DR. PAULA ANDREA VINCENT (Orcid ID : 0000-0002-4623-0085)

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# *Pseudomonas stutzeri* MJL19, a rhizosphere-colonizing bacterium that promotes plant growth under saline stress.

María Jesús Lami[1,2#], Conrado Adler[1#], María Carolina Caram-Di Santo[1], Ana María Zenoff[1], Ricardo E. de Cristóbal[1], Manuel Espinosa-Urgel[2]and Paula A. Vincent[1*]

[1] *Instituto Superior de Investigaciones Biológicas (INSIBIO-CONICET-UNT), Instituto de Química Biológica "Dr. Bernabé Bloj", Tucumán, Argentina.*

[2] *Department of Environmental Protection, Estación Experimental Del Zaidín. CSIC. Granada, Spain.*

**Running title:***P. stutzeri* MJL19 as PGPR in saline conditions

*Corresponding author: Paula A. Vincent. Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT and Instituto de Química Biológica "Dr. Bernabé Bloj", Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán. Chacabuco 461, CP 4000, San Miguel de Tucumán, Tucumán, Argentina. Tel: +54 381 4248921; email: vincent.paula@gmail.com.

[#] These authors contributed equally to this work

**ABSTRACT**

**Aims:** The aim of this study was to find and use rhizobacteria able to confer plants advantages to deal with saline conditions.

**Methods and Results:** We isolated 24 different bacterial species from the rhizosphere of halophyte plants growing in Santiago del Estero, Argentina salt flat. Four strains were selected upon their ability to grow in salinity and their biochemical traits associated with plant growth promotion. Next, we tested the adhesion on soybean seeds surface and root colonization with the four selected isolates. Isolate 19 stood out from the rest and was selected for further experiments. This strain showed positive chemotaxis towards soybean root exudates and a remarkable ability to form biofilm both *in vitro* conditions and on soybean roots.Interestingly, this trait was enhancedin high saline conditions, indicating the extremely adapted nature of the bacterium to high salinity. In addition, this strain positively impacted on seed germination, plant growth and general plant health status also under saline stress.

**Conclusions:** A bacterium isolate with outstanding ability to promote seed germination and plant growth under saline conditions was found.

**Significance and impact of Study:** The experimental approach allowed us to find a suitable bacterial candidate for a biofertilizer intended to alleviate saline stress on crops. This would allow the use of soil now considered inadequate for agriculture and thus prevent further advancement of agriculture frontiers into areas of environmental value.

Keywords: *Pseudomonas stutzeri,* rhizosphere, plant growth, saline, stress

**INTRODUCTION**

According to data from the Food and Agriculture Organization (FAO), an increase in crop production of around 70% will be required by 2050 to meet the needs of the rising world population(www.fao.org). However, incorrect land management and environmental factors related to climate change are reducing productive agricultural areas by 1-2% every year. Pressing problems include soil exhaustion and salinization, and dispersal of plant pathogens formerly confined to small

areas. Soil salinization, for example, affects around 7% of the land worldwide, with a deteriorating trend in Eurasia and South America (Pennock and McKenzie 2015). Salinization of agricultural lands is particularly widespread in arid and semiarid environments, where crop production requires irrigation schemes. At least 20% of all irrigated lands are salt-affected, with some estimates being as high as 50% (Pitman and Läuchli 2002).Soil salinity is a major concern for crop production: by reducing water availability, salinity creates a mineral nutrient imbalance caused by the elevated levels of $Na^+$ and $Cl^-$ ions (Leidi and Pardo 2002), thus altering normal plant growth and in consequence reducing crop yields. Even though salt tolerance in plants has been studied in detail; the knowledge gained has been limitedly transferred into crop improvement (Yuan *et al.* 2016).

As an alternative approach, plant growth promoting rhizobacteria (PGPR) have shown promising results in terms of improving production parameters for a series of agronomically relevant crops (Dodd and Pérez-Alfocea 2012). These microorganisms colonize the root surface and adjacent soil area (the rhizosphere), taking advantage of the nutrients present in root exudates, and they can improve nutrient and water uptake, modulate plant metabolism and/or protect plants against biotic and abiotic stress (Lugtenberg and Kamilova 2009).Their use could allow cultivation in less productive areas, reducing the input of chemical fertilizers and improving plant adaptation to hostile conditions. Microbial properties that are usually considered as indicators of plant growth promoting potential include nitrogen fixation, phosphate solubilization, ACC deaminase activity, production of siderophores and of phytohormones such as indole acetic acid (Goswami *et al.* 2016; Mishra *et al.* 2016).

