18:1}\omega7c$ (15.1 %), $C_{12:0}$ 3-OH (8.8 %), $C_{16:1}\omega9c$ (7.8 %) and $C_{17:1}\omega8c$ (5.9 %). The presence of major amounts of $C_{16:1}\omega7c$, $C_{17:1}\omega8c$ and $C_{18:1}\omega7c$ in the fatty acid profiles of strains E48^T and E49^T was similar to the recognized strains of species of the genus *Rheinheimera* (Table S1).

The type strains *Rheinheimera nanhaiensis* E407-8^T, *Rheinheimera baltica* OSBAC1^T and *Rheinheimera chironomi* K19414^T were not able to grow on LG medium, whereas *Rheinheimera pacifica* KMM 1406^T and *Rheinheimera aquimaris* SW-353^T presented growth after 48 h under optimal temperature growth conditions.

The *nifH* gene amplification for strains E49^T, *Rheinheimera pacifica* KMM 1406^T and *Rheinheimera aquimaris* SW-353^T did not yield any PCR products using the PolF and PolR primers with the original protocol (Poly *et al.*, 2001) or with changes in the annealing temperature, $MgCl_2$ concentration, primer concentration and/or DNA concentration. *A. vinelandii* DSM 2289^T and *Rhizobium leguminosarum* DSM 6044 DNA extractions were used as controls and amplified the expected size band (360 bp) when modification and/or original protocol were tested (data not shown).

The *nifH* gene sequence of strain E48^T (360 bp) showed the highest similarity (99 %) with an uncultured bacterium (GenBank accession number JX878668). Among the *nifH* sequences of species with validly published names, that of *Rhizobium rosettiformans* (GQ241353) was most similar at 96 %. Results from the reconstructed neighbour-joining phylogenetic tree based on *nifH* gene and amino acid sequences showed that strain E48^T was located in an internal subcluster together with *Burkholderia xenovorans* and *Herbaspirillum seropedicae* inside a mixed cluster with *nifH* gene sequences of members of the classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Betaproteobacteria* (Figs S5 and S6). The genus *Rheinheimera* belongs to the class *Gammaproteobacteria* based on 16S rRNA gene sequence phylogeny. The *nifH* gene phylogenetic clusters of members of the phylum *Proteobacteria* are generally well defined for the classes *Alphaproteobacteria* and *Gammaproteobacteria* (Zehr *et al.*, 2003). The incongruence of the phylogenies of the *nifH* and the 16S rRNA gene sequences of the strain E48^T can be explained by lateral gene transfer of the *nifH* gene (Raymond *et al.*, 2004).

The acetylene reduction assay showed that strains E48^T, E49^T, *Rheinheimera pacifica* KMM 1406^T and *Rheinheimera aquimaris* SW-353^T were not able to reduce acetylene to ethylene under these conditions, either on Nfb-medium or LG agar. Under all conditions, *A. vinelandii* DSM 2289^T was able to reduce acetylene to ethylene (data not shown).

Isolates able to grow in nitrogen-free media that are not able to reduce acetylene (Hill & Postgate, 1969), and also do not carry the *nifH* gene, have been reported by Boström *et al.* (2007). The authors speculate that the bacteria presenting under these growth features are nitrogen

scavengers, bacteria able to take low levels of reduced nitrogen from the atmosphere.

Description of *Rheinheimera hassiensis* sp. nov.

Rheinheimera hassiensis (has.si.en′sis. N.L. fem. adj. *hassiensis* pertaining to Hassia, the latin name for Hessen, the state from which the organism was first isolated).

Cells are Gram-staining-negative rods (0.7–2 μm long and 0.4–0.8 μm wide), non-spore-forming, motile by the presence of a single-polar flagella, and grow under aerobic and microaerophilic conditions. Colonies on LG agar are 1–1.5 mm in diameter, transparent, non-pigmented, circular and slightly convex after 48 h of incubation at 28 °C. After 24 h of incubation on TSA, colonies are 2.0 mm in diameter, circular, slightly convex, smooth and greyish yellow. Growth occurs between 4 and 37 °C with an optimal growth temperature between 25 and 30 °C, in the absence of NaCl up to 6 % (w/v) NaCl on TSA and within a pH range of 6.5 to 10 with optimal growth between pH 7.0 and 8.0. Positive for starch and Tween 80 hydrolysis. Oxidase-positive and negative for cellulose and gelatin hydrolysis, and catalase activity. Positive results from the API 20NE system for aesculin hydrolysis, and assimilation of citrate and maltose. Negative results for nitrate reduction, indole production, D-glucose fermentation, arginine dihydrolase, β-galactosidase and urease activities, gelatin hydrolysis, and the assimilation of *N*-acetylglucosamine, D-glucose, arabinose, mannose, mannitol, gluconate, caprate, adipate, malate and phenylacetate. With the API ZYM system, positive results for alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. Negative results for C14 lipase, valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities. Major fatty acids are $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{17:1}\omega8c$. Susceptible to tetracycline (30 μg) and erythromycin (15 μg), slightly susceptible to chloramphenicol (30 μg) and kanamycin (30 μg), resistant to ampicillin (10 μg) and penicillin G (10 μg).

The type strain, E48^T (=LMG 27268^T=KACC 17070^T), was isolated from the rhizosphere of *Hordeum secalinum* in a natural salt meadow in a nature protection area near Münzenberg, Hessen, Germany. The DNA G+C content of the type strain is 50.5 mol%.

Description of *Rheinheimera muenzenbergensis* sp. nov.

Rheinheimera muenzenbergensis (muen.zen.berg.en′sis. N.L. fem. adj. *muenzenbergensis* pertaining to Münzenberg, the village near the place the organism was first isolated).

Cells are Gram-staining-negative rods (1.3–2.4 μm long and 0.5–0.8 μm wide), non-spore-forming, motile by the presence of a single-polar flagella and grow under aerobic and microaerophilic conditions. Colonies on LG agar are 1–1.5 mm in diameter, transparent, non-pigmented, circular and slightly convex after 48 h of incubation at 28 °C. Colonies on TSA after 18 h of incubation are 2.0 mm in diameter, non-pigmented, circular, slightly convex, smooth and greyish yellow and after 24 h of incubation the colonies become slimy with a pale orange colour. Growth occurs between 4 and 37 °C with an optimal growth temperature between 25 and 30 °C, in the absence of NaCl up to 5 % (w/v) NaCl on TSA and within a pH range of 6.5 to 9.5 with optimal growth between pH 7.0 and 8.0. Positive for starch, gelatin and Tween 80 hydrolysis and oxidase activity and negative for cellulose hydrolysis and catalase activity. Positive results from the API 20NE system for aesculin and gelatin hydrolysis. Negative results for nitrate reduction, indole production, D-glucose fermentation, arginine dihydrolase and urease activities and the assimilation of D-glucose, arabinose, mannose, mannitol, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate. With the API ZYM system, positive results for alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. Negative results for C14 lipase, valine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities. Major fatty acids are $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{18:1}\omega7c$. Susceptible to tetracycline (30 μg) and kanamycin (30 μg), slightly susceptible to chloramphenicol (30 μg) and erythromycin, (15 μg), resistant to ampicillin (10 μg) and penicillin G (10 μg).

The type strain, E49^T (=LMG 27269^T=KACC 17071^T), was isolated from the rhizosphere of *Hordeum secalinum* in a natural salt meadow in a nature protection area nearby Münzenberg, Hessen, Germany. The DNA G+C content of the type strain is 50 mol%.

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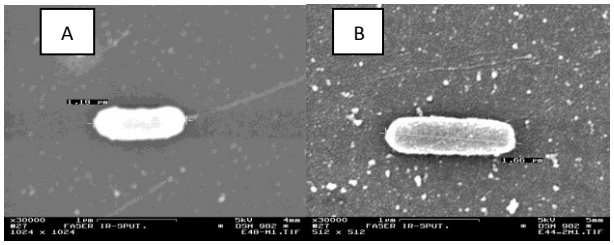
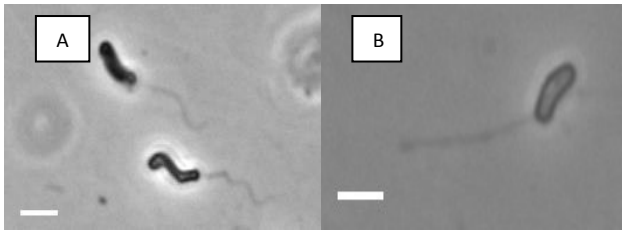
Supplementary material for

Rheinheimera hassiensis* sp. nov. and *Rheinheimera muenzenbergensis* sp. nov., two new isolates from the rhizosphere of *Hordeum secalinum

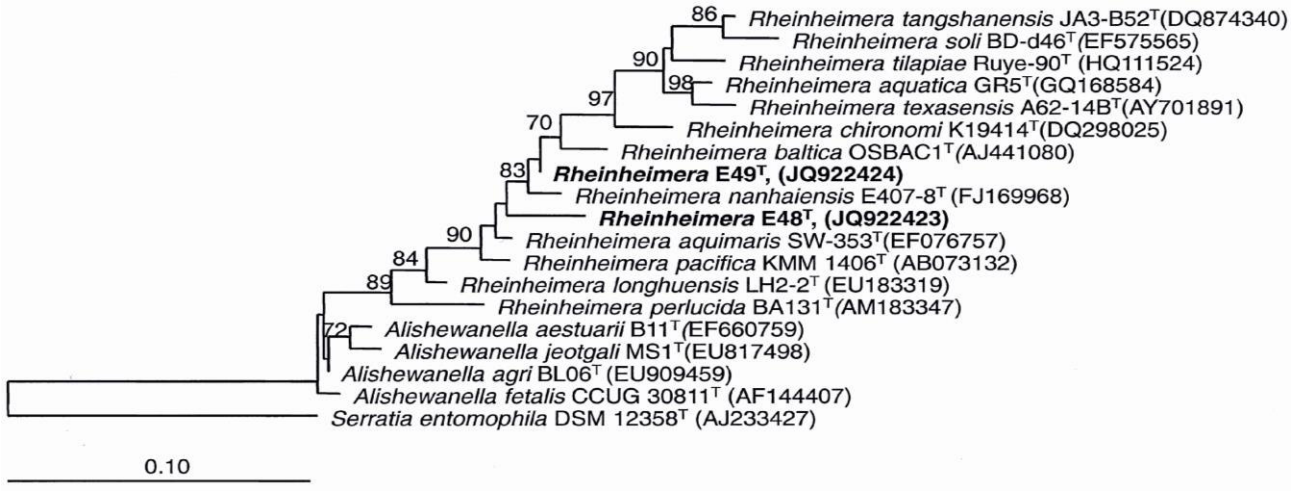
Christian Suarez[1], Stefan Ratering[1, 2*], Rita Geissler-Plaum[1] and Sylvia Schnell[1]



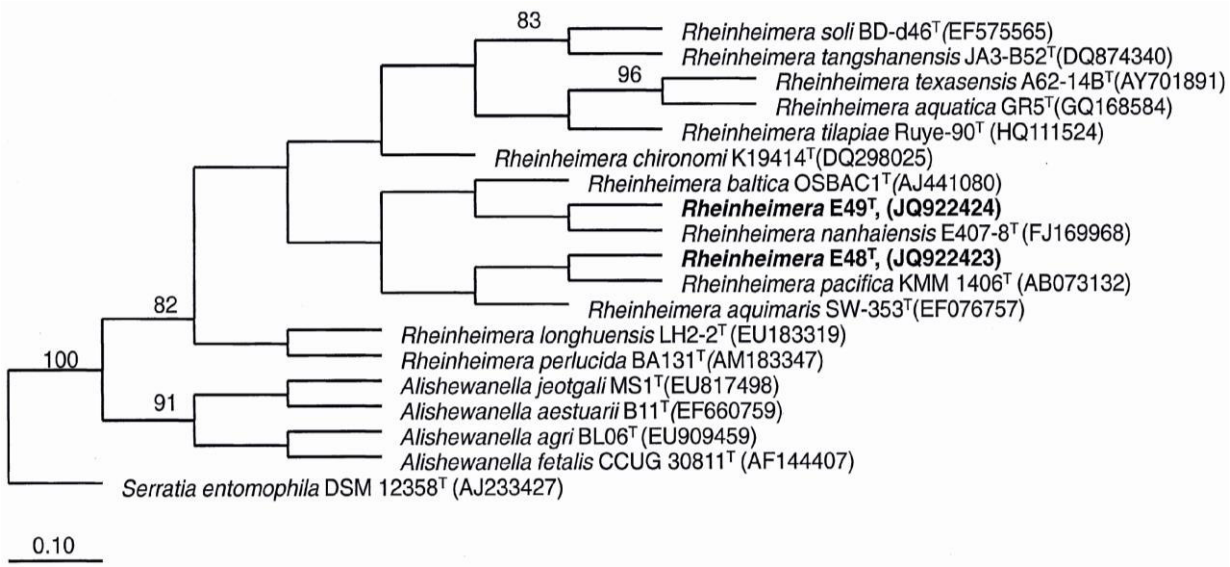
Supplementary Fig. S1. Electron micrographs of cells of strain A. E48^T, B. E49^T. Bar, 1 µm



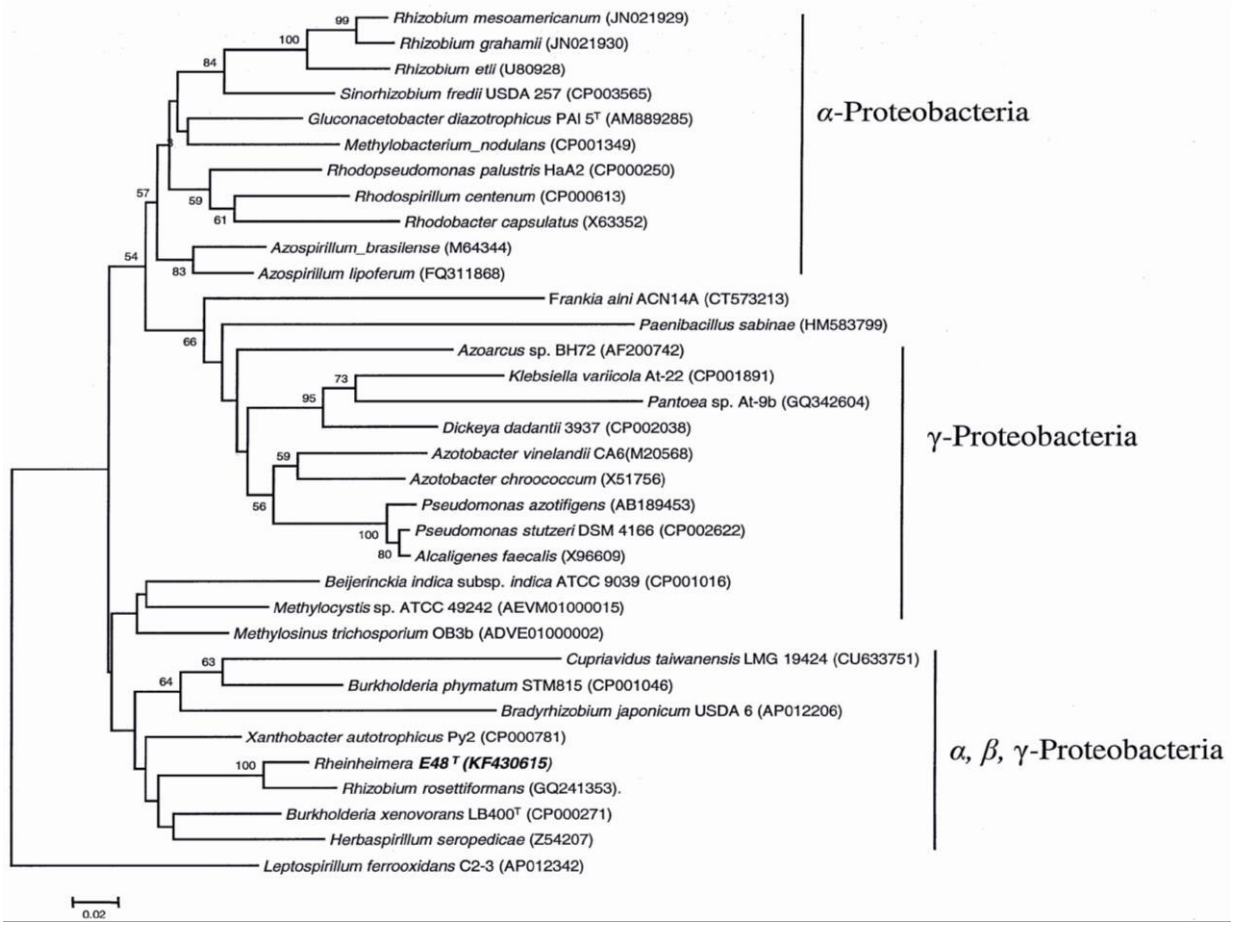
Supplementary Fig. S2. Light microscopy of flagella staining. A. Strain E48^T, B. Strain E49^T showing both a single polar flagella. (Bars, 2 µm.).



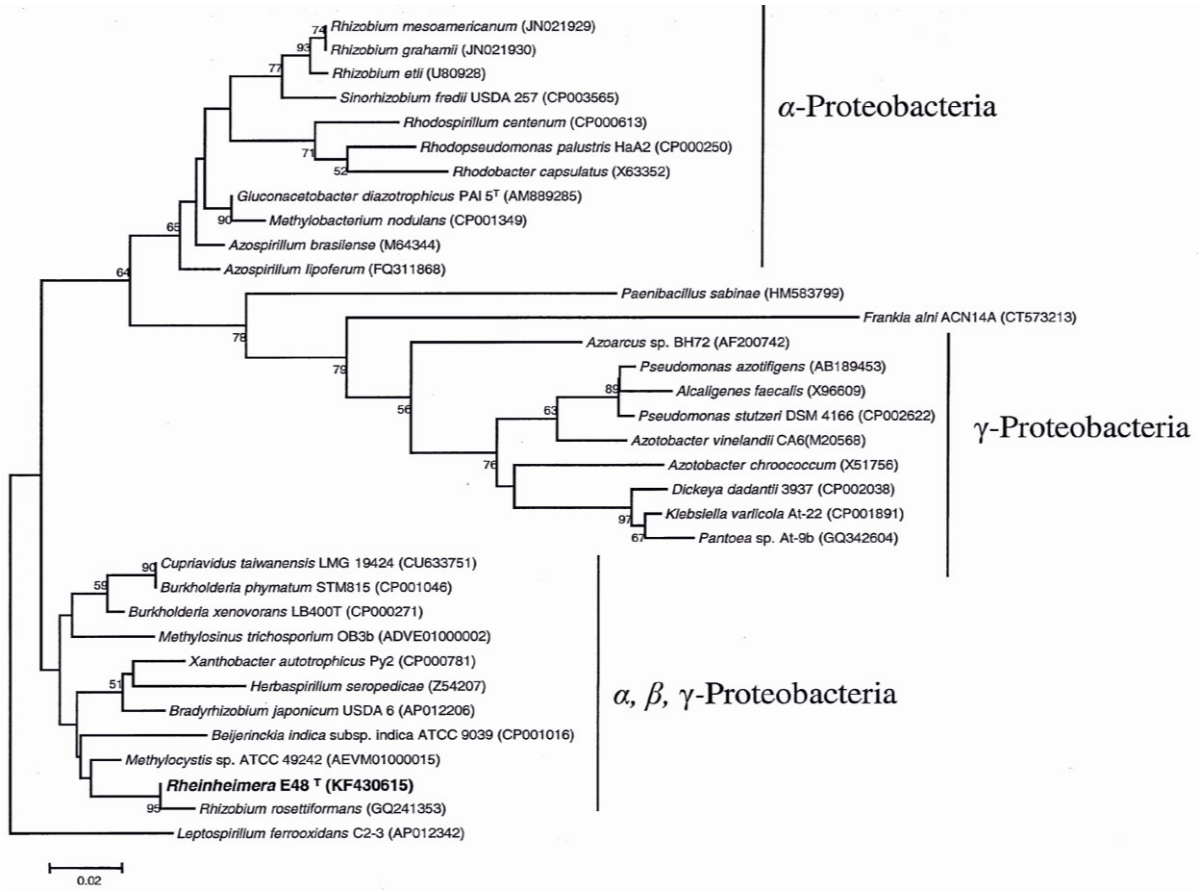
Supplementary Fig. S3. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences for the strains E48^T, E49^T, members of the recognized *Rheinheimera* genus and some other related genera. Bootstrap percentages higher than 70 (based on 1000 replicates) are showed at nodes. Bar, 0.10 substitutions per nucleotide position.



Supplementary Fig. S4. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences for the strains E48^T, E49^T, members of the recognized *Rheinheimera* genus and some other related genera. Bootstrap percentages higher than 70 (based on 1000 replicates) are showed at nodes. Bar, 0.10 substitutions per nucleotide position



Supplementary Fig. S5. Neighbor-joining tree based on *nifH* gene sequences showing relationships between strains E48^T and *nifH* gene sequence containing species based on 344 bp sequences length. *Leptospirillum ferrooxidans* C2-3 was set as outgroup. Bootstrap percentages higher than 50 (based on 1000 replicates) are showed at nodes. Bar, 0.02 substitutions per nucleotide position.



Supplementary Fig. S6. Neighbor-joining tree based on amino acids sequences showing relationships between strains E48^T and *NifH* sequence containing species based on 115 amino acids sequences length alignment. *Leptospirillum ferrooxidans* C2-3 was set as outgroup. Bootstrap percentages higher than 50 (based on 1000 replicates) are showed at nodes. Bar, 0.02 substitutions per amino acid position.

Table S1. Fatty acid compositions of strains E48^T, E49^T and type strains of other *Rheinheimera* species. Strains: 1. Strain E48^T; 2, Strain E49^T; 3, *R. chironomi* K19414^T (Halpern *et al.*, 2007). 4, *R. pacifica* DSM 17616^T (Romanenko *et al.*, 2003); 5, *R. baltica* OSBAC1^T (Brettar *et al.*, 2002); 6, *R. aquimaris* JCM 14331^T (Yoon *et al.*, 2007); 7, *R. soli* BD-d46^T (Ryu *et al.*, 2008); 8, *R. tangshanensis* JA3-B52 ^{T}(Zhang *et al.*, 2008); 9, *R. texasensis* A62-14B^T (Merchant *et al.*, 2007); 10, *R. perlucida* BA131^T (Brettar *et al.*, 2006); 11, *R. nanhaiensis* E407-8^T (Li *et al.*, 2011); 12, *R. aquatica* GR5^T (Chen *et al.*, 2010); 13, *R. longhuensis* LH2-2^T (Liu *et al.*, 2012); 14, *R. tilapiae* Ruye-90^T (Chen *et al.*, 2013). -, not detected or not reported; tr, trace (<1.0%); MA, Marine Agar; TS, trypticase soy agar; HMA, half concentration marine agar; R2A, R2A agar; §, results from this study. Summed features are groups of two or three fatty acids that it is not possible to be separated by GLC with the MIDI System. Summed reported as: a, Summed feature 1 ($C_{13:0}$ 3-OH and/or iso-$C_{15:1}$ H); b, Summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I); c, summed feature 3 ($C_{16:1}{}^{\omega 7c}$ and/or iso-$C_{15:0}$ 2-OH).

	E48^{T§}		E49^{T§}		3		4		5	6	7	8	9	10	11	12	13	14
	TS	MA	TS	MA	TS§	TS Halpern *et al.* (2007)	MA§	Li *et al.* (2011)	HMA	MA	MA	TSA	R2A	HMA	MA	R2A	MA	R2A
$C_{10:0}$	-	-	-	-	1.6	1.2	-	0.1	-	0.1	3.7	2.2	-	-	0.2	-	tr	1.0
$C_{11:0}$	0.6	-	-	-	-	-	0.6	-	-	-	-	-	-	-	-	-	tr	-
$C_{11:0}$ 3-OH	1.0	tr	-	-	2.0	5.5	1.0	0.4	-	1.5	4.6	3.3	-	1.2	2.1	2.1	2.3	1.1
$C_{12:0}$	2.2	3.0	1.2		1.4	1.1	3.5	2.8	1.4	2.1	0.5	-	2.0	1.1	1.3	2.9	tr	1.3
$C_{12:0}$ 3-OH	3.9	5.2	8.8	11.2	10.4	8.5	5.4	4.7	5.3	3.6	9.5	7.2	12.8	3.1	3.9	12.5	2.6	7.0
$C_{14:0}$	1.1	1.7	1.6	2.16	1.2	0.9	0.7	1.4	2.3	0.9	1.4	-	-	1.8	0.7	1.9	tr	2.1
$C_{15:0}$	2.0	1.4	-	-	1.3	6.9	1.8	1.0	1.7	3.2	6.0	2.1	-	3.0	2.8	-	-	-
$C_{15:1}{}^{\omega 8c}$	2.2	1.0	1.2		1.2	2.8	2.9	1.3	1.7	3.8	2.9	3.0	-	2.0	4.5	1.7	1.9	1.4
$C_{16:0}$	18.7	25.1	26.8	30.1	16.7	14.8	17.1	19.9	19.5	17.7	10.8	7.8	19.0	17.1	11.9	16.6	12.7	19.3
iso-$C_{16:0}$	1.6	1.4	1.0	-	1.5	1.5	3.1	1.5	0.5	2.1	0.8	-	-	1.2	3.7	1.2	tr	-
$C_{16:1}{}^{\omega 9c}$	1.1	2.0	7.8	-	-	-	1.2	2.1	1.7	2.1	0.7	-	-	-	0.6	-	-	-
17:0 ANTEISO	1.3	tr	1.7	-	0.8	-	1.1	-	-	-	-	-	-	-	-	-	-	-
$C_{17:0}$	8.6	4.4	3.8	-	2.2	5.8	4.7	4.1	3.3	8.1	4.2	-	-	3.4	8.6	1.9	8.0	1.1
$C_{17:1}{}^{\omega 8c}$	15.8	7.8	5.9	2.5	4.9	7.6	8.7	5.6	5.7	11.6	12.5	15.2	3.8	13.9	14.5	4.8	18.9	2.7
$C_{17:1}{}^{w6c}$	1.1	-	-	-	-	-	1.0	-	-	-	-	-	-	-	-	-	1.1	-
$C_{18:0}$	1.1	1.1	3.0	1.9	0.8	0.3	0.7	1.5	0.5	0.8	0.4	-	2.0	0.4	0.6	1.7	tr	2.9
iso-$C_{18:0}$	tr	-	-	-	-	-	1.0	0.6	-	0.6	-	-	-	-	1.2	-	-	-
$C_{18:1}{}^{\omega 7c}$	15.0	17.5	15.1	14.5	11.9	6.7	14.1	15.9	15.9	8.1	7.1	13.4	7.7	12.4	10.0	9.1	16.4	8.4
$C_{18:1}{}^{\omega 9c}$	-	-	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{13:0}$ 3-OH	2.3a	1.3a	-	-	-	1.9a	1.5a	1.0	1.0	2.1a	1.3a	-	-	0.7	2.7a	-	2.3a	-
$C_{14:0}$ 3-OH	2.2b	3.3b	-	-	-	0.3b	2.33b	3.8b	1.6	2.1b	0.3b	-	-	0.5	2.0b	-	2.8b	-
$C_{16:1}{}^{\omega 7c}$	17.2c	22.1c	20.2c	27.5c	38.5c	25.8c	26.8c	28.5c	33.6	23.4c	24.0c	33.7	38.6	33.4	21.1c	36.3	21.9c	41.3

Chapter 6

Hartmannibacter diazotrophicus gen. nov., sp. nov., a novel phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt meadow plant

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Hartmannibacter diazotrophicus gen. nov., sp. nov., a phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt-meadow plant

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A phosphate-mobilizing, Gram-negative bacterium was isolated from rhizospheric soil of *Plantago winteri* from a natural salt meadow as part of an investigation of rhizospheric bacteria from salt-resistant plant species and evaluation of their plant-growth-promoting abilities. Cells were rods, motile, strictly aerobic, oxidase-positive and catalase-negative. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain E19^T was distinct from other taxa within the class *Alphaproteobacteria*. Strain E19^T showed less than 93.5 % 16S rRNA gene sequence similarity with members of the genera *Rhizobium* (⩽93.5 %), *Labrenzia* (⩽93.1 %), *Stappia* (⩽93.1 %), *Aureimonas* (⩽93.1 %) and *Mesorhizobium* (⩽93.0 %) and was most closely related to *Rhizobium rhizoryzae* (93.5 % 16S rRNA gene sequence similarity to the type strain). The sole respiratory quinone was Q-10, and the polar lipids comprised phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, an aminolipid and an unidentified phospholipid. Major fatty acids were $C_{18:1}\omega7c$ (71.4 %), summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; 8.3 %), $C_{20:0}$ (7.9 %) and $C_{16:0}$ (6.1 %). The DNA G+C content of strain E19^T was 59.9±0.7 mol%. The capacity for nitrogen fixation was confirmed by the presence of the *nifH* gene and the acetylene reduction assay. On the basis of the results of our polyphasic taxonomic study, the new isolate represents a novel genus and species, for which the name *Hartmannibacter diazotrophicus* gen. nov., sp. nov. is proposed. The type strain of *Hartmannibacter diazotrophicus* is E19^T (=LMG 27460^T=KACC 17263^T).

The orders *Rhodobacterales* and *Rhizobiales*, members of the class *Alphaproteobacteria*, are circumscribed solely on the basis of phylogenetic analysis of 16S rRNA gene sequences (Garrity *et al.*, 2005). According to the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity *et al.*, 2005), the order *Rhodobacterales* contains the family *Rhodobacteraceae*, and the order *Rhizobiales* comprises ten families, *Rhizobiaceae*, *Bartonellaceae*, *Brucellaceae*, *Phyllobacteriaceae*, *Methylocystaceae*, *Beijerinckiaceae*, *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae* and *Rhodobiaceae*. In recent years, three additional new families have been proposed, *Aurantimonadaceae*, *Xanthobacteraceae* and *Cohaesibacteraceae*, as well as three genera that have not been assigned to a family, *Amorphus*, *Bauldia* and *Vasilyevaea*, according to the *List of Prokaryotic Names with Standing in Nomenclature* (http://www.bacterio.net/index.html).

Salinization of agricultural soil by irrigation causes major problems for crop productivity in arid lands. An increase in the salt concentration in soils above 0.5–1 % is generally toxic to plants. Specialized plant species have adapted to higher salt concentrations by several mechanisms, including enhancement of tolerance to salt stress by the presence of adapted plant-growth-promoting rhizobacteria (PGPR). PGPR help plants to tolerate abiotic stress by increasing nutrient uptake from soils, reducing susceptibility to plant diseases and inducing systemic tolerance (Yang *et al.*, 2009). The use of PGPR is a promising agricultural practice to help less salt-tolerant horticultural crops to tolerate higher salt concentrations. In a meta-analysis of publicly available 16S rRNA gene sequences, it was found that, in saline soil habitats, less than 25 % of the bacterial diversity has been recovered using molecular techniques (Ma & Gong, 2013), and only a small part has been isolated. Strain E19^T was isolated as part of a study to look for PGPR from salt-resistant plant species from a natural salt meadow in

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PGPR, plant-growth-promoting rhizobacterium; PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial *nifH* gene sequence of strain E19^T are respectively KC567245 and KF640642.

Five supplementary figures and two supplementary tables are available with the online version of this paper.

order to find isolates able to increase the salt tolerance of horticultural crops.

Strain E19^T was isolated from a natural salt meadow in a nature protection area near Münzenberg, Hesse, Germany (50° 27′ 36″ N 8° 44′ 35″ E). Root systems of greater plantain (*Plantago winteri*) from the salt meadow were washed thoroughly by vigorous shaking in sodium pyrophosphate (100 mM). Subsequently, the suspensions were diluted with sterile saline using a standard dilution method and then incubated at 28 °C for 2–3 days on calcium phosphate (CP) agar containing 1 % sucrose, 0.01 % NaCl, 0.05 % $MgSO_4 . 7H_2O$, 0.02 % yeast extract, 0.05 % NH_4Cl, 0.01 % $MnSO_4 . H_2O$, 0.5 % $Ca_3(PO_4)_2$, 0.5 % (v/v) bromocresol green (0.5 % in 0.2 M KOH) and 1.5 % agar. Strain E19^T was isolated using dilution streaking and then stored as 20 % glycerol stocks at −20 °C. Unless otherwise stated, subcultures were grown in half-concentration marine agar (Roth) for handling and analysis.

Cell morphology, Gram-staining (Gerhardt *et al.*, 1994) and the presence of flagella (Heimbrook *et al.*, 1989) and poly-β-hydroxybutyrate (PHB) granules (Rohde, 2011) were evaluated by light microscopy using a Zeiss microscope with ×1000 magnification from 24 and 48 h-grown colonies. Growth of strain E19^T was tested at 4, 10, 16, 22, 28, 37, 45 and 50 °C and under anaerobic and microaerobic conditions (Anaerocult A and C; Merck). Growth in different salt concentrations was evaluated on marine medium agar (0.5 % peptone, 0.1 % yeast extract, 0.01 % ferric citrate, 0.59 % $MgCl_2 . 6H_2O$, 0.32 % $MgSO_4 . 7H_2O$, 0.18 % $CaCl_2 . 2H_2O$, 0.06 % KCl, 0.008 % KBr, 0.002 % H_3BO_3, 1.5 % agar) using 0–10 % (w/v) NaCl at intervals of 1 %. Catalase activity was determined by bubble formation in 3 % (v/v) H_2O_2 and oxidase activity using oxidase test strips (Merck). Other physiological and biochemical tests were performed using API 20NE and API ZYM strips (bioMérieux). Cells for inoculation of the API ZYM and API 20NE strips were suspended in a solution containing 0.3 % $MgCl_2$ and 3 % NaCl. Hydrolysis of CM-cellulose and starch (0.2 %) under saline conditions was respectively determined with Congo red dye and an iodine solution following the protocol of Kasana *et al.* (2008). Sensitivity to antibiotics of strain E19^T was tested with the disc diffusion method by spreading a cell suspension (0.5 McFarland; 28 °C for 48 h) on agar plates with discs (Oxoid) containing the following antibiotics: ampicillin (10 µg), chloramphenicol (10 µg), kanamycin (30 µg), penicillin G (10 µg), erythromycin (15 µg) and tetracycline (30 µg). The diameters of the inhibition zones were measured after 72 h at 28 °C to determine susceptibility, slight susceptibility or resistance.

Plant growth-promoting abilities of strain E19^T were tested in different PGPA media under optimal growth conditions. Growth was tested on nitrogen-free LG agar (Turner & Gibson, 1980) and on DF medium agar supplemented with 1-aminocyclopropane-1-carboxylate (ACC) as sole nitrogen source (Penrose & Glick, 2003). Detection of induction of indole-3-acetic-acid (IAA) biosynthesis and siderophore production was done according to Ribeiro & Cardoso (2012). Phosphate solubilization was tested on agar plates containing 1 % sucrose, 0.01 % NaCl, 0.05 % $MgSO_4 . 7H_2O$, 0.02 % yeast extract, 0.05 % NH_4Cl, 0.01 % $MnSO_4 . H_2O$, 0.5 % (v/v) bromocresol green (0.5 % in 0.2 M KOH) and 1.5 % agar supplemented with 0.5 % $Ca_3(PO_4)_2$, 0.5 % $AlPO_4$ and 0.2 % $FePO_4$. Phytate mobilization was tested on inositol hexaphosphate (IHP) agar (Unno *et al.*, 2005). The acetylene reduction assay was performed using cells grown in LG medium with 0.5 % NaCl and incubated for 48 h at 28 °C (Suarez *et al.*, 2014a). *Azotobacter vinelandii* DSM 2289^T was used as a control for all tests of acetylene-reducing activity. Phosphatase activity was tested using the methods of Tabatabai & Bremner (1969) after 15 days of growth of strain E19^T on SRSM1 medium (Vazquez *et al.*, 2000) with 0.5 % $Ca_3(PO_4)_2$ at 28 °C.

Genomic DNA from cells of strain E19^T was extracted following the protocol of Moré *et al.* (1994). The G+C content of genomic DNA was measured according to González & Saiz-Jimenez (2002). Presence of the *nifH* gene was evaluated according to Bürgmann *et al.* (2004). The almost-complete 16S rRNA gene sequence (1426 bp) was obtained by PCR according to Kampmann *et al.* (2012) and sequencing of both strands of the 16S rRNA gene and the *nifH* gene was done as described previously (Suarez *et al.*, 2014b). The most similar sequences to the new 16S rRNA gene sequence of strain E19^T were retrieved from the GenBank database using the BLAST algorithm (Altschul *et al.*, 1990) and the EzTaxon database of type strains of species with recognized prokaryote names (Kim *et al.*, 2012).

The 16S rRNA gene sequence was screened for chimeras using the toolset DECIPHER (version 1.6.0; Wright *et al.*, 2012), aligned online with the nearest relatives with the alignment tool SINA (version 1.2.9; Pruesse *et al.*, 2012) and merged with the pre-aligned 16S rRNA gene online database LTPs111 (February 2013 release) (Yarza *et al.*, 2008) using ARB version 5.2 (Ludwig *et al.*, 2004). Similarity was determined and phylogenetic analysis was performed using ARB version 5.2. Maximum-likelihood (ML), neighbour-joining (NJ) and maximum-parsimony (MP) trees were reconstructed using bootstrap analysis (1000 replicates) and a filter of termini of the 16S rRNA gene sequences between positions 123 and 1409 (*Escherichia coli* numbering; Brosius *et al.*, 1978). The *nifH* gene sequence (335 bp) was screened for chimeras using the UCHIME algorithm (Edgar *et al.*, 2011). Reported *nifH* nucleotide sequences were extracted from the functional gene pipeline and repository (FunGene) from the Ribosomal Database Project (Cole *et al.*, 2009). An NJ phylogenetic tree for the *nifH* gene nucleotide sequence corrected with Kimura's two-parameter model was reconstructed using MEGA version 5.0 (Tamura *et al.*, 2011).

For fatty acid methyl ester analysis, cell biomass of strain E19^T was grown aerobically on marine agar (Difco BD) at 28 °C for 48 h. Inoculation, incubation, cell harvesting,

extraction and analysis were carried out by the Identification Service of the BCCM/LMG, Belgium. Table MIDI TSBA 5.0 was used for peak naming. Analyses of respiratory quinones and polar lipids were carried out by the Identification Service of the DSMZ (Braunschweig, Germany) from lyophilized cell biomass grown in half-concentration marine broth at 28 °C for 48 h.

Strain E19^T was a Gram-negative, asporogenous, strictly aerobic, oxidase-positive, catalase-negative, non-pigmented, rod-shaped bacterium (1.9 × 0.5 μm), motile by a single lateral flagellum (Fig. S1, available in the online Supplementary Material), that did not produce intracellular PHB inclusions. Morphological, physiological and biochemical characteristics of strain E19^T are given in the genus and species descriptions. After 48 h at 28 °C, colonies were 1.0–2.0 mm in diameter, circular, colourless, non-mucoid and smooth when grown on half-concentration marine agar. Under the same incubation conditions on CP agar, colonies were 1.5–2 mm in diameter, dark blue–green, non-mucoid and smooth with a solubilization halo and, on LG agar, colonies were 1.0–2.0 mm in diameter, brilliant, transparent, non-mucoid and smooth. Strain E19^T grew in 1–3 % (w/v) NaCl and at 22–37 °C. No growth was observed at 4 % NaCl or at 16 or 45 °C. The isolate is clearly differentiated from all closely related genera by the absence of catalase activity (Table 1). Other characteristics of strain E19^T are presented in the species description.

Positive growth results of strain E19^T on different PGPA media showed its potential as a PGPR under plant stress conditions. Growth on ACC-supplemented medium indicated ethylene reduction and thus its ability to diminish plant stress reactions. Different phosphate sources were used including organic (IHP) and insoluble [$Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$] phosphate sources. The highest phosphatase activity was 0.043 μmol *p*-nitrophenol min^{-1}, measured after 7 days of growth. As a result of this ability, difficult phosphorus sources can become available to the plant. Moreover, strain E19^T could provide the plant with nitrogen, as shown in the acetylene reduction assays at a calculated rate of 0.18 μmol N_2 (mg protein)$^{-1}$ h^{-1}. Negative PGPA included no IAA and no siderophore production.

Growth on DF medium agar supplemented with ACC and IHP was positive, and no growth was observed on LB agar supplemented with 0.1 % g L-tryptophan. Growth on agar containing $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ as insoluble phosphate sources was positive in all cases. Production of siderophores was negative. Positive results of growth of strain E19^T on different PGPA media, acetylene reduction activity, phosphatase activity and salt tolerance made it a potential candidate to be tested as a PGPR under salt stress conditions (data not shown).