PGPR-mediated improvement of plant-water relationships, ionic homeostasis and photosynthetic efficiency under salt stress have also been investigated, but a thorough understanding of the mechanisms involved remains elusive. Inoculation of plants with certain bacteria can result in alteration of plant responses to abiotic stress through direct and indirect mechanisms, leading to the so-called induced systemic tolerance (ITS; Yang *et al.* 2009).These mechanisms involve, for example, modulation of stomatal conductance, water and nutrient uptake, ion transport, root remodeling, production of osmolites, changes in the phytohormonal status, microbial production of exopolysaccharides that retain water and trap cations, etc. (Ilangumaran and Smith 2017).However,

the effectiveness of bacteria as bioinoculants requires any of these potentially plant-beneficial properties to be accompanied by efficient rhizosphere colonization and persistence in the presence of saline stress.

In this work we aimed to identify bacteria that could be potential candidates for the development of a bioinoculant for plant growth promotion under saline conditions. We have isolated bacteria associated to halophyte plants, assessed their ability to cope with high salt concentrations and tested properties associated with PGPR traits. One of these isolates, showing halotolerance and promising biochemical activities, has been characterized in detail. This strain, belonging to the *Pseudomonas stutzeri* species, stimulates growth of soybean plants under saline stress conditions and shows excellent colonization efficiency, likely as the result of its strong chemotactic response to root exudates and its biofilm formation capacity being enhanced in the presence of high salt concentrations.

## MATERIALS AND METHODS

### Sample collection and isolation of bacteria

Samples were collected from *Sesuvium portulacastrum* L. plants growing in a salt flat (29°48'10''S, 64°43'00''W) in Santiago del Estero province, Argentina. To isolate bacteria from the rhizosphere, soil loosely adhered to roots was removed by gently shaking. Roots having firmly adhered soil particles were cut and placed into sterile 50 mL screw-cap tubes containing 3 ml M9 minimal salts medium (Sigma Aldrich, Munich, Germany) (Sambrook and Russell 2001) supplemented with 0.2% casamino acids, 0.2% glucose, 1 mM $MgSO_4$ and 1 mg/ml B1 vitamin. The tubes were vortexed with 4 g of glass beads (3 mm diameter) for 2 min and serial dilutions were plated on selective medium (M9 supplied with sodium benzoate 10mM as a carbon source). The plates were incubated at 30°C overnight. Individual colonies were picked and streaked on fresh LB plates in order to be analyzed.

### Taxonomical identification of the isolates

For the rizospheric isolates identification, 16S rDNA were amplified with the universal oligonucleotide primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-

GGTTACCTTGTTACGACTT-3') described by Lane (1991). The sequences obtained were analyzed using the Targeted Loci Nucleotide BLAST tool available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

**Characterization of PGP-related activities**

*Total indole production.* Total indole production was detected with the method described by Glickmann and Dessaux (1995). Bacterial cultures were grown in M9 supplemented with 0.2% casaminoacids, 0.2% glucose, 1 mM $MgSO_4$ and B1 vitamin 1 mg/ml and L-tryptophan 0.1mg/mL for 48 h at 30°C. The cultures were centrifuged at 5000 rpm for 10 min. The supernatant was mixed with Salkowski reagent ($FeCl_3$ 12.5 g, $H_2SO_4$ 7.5 M) (1:1) and left in the darkness during 30 min. Development of pink colour indicates indole production. Optical density was measured with spectrophotometer at 540 nm. Concentration of indoles was measured using IAA (Sigma-Aldrich, USA) as standard, in the range of 3-30 µg/mL.

*Siderophore production.* Production of siderophores by plant growth promoting rhizobacteria was detected via the chrome azurol S assay (Schwyn and Neilands 1987). The 24 isolates were spotted on chrome azurol S agar plates (10 µL of $10^6$ CFU/mL), and development of a yellow-orange halo around the colony, indicative of siderophore production was analyzed after 48 h (Pérez-Miranda *et al.* 2007). Experiments were repeated three times each. Siderophore production was normalized with respect to colony growth and expressed as $[(Hd–Cd)/Cd] \times 100$, where Hd and Cd are halo and colony diameter respectively (Edi-Premono *et.al*. 1996).