BLAST analysis of the 16S rRNA gene sequence (1426 bp) of strain E19^T revealed 99 % identity to 16S rRNA gene sequences from sediment bacteria isolates 23-01 (GenBank accession no. EU167992) and 23-02 (EU167993) (Hilyard *et al.*, 2008). Among strains from species with validly published names, the 16S rRNA gene sequence of strain E19^T was most similar to those of *Rhizobium rhizoryzae* J3-AN59^T (93.5 %), *Labrenzia suaedae* YC6927^T (93.1 %), *Aureimonas ureilytica* 5715S-12^T (93.1 %) and *Stappia stellulata* IAM 12621^T (93.1 %). Similarity of less than 93 % was obtained for other described bacteria belonging to the *Alphaproteobacteria* (Table S1).

As a general statement, values of 16S rRNA gene sequence similarity below the mean (96.4 ± 0.2 %) or minimum (94.9 ± 0.4 %) identity to the type species may lead to circumscription of a new genus (Yarza *et al.*, 2008). The NJ phylogenetic tree (Fig. 1) showed that strain E19^T formed a monophyletic group with a bootstrap value of 87 % with the sediment bacteria 23-01 and 23-02, and belonged to the monophyletic group of the *Alphaproteobacteria*. Furthermore, there were no closer affiliations to members of the 13 families of the order *Rhizobiales* or the family *Rhodobacteraceae*. Similar results were obtained with the ML and MP trees (Figs S2 and S3). Based on these results, strain E19^T could not be clearly affiliated to any classified genus with any of the algorithms. The *nifH* gene sequence of strain E19^T (335 bp) showed the highest similarity among reported *nifH* gene sequences in GenBank to that of *Methylobacterium* sp. 4-46 (GenBank accession no. CP000943.1) (91 %). Similar values were obtained for reported *nifH* gene sequences of the genus *Azospirillum*. Results from the reconstructed NJ phylogenetic tree showed that the *nifH* gene sequence of strain E19^T was located in an internal low-bootstrap-support subcluster together with species of genus *Methylobacterium* (Fig. S4). The DNA G+C content of strain E19^T was 59.9 ± 0.7 mol%.

Major fatty acids of strain E19^T (>5 %) were $C_{18:1}\omega7c$ (71.4 %), summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; 8.3 %), $C_{20:0}$ (7.9 %) and $C_{16:0}$ (6.1 %) (Table S2). The predominance of octadecenoic acids as major fatty acids in the fatty acid profile of strain E19^T revealed the affiliation of the isolate to the *Alphaproteobacteria*, whereas the low content of cyclic $C_{19:0}$ was in contrast to the predominance of this fatty acid in members of the order *Rhizobiales* (Moreno *et al.*, 1990; Tighe *et al.*, 2000; Wilkinson, 1988). The predominance of $C_{20:0}$ as a major fatty acid differentiated strain E19^T from most phylogenetically closest related taxa (Table 1), from the nitrogen-fixing genera (*Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mycoplana* and *Methylobacterium*; Urakami *et al.*, 1990; Tighe *et al.*, 2000; Schauer *et al.*, 2011) among the order *Rhizobiales* and from the genera *Roseibium*, *Pannonibacter* and *Nesiotobacter* in the order *Rhodobacterales* (Donachie *et al.*, 2006; Biebl *et al.*, 2007).

Ubiquinone 10 (Q-10) as the major quinone is a characteristic of members of the *Alphaproteobacteria* (Collins & Jones, 1981; Yokota *et al.*, 1992). Moreover, E19^T contained Q-10 as the sole major respiratory quinone, in contrast to members of the families *Aurantimonadaceae*, *Phyllobacteriaceae*, *Rhodobiaceae*, *Bradyrhizobiaceae*, *Methylocystaceae*

Table 1. Differential phenotypic characteristics that distinguish strain E19^T from related genera

Taxa: 1, *Hartmannibacter diazotrophicus* gen. nov., sp. nov. E19^T (data from this study); 2, *Rhizobium rhizoryzae* J3-AN59^T (data from Zhang *et al.*, 2014); 3, *Rhizobium* (Tighe *et al.*, 2000; Kaiya *et al.*, 2012; Xu *et al.*, 2013; Zhang *et al.*, 2014); 4, *Stappia* (Uchino *et al.*,1998; Biebl *et al.*, 2007; Lai *et al.*, 2010; Kämpfer *et al.*, 2013); 5, *Labrenzia* (Biebl *et al.*, 2007; Bibi *et al.*, 2014); 6, *Mesorhizobium* (Jarvis *et al.*, 1997; Choma & Komaniecka, 2002; Zhou *et al.*, 2010; Laranjo & Oliveira, 2011); 7, *Pleomorphomonas* (Xie & Yokota, 2005; Im *et al.*, 2006; Madhaiyan *et al.*, 2013b); 8, *Methylopila* (Doronina *et al.*, 1998; Li *et al.*, 2011); 9, *Phyllobacterium* (Lambert *et al.*, 1990; Mergaert *et al.*, 2002; Valverde *et al.*, 2005; Mantelin *et al.*, 2006; Flores-Félix *et al.*, 2013); 10, *Aureimonas* (Jurado *et al.*, 2006; Weon *et al.*, 2007; Rathsack *et al.*, 2011; Madhaiyan *et al.*, 2013a). ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Pleomorphic rods	Rods	Rods	Rods
Motility	+	ND	+	+	+	+	–	+	+	+
Flagella	Single lateral	ND	Polar, subpolar, peritrichous	Single polar	Monotrichous	Polar, subpolar, peritrichous	–	Subpolar single, lateral	Polar, subpolar or lateral	Polar
PHB granules	–	ND	+	ND	ND	+	+	+	ND	+
Root nodules produced	–	–	+	–	–	+	–	–	+	–
Major fatty acids*	16:0, 18:1ω7c 20:0	16:0, 18:1ω7c 18:1ω6c	16:0, 18:1ω7c SF3, 19:0 cyclo ω8c	16:0, 18:1ω7c 11-Me 18:1ω7c	18:0, 18:1ω7c 11-Me 18:1ω7c 20:1ω7c	16:0, 18:1ω7c 11-Me 18:1ω7c 19:0 cyclo ω8c	16:0, 18:1ω7c 19:0 cyclo ω8c	16:0, 18:1ω7c 18:0	16:0, 18:1ω7c 19:0 cyclo ω8c, 20:0	16:0, 18:1ω7c 18:1ω7c 18:1, 19:0 cyclo ω8c
Polar lipids†	PC, PG, PL, PE, AL	PC, PG, DPG, PE, PL	PC, PG, DPG, PE	PC, PG, DPG, PE, PMME, AL	PC, PG, DPG, PE, PMME, AL, PL, SQDG	PC, PG, DPG, PE, PMME, OL	PC, PG, DPG, PE, PMME, PDE, AL	PE, PC, PG, PME	ND	PE, PC, PG, PMME, DPG, AL, SQDG
DNA G+C content (mol%)	59.9	55.7	57–66	59–65.9	56–60	59–64	62.1–65.1	66–70.4	51–61	63.9–71.8
Quinone system	Q-10	ND	Q-10	Q-10	Q-10	Q-10	Q-10, Q-8	Q-10	Q-10, Q-9	Q-10, Q-9
NaCl concentration for growth (%, w/v)	1–3	1–4	1–5	1–10	1–10	1–5	1–2	1–2.5	1–3	0–3
Oxidase	+	–	+	+	+	+	+	+	+	+
Catalase	–	ND	+	+	+	+	+	+	+	+
Nitrogen fixation	+	–	+	–	–	+	+	+	–	–

*Me, Methyl; SF3, summed feature 3 (one or more of unknown ECL 10.928, $C_{14:0}$ 3-OH and iso-$C_{16:1}$).

†DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PDE, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PMME, phosphatidylmonomethylethanolamine; SQDG, sulphoquinovosyldiacylglycerol; AL, unidentified aminolipid; OL, ornithine-containing lipid; PL, unidentified phospholipid.

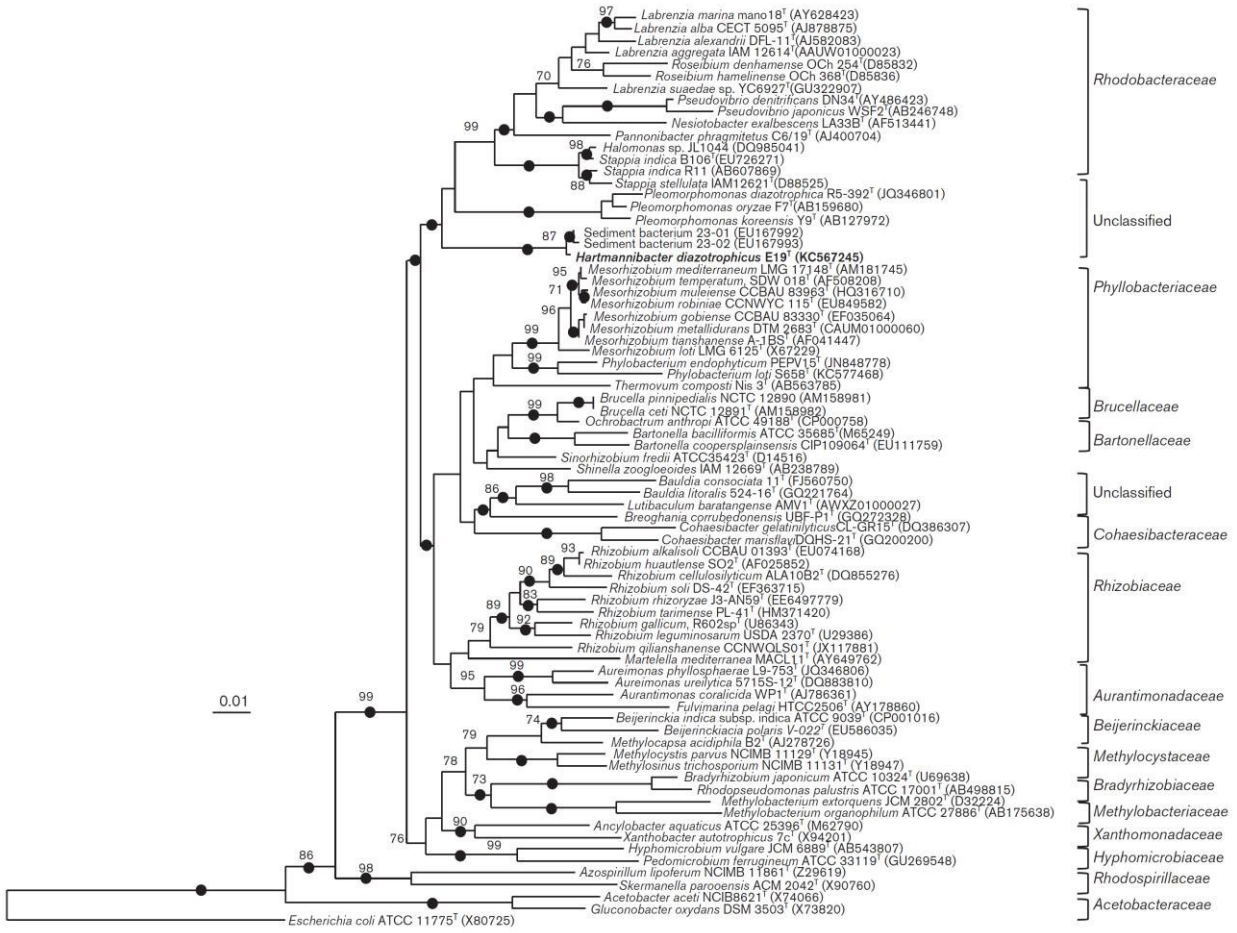

Fig. 1. NJ phylogenetic tree based on 16S rRNA gene sequences (1426 bp) of strain E19^T, members of related families of the orders *Rhizobiales* and *Rhodobacterales* and representatives of the class *Alphaproteobacteria*. Bootstrap percentages (based on 1000 replicates) are shown above nodes. Bootstrap values lower than 70 % are not shown. Filled circles indicate that the same branch topology was formed with the NJ, ML and MP methods. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

and *Hyphomicrobiaceae* (Hwang & Cho, 2008). Related genera from the family *Rhodobacteraceae* present Q-10 as the dominant respiratory lipoquinone (Cho & Giovannoni, 2004; Biebl *et al.*, 2007).

The polar lipids comprised an unidentified phospholipid, phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and an aminolipid (Fig. S5). The presence of an unidentified phospholipid as a major polar lipid and the absence of phosphatidylmonomethylethanolamine (PMME) and sulphoquinovosyldiacylglycerol (SQDG) distinguish strain E19^T from most phylogenetically closely related genera (Table 1). The presence of PG, PE and PC as membrane polar lipids was consistent with the polar lipid compositions of genera of different families of the order *Rhizobiales* (Kaneshiro & Marr, 1962; Thompson *et al.*, 1983; Miller *et al.*, 1990; Kämpfer *et al.*, 1999; Choma & Komaniecka, 2002; Hwang & Cho, 2008). The absence of PMME distinguished strain E19^T from genera belonging to the families *Brucellaceae*, *Aurantimonadaceae*, *Phyllobacteriaceae*, *Rhizobiaceae*, *Methylocystaceae* and *Cohaesibacteraceae* (Hwang & Cho, 2008). The absence of SQDG and PMME respectively distinguished strain E19^T from members of the genera *Roseibium* and *Labrenzia* and the genus *Stappia* (Biebl *et al.*, 2007).

All genetic, phylogenetic and phenotypic data analysed in this study indicated that strain E19^T could not be characterized as a member of any recognized genus within the *Alphaproteobacteria*. Therefore, the name *Hartmannibacter diazotrophicus* gen. nov., sp. nov. is proposed to accommodate strain E19^T.

Description of *Hartmannibacter* gen. nov.

Hartmannibacter [Hart.man.ni.bac′ter. N.L. masc. n. *bacter* (from Gr. n. *bakterion*) a rod-shaped bacterium; N.L. masc.

n. *Hartmannibacter* Hartmann's rod-shaped bacterium, referring to Anton Hartmann, a German microbiologist, in recognition of his many contributions to rhizosphere microbiology].

Cells are Gram-reaction-negative, motile, non-spore-forming and diazotrophic. PHB granules are not accumulated. Catalase is negative and oxidase is positive. The DNA G+C content of the type strain of the type species is 59.9±0.7 mol%. Predominant fatty acids are $C_{18:1}\omega7c$, summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$), $C_{20:0}$ and $C_{16:0}$. The major polar lipids are an unidentified phospholipid, PC and PG. The sole respiratory quinone is Q-10. Phylogenetically, the genus is a member of the class *Alphaproteobacteria*. The type species is *Hartmannibacter diazotrophicus*.

Description of *Hartmannibacter diazotrophicus* sp. nov.

Hartmannibacter diazotrophicus (di.a.zo.tro′phi.cus. Gr. pref. *di* two, double; Fr. n. *azote* nitrogen; Gr. adj. *trophikos* nursing, tending or feeding; N.L. masc. adj. *diazotrophicus* feeding on dinitrogen, diazotrophic).

Colonies on half-concentration marine agar are 1.0–2.0 mm in diameter, circular, colourless, non-mucoid and smooth after 48 h at 28 °C. Cells are short rods (1.0–2.7×0.5–0.7 μm), motile by the presence of one single lateral flagellum. Growth occurs at 22–37 °C (optimum, 28 °C). No growth occurs under microaerobic or anoxic conditions. Growth occurs at 1–3 % (w/v) NaCl. Positive in growth tests for casein and negative for starch and CM-cellulose. Positive results from the API 20NE system are nitrate reduction, urease activity, aesculin hydrolysis and assimilation of D-glucose, arabinose, mannose and mannitol. Negative results are obtained for gelatin hydrolysis, indole production, D-glucose fermentation, activities of arginine dihydrolase and β-galactosidase and assimilation of *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate. With the API ZYM system, positive results are obtained for C8 esterase lipase, C14 lipase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase activities. Negative results are obtained for alkaline phosphatase, C4 esterase, leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase activities. Susceptible to kanamycin (30 μg), chloramphenicol (30 μg), penicillin G (10 μg), tetracycline (30 μg) and ampicillin (10 μg) and intermediately susceptible to erythromycin (15 μg). Polar lipids, respiratory lipoquinone and fatty acids are consistent with those listed in the genus description. In addition, polar lipids in minor amounts are phosphatidylethanolamine and an aminolipid. Fatty acids present in minor amounts (<2 %) are $C_{16:0}$ 3-OH, $C_{19:0}$ cyclo $\omega8c$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:1}\omega11c$, $C_{10:0}$ 3-OH and $C_{8:0}$ 3-OH.

The type strain, E19^T (=LMG 27460^T=KACC 17263^T), was isolated from the rhizosphere of *Plantago winteri* in a salt meadow in a nature protection area near Münzenberg, Hesse, Germany.

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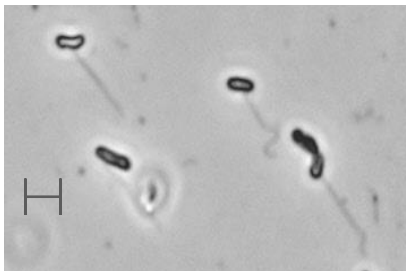
Supplementary material

"*Hartmannibacter diazotrophicus* gen. nov., sp. nov., a novel phosphate-solubilizing and nitrogen-fixing alphaproteobacterium isolated from the rhizosphere of a natural salt meadow plant" Suarez et al. (2014)

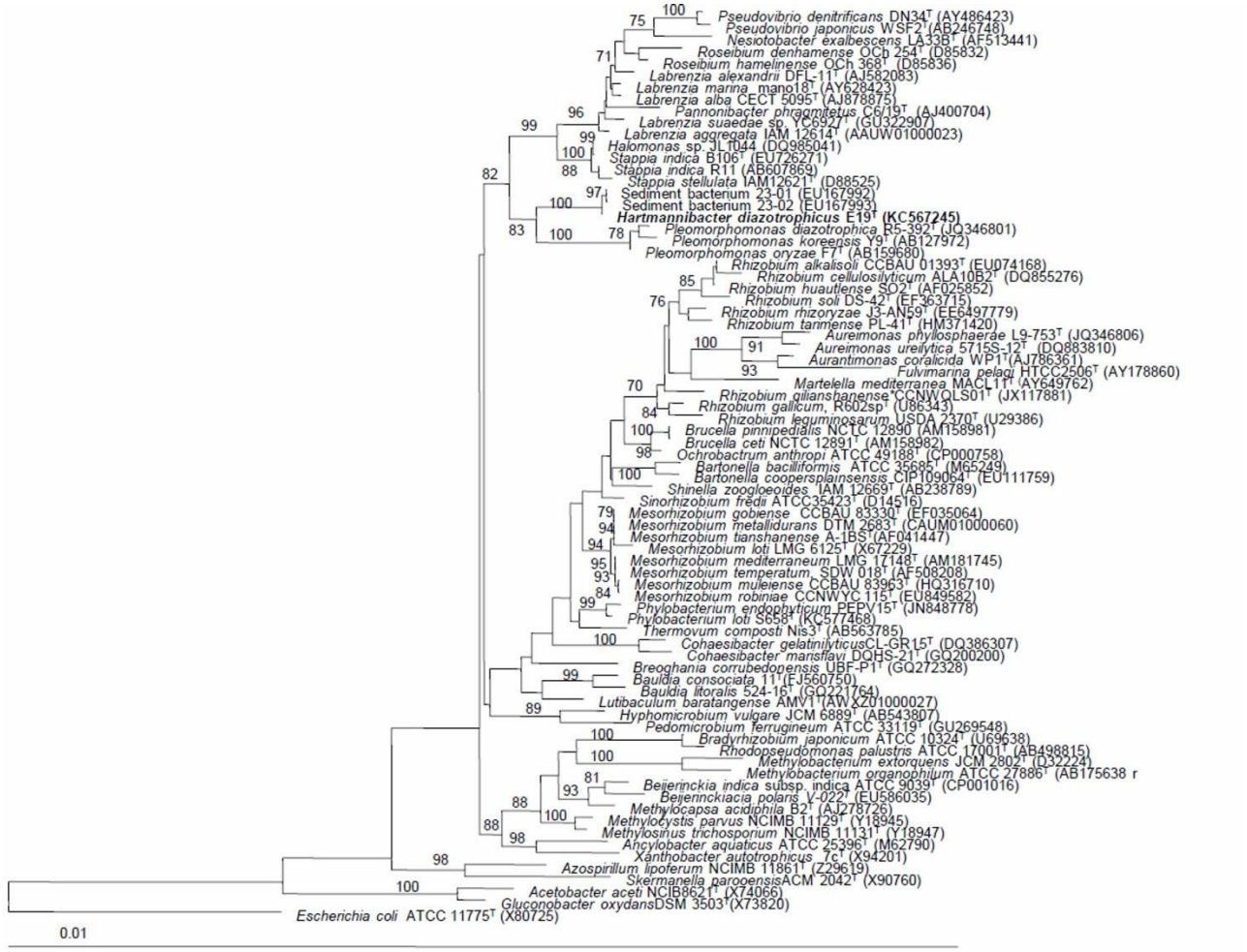


Supplementary Fig. S1. Light microscopy of flagella staining. Strain E19^T showing a single lateral flagellum. (Bar, 2 μm).

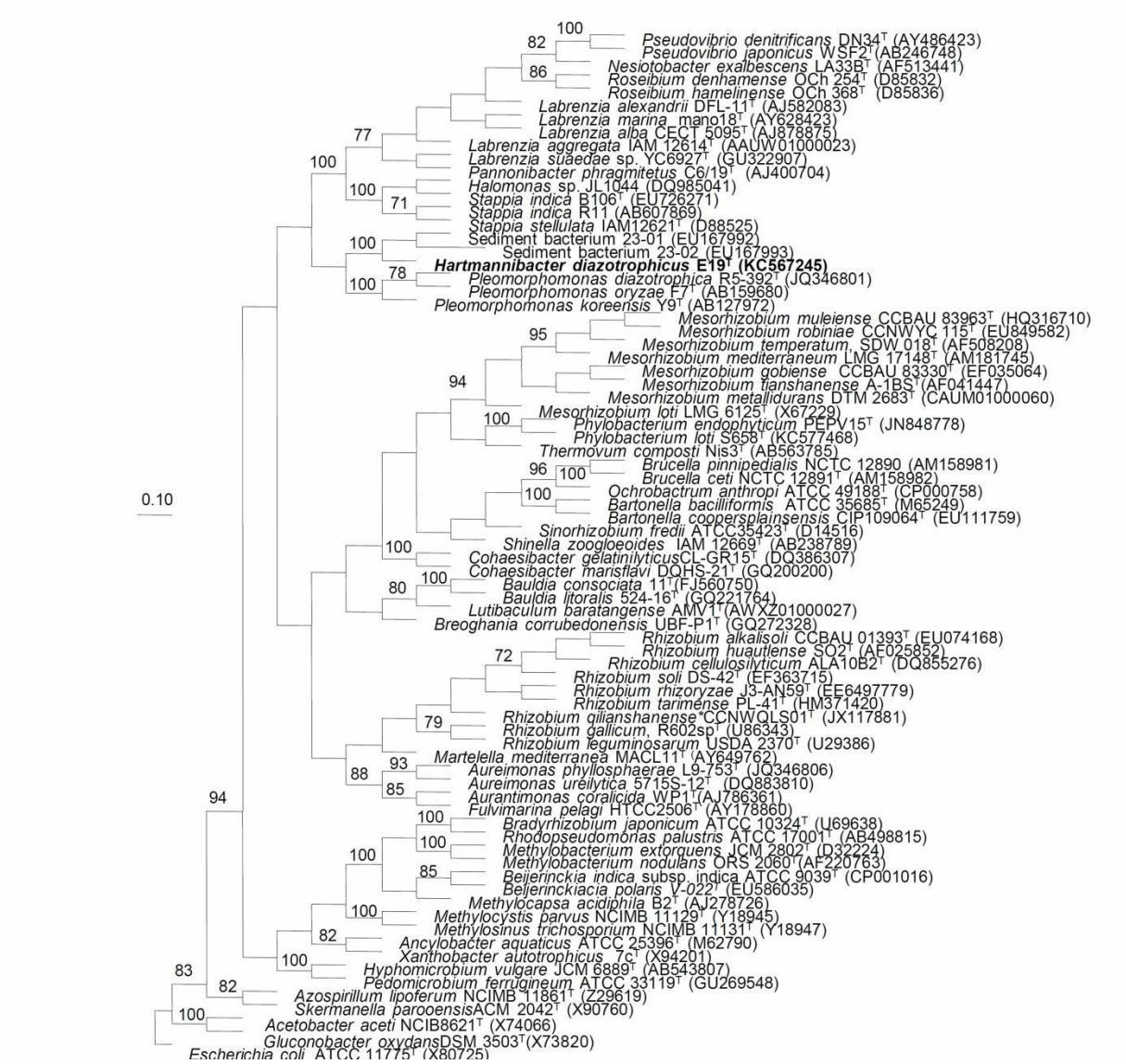
Supplementary Table S1.The 16S rRNA gene similarity values for the strain E19^T with closest related16S rRNA gene bacteria sequences retrieved from GenBank database and EzTaxon database of type strains of recognized prokaryotic names. Similarity analysis was performed using ARB version 6.

Name	Strain	Accesionnumber	Similarity %
Hartmannibacter diazotrophicus	E19^T	KC567245	100
Sediment bacterium	23-01	EU167992	99,8
Sediment bacterium	23-02	EU167993	99,7
Rhizobium qilianshanense	CCNWQLS01^T	JX117881	93,5
Rhizobium rhizoryzae	J3-AN59^T	EF649779	93,5
Labrenzia suaedae	YC6927^T	GU322907	93,1
Stappia stellulata	IAM12621^T	D88525	93,1
Stappia indica	R11	AB607869	93,1
Halomonas sp.	JL1044	DQ985041	93,1
Rhizobium sp.	ORS 1465	AY500261	93,1
Rhizobium tarimense	CCBAU 83306^T	EF035058	93,0
Aureimonas phyllosphaerae	L9-753^T	JQ346806	93,0
Mesorhizobium robiniae	CCNWYC 115^T	EU849582	93,0
Stappia indica	B106^T	EU726271	92,9
Mesorhizobium mediterraneum	LMG 17148^T	AM181745	92,9
Methylopila jiangsuensis	JZL-4^T	FJ502233	92,8
Labrenzia aggregata	IAM 12614^T	AAUW01000023	92,8
Pseudoxanthobacter soli	CC4^T	EF465533	92,8
Aureimonas ureilytica	5715S-12^T	DQ883810	92,8
Albibacter methylovorans	DSM 22840^T	FR733694	92,7
Rhizobium soli	DS-42^T	EF363715	92,7
Mesorhizobium temperatum	SDW 018^T	AF508208	92,7
Methylopila musalis	CCUG 61696^T	JQ173144	92,6
Rhizobium gallicum	R-602^T	U86343	92,6
Rhizobium cellulosilyticum	ALA10B2^T	DQ855276	92,6

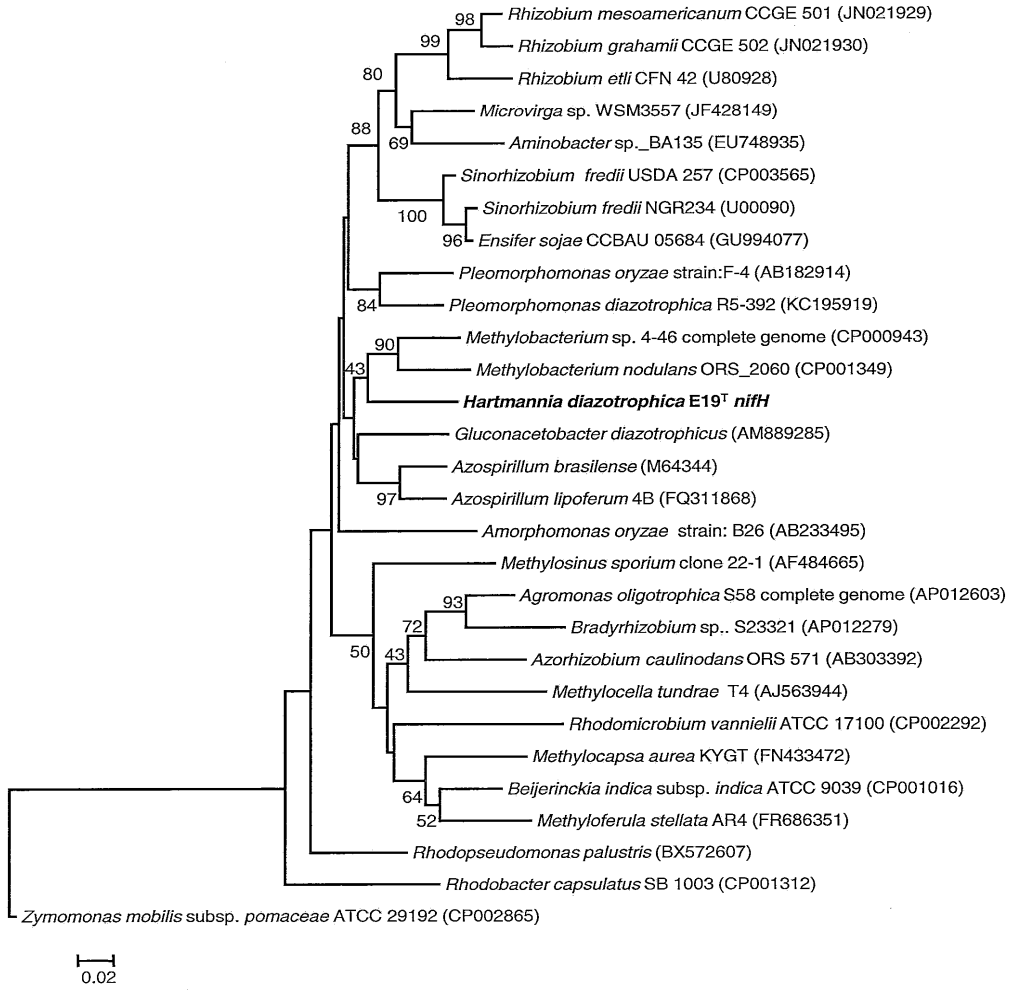
Rhizobium huautlense	S02 ^{T}	AF025852	92,6
Rhizobium alkalisoli	CCBAU 01393^{T}	EU074168	92,6
Aliihoeflea aestuarii	N8^{T}	EF660756	92,6
Mesorhizobium tianshanense	A-1BS ^{T}	AF041447	92,6
Mesorhizobium muleiense	CCBAU 83963 ^{T}	HQ316710	92,6
Shinella zoogloeoides	IAM 12669 ^{T}	AB238789	95,5
Mesorhizobium metallidurans	CFBP 7147 ^{T}	AM930381	92,5
Hansschlegelia zhihuaiae	S 113 ^{T}	DQ916067	92,4
Kaistia soli	5YN9-8 ^{T}	EF592609	92,4
Labrenzia alba	5OM6 ^{T}	AJ878875	92,4
Rhizobium mongolense	USDA 1844^{T}	U89817	92,4
Phyllobacterium trifolii	PETP02^{T}	AY786080	92,4
Mesorhizobium tamadayense	Ala-3 ^{T}	AM491621	92,4
Labrenzia alexandrii	DFL-11^{T}	AJ582083	92,3
Pleomorphomonas oryzae	F7 ^{T}	AB159680	92,3
Pleomorphomonas koreensis	Y9 ^{T}	AB127972	92,3
Mesorhizobium caraganae	CCBAU 11299^{T}	EF149003	92,3
Hoeflea anabaenae	WH2K ^{T}	DQ364238	92,2
Mesorhizobium gobiense	CCBAU 83330 ^{T}	EF035064	92,2
Mesorhizobium opportunistum	WSM2075 ^{T}	AY601515	92,2
Mesorhizobium huakuii	IFO 15243 ^{T}	D13431	92,2
Mesorhizobium amorphae	ACCC 19665^{T}	AF041442	92,2
Thermovum composti	Nis3 ^{T}	AB563785	92,1
Labrenzia marina	mano18^{T}	AY628423	92,1
Sinorhizobium fredii	ATCC 35423 ^{T}	D14516	92,1
Phylobacterium endophyticum	PEPV15 ^{T}	JN848778	92,1
Thermovum composti	Nis3 ^{T}	AB563785	92,1
Mesorhizobium tarimense	CCBAU 83306 ^{T}	EF035058	92,1
Hansschlegelia plantiphila	S113 ^{T}	DQ404188	92,1
Mesorhizobium septentrionale	SDW 014 ^{T}	AF508207	92,1
Mesorhizobium plurifarium	LMG 11892 ^{T}	Y14158	92,1
Rhizobium leguminosarum	USDA 2370 ^{T}	U29386	92
Martelella mediterranea,	MACL11 ^{T}	AY649762	92
Breoghania corrubedonensis	UBF-P1 ^{T}	GQ272328	92
Pannonibacter phragmitetus	C6/19 ^{T}	AJ400704	91,9
Phyllobacterium brassicacearum	STM 196 ^{T}	AY785319	91,9
Mesorhizobium chacoense	PR5 ^{T}	AJ278249	91,9
Daeguiacaeni	K107 ^{T}	EF532794	91,9
Phyllobacterium ifriqiyense	STM 370 ^{T}	AY785325	91,8
Phyllobacterium bourgognense	STM 201 ^{T}	AY785320	91,8
Nitratireductor lucknowense	IITR-21 ^{T}	HQ658355	91,8
Phyllobacterium myrsinacearum	STM 948 ^{T}	AY785315	91,7
Phyllobacterium leguminum	ORS 1419 ^{T}	AJ968695	91,7
Hoeflea phototrophica	DFL-43 ^{T}	ABIA02000018	91,7
Hoeflea suaedae	YC6898^{T}	HM800935	91,7
Mesorhizobium ciceri	UPM-Ca7 ^{T}	U07934	91,6
Mesorhizobium australicum	WSM2073 ^{T}	AY601516	91,6
Phyllobacterium catacumbae	CSC19 ^{T}	AY636000	91,6
Hoeflea alexandrii	AM1V30 ^{T}	AJ786600	91,6
Brucella ceti	NCTC 12891 ^{T}	AM158982	91,6
Brucella pinnipedialis	NCTC 12890 ^{T}	AM158981	91,6
Mesorhizobium loti	LMG 6125 ^{T}	X67229	91,5
Mesorhizobium shangrilense	CCBAU 65327 ^{T}	EU074203	91,5
Lutibaculum baratangense	AMV1 ^{T}	AWXZ01000027	91,5



Supplementary Fig. S2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences for strain E19^T (1426 bp), members of related families of the order *Rhizobiales* and *Rhodobacterales*, and representatives of class *Alphaproteobacteria*. Bootstrap percentages higher than 70 (based on 1000 replicates) are showed at nodes. *Escherichia coli* ATCC 11775^T was used as out-group.Bar, 0.01 substitutions per nucleotide position.



Supplementary Fig. S3. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences for strain E19^T (1426 bp), members of related families of the order *Rhizobiales* and *Rhodobacterales*, and representatives of class *Alphaproteobacteria*. Bootstrap percentages higher than 70 (based on 1000 replicates) are showed at nodes. *Escherichia coli* ATCC 11775^T was used as out-group.Bar, 0.10 substitutions per nucleotide position.

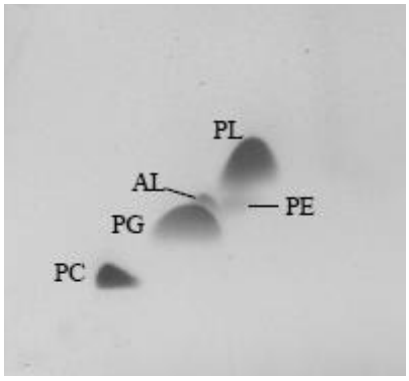


Supplementary Fig. S4. Neighbor-joining tree based on *nifH* gene sequences showing relationships between strain E19^T and *nifH* gene sequence containing species based on 324 bp sequences length. *Zymomonas mobilis* subsp. *pomaceae* ATCC 29192 was set as out-group. Bootstrap percentages higher than 40 (based on 1000 replicates) are showed at nodes. Bar, 0.02 substitutions per nucleotide position.

Supplementary Table S2. Fatty acid composition of strain E19^T and *Stappia stellulata* LMG 23382^T. Cells were grown on marine agar at 28 °C for 48 h. Values are percentages of total fatty acids

Fatty acid	E19^T	*St. stellulata* LMG 23382^T
$C_{8:0}$ 3-OH	0.2	-
$C_{10:0}$ 3-OH	0.4	-
$C_{14:0}$	-	0.2
Sum in feature 2	8.3	3.2
$C_{16:1}$ ω11c	0.8	0.4
Sum in feature 3	1.1	1.1
$C_{16:0}$	6.1	7.2
$C_{16:0}$ 3-OH	1.8	0.1
$C_{18:1}$ ω9c	-	0.6
$C_{18:0}$	-	2.0
$C_{18:1}$ ω7c	71.4	62.1
$C_{18:1}\omega$7c11 methyl	-	16.9
Sum in feature 7	-	1.9
$C_{19:0}$ 10 methyl	-	0.2
$C_{19:0}$ cycloω *8c*	1.5	2.1
$C_{18:0}$ 3-OH	-	1.3
$C_{20:1}\omega 7^c$	-	0.2
$C_{20:0}$	7.9	0.3

Summed features are groups of two or three fatty acids that it is not possible to be separated by GLC with the MIDI System: Summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I); summed feature 3 ($C_{16:1}\omega 7$cand/or iso-$C_{15:0}$ 2-OH); summed feature 7 (unknown 18.846 and/or $C_{19:1}\omega$6c)



Supplementary Fig. S5. Polar lipids profile ***Hartmannibacter diazotrophicus*** E19^T. **Unidentified** phospholipid (PL), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and aminolipid (AL).

Chapter 7

Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare*, L.) under salt stress

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Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare*, L.) under salt stress

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Abstract

Soil salinization is a major concern of modern agriculture, specifically regarding irrigation and arid regions. However, plant growth promoting rhizobacteria (PGPR) can increase plant resistance to abiotic stress and represent an environmental friendly approach to alleviate salt stress in crops. The plant growth promoting (PGP) activities of *Hartmannibacter diazotrophicus* E19^T, a new genus recently isolated from the rhizosphere of *Plantago winteri* from a natural salt meadow, were assessed in pure culture experiments and in *vivo* assays. ACC-deaminase activity for strain E19^T at 1, 2 and 3 % NaCl were 0.56 ± 0.20, 1.29 ± 0.82 and 2.60 ± 1.2 µmol α-ketobutyrate mg protein^{-1} h^{-1} respectively, and production of IAA was not detected. *H. diazotrophicus* E19^T inoculated summer barley seedlings exposed for 2 h to 200 mM and 400 mM NaCl stress showed reduced ethylene emission in comparison to uninoculated plantlets exposed to same conditions. Inoculation of barley plants (*Hordeum vulgare* L.) with strain E19^T in non-sterile soil under salt stress conditions significantly increased root (308 %) and shoot (189 %) dry weight. The relative increase of water content in the root system was 378 % than the control treatment, and the root-to-shoot ratio more than double compared to control. *H. diazotrophicus* inoculation showed no effect on both Na^+ and K^+ concentration in leaf blades or sheaths, but decreased root surface sodium uptake. The capability of strain E19^T to colonize barley roots under salt stress conditions was revealed with a specific designed fluorescence in situ hybridization (FISH) probe. *H. diazotrophicus* strain E19^T positively promotes barley growth under salt stress conditions, and indicates that the mode of action is based on ACC-deaminase production.

Keywords: *Hartmannibacter diazotrophicus*, PGPR activity, FISH, 1-Aminocyclopropane-1-Carboxylate (ACC)-deaminase, salt stress

1. Introduction

Salinity affects about 20 % of all irrigated agricultural fields and over 7 % of the world land surface (Szabolcs, 1994), by reducing the ability of crops to take up water and by ion toxicity. The physiological consequences of this is the loss of the crops by inhibition of seed germination, seedling growth, flowering and fruit set (Sairam and Tyagi, 2004). Economical global annual costs by loss in crop production caused by salt-induced land degradation were estimated to be US$ 27.3 billion (Qadir et al., 2014). Moreover future climate change-predicted scenarios show the increasing risk of salinization at different latitudes, and therefore a special effort will be required for maintaining crop production under salt stress (Turral et al., 2011).

It is widely reported that the application of plant growth promoting rhizobacteria (PGPR) influences plant growth by different mechanisms such as fixation of atmospheric nitrogen, solubilization and mobilization of phosphorus, sequestration of iron by siderophores, production of phytohormones, 1-aminocyclo-propane-1-carboxylate (ACC) deaminase, antibiotics, hydrogen cyanide, organic molecules such as vitamins, amino acids and volatile compounds and synthesis of hydrolytic enzymes (Babalola, 2010). Moreover, PGPR can also help plants to tolerate abiotic stresses such as salinity, drought, waterlogging and heavy metals e.g. by inducing systemic tolerance (Yang et al., 2009). Bacterial mediated plant tolerance to salt stress has been reviewed and includes diverse functional and taxonomical groups of bacteria (Dimkpa et al., 2009).