*Phosphate solubilization.* The ability to solubilize inorganic phosphate was tested by growing the bacterial isolates on NBRIP agar plates in triplicate (Nautiyal 1999) for 6 days at 30°C as described by Gaur (1990). The formation of a transparent halo around the colonies was indicative of inorganic phosphate solubilization. The diameter of the halos was normalized with respect to colony growth and expressed as above.

*NaCl tolerance assays.* Salt stress tolerance was initially assessed on LB plates supplied with 0.5 M NaCl, where serial dilutions of overnight cultures of the isolates, diluted to an $OD_{600}$ = 1 were

spotted in triplicate. Growth was evaluated after 24h. Further analyses were done by following growth in liquid LB medium supplied with 0.5 M NaCl.

***Pseudomonas stutzeri* MJL19 genome sequencing and analysis**

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk) which is supported by the BBSRC (grant number BB/L024209/1).The genomic data of *P. stutzeri* MJL19 was obtained using Illumina next-generation sequencing with 30x coverage. Genome analysis and automated annotation were done with the PATRIC platform (Wattam *et al.* 2017; Brettin *et al.*, 2015), from the Bacterial Bioinformatics Resource Center (http://patricbrc.org). Sequence data have been deposited in GenBank (www.ncbi.nlm.nih.gov; BioProject ID: PRJNA610517; Accession number to be assigned).

**Bacterial adhesion to seeds and root colonization**

Soybean plants (*Glycine max* var. Munasqa), obtained from Estación Experimental Agroindustrial Obispo Colombres (Tucumán, Argentina) were used in the present study.

Adhesion to soybean seeds was evaluated as previously described (Espinosa-Urgel *et al.* 2000) with some modifications. Soybean (*Glycine max* L.) seeds were surface disinfected with 70% (vol/vol) ethanol for 30 s followed by immersion in 20% (vol/vol) sodium hypochlorite for 30 s and three successive rinses in sterile distilled water. Seeds were dried on a sterile plate for 30 min (De Gregorio *et al*. 2017).Overnight cultures of the strains under study were grown at 30°C in M9 medium supplemented with 0.2 mM glucose and diluted to an $OD_{600}$ of 1 (equivalent to approximately $10^9$ CFU/mL). These suspensions were used for seed inoculation (N=10) by immersion of the seeds for 10 min. After inoculation, seeds were washed briefly with distilled water to remove the bacteria that may not have been tightly attached to the seed surface. Seeds were then transferred to tubes containing 10 mL of sterile saline solution (M9 medium) and 4 g of glass beads (diameter, 3 mm). Cells attached to the seeds were removed by vortexing for 2 min, and serial dilutions of the suspension were then plated on M9 medium supplemented with sodium citrate as carbon source. Then the number of viable cells was quantified and informed as CFU/g seed. Two independent experiments were performed with three replications per treatment.

To determine soybean root colonization, seeds were disinfected and inoculated as described, and sown in pots with sterile sand (1.5 Kg capacity). Seedlings were maintained in a controlled chamber at 24°C with a daily light period of 16 h and irrigated with distilled water. The first irrigation was with 100 mL and the following irrigations were with 50 mL each 48 h. Plants were removed from the pots 10 days after seeding, and roots were cut, weighed and transferred to 50 mL Falcon tubes containing 10 mL of sterile saline solution and 4 g of glass beads. The tubes were vortexed for 2 min, the suspensions were collected and serial dilutions of the suspension were plated on M9medium supplemented with sodium citrate as carbon source. Then the number of viable cells was quantified and informed as CFU/g root. Two independent experiments were performed with four replicas per treatment.

**Chemotaxis assays**

The chemotactic response of *P. stutzeri* MJL19 ~~and *P. putida* KT2440~~ to soybean exudates was assayed first by depositing germinating seeds (previously disinfected as explained above) on 0.3% agar plates, close to the border. After 48h the seeds were extracted and 10 µl of a bacterial suspension ($OD_{600}$=1) were inoculated in the center of the plate. As controls, 10 µL of10% glucose or10% casaminoacids were used. Bacterial movement was analyzed after 5 days of incubation at 30°C.