One of the mechanisms of PGPR to alleviate salt stress is the synthesis of ACC deaminase in the rhizosphere, which lowers the level of ethylene accumulation in stressed plants (Glick et al., 1998; Mayak et al., 2004). Ethylene is a modulator of growth and development in plants and is involved in the response of plants to stress. A sustained high level of ethylene, due to stress, would inhibit root elongation leading to an abnormal root growth, which would affect plant growth and development (Babalola, 2010). Bacteria producing ACC deaminase are able to promote root elongation and plant growth by lowering ethylene levels in the roots of developing plants (Dey et al., 2004). Diversity of rhizobacteria-mediated plant tolerance to salinity stress involving ACC deaminase activity in different plant species have been reviewed by Nadeem et al. (2010) and Paul and Lade (2014). ACC deaminase production has been reported in strains belonging to

Proteobacteria, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Glick, 2014; Nadeem et al., 2010). Nevertheless, no particular bacterial genus and species have the same genetic makeup and metabolic capabilities to consider a specific bacterial group as PGPR (Glick, 2014).

The use of PGPR is a promising agricultural practice to help salt affected crops to maintain an acceptable level of productivity under higher salt concentrations (Nadeem et al., 2012; Singh et al., 2011). Amelioration of adverse effect of salt stress by different rhizobacteria on several crops is reviewed by Paul and Lade (2014). Barley (*Hordeum vulgare* L.) is one of the world´s most extensively cultivated crops and the most salt tolerant cereal, reported to become seriously damaged only after extended periods at salt concentrations higher than 250mM NaCl (equivalent to 50 % seawater) (Munns et al., 2006). To our knowledge not many studies have been done in plant growth promotion by PGPR on barley (*Hordeum vulgare* L.) (Omar et al., 2009; Chang et al., 2014; Hmaeid et al., 2014; Nabti et al., 2014) probably due to its natural salt tolerance compared to other agricultural crops such as corn, soybean, wheat, tomato and rice. Therefore, increasing research on PGPR strains to ameliorate salt plant stress on barley will help to improve its natural tolerance on fields and also its potential use in remediation of salt-impacted soils (Chang et al., 2014).

Salt impacted environments are sources of potential PGPR able to ameliorate salt stress in agriculture. Meta-analysis of publicly available 16S rRNA gene sequences suggested that in saline soil habitats less than 25 % of bacterial diversity has been recovered using molecular techniques (Ma and Gong, 2013) and only a small part has been isolated. Recent studies on PGPR from salt environments reported the presence of a possible new species *´Haererehalobacter´*, (Jha et al., 2012) and new species, with plant-growth promotion potential (Suarez et al., 2014a, 2014b, 2014c; Gontia et al., 2011)

We hypothesized that a bacterium naturally occurring in a naturally salinized soil and coevolved over long time with salt resistant plants could be a suitable alleviator of salt stress on crops such as barley. Therefore, we tested the *in vitro* and *in vivo* PGP activities of a new genus recently isolated in our laboratory from a natural salt meadow (Suarez et al., 2014a). We investigated the effect of *H. diazotrophicus* on growth of summer barley (*H. vulgare* L.) in a greenhouse experiment with non-sterile soil and under high salinity.

2. Material and methods

2.1 Bacterial strain and cultivar

H. diazotrophicus E19^T (Suarez et al., 2014a) was grown routinely in half concentrated marine broth (Carl Roth GmbH, Germany) at 28 °C. Liquid cultures were incubated 48 h in an orbital shaker, at 28 °C and 150 rpm. The strain does not grow on NaCl < 1%. Optimal growth occurs at NaCl concentration of 1-3%. The strain is available by the LMG and KACC culture collection under the code LMG 27460^T and KACC 17263^T. For all plant assays, the cultivar Propino of summer barley (*Hordeum vulgare* L.) was used.

2.2 Plant growth promotion activities *in vitro*

ACC-deaminase activity was determined by the amount of α-ketobutyric acid generated from the cleavage of ACC following the protocol proposed by Penrose and Glick (2003) with following modifications: Strain E19^T was growth in 20 ml half marine broth (Roth) for 48 h at 28 °C and in order to evaluate enzyme activity under different salt concentration DF minimal salt medium supplemented with ACC (Penrose and Glick, 2003) was adjusted at 1, 2 and 3 % NaCl final concentration. Reaction was determined at 540 nm absorbance comparing the values of the samples to a standard curve of α-ketobutyrate ranging between 0.1 and 1 µM. Protein concentration was determinated by Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) as standard protein for ACC-deaminase activity. Indole acetic acid (IAA) production was previously reported as negative for this strain by growth on Luria Bertani (LB) agar supplemented with L-tryptophan and it was qualitatively confirmed in this study by inoculating 200 µl of liquid E19^T culture grown for 48 h at 28 °C in 10 ml (LB) broth supplemented with L-tryptophan (100 µg ml^{-1}) and adjusted at 1, 2 and 3 % NaCl final concentration. Grown culture (1.5 ml) was centrifuged at 16200 g for 5 min and 50 µl supernatant were added to 100 µl Salkowsky reagent (Gordon and Weber, 1951). After 30 min incubation at room temperature IAA concentration was measured at 530 nm absorbance comparing the values of the samples to a standard curve of IAA (5, 15, 25, 35 and 45 µg ml^{-1}). A *Pseudomonas* species (isolate E8, isolated in our laboratory and producing a high amount of ACC deaminase and IAA; unpublished) was used as positive control.

2.3 ACC deaminase activity *in vivo*

Barley plants ethylene emission was measured following the protocol of Siddikee et al. (2011) with modification. Strain E19^T cells were grown in half marine broth, centrifuged at 3345 g for 10 minutes, re-suspended in ACC supplemented DF minimal salt medium (1, 2 and 3 % NaCl final concentration) and incubated for 48 h at 28°C in agitation (120 rpm). Cells were harvested, washed and re-suspended in sterile 30 mM $MgSO_4$ solution to reach a concentration of 10^7– 10^8 CFU ml^{-1}. Sterilized barley seeds (*H. vulgare* L, cv. Propino) were incubated for 1 hour with the bacterial suspension, seeded into rectangular plastic pots (45.5 cm x 27.5 cm x 5 cm depth, 40 seeds per pot) filled with ~4500 ml (~1620 g dry weight) non-sterile classic clay substrate ED 73 (Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co.KG, Sinntal – Altengronau, Germany). Three replicate pots per treatment were prepared. Physico-chemical properties of the substrate are: pH ($CaCl_2$) 5.8, KCl 2.5 g l^{-1}, EC 0.3–0.9 dS m^{-1}, nitrogen ($CaCl_2$) 250 mg l^{-1}, phosphate 300 mg l^{-1}, potassium 400 mg l^{-1}, sulphur 200 mg l^{-1}, magnesium 700 mg l^{-1}. The maximum water holding capacity (WHC) was estimated to be 2000 ml, and each pot was irrigated with 1500 ml rain water (~75% m. WHC). Growth conditions were daylight from 06:00 to 22:00 (artificial light switched on when natural light was less than 10 Klx), and temperature of 20 °C and 16 °C (day and night, respectively).

Eight days after seeding, the soils were irrigated with 0, 200 and 400 mM NaCl solved in deionized water, to impose different salt stress levels. Not inoculated seeds, with and without salt stress, were used as control treatments. From each pot, 30 seedlings were uprooted, washed in order to remove soil from roots using respective NaCl solution and placed in 120 ml flasks (Schott, Mainz, Germany). After 30 minutes, the flasks were sealed using butyl rubber stoppers. One milliliter air samples from head-space were sampled after 4 h and 24 h of incubation at room temperature. Ethylene concentrations were measured using a Perkin Elmer Auto system XL chromatograph equipped with a thermal conductivity detector adjusted at 150°C and a Hayesep R column (2 m length, 2 mm diameter) at a helium (quality 5.0) flow of 28 ml min^{-1} and 50 °C oven temperature. Three measures were done for each treatment, and the average was used. Ethylene emission results are expressed as nmol of ethylene g^{-1} fresh weight h^{-1} by comparing the values of the samples to a standard curve generated with ethen 2.5 (Westfalen AG, Germany).

2.4 Plant-growth promotion *ad planta* under salt stress

Strain E19^T was tested for its efficiency in alleviating salt stress on barley (*Hordeum vulgare* L, cv. Propino) *in vivo*, under greenhouse conditions. Barley seeds were surface sterilized using a solution with final 3 % bleach concentration (Hurek et al., 1994) and washed seven times under gentle agitation for 10 min each with sterile water. The bacterial inoculum of strain E19^T used for all plant experiments was prepared as follows: strain E19^T in exponential growth phase was centrifuged at 3345 g in 50 ml screw-cap tubes, and resuspended in 30 mM $MgSO_4$ solution to reach a concentration of 1.5 10^9 CFU ml^{-1}.

Squared plastic pots (13 cm x 13 cm) were filled with ~750 ml (~270 g dry weight) non-sterile classic clay substrate ED 73. The maximum water holding capacity (WHC) was estimated to be 233 ml, and each pot was irrigated with 180 ml rain water (~77% WC). This amount of water allowed the whole substrate in the pot to be moistened, yet avoiding extensive percolation. Five pots per bacterial treatment and uninoculated control were prepared and arranged in a greenhouse with a randomized complete block design (RCBD; Clewer and Scarisbrick, 2001) to account for possible gradient effects in the greenhouse.

Fifteen disinfected barley seeds incubated for 1 hour with the suspension of strain E19^T were placed on each pot and covered with a one cm-layer of soil substrate. Germination was monitored until eight days after sowing and then each pot was rarified to 5 plants. Barley seeds incubated with sterile $MgSO_4$ solution served as negative control (treatment "S+B-"). Additional barley seeds inoculated with an autoclaved aliquot of the strain E19^T (treatment "S+D") was used to evaluated the influence of dead bacterial biomass on barley growth under salt stress.

To assess the effect of salt stress on the germination, the pots were irrigated three times before seeding with 150 mM NaCl solution in rain water. NaCl concentration at the seeding stage was 1.75 %, and it was increased by further irrigation with 150 mM NaCl solution in rain water, until a final NaCl concentration of 4.4 % reached 15 days after germination. At this NaCl concentration, the electrical conductivity of one part dry soil + 5 parts water (EC 1:5) was 22.40 ± 0.7 dS m^{-1}. Uninoculated seeds without salt stress (treatment "S- B-") received the same amount of rain water without NaCl.

After 42 days of growth under the same conditions described above for ethylene emission experiment, sheaths, leaf blades and roots of the 5 plants of each pot were separated and collected in three paper bags, and their fresh weights were recorded. Dry biomass accumulation and water content were assessed after 3 days drying at 80 °C. As growth parameter to evaluate the effect of the bacterial inoculation on the plant fitness, the percentage on relative increase (RI; Crane-Droesch et al., 2013) was used on both accumulated biomass (the dry weight) and water content (difference between fresh weight and dry weight).

2.5 Cation analysis in plant samples

The concentration of Na, Ca, K and Mg (mg g^{-1}) were determined in dry and ground samples from leaf blades, sheaths and roots after dry-ashing (550 °C) and measured by means of flame photometry (Varian FS 220) (Steffens and Schubert, 2011), under a full-factorial design 2X3: two inoculation treatments (inoculated/not inoculated) and three plant habitats (leaf blades/sheats/roots). Cation uptake at the root surface was calculated as ratio of total plant cation content divided to the root dry weight (Schubert et al., 2009). Root-to-shoot translocation was calculated as the total cation content of the shoots (leaf blades+sheaths) divided to the total cation content of the roots. This value was normalized to the biomass partition (shoots DW/roots DW) in order to eliminate the bias due to the different effects of the bacterium on the growth of shoots and roots (Saqib et al., 2005).

2.6 Probe design for *H. diazotrophicus* for fluorescence *in situ* hybridization (FISH)

Specific probe E19.2-FISH (5' AT TAG CTG ACC CTC GCA GGT 3') labeled with fluorescein isothiocyanate (FITC) at the 5' end was designed based on the 16S rRNA gene sequence of *H. diazotrophicus* (KC567245) aligned together with the next relatives with the alignment tool SINA (v1.2.9) (Pruesse et al., 2012) and merged with the pre-aligned 16S rRNA gene online database (LTPs111, Feb 2013) (Yarza et al., 2008) using ARB version 5.2 (Ludwig et al., 2004). Specificity of the probe was checked using Probe Check (Loy et al., 2008) and Probe Match tools, provided by the Ribosomal Database Project (RDP) (Cole et al., 2009). Annealing site is located in an accessible position according to Behrens et al., (2003). In order to ensure the specificity of the probe, an unlabeled competitor probe was designed with one mismatch (E19.2 comp 5' AT TAG CTY ACC CTC GCA GGT 3').

Specificity of the E19.2-FISH probe was tested against *Marinobacterium jannaschii* LMG 6239^T (AB006765), a bacterium with one mismatch in the E19.2-FISH target sequence, by using the probe competitor. For cells fixation, 1 ml of bacterial pure liquid cultures were centrifuged (16200 g, at 4 °C for 5 min), resuspended in 1X phosphate buffer saline (PBS), mixed with 4 % paraformaldehyde (PFA) solution (3 vol. PFA + 1 vol. sample) and incubated for 4 h at 4 °C. Fixed cells were washed twice by centrifugation (16200 g, at 4 °C, 5 min) and the final pellets were resuspended with ice cold 1x PBS. Immediately, 1vol of ice-cold 96 % ethanol was added, and the fixed cells were stored at -20 °C until FISH staining.

Fixed cells samples (15 µl of each) were placed and dried at 41 °C for 15 min on a poly-L-lysine coated microscopy slide. Cells were permeabilized by incubation in lysozyme (1 mg ml^{-1}) for 10 minutes at room temperature. Three minutes-stepwise incubation in ethanol (50 %, 80 % and 100 %) was used to dehydrate the samples. Probe binding profiles were tested using hybridization buffer (0.9 M NaCl, 0.01 % SDS, 20 mM Tris-HCl, pH 7.6) containing 0 to 50 % formamide (10 % stepwise increase), in order to determine the optimal stringency condition of the E19.2-FISH probe to strain E19^T, as previously described (Daims et al., 2005). Probe EUB338 (Amann et al., 1990) labeled with Rhodamine was used as counterstaining and positive control for FISH. All oligonucleotide probes were purchased from biomers (Ulm, Germany).

2.7 Bacterial detection of *H. diazotrophicus* by FISH in plant roots

Surface sterilized barley (cultivar Propino) seeds were germinated on sterile filter paper supplemented with 3 ml of sterile water and incubated for 72 h at 25 °C in darkness. Plantlets were placed into 50 ml tubes containing 25 ml of strain E19^T suspension, and incubated for 1 h at 28 °C in darkness under slow agitation. Inoculated plantlets were transferred to sterile rubber plugs (1.7 cm, 2 cm length), with a central hole of approximately 0.5 cm, placing the root system at the lower part of rubber plug to protect it from light influence, and immediately sealed with sterile agar 1.5 % (50 °C). Four rubber plugs with the fixed plantlets were transfer to round grey plastic racks (4.5 cm diameter x 3 cm depth) and placed in sterile transparent plastic boxes (9.5 x 8.0 x 12 cm) filled with 175 ml of half concentrated plant nutrient solution (Hatzig et al., 2009) adjusted at final 1 %

NaCl concentration (Fig. 2 supplement) in order to both generate the salt stress to the plantlets and maintain the optimal NaCl concentration for the growth of strain E19^T. The boxes were placed in greenhouse for 8 days under the same conditions described above. Non-inoculated control plantlets were also prepared and used as negative controls. Samples of the root system were washed with PBS 1X, fixed by using 4 % paraformaldehyde/PBS (3:1 vol/vol), incubated at 4 °C for 4 h, washed twice with PBS and then stored in PBS/ ethanol (1:1) at -20 °C until FISH staining.

Root fixed samples were cut in fragments of 4 to 5 mm, washed twice with PBS and in tube-FISH was performed as described by Cardinale et al., (2008). FISH probes used for the hybridization step were E19.2-FISH for *H. diazotrophicus* E19^T labeled with FITC, unlabeled competitor (E19.2 comp), and EUB338 (Amann et al., 1990) EUB338II and EUB338III (Daims et al., 1999) labeled with Rhodamine for eubacteria detection. Hybridization was performed at 20 % formamide concentration and 41 °C, for 2 h followed by a washing step at 44 °C for 15 min and then a rinse with ice-cold water. Negative FISH control was obtained by hybridizing the samples with the non-sense FISH-probe NONEUB labeled with the same dyes used for the positive probes, in order to detect possible non specific staining of both the probes and the fluorochromes.

FISH stained root sections were placed on regular microscope glass slide, dried with compressed filtered air and immediately mounted with Citifluor AF1 mounting medium (Citifluor Ltd., London, UK). Samples were visualized with the epifluorescence microscope Zeiss Axioplan (Zeiss Jena, Germany) using the Zeiss filter sets 9 and 15 to observe FITC and Rhodamine respectively. Image overlap was done with imageJ version 1.48.

2.8 Statistical analysis

Statistical differences of plant growth parameters between inoculated and uninoculated plants were determined by ANOVA followed by Tukey HSD post-hoc test at $P<0.05$. Statistical differences of ethylene emissions and cation concentration between inoculated and uninoculated plants were determined by ANOVA and factorial ANOVA, respectively, followed by Fisher LSD post-hoc test at $p<0.05$. Statistical differences of both root surface uptake and root-to-shoot translocation between inoculated and uninoculated plants were

assessed by Student's T-Test. All statistical tests were performed with the software Statistica (Statsoft Inc., Tulsa, USA) version 12.0.

3. Results

3.1 Plant growth promotion abilities *in vitro*

Strain *Hartmannibacter diazotrophicus* E19^T isolation, as well as its complete phenotypic, chemotaxonomic, phylogenetic and genomic characterization was previously described (Suarez et al., 2014a). ACC-deaminase activity of strain E19^T in DF minimal salt medium supplemented with ACC under different salt concentration of 1, 2 and 3 % NaCl were 0.56 ± 0.20, 1.29 ± 0.82 and 2.60 ± 1.2 μmol α-ketobutyrate mg protein^{-1} h^{-1}. Strain E19^T presented very limited growth in LB broth supplemented with L-tryptophan (100 μg ml^{-1}), independently from NaCl concentration, and no IAA production was observed.

3.2 Monitoring ethylene emission of plantlets

Plantlets treated with 400 mM NaCl solution without bacterial inoculum presented the highest values of ethylene emission, whereas lower emission values were obtained from plants treated with deionized water or 200mM NaCl, with and without bacterial inoculum, after both 4 and 24 h incubation (Table 1). After 4 hours incubation, inoculation with E19^T had no effect onto non-stressed plants. Plantlets inoculated with *H. diazotrophicus* at 200 mM NaCl emitted significantly less ethylene ($F_{1, 16} = 8.54$, $P = 0.01$) with respect to uninoculated controls, while no significant ethylene emission difference from inoculated and uninoculated plantlets was observed at 400 mM NaCl.

Ethylene production after 24 h incubation increased by 28 % and 36 % in uninoculated plantlets treated with 200 mM and 400 mM NaCl, respectively, compared to non-stressed uninoculated plantlets (Table 1). Plantlets inoculated with *H. diazotrophicus* increased by about 1 % and 22 % ethylene emission, at 200 mM and 400 mM NaCl, respectively. At both 200 mM and 400 mM NaCl the reduction of ethylene emission due to the inoculation with strain E19^T was statistically significant ($F_{1, 36} = 13.61$, $P < 0.001$).

3.3 Plant-growth promotion potential under salt stress

H. diazotrophicus strain E19^T was tested *in vivo* for the ability to alleviate salt stress on summer barley (cultivar Propino) under greenhouse conditions. Salt stressed barley plants inoculated with the strain E19^T presented not significant difference in fresh or dry weight on leaf blades (Table 2). Sheaths dry weight was significantly higher than uninoculated plants ($F_{2, 12} = 9.64$, P = 0.003). A clear significant difference was also observed in the root fresh ($F_{2, 12} = 12.31$, P = 0,001) and dry weight ($F_{2, 12} = 64.05$, P < 0.001) when compared plants treated with E19^T dead biomass ("S+D") and uninoculated plants ("S+B-"). A significant increase value of 333 % in the root biomass and 109 % in sheath biomass, and a relative increase in water content of 206 % in root system compared to plants inoculated with E19^T dead biomass was observed (Table. 2). The root-to-shoot ratio was significantly ($F_{2, 12} = 24.45$, P < 0.001) increased more than double when compared inoculated plants to uninoculated or inoculated with dead biomass of strain E19^T. Results clearly showed that active growth promotion of *H. diazotrophicus* is due to its PGP activities, instead of nutrient addition from dead cell biomass.

3.4 Cations concentration of plant tissue and parameters of Na^+ exclusion

Salt stressed barley plants inoculated with the strain E19^T showed no significant differences in the cation concentrations ($F_{4,20} = 2.36$, P = 0.09; Table 3) and there was no significant interaction between inoculation and habitat ($F_{8,40} = 0.53$, P = 0.83).
Sodium uptake at the root surface was significantly lower ($t_7 = -6.08$, p = 0,0005) in salt stressed barley plants inoculated with the strain E19^T (65.3 ± 19.7 mg Na^+ g root DW^{-1}) in comparison with uninoculated plants (157.4 ± 25.9 mg Na^+ root DW^{-1}). Similarly, the root uptake of the other analyzed cations was reduced by strain E19^T (data not shown). Root to shoot translocation (normalized to the biomass partition) of all analyzed cations was not significantly modified by inoculation of strain E19^T.

3.5 Design of a specific FISH probe for *H. diazotrophicus* strain E19^T

A 20 nucleotides length probe, named E19.2-FISH, designed from a pre-aligned 16S rRNA gene online database (LTPs111, Feb 2013) containing the 16S rRNA gene sequence of *H.*

diazotrophicus (KC567245) was checked on Probe Check and Probe Match databases. The probe has a G-C content of 55 % and annealed between positions 1262 to 1281 of the *E coli* 16S rRNA gene sequence (Brosius *et al.*, 1978).

Results revealed that the probe was specific for *H. diazotrophicus* strain E19^T. Specificity of E19.2-FISH hybridization on cells of strain E19^T compared with cells of *M. jannaschii* LMG 6239^T was reached at 20 % formamide. Hybridization of both E19^T and *M. jannaschii* cells with NON-EUB probe showed no signal.

3.6 Root colonization abilities of E19^T on barley plantlets

Experiments in liquid growth system confirmed the presence of strain E19^T on root surface of 8 days barley plantlets grown in half concentrated plant nutrient solution adjusted to 1 % NaCl concentration. FISH images showed the green signal of the E19.2-FISH probe (Fig.1A, arrows) perfectly colocalized with the red fluorescence (EUB338 probe; Fig.1B, arrows). The overlap of the two images resulted in yellow *H. diazotrophicus* E19^T (Fig.1C, arrows). E19^T cells were mostly localized on the surface of the root and no presence of strain E19^T cells were found on root tips. Presence of other bacterial cells beside *H. diazotrophicus* was visualized in the root system of inoculated plantlets (Fig 1A-C, red only cells; dotted circles). Similar cells were also observed on uninoculated plants roots, where no *H. diazotrophicus* cells were detected, suggesting that certain bacteria colonize the internal tissues of the barley seeds.

4. Discussion

PGPR efficiently interact and alleviate abiotic stress conditions that affect plant growth, including salt stress (Babalola, 2010; Dimkpa et al., 2009). *H. diazotrophicus* strain E19^T promoted positively the plant growth of barley under salt stress conditions. Previously described PGP activities for strain E19^T (Suarez et al., 2014a) involved in plant growth-promotion included ability to grow on agar containing different kinds of insoluble phosphate, growth on nitrogen free media and growth on media with ACC as only nitrogen source.

H. diazotrophicus ACC deaminase activity (0.56 to 2.60 μmol α-ketobutyrate mg protein^{-1} h^{-1}) in DF medium at 1, 2 and 3 % NaCl was comparable with reported salt tolerant

rhizobacteria with ACC deaminase activity like *Bacillus licheniformis*, *Brevibacterium iodinum*, *Zhihengliuella alba* (Siddikee et al., 2011), *Achromobacter xylosoxidans* (Karthikeyan et al., 2012) performing ACC deaminase activity between 1.38 and 4.24 µmol α-ketobutyrate mg protein^{-1} h^{-1}. In addition, these values were higher than ≥ 0.02 µmol α-ketobutyrate mg protein^{-1} h^{-1}, an estimated value of ACC deaminase activity considered sufficient to permit a bacterium to act as PGPR under abiotic stress (Penrose and Glick, 2003).

Indeed, the results of the ethylene emission experiment (Table 1) revealed rising of ethylene production by increasing salt concentration and significant reduction of ethylene production of barley plantlets by the ACC deaminase activity of *H. diazotrophicus* E19^T under the same salt stress conditions. These findings are in accordance with similar experiments of ethylene emission reduction by inoculation of a ACC deaminase producing bacterium *Achromobacter piechaudii* on tomato plants (Mayak et al., 2004), *Achromobacter xylosoxidans* on Madagascar periwinkle (*Catharanthus roseus*) (Karthikeyan et al., 2012) and *Bacillus licheniformis*, *Brevibacterium iodinum* and *Zhihengliuella alba* on red pepper seedlings (Siddikee et al., 2011). Beside the effect of lowering the ethylene concentration and thereby the stress signal for the plant, Glick (2014) suggested a cross-talk between IAA and ACC deaminase: by lowering plant ethylene levels, ACC deaminase facilitates the stimulation of plant growth by IAA. In strain E19^T no indole acetic acid production was detected suggesting that stimulation on plant root growth did not occur by a coordinated stimulation of IAA and ACC deaminase. However, significantly higher root and sheath dry weights of barley plants treated with *H. diazotrophicus* E19^T under salt stress after 42 days growth was observed (Table 2) revealing a reduction of the salt stress effect. Similar results were reported by Chang et al. (2014) showing an enhancement of barley root and shoot biomass in greenhouse experiment by inoculation of *Pseudomonas corrugata* CMH3 and *Acinetobacter* sp. CMH2 isolated from the rhizosphere of monocotyledonous grasses from soil with high salinity. Although these strains produce similar effects as *H diazotrophicus* under similar conditions, both are ACC deaminase and IAA producers, and not only ACC deaminase as strain E19^T.

Root-to-shoot ratio of barley plants under salt stress inoculated with *H. diazotrophicus* increase remarkably compared to uninoculated plants (Table 2). Under salt stress

conditions root-to-shoot ratio increases due to the importance of the root biomass to allow proper development and water uptake; it is considered as an adaptive response to survive salt stress in order to enable the capacity to divert photoassimilates to the most limiting organ for growth (Pérez-Alfonseca et al., 1996). Increases on root-to-shoot under salt stress have been reported in *Citrus* spp, olive and barley (Zekri and Parsons, 1989; Perica et al., 2008; Pérez-López et al., 2013) and are in accordance to Munns and Tester, (2008) general statement that root biomass is less affected by excess salinity than above ground organs. In addition the use of PGPR under salt stress help to increase even more the root-to-shoot ratio indicating the root behavior to growth as if there was not stress. Root-to-shoot increase by PGPR under salt stress have been reported on wheat by *Pseudomonas putida* (N21), *Pseudomonas aeruginosa* (N39) and *Serratia proteamaculans* (M35) (Zahir et al., 2009), on red pepper seedlings by *Bacillus licheniformis*, *Brevibacterium iodinum* and *Zhihengliuella alba* (Siddikee et al., 2011) and on barley by *Pseudomonas corrugate* CMH3 and *Acinetobacter* sp. CMH2 (Chang et al., 2014). PGPR promote root development providing more surface area enhancing both water and nutrients uptake from soil. Morphological changes in shoot and root elongation in certain plant species by different ACC deaminase producer PGPR strains was also summarized by Bhattacharyya and Jha (2012).

Plants exposed to salt stress absorb large amount of Na^+ leading to limited transport, assimilation and distribution of mineral nutrients and as well to nutrient imbalance in plants organs. Osmotic stress tolerance, Na^+ and Cl^- exclusion and tolerance of tissue to accumulated Na^+ and Cl^- are the three distinct types of plant adaptations to salinity (Munns and Tester, 2008). *Hordeum vulgare* spp. *vulgare* excludes Na^+ on the root surface, increase K^+/Na^+ selectivity, compartmentalize Na^+ into vacuoles, and increases organic solutes to regulate osmolarity in cells cytosol (Tester and Davenport, 2003). Inoculation of *H. diazotrophicus* in salt stressed barley plants showed no effect on leaf blades or sheaths Na^+ concentration. These findings are in accordance with Mayak et al. (2004) who reported no influence on Na^+ in leaves by *Achromobacter piechaudii* inoculation on tomato plants under two different salt stress levels. $E19^T$ inoculation in salt stressed barley plants decreased root surface sodium uptake compared to uninoculated plants, indicating an adding effect to the barley root Na^+ exclusion mechanism (Garthwaite et al., 2005; Munns, 2002). Interestingly, the root surface uptake of all analyzed cations was significantly reduced, suggesting a general effect of strain $E19^T$, possibly due to production of surface

polysaccharides that might reduce the availability of cations to plants, as suggested by Siddikee et al. (2011). The root-to-shoot translocation was not altered by the inoculation of strain E19^T. Although there was a much higher Na^+ accumulation in the roots of the E19^T-inoculated plants (4.7 ± 0.44 mg pot^{-1}) compared to the uninoculated ones (1.3 ± 0.27 mg pot^{-1}), this was not due to lower translocation to the shoots, but instead to the stronger growth promoting effect of strain E19^T on the root compared to the effect on the shoots.

Pseudomonas putida, *Enterobacter cloacae*, *Serratia ficaria* and *Pseudomonas fluorescens* have been reported to increase the K^+/ Na^+ ratio by increasing K^+, effectively influencing salinity tolerance in wheat (Nadeem et al., 2012). Inoculation of *H. diazotrophicus* in salt stressed barley plants showed no significant effect on K^+ concentration neither in leaf blades, sheaths nor roots, but a slight, yet not significant, increase of Mg^{2+} in sheaths, Ca^{2+} in leaf blades and an opposite effect in roots by a slight reduction of Mg^{2+} and Ca^{2+} concentration. Increased uptake of Mg^{2+} and Ca^{2+} in leaves and roots by K^+ content increasing in cotton leaves by inoculation of *Pseudomonas putida* Rs-198 have been reported by Yao et al. (2010).

The presence of *H. diazotrophicus* on barley root system was confirmed by FISH using the newly designed probeE19.2-FISH. Occurrence of PGPR in plant root systems have to be confirmed, since many plant associated bacteria with putative plant growth-promoting traits fail to confer these beneficial effects due to insufficient rhizo and/or endosphere colonization (Compant et al., 2010). In fact, successful root colonization and persistence in plant rhizosphere are required to exert beneficial effect on the plant (Bhattacharyya and Jha, 2012).

FISH images demonstrated high density of *H. diazotrophicus* on root surface; however no cells were visualized at the root tips. Surfaces colonization of root systems and their respective pathways have been reported for different kind of bacteria (Compant et al., 2010), which may show different colonization patterns on different plants, as demonstrated with *Azospirillum brasilense* Cd on tomato, pepper and cotton (Bashan et al., 1991).

Visualization of other bacterial cells, beside *H. diazotrophicus,* on the root system in the liquid system corresponded to bacteria that survive the seed surface sterilization. Beside the presence of other bacteria, *H. diazotrophicus* showed to be efficient in colonization and

establishment on the root surface. *H. diazotrophicus* possess lateral flagella considered a useful trait for root colonization enabling movement of bacteria along the root probably playing an important role in long-term colonization as suggested for *Azospirillum brasilense* (Moens et al., 1995; Merino et al., 2006). The presence of bacterial endophytes inside the seeds is a known phenomenon that surely deserves more attention. Such endophytes in fact may play important roles for the seed germination and the plant growth, and, since they are vertically transmitted to the next generation, might eventually co-evolve with the host and establish intimate associations and interactions. Moreover, also certain bacterial phytopathogens, such as *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, are known to be transmitted by seed (Camara et al. 2009).

5. Conclusions

H. diazotrophicus strain E19^T promotes plant growth in barley plants under salt stress. The mechanism of action is based on the production of ACC deaminase. The specific FISH probe designed will be useful for further *in situ* analyses of *H. diazotrophicus* in the host plant.

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Table 1. Ethylene biosynthesis by 8 days old summer barley (*Hordeum vulgare* L.) plantlets under different salt stress conditions, with and without inoculation of *H. diazotrophicus* strain E19^T. Average ± standard error from 3 replications. Different letters indicate significantly different means (LSD $p<0.05$) within incubation times.

Treatment	Ethylene emission (nmol ethylene g FW $^{-1}$ h $^{-1}$)					
	4 h incubation			24 h incubation		
NaCl concentration	0 mM	200 mM	400 mM	0 mM	200 mM	400 mM
Not inoculated	0	73.67 ± 8.45 b	75.07 ± 1.48 b	8.42 ± 0.6 a	10.81 ± 1.2 bc	11.48 ± 0.96 c
H. diazotrophicus	0	60.18 ± 1.72 a	72.76 ± 8.34 b	8.54 ± 1.1 a	8.57 ± 0.81 a	10.46 ± 0.74 b

Table 2. Effects of *H. diazotrophicus* strain E19^T on summer barley (*Hordeum vulgare* L.) in saline, non-sterile soil, after 42 days growth. Average ± standard error from 5 replications. Different letters indicate significantly different means (Tukey HSD, $p<0.05$) within rows. S+B-, barley in saline soil without bacterial inoculum; S+D-, barley in saline soil inoculated with autoclaved strain E19^T biomass; RI, relative increase.

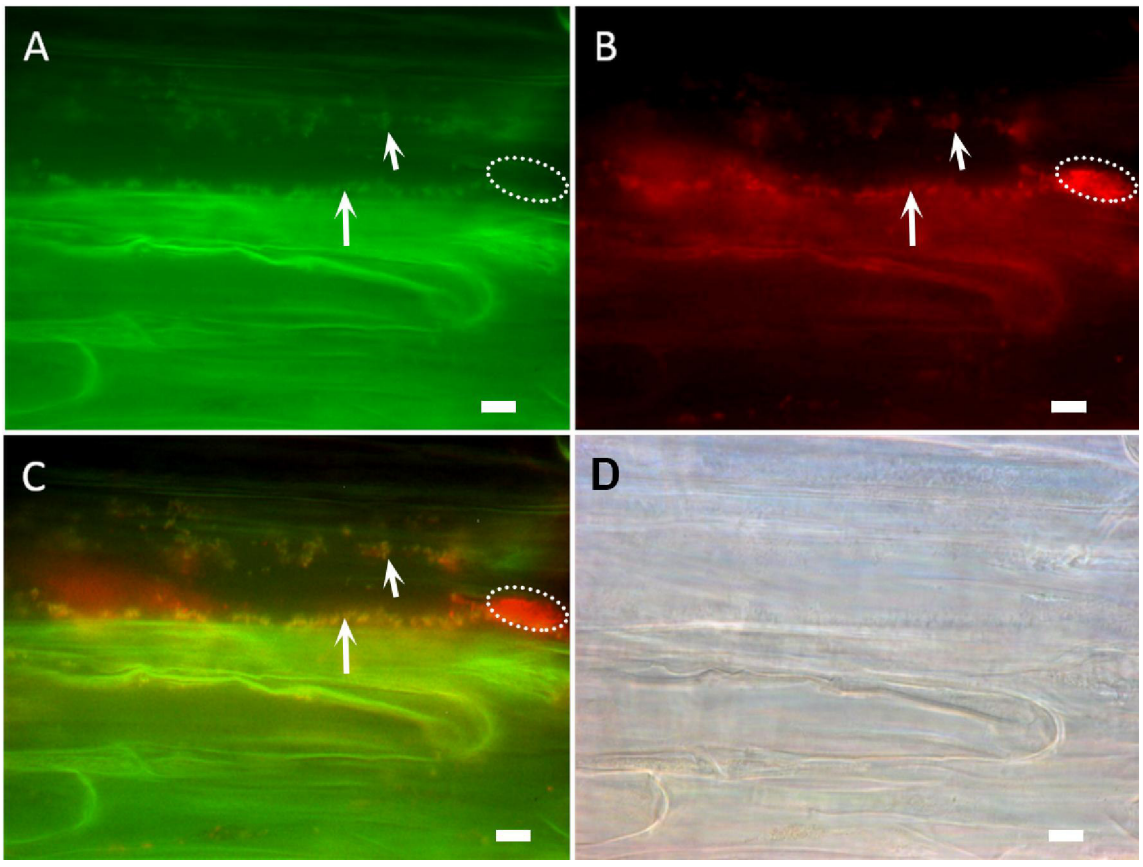
Growth parameter	E19^T	S+B-	S+ D
Leaf blades fresh weight (g pot^{-1})	3.06 ± 0.69 a	2.68 ± 0.85 a	3.14 ± 1.28 a
Sheaths fresh weight (g pot^{-1})	2.07 ± 0.53 a	1.52 ± 0.59 a	1.80 ± 0.87 a
Roots fresh weight (g pot^{-1})	1.73 ± 0.57 a	0.43 ± 0.15 b	0.74 ± 0.47 b
Leaf blades dry weight (g pot^{-1})	0.42 ± 0.10 a	0.35 ± 0.15 a	0.41 ± 0.23 a
Sheaths dry weight (g pot^{-1})	0.53 ± 0.08 a	0.23 ± 0.11 b	0.28 ± 0.15 b
Roots dry weight (g pot^{-1})	0.40 ± 0.04 a	0.08 ± 0.03 b	0.13 ± 0.06 b
Root /shoot ratio	0.43 ± 0.07 a	0.16 ± 0.07 b	0.20 ± 0.06 b
RI biomass leaf blades (%)	20.11 ± 20.55 a	0 ± 38.66 a	16.35 ± 65.09 a
RI biomass sheaths (%)	130.59 ± 34.73 a	0 ± 41.68 b	22.01 ± 64.99 b
RI biomass roots (%)	384.75 ± 52.54 a	0 ± 34.90 b	51.57 ± 77.01 b
RI water content leaf blades (%)	13.17 ± 29.24 a	0 ± 17.08 a	17.34 ± 44.94 a
RI water content sheaths (%)	19.56 ± 34.75 a	0 ± 23.87 a	18.18 ± 56.35 a
RI water content roots (%)	280.18 ± 150.16 a	0 ± 35.04 b	74.56 ± 116.99 b

Table 3. Cations concentration in leaf blades, sheats and roots of *H. diazotrophicus* strain E19^T inoculated and uninoculated summer barley (*Hordeum vulgare* L.) under salt stress after 42 days growth in non-sterile soil. Average ± standard error from 5 replications. Different letters indicate significantly different means (LSD $p<0.05$) within columns. S+B-, barley in saline soil without bacterial inoculum.

Plant part	Treatment	Cations concentration (mg g DW^{-1})			
		Na^+	Ca^{2+}	K^+	Mg^{2+}
Leaf blades	E19	21.15 ± 4.62 ab	14.42 ± 2.16 a	39.88 ± 5.24 a	2.74 ± 0.30 a
	S+B-	21.69 ± 5.87 ab	12.46 ± 1.80 a	41.87 ± 1.10 a	2.66 ± 0.27 a
Sheaths	E19	26.20 ± 9.92 a	8.68 ±2.20 b	36.96 ± 3.55 a	3.00 ± 0.74 a
	S+B-	27.40 ± 8.30 a	8.72 ± 0.24 b	39.75 ± 3.88 a	2.84 ± 0.22 a
Roots	E19	11.80 ± 2.00 c	6.60 ± 1.49 b	10.50 ± 3.17 b	1.32 ± 0.26 b
	S+B-	15.13 ± 3.25 bc	7.65 ± 5.34 b	15.74 ± 5.50 b	1.78 ± 1.15 b

Figure legends

Figure 1. Fluorescence *in situ* hybridization (FISH) images of barley root colonization by *H. diazotrophicus* strain E19^T. Summer barley was inoculated with strain E19^T in liquid growth solution, and roots were stained after 8 days of growth. A) EUB338 (universal bacterial probe) labelled with Rhodamine (red); B) E19.2-FISH (E19^T -specific probe) labelled with FITC (green); C) overlap of panel B and C (E19^T cells appear orange/yellow, other bacteria appear red); D) Phase contrast image. Arrows indicate *H. diazotrophicus* E19^T cells. Dotted circles indicate other unidentified bacterial cells stained with the FISH probe EUB338 only. Scale bars indicate 5 μm.



Chapter 8

The Draft Genome Sequence of the Plant Growth- Promoting Bacterium *Hartmannibacter diazotrophicus* E19^{T}

Publication draft version

The Draft Genome Sequence of the Plant Growth-Promoting Bacterium *Hartmannibacter diazotrophicus* E19^T.

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Hartmannibacter diazotrophicus E19^T is a Plant Growth-Promoting Rhizobacterium (PGPR), that was isolated from the rhizospheric soil of *Plantago winteri* from a natural salt meadow. The draft genome sequence (estimated size 5.43 MB, 4,983 coding sequences (CDSs)) will help to decipher the molecular interaction between plant and bacteria under salt stress conditions of the plant.

The term Plant Growth-Promoting Rhizobacteria is used to define bacteria that colonize the rhizosphere and stimulate plant growth (Kloepper and Schroth, 1981), also under abiotic stress (Yang et al., 2009). *Hartmannibacter diazotrophicus* E19^T was isolated as a part of an investigation on rhizospheric bacteria from salt resistant plant species (Cardinale et al. 2015, submitted). Plant growth-promoting abilities of *H. diazotrophicus* were evaluated (Suarez et al., 2014). Strain E19^T is able to solubilize insoluble phosphate, fix nitrogen and produce ACC deaminase. Plant growth promoting effect of E19^T has been reported in summer barley plants under salt stress (Suarez et al. 2015, submitted).

Genomic DNA of *H. diazotrophicus* PGPR E19^T was isolated using PureLink Genomic DNA Mini Kit (Life Technologies, USA). A Nextera XT paired-end library was prepared and sequenced on a MiSeq system using v2 chemistry, according to protocols provided by the manufacturer (Illumina, Netherlands). Sequencing reads were analyzed using CLC Genomics Workbench 7.0.4 (CLC bio, Denmark) and gave following metrics: 2,455,240 reads in pairs, with an average length of 210.78 bases, were assembled to a gapped genome of a total length of 5,436, 725 bp with an average coverage of 95.19. The genome was then manually curated using the CLC Microbial finishing tool (CLC bio) to two contigs. Manual and automatic annotations, and comparative genome analyses were done using software GenDB 2.4 (Meyer et al., 2003) and EDGAR (Blom et al., 2009) respectively.

The draft genome sequence of strain E19^T consisted of a single circular chromosome of 5,432,335 bp with a GC content of 63.98 %. A total of 4,983 genes (coding sequences [CDS]), 3 rRNA operons and 46 tRNAs were found. *H. diazotrophicus* E19^T genome based relationship on average amino acid identity (AAI) mean values (Table 1) is close to *Pleomorphomonas koreensis* DSM23070 (71.57 %), *Pleomorphomonas oryzae* DSM 16300 (71.54 %) and *Stappia stellulata* DSM 5886 (69.17 %).

Among different reported genes involved in plant growth-promotion (Table 2) (Bruto et al., 2014; Gupta et al., 2014) E19^T genome includes genes involved in phosphate solubilization and phosphate transport system *pqqBCDE, pstABCS, phoBHU,* nitrogenase-encoding *nifHDK* and ACC deaminase production *rimM* . Also, present are genes *luxS, lsr* involved in *quorum sensing, flgIHG fliGMPI* involved in motility by flagella, *cysCJIN* for H_2S production, *soxB* involved in glycine-betaine production, *katG* for catalase and *sodB* for superoxide dismutase. No presence of genes *ipdC/ppdC* involve in synthesis of indole acetic acid (IAA), *nirK* involve in formation of the NO root-branching signal, *hcnABC* for hydrogen cyanide production, *phlACBD* for synthesis of 2,4-diacetylphloroglucinol, *budAB* for acetoine synthesis, *budC* 2,3-butanediol synthesis,

pvd, *fpvA*, *mbtH*, *fhu* involved in siderophore production, *pelF*, *alg8*, *pslA* involved in exopolysaccharides synthesis, *osmC*, *phzF* involved in phenazone production, and *ubiC* in 4-hydroxybenzoate production.

E19^T contains gene clusters involved in nitrogen fixation and phosphate solubilization, well know PGP traits and most common traits found in a comparative genome analysis of PGPR strains belonging to different genera of *Proteobacteria* (Bruto et al., 2014). Also, present is the gene cluster for H_2S production reported as a biological active gas able to increase plant growth and seed germination (Dooley et al., 2013). Genes related with reported PGP traits under salt stress contained in E19^T encode for enzymes such as catalase and superoxide dismutase, involved in management of oxidative stress in plants, ACC deaminase, reported to lower the level of ethylene accumulation due to stress, and for synthesis of osmoprotectant glycine betaine (Paul and Lade, 2014). Genes for *quorum sensing* and motility are present and reported to be involved in colonization and establishment processes in rhizosphere by PGPR (Ryan et al., 2009; Merino et al., 2006). In E19^T genome were not founded genes involved in synthesis of hydrogen cyanide, 2,4-diacetylphloroglucinol, acetoine, 2,3-butanediol, phenazine and 4-hydroxybenzoate reported as antimicrobial compounds produced by PGPR strains used in biocontrol (Loper et al., 2012; Babalola, 2010). Genes encoding for other known PGP traits such as siderophore production, synthesis of IAA, NO root-branching signal and synthesis in exopolysaccharides are not content. E19^T sequence and annotation will be deposited in EMBL.

Table 1. Genome retrieved *Hartmannibacter diazotrophicus* E19^T means of the average amino acid identity (AAI) and 16S rRNA gene sequence (1472 bp) similarities to next relative available genome sequences. Similarity values were transferred from Suarez et al. (2014)..

Next relative bacteria	AAI (Mean)	Similarity (%)
Pleomorphomonas koreensis Y9^T (AB127972)	70.87	92.3
Pleomorphomonas oryzae F7^T (AB159681)	70.80	92.3
Stappia stellulata IAM12621^T (D88525)	68.33	93.2
Labrenzia aggregata IAM 12614^T (AAUW01000023)	67.93	92.8
Mesorhizobium loti LMG 6125^T (X67229)	65.76	91.6
Mesorhizobium_amorphae ACCC 19665^T (AF041442)	65.58	92.1
Mesorhizobium metallidurans STM 2683^T (CAUM01000060)	65.49	92.5
Brucella pinnipedialis B2 94^T (CP007743)	65.45	91.5
Lutibaculum baratangense AMV1^T	65.01	91.5
Aureimonas ureilytica 5715S-12^T (DQ883810)	64.61	92.8

Table 2. *Hartmannibacter diazotrophicus* E19^T gene content in draft genome involved in plant growth-promotion. +, indicates presence of genes; -, indicates absence of gene.

Plant growth promotion traits	**Genes**	***H. diazotrophicus* E19^T**
Nitrogen fixation	*nifHDK*	+
Phosphate solubilization	*pqqBCD*	+
	pqqFG	-
	pqqE	+
	pstABCS	+
	phoBU	+
	phoR	-
	phoCDET	-
	phoH	+
IAA production	*ipdC*	-
	ppdC	-
Siderophore production	*pvd*	-
	fpvA	-
	mbtH	-
	acrB	-
	fhu	-
ACC deaminase	*acdS*	-
	rimM	+
	dcyD	-
H_2S production	*cysCJIN*	+
Quorum sensing	*luxS*	+
	lsr	+
Motility	*flgIHG*	+
	fliGMPI	+
Heat shock proteins	*dnaJ*	+
	dnaK	+
	groE	-
Glycine-betaine production	*soxB*	+
Catalase	*katG*	+
Superoxide dismutase	*sodB*	+
2,4-Diacetylphloroglucinol synthesis	*phlABCD*	-
Hydrogen cyanide synthesis	*hcnABC*	-
Acetoin & butanodiol synthersis	*als*	-
	budABC	-
	poxB	-
Phenazone production	*phzF*	-
4-hydroxybenzoate production	*ubiC*	-
Exopolysaccharides synthesis	*pelF*	-
	alg8	-
	pslA	-

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Supplementary Material

Fig S1.Venn diagram showing the number of shared CDS between *Hartmannibacter diazotrophicus* E19^{T} *Pleomorphomonas koreensis* DSM 23070 *and Pleomorphomonas oryzae* DSM 16300. The overlapping part in the middle shows the number of CDS shared by all three bacteria; the other overlaps show CDS shared between two bacteria, but not the third. *Pleomorphomonas koreensis* DSM 23070 and *Pleomorphomonas oryzae* DSM 16300 share more common CDS in between that individually with *Hartmannibacter diazotrophicus* E19$^{T.}$

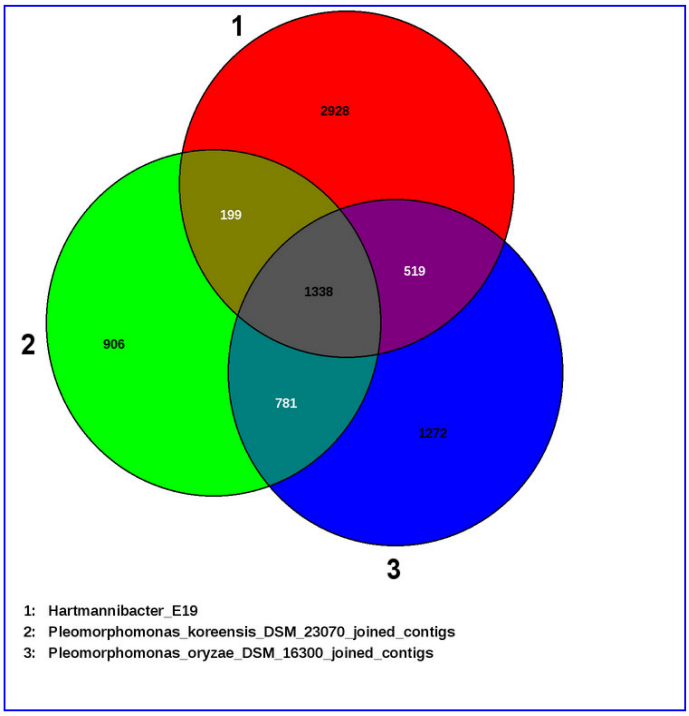


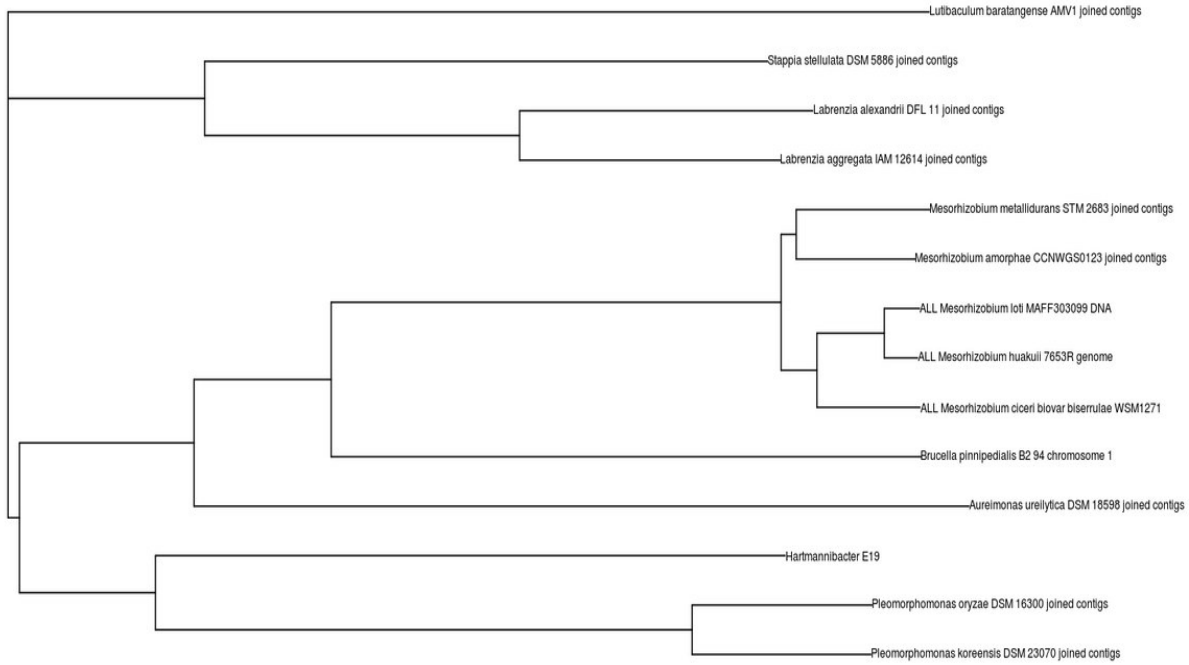
Table S1. Matrix of the mean values of the average amino acid identity (AAI) of *H. diazotrophicus* E19^{T} to next relative available genome sequences.

AAI matrix MEAN values

Heatmap colors for a range of values from [60 .. 100]

NAME/ID	SID_17	SID_1	SID_4	SID_9	SID_11	SID_13	SID_18	SID_25	SID_26	SID_29
ALL_Mesorhizobium_loti_MAFF303099_DNA **(SID_17)**	100.00 (0.00)	67.66 (11.36)	65.06 (11.39)	65.18 (11.20)	62.76 (10.98)	93.11 (4.73)	92.75 (5.09)	63.89 (11.20)	63.69 (11.28)	65.15 (11.12)
Aureimonas_ureilytica_DSM_18598_joined_contigs **(SID_1)**	67.66 (11.36)	100.00 (0.00)	63.62 (13.10)	63.37 (13.09)	61.98 (12.11)	67.55 (11.55)	67.30 (12.20)	62.71 (12.98)	62.31 (13.65)	63.78 (12.43)
Hartmannibacter_E19 **(SID_4)**	65.06 (11.39)	63.69 (12.89)	100.00 (0.00)	66.99 (12.01)	64.42 (12.11)	64.88 (11.85)	64.75 (12.13)	70.34 (10.88)	70.23 (11.23)	67.42 (11.34)
Labrenzia_aggregata_IAM_12614_joined_contigs **(SID_9)**	65.18 (11.20)	63.50 (12.69)	67.01 (11.98)	100.00 (0.00)	64.20 (11.80)	64.93 (11.73)	64.81 (11.75)	64.83 (11.76)	64.80 (11.83)	73.87 (10.62)
Lutibaculum_baratangense_AMV1_joined_contigs **(SID_11)**	62.77 (10.98)	62.01 (12.08)	64.42 (12.10)	64.21 (11.78)	100.00 (0.00)	62.61 (11.67)	62.56 (11.70)	63.30 (11.40)	62.88 (11.97)	65.01 (10.99)
Mesorhizobium_amorphae_CCNWGS0123_joined_contigs **(SID_13)**	93.11 (4.73)	67.54 (11.57)	65.00 (11.38)	65.09 (11.21)	62.75 (11.19)	100.00 (0.00)	93.46 (6.11)	63.74 (11.40)	63.58 (11.45)	65.18 (11.07)
Mesorhizobium_metallidurans_STM_2683_joined_contigs **(SID_18)**	92.75 (5.09)	67.45 (11.67)	64.84 (11.86)	64.96 (11.23)	62.62 (11.47)	93.46 (6.11)	100.00 (0.00)	63.77 (11.18)	63.59 (11.26)	65.07 (11.29)
Pleomorphomonas_koreensis_DSM_23070_joined_contigs **(SID_25)**	63.89 (11.20)	62.84 (12.57)	70.34 (10.88)	64.76 (12.00)	63.24 (11.63)	63.65 (11.66)	63.62 (11.68)	100.00 (0.00)	90.57 (6.73)	65.42 (11.75)
Pleomorphomonas_oryzae_DSM_16300_joined_contigs **(SID_26)**	63.69 (11.27)	62.58 (12.73)	70.21 (11.28)	64.73 (12.08)	62.87 (11.98)	63.49 (11.71)	63.43 (11.76)	90.57 (6.73)	100.00 (0.00)	65.24 (11.84)
Stappia_stellulata_DSM_5886_joined_contigs **(SID_29)**	65.14 (11.13)	63.83 (12.23)	67.42 (11.34)	73.87 (10.62)	64.89 (11.46)	65.01 (11.61)	64.98 (11.58)	65.49 (11.49)	65.19 (12.04)	100.00 (0.00)

Fig S2. Phylogenetic tree based on core genes sequences of strain E19^T and available closest gene sequence of members of related families of the orders *Rhizobiales* and *Rhodobacterales* and representatives of the class Alphaproteobacteria. Genome sequences were aligned by MUSCLE and non matching parts of the alignment were masked by GBLOCKS and subsequently removed. The remaining parts of all alignments were concatenated in to one alignment and the phylogenetic tree construction was done with PHYLIP. All previously described step were done in EDGAR (Blom et al., 2009).



GENERAL DISCUSSION

In this halotolerant PGPR screening study, 100 potential PGPR were isolated on selective enrichment media. Most of them belonging to diazotrophic, P-mobilising bacteria and ACC deaminase producer (Chapter 2, Fig.1B). The bacteria were isolated from the rhizospheric soil of the natural salt tolerant plants *Hordeum secalinum* (meadow barley) and *Plantago winteri* (great plantain). These halotolerant PGPR isolates were potential candidates to be tested under salt stress because they have coevolved and are adapted to the rhizosphere of natural salt resistant inhabitant plants sampled from a natural salt meadow. It is known that, microbial communities are selectively influenced by roots exudates, biotic and/or abiotic stresses, nutritional deficiencies and are even different between plant species and cultivar (Berg and Smalla, 2009; Hartmann et al. 2008). Therefore, the specific biotic and abiotic conditions of the natural salt meadow allowed *H. secalinum* and *P. winteri* to coevolve with salt adapted microbial communities. Among these rhizospheric microbial communities, natural adapted rhizobacteria with PGPA would have also coevolved and they could be capable to perform a highly efficient plant promotion under salt stress. As a promising agricultural practice to help salt affected crops and to ameliorate adverse effect of salt stress, there is an increasing interest in the isolation of halotolerant rhizospheric bacteria from natural salt tolerant (Paul and Lade, 2014) and halophytes plant species (Ruppel et al., 2013).

Crop cultivars Morex and Propino of *Hordeum vulgare* were chosen in this study as model plant to test *ad planta* PGPR candidates under salt stress. These plant models are related to the plant species *H. secalinum* and therefore the halotolerant PGPR candidates would have better chances to positively promote their growth. Nevertheless, the ability of PGPR strains to promote growth in a plant species different from where they were originally isolated is a desirable characteristic. For instance, this is the case of a well-studied IAA and ACC deaminase producer PGPR *Pseudomonas*. sp. UW4 isolated from reeds and reported to promote canola seedling root elongation in growth pouches under gnotobiotic conditions (Glick et al., 1995). Therefore also *Plantago winteri* was chosen as isolation source.

In order to evaluate *ad planta* growth effects of the halotolerant PGPR candidates on *H. vulgare* under salt stress, different approaches were used. Several considerations were taken into account for the procedure to impose salt stress to the systems, and the plant-

bacteria growth conditions. These interactions were evaluated using both a gnotobiotic plant growth liquid system and a non-sterile pot system using two different types of soils.

A gnotobiotic plant growth liquid system was designed to study colonization, establishment and plant growth effect of selected isolates on *H. vulgare* cv. Morex seedlings using plant nutrient solution (PNS) under salt stress conditions (Chapter 3, Fig.2). The system was successful in order to impose salt stress and allow colonization of the inoculated bacterium, but unsuccessful to keep gnotobiotic conditions. Apart from inoculated strain E50^T colonies, other kinds of colonies were isolated from colony forming unit (CFU) analysis at the end of the experiment, displaying the failure to maintain the expected gnotobiotic condition (Chapter 3, Table.4). Additionally, similarity of predominant colonies isolated from CFU analysis at the end of experiment to colonies isolated at the seed surface sterilization procedure was confirmed (Chapter 3, Table.4). This is the case of isolates C5EA and IA3, which presented identical morphological characteristics and closest Blast similarity of their 16S rRNA gene to *Curtobacterium flaccumfaciens* A4-16 (JF496347). Members of the genus *Curtobacterium* have been frequently reported as bacterial seed endophytes (Truyens et al., 2014), vertically transmitted and able to remain viable for long periods in stored seeds (EPPO/CABI, 1997; Camara et al., 2009).

These arguments lead to hypothesize that isolates C5EA and IA3 correspond to a *Curtobacterium flaccumfaciens* strain vertically transmitted in *H. vulgare* cultivar Morex seeds, able to resist the seed surface sterilization procedure. Moreover, presence of bacterial growth on SG tube agar, used to check the seed surface sterilization, was detected after 15 days of incubation and not after 72 h, as expected (Chapter 3, Fig. 4). The slow bacterial growth and its presence on the area where the radicular system was developed may be explained by the presence of endophytic bacteria that were partially or not affected in the seed surface sterilization procedure. Several attempts, with unsuccessful results, were done by using antibiotics to eliminate possible endophytic bacteria (data not shown).

Even though, nowadays there is a particular interest in the plant growth-promoting effect of bacterial seed endophytes (Partida-Martínez and Heil, 2011; Truyens et al., 2014), the use of germ free seeds or seedlings obtained from sterile plant tissue´s cultures is of utmost importance in gnotobiotic studies. Gnotobiotic systems have been used in/with

different plants such as tomato, radish, potato, cucumber, wheat, grape, rice and grass in order to study bacterial root colonization and to determine bacterial root concentration (Hurek et al., 1994; Simons et al. 1996; Bloemberg et al. 2000; Compant et al., 2005). Such kind of studies have used seeds, seedlings or roots developed from sterile stem cuttings (de Weger et al., 1987) depending on the plant and purpose of the research. Bacterial growth on plant material used in gnotobiotic studies, mainly in seeds, are usually checked after seed surface sterilization protocols on different kinds of nutrient agars and incubated for short period time (Hurek et al., 1994; Simons et al., 1996; Kutter et al., 2006). Moreover, the so called gnotobiotic conditions have been assumed to be reached by the use of seed surface sterilization protocols overlooking or no mentioning endophytic bacterial growth (Yim et al., 2009; Ahmad et al., 2013; Bal et al., 2013; Palaniyandi et al., 2014). In order to study plant microbe interaction under gnotobiotic conditions the presence of endophytes after seed surface sterilization protocols must not be overlooked.

Improvement of sterilization protocols by combining disinfectants and antibiotics, verifying absence of bacterial growth by cultivation methods and confirming absence of bacterial cells by microscopic techniques to plant material is required in plant-microbe gnotobiotic studies. For instance, Nabti et al. (2010) and Buddrus-Schiemann et al. (2010) used combination of disinfectant and antibiotics for seed surface sterilization and confirmed the axenic gnotobiotic condition in plant roots by testing bacterial growth on nutrient agar media and microscopic visualization using an eubacteria specific FISH probe.

After analysis of different seed batches it was decided to use *H. vulgare* cv. Propino instead of *H. vulgare* cv. Morex, originally used for its natural salt tolerance (Witzel et al., 2009), for further experimentation. This decision was mainly taken because of the presence of endophytic bacteria, which could not be eliminated with the seed surface sterilization protocol, especially strains of *C. flaccumfaciens* (see above). These reported ecological roles could have an undesirable positive or negative influence in a plant growth promotion study. Furthermore, summer barley *H. vulgare* cv. Propino was selected for further experimentation because it is a commercial available variety, widely cultivated, and has a high malt content, desirable for the brewery industry (Syngenta, 2014).

Further improvement on the designed plant growth liquid system and on the seed surface sterilization protocol must be done in order to maintain the desirable gnotobiotic

conditions. Gnotobiotic systems experiments to evaluate PGP by rhizobacteria using quartz sand, plant nutrient solution and/or sterilized soil generally lead to different results than systems using non-sterile soils. Indeed, functional bacterial plant growth promoting mechanisms in a gnotobiotic system could be less or no functional in non-sterile soil experiments by the presence of indigenous organisms (Ryu et al., 2005). To sum up, it must be regarded that a plant completely free of microorganism is an exotic exception rather than a natural plant growth condition (Partida-Martínez and Heil, 2011).

In parallel to gnotobiotic experiments, non-sterile soil experiments were settled to determine *ad planta* growth effect of the halotolerant PGPR candidates on summer barley *H. vulgare* cv. Propino under salt stress. As first approach in non-sterile soil, mixtures of subsoil, sand and mineral salts were used to set different plant growth conditions. Such conditions were adjusted to analyze nutritional deficiencies, different P fertilization sources and salt stress in Mitscherlich pots under growth chamber conditions (Chapter 3, Fig.5.8). In all cases the different experimental variables used to impose salt stress and to test nutritional deficiencies and fertilization effects on the plants were statistically significant. On the contrary, no statistical significant interaction by the inoculation of any evaluated PGPR candidate on the growth of *H. vulgare* cv. Propino under any of the different conditions settled in subsoil experiments was observed. For instance, no plant growth effects were observed in strain E19^T (Chapter 3) and strains E64I, E22, E55, E51 tested in the master thesis of Ana Maria Zapata (data not part of this work). Plant growth promotion by PGPR has been reported to have a prominent beneficial effect on limited resources in poor soils (Ramos Solano et al., 2006). Subsoil contains low organic-matter, restricted microbial activity, and provides low nutritional conditions that can be adjusted by mineral salt or fertilizers supplementation (Steffens et al., 2010). Therefore, it was expected to provide proper conditions in order to evaluate PGP by the PGPR candidates under nutrient limitation and with and/or without salt stress.

Even though PGPR candidates revealed no PGP effects neither in the gnotobiotic plant liquid system nor in non-sterile subsoil experiments, these two methodologies were successful in imposing the desirable plant salt stress and can be useful for further experimentation. Unfortunately, these methodologies did not allow to easily test several isolates at once. Therefore, a second methodological approach using an organic non-sterile

soil to test the PGPR isolates on *H. vulgare* cv. Propino under salt stress was settled (Chapter 2. Plant assay 1).

Due to the large number of halotolerant PGPR candidates to be tested, twenty-two isolates out of the one hundred were selected for further experimentation based on their isolation medium and taxonomical diversity. The selected isolates were fully characterized for their PGPA (*in pure culture*) and their (*ad planta*) effects on *H. vulgare* cv. Propino growth under salt stress tested in the organic non-sterile soil under greenhouse conditions (Chapter 2. Plant assay 1). A very low proportion of the isolates containing all or almost all PGPA activities *in pure culture* presented successful results in their *ad planta* experiment (Chapter 2, Table 2). Strains E108 and E110, identified as *Curtobacterium flaccumfaciens* (AJ312209) and *Ensifer garamanticus* (AY500255) respectively (Chapter 2, Supplement 4), showed statistical significant effects on *H. vulgare* cv. Propino growth under salt stress in the first plant assay (Chapter 2, Fig. 2, 3). These two strains were therefore selected for a second plant assay, performed under stronger salt stress conditions confirming their positive plant effect on *H. vulgare* cv. Propino under salt stress (Chapter 2, Fig. 4-6). Surprisingly, strain E108 showed the best results on *ad planta* experiment but did not present several PGPA activities in pure culture.

Classically, approaches for screening PGPR have always looked for best quantitatively and/or qualitatively PGPA in pure culture to select few among several isolates to test their effect on plant growth (*ad planta*) (Franco-Correa et al., 2010; Bal et al., 2013;Goswami et al., 2014). Consequently, potential efficient *ad planta* PGPR isolates, able to colonize the root system and promote plant growth, could be underestimated by their low performance in pure culture PGPA tests. This phenomenon may occur because the effect of a PGPR to promote plant growth also depends on its ability to colonize the root and to interact with other soil or endophytic bacteria (Compant et al., 2010). Not many studies use *ad planta* experiments as a screening method in order to test PGPR effect on plants. One example of the use of this approach was reported by Etesami et al. (2013), who proposed a rapid screening method to select PGPB. Endophytes (80) and rhizospheric (120) isolates of berseem clover (*Trifolium alexandrinum*) were inoculated in mixtures to rice plants. Then, thirty-four re-isolated bacteria from the rice roots systems were positively tested for several PGP traits and among them several were successful to promote rice plantlets in gnotobiotic conditions. Based on the results of this study and previously exposed considerations, *ad*

planta tests could represent a more realistic field growth condition and a less time and money consuming selection parameter. Furthermore, the possible low efficiency of classical PGPR in pure culture screening could have been undetectable because normally just successful results using similar approaches have been published.

Interestingly and supporting the previous discussion, classical screening methods based on pure culture PGPA screening test (Chapter 2, Table. 1) would have excluded strain E19^T from *ad planta* experiments. Although, due to its taxonomical interest, salt tolerance, in pure culture ACC deaminase activity and the slightly increasing effect on the water content of *H. vulgare* cv. Propino leaves in the screening *ad planta* experiment (Chapter 2, Fig. 3C), it was included in further experimentation.

Plant growth promotion of strain E19^T on summer barley cv. Propino under salt stress using non-sterile soil (Chapter 7) was demonstrated by the significantly increasing root and shoot dry weights, water content in the root system and root-to-shoot ratio, and by decreasing root Na^+ concentration and root surface sodium uptake. The significant ACC deaminase *ad planta* activity on barley plantlets under salt stress and the lack of IAA production and/or remarkable qualitatively PGP in pure culture able to influence PGP revealed that the main mechanism of strain E19^T for PGP under salt stress is based on the production of ACC deaminase. The presence of *H. diazotrophicus* on barley root system under salt stress was demonstrated by FISH using a newly designed probe for strain E19^T. Results from this study, caused the inclusion of *H. diazotrophicus* E19^T in the list of rhizobacteria reported in the literature as PGPR involving ACC deaminase activity in different plant species (Chapter 1, Table.3). Moreover, the lack on IAA production of strain E19^T shows the individual effect of this trait without the synergistically linked effect of IAA in PGP (Glick, 2014).

Visualization of other bacterial cells by FISH staining, besides *H. diazotrophicus,* on the root system corresponded to bacteria that survived the seed surface sterilization of *H. vulgare* cv. Propino seeds (Chapter 7, Fig. 1). Contrary to the desirable absence of endophytic bacteria in plant microbe interaction on gnotobiotic conditions, the presence of endophytic bacteria in *H. vulgare* cv. Propino was expected to interact with strain E19^T on the root system. Besides the presence of other bacteria, *H. diazotrophicus* was able to

confer its beneficial effect and showed to be efficient to colonize and establish on barley cv. Propino root surface.

Apart from the objective to isolate halotolerant PGPR promoting growth of barley under salt stress, this study was useful to analyze the cultivable microbial diversity of the rhizosphere of the natural salt tolerant plants *H. secalinum* and *P. winteri*. Partial 16S rRNA (400-600 bp approx.) (Chapter 2, Supplement 4) was used for an initial taxonomic affiliation of new isolates and also for identification of potential candidates of taxonomical interest. Results revealed that the rhizosphere of *H. secalinum* and *P. winteri* plants is a source of high bacterial diversity, where most common isolate taxa affiliation was to phyla *Proteobacteria* and *Actinobacteria.* Likewise, the most common bacterial taxa present in saline and non-saline soils, belong to phyla *Proteobacteria* and *Actinobacteria* (Canfora et al., 2014; Ma and Gong, 2013). The rhizospheric bacterial genera diversity of *P. winteri* (29 genera) was higher than of *H. secalinum* (21 genera) (Chapter 2, Supplement 4**)**. However, in both cases, isolates most common genera affiliation corresponded to *Streptomyces*, *Pseudomonas*, *Rhizobium* and *Bacillus,* which are recognized genera containing strains with PGPA, confirming their isolation prevalence on PGP qualitative selective media screening (Paul and Lade, 2014; Dimkpa et al., 2009).

Eleven out of one hundred isolates were considered for further taxonomical characterization, including the almost complete 16S rRNA gene sequence. In general, two organisms with 16S rRNA gene sequence pairwise similarities lower than 98.7% may not belong to the same species (Stackebrandt and Ebers, 2006). Even though, for isolates belonging to *Streptomyces* (Rainey, 2011), *Rhizobium* (Puławska et al., 2012) and *Pseudomonas* (Cámara et al., 2007), another phylogenetic markers besides their 16S rRNA gene sequence must be used in order to consider them as possible new taxa description.

In order to characterize new taxa isolates, a polyphasic experimental approach was done following previously related taxa rank publications, the recommended key elements outline for prokaryotes characterization (Tindall et al., 2010), and focusing on new metabolic activities. So far and as part of this work new species *Cellvibrio diazotrophicus* E50^T (Suarez et al., 2014c), *Rheinheimera hassiensis* E48^T and *R. muenzenbergensis* E49^T (Suarez et al., 2014b) and new genus and species *Hartmannibacter diazotrophicus* E19^T (Suarez et al., 2014a) were proposed and accepted. For these new species proposals, the

mandatory DNA-DNA hybridization with closest relatives, the almost complete 16S rRNA gene sequence, the major fatty acids membrane profile and their analysis of phenotypical traits were analyzed. *C. diazotrophicus* polyphasic study was done with two different isolates, strains E20 and E50^T, differently than new description of *Rheinheimera* species and new genus and species *H. diazotrophicus*. For new species description the use of more than one strain is desirable in order to evidence intraspecific diversity, although sometimes impossible (Rosselló-Mora and Amann, 2001). Furthermore, emendation of the genus *Cellvibrio* was accepted based on the confirmed capability of fixing nitrogen and growth in presence of up to 5% NaCl (w/v) not reported in previously species descriptions. Concerning the singularities of *R. hassiensis* E48^T and *R. muenzenbergensis* E49^T descriptions, this is the first descriptive work to explore diazotrophy among described species of genus *Rheinheimera*. Strain E48^T and E49^T are able to grow in nitrogen free media but they are not able to reduce acetylene. The *nifH* gene presence was detected only in strain E48^T and not in other tested *Rheinheimera* species able to grow in nitrogen free media in this study.

H. diazotrophicus E19^T belongs to *Alphaproteobacteria* but it could not be characterized neither as a member of any recognized genus nor any family of the orders *Rhizobiales* or *Rhodobacterales*. These orders, members of the class *Alphaproteobacteria*, are circumscribed solely on the basis of phylogenetic analysis of 16S rRNA gene sequences (Garrity et al., 2005). Strain E19^T showed less than 93.5% 16S rRNA gene sequence similarity with members of genera among *Alphaproteobacteria.* Also, this value is below of genus, mean (96.4 % ± 0.2) or minimum identity (94.9 % ± 0.4), and above family, mean (90.1 % ± 1.1) or minimum identity (87.5 % ± 1.3), proposed boundaries (Yarza *et al.*, 2008). Similarly to 16S rRNA, chemotaxonomic and phenotypic analysis presented enough criteria for the new genus proposal and eventually could lead to a higher rank classification proposal. Consistently, the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/index.html) included *H. diazotrophicus* E19^T in the list of ¨validly published names of genera and taxa above the rank of genus up to and including class¨.

In light of the above, the natural salt tolerant plant inhabitant *H. secalinum* and *P. winteri* growing in the salt meadow are source of diversity of halotolerant and halophylic bacteria, and potential novel taxa. One of the major factors influencing bacterial community

composition and diversity in soil is salinity (Canfora et al., 2014; Ma and Gong, 2013). It has been suggested that less than 25 % bacterial diversity from saline soil habitats has been recovered using molecular techniques (Ma and Gong, 2013) and only a small part has been isolated. Also, undisturbed environmental conditions of the natural protected area, where the salt meadow is located and the coevolution of microbial communities with the sampled plants rhizospheres would have been driving forces influencing bacterial diversity. For instance, Chen et al. (2010) reported abundant diversity and potentially novel taxa description of halophilic and halotolerant isolated bacteria from non-saline soil from natural protected areas by cultivable dependent methods.

In summary, the rhizospheres of *H. secalinum* and *P. winteri* are sources of new halotolerant PGPR and isolates with taxonomical interest. Similarly, Jha et al. (2012) reported the isolation of a possible novel species description of genus *Haererehalobacter* and accepted novel species description *Brachybacterium saurashtrense* (Gontia et al., 2011) as part of a screening of new halotolerant diazotrophic bacteria with PGP potential from roots of the halophyte *Salicornia brachiata.*.

Due to its PGP effect on *H. vulgare* cv. Propino under salt stress (Chapter 7) and its described taxonomical singularity (Chapter 6), the draft genome of *Hartmannibacter diazotrophicus* E19^T was sequenced and assembled (Chapter 8). As part of its taxonomical analysis, the average amino acids identity (AAI) of the strain E19^T genome sequence was calculated using EDGAR (Blom et al., 2009). Strain E19^T AAI comparison with other sequenced bacterial genomes showed as closest relatives two species of the unclassified genus *Pleomorphomonas, Pleomorphomonas koreensis* DSM23070 (71.57 %) and *Pleomorphomonas oryzae* DSM 16300 (71.54 %), belonging to *Alphaproteobacteria*. Other sequenced bacterial genomes belonging to genera of *Rhodobacterales* and *Rhizobiales* orders showed AAI values lower than 70% in comparison to strain E19^T (Chapter 8, Table.S1), confirming its taxonomical singularity among *Alphaproteobacteria*. Nevertheless, average nucleotide identity (ANI) and AAI are genome sequences pair-wise similarities analysis that typically give good resolution at species and sub species level (Konstantinidis and Tiedje, 2005). Moreover, they do not have enough resolution for genus delimitation because just about 10 % of the whole genome DNA sequence is used when distant genetic organisms are compared. Recently proposed percentage of conserved proteins (POCP) showed much less overlap in genus and family/order ranks and could be a

more suitable analysis for taxonomic delimitation (Qin et al., 2014). Furthermore, even the strong correlation between AAI values and 16S rRNA gene sequence, genome retrieved *Hartmannibacter diazotrophicus* E19^T 16S rRNA gene sequence (1472 bp) similarity and mean of (AAI) to next relative available genome sequences showed different rank order (Chapter 8, Table.1). In order to characterize the taxonomical rank of strain E19^T, isolation of related organisms and their complete genome available sequences, as well as suitable genome sequences pair-wise similarities analysis are needed.

The manual annotation of genes of strain E19^T draft assembly genome sequence is currently done. Encoding genes in genome consistently confirmed the pure culture PGP activities of E19^T for phosphate solubilization (genes *pqqBCDE, pstABCS, phoBHU*), nitrogen fixation (genes *nifHDK*), ACC deaminase production (gene *rimM*), motility (genes *flgIHG, fliGMPI*), catalase (gene *katG)* and superoxide dismutase (gene *sodB*). No genes have been found for siderophore production and IAA production. The presence of ACC deaminase gene was not detected by original and/or modified PCR protocol approach using specific primers for *acdS* gene (data not shown) in strain E19^T although it was reported to encode for ACC deaminase among *Proteobacteria* (Blaha et al., 2006). Interestingly, genome sequence confirmed absence of *acdS* gene but presence of *rimM,* which also codes for ACC deaminase (Gupta et al., 2014). The presence of *rimM* gene is likely to explain the ACC deaminase activity of the strain E19^T in pure culture and in the plant-bacteria interaction experiment (Chapter 7, Table.1). Complete annotation of genes will be useful in order to identify the presence of genes contributing directly or indirectly to enable PGPR effects on plants, salt tolerance, rhizocompetence, colonization and elucidation of metabolic pathways aiming. Sum of these results will be useful to better understand the mode of interaction of the bacterium and plants. Furthermore, it will be helpful for a comparative genome study with other PGPR strains sequenced genomes to analyze singularity and/or co-ocurrance of genes involved in plant growth promotion.

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Abbreviations

16S rRNA gene	16S ribosomal ribonucleic acid gene
AAI	Average amino acid identity
ABA	Abscisic acid
ACC	1-Aminocyclopropopane-1-carboxylic acid
ANOVA	Analysis of variance
ANI	Average nucleotide identity
BCCM	Belgian Coordinated Collections of Microorganisms
BLAST	Basic Local Alignment Search Tool
BNF	Biological nitrogen fixation
BSA	Bovine Serum Albumin
CAS	Chrome azurol S
CDS	Coding sequences
CFU	Colony-forming unit
CMC	Carboxymethyl-cellulose
CLSM	Confocal laser scanning microscopy
CMR	Comprehensive Microbial Resource
DAPI	4′,6-Diamidin-2-phenylindol
DDH	DNA–DNA hybridization
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DW	Dry weight
EC	Electrical conductivity
EDTA	Ethylene diamine tetra-acetate
EDGAR	Efficient Database framework for comparative Genome Analyses using BLAST score Ratios
ePGPR	Extracellular plant growth promoting rhizobacteria
ET	Ethylene
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
FW	Fresh weight
GC	Gas Chromatograph
GAs	Gibberellins
HGT	Horizontal gene transfer
IAA	Indole-3-acetic acid
IHP medium	Inositol hexaphosphate
iPGPR	Intracellular plant growth promoting rhizobacteria
IST	Induced systemic tolerance
JA	Jasmonic acid
KACC	Korean Agricultural Culture Collection
LBT	Luria Bertani Broth supplemented with L-tryptophan
LTP	Living Tree Project
L-TRP	L-tryptophan
MANOVA	Multivariate Analysis of Variance
MEGA	Molecular Evolutionary Genetics Analysis
mM	miliMolar

MPN	Most probable number
MLSA	Multi locus sequence analysis
NFb	N free malate
OD	Optical Density
P	Phosphorous
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PGPA	Plant growth promoting abilities
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PNS	Plant nutrient solution
PSB	Phosphate solubilizing bacteria
RDP	Ribosomal Database Project
ROS	Reactive oxygen species
SA	Salicylic acid
SRSM1	Sundara Rao and Sinha Modified 1
TSB	Tryptic Soy Broth
VOCs	Volatile organic compounds

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