Soybean root exudates were obtained from plants grown hydroponically for 6 days in 5 mL MS medium (Murashige and Skoog 1962) without or with 0.1 M NaCl, under sterile conditions. Exudates were collected and filtered through a 0.22 µm membrane, and used for quantitative chemotaxis assays, performed based on the microcapillary method of Martín-Mora *et al.* (2016) with some modifications. Cultures were grown in LB medium diluted 1/10 to an $OD_{600}$=0.4, washed and resuspended in chemotaxis buffer (30 mM $K_2HPO_4$, 19 mM $KH_2PO_4$, 20 µM EDTA and 0.05% glycerol (v/v), pH 7.0), to a final $OD_{600}$=0.1. 230 µl of the bacterial suspension was placed in a polystyrene multi-well plate. Capillaries (32 mm long, 1 µl capacity Microcaps capillaries; Drummond Scientific, USA) were thermally sealed at one end, filled with the obtained root exudates and immersed in the bacterial suspension by the open side. After 30 min of incubation, the capillary was removed, washed on the outside with water and emptied into an eppendorf tube containing 1 ml of M9

medium. Serial dilutions were plated in LB to determine the number of bacteria that had moved into the capillary (CFU/ml). Positive and negative controls were included (0.1% casaminoacids and chemotaxis buffer, respectively). The data are the means of three experiments performed independently and in triplicate.

**Biofilm formation assays**

Biofilm development in borosilicate glass tubes was followed during growth in liquid LB or LB supplied with 0.1 or 0.5M NaCl. In both cases, the $OD_{600}$ of the cultures was adjusted to 0.05at the start of the experiment, and tubes were incubated with orbital rotation at 40rpm at 30°C. At the indicated times, the liquid was removed and non-adherent cells were washed away by rinsing with distilled water. The biofilms were stained with 1% crystal violet (Sigma) for 15 min, followed by rinsing twice with distilled water. Photographs were taken, and the cell-associated dye was solubilized with 30% acetic acid and quantified by measuring the absorbance at 580 nm ($A_{580}$). Assays were performed in triplicate.

**Fluorescence microscopy**

Plasmid pCrdA::*gfp*, a fluorescence-based biosensor for the intracellular second messenger c-di-GMP (Rybtke*et al.* 2012) was introduced in MJL19 by electroporation, and used to visualize biofilms by fluorescence microscopy, using a Leica M165FC epifluorescence stereomicroscope, and to determine the influence of salt concentration on c-di-GMP contents.

**Plant growth promotion assays**

Plant growth promotion assays were done by inoculating surface sterilized soybean seeds with a suspension of MJL19, as described above. Seeds were immersed in the bacterial suspension ($10^9$cfu/mL) during 10 seconds and transferred to 0.5 kg sand pots. Two treatments, saline and not saline condition, were carried out irrigating with 100 mM NaCl solution and distilled water respectively. Plants were maintained in a growth chamber (Panasonic MLR) with 16 h light/ 8 h night photoperiod, 25°C and 80% RH. Six days after inoculation, seedlings were recovered and the following growth parameters were measured: shoot and root length, fresh and dry weight,

germination rate [GR% = (number of germinated seeds/total seed number used in the test) × 100] and the vigor index [(mean root length + mean shoot length) × GR] (Gholami*et. al.* 2009) were calculated. The experiment was done with 15 seeds per treatment and repeated three times. Data presented correspond to averages and standard deviations.

## RESULTS

### Isolation and characterization of rhizosphere halotolerant bacteria

Bacteria associated to the rhizosphere of halophite plants (*Sesuviumportulacastrum* L.) growing in the "Salinas Grandes"salt flat located in the Argentinean province of Santiago del Estero were isolated as indicated in the Experimental procedures section. Initially, 24 isolates were chosen based on different cultural features such as colony size, morphology, color and growth rate. Sequencing of the 16S rDNA, comparison with databases and phylogenetic analyses indicate that the 24 isolates belong to 7 genera, namely *Pseudomonas, Halomonas, Acinetobacter,Cronobacter, Enterobacter, Siccibacter* and *Bacillus*(Table 1 and Figure S1).

To select strains that could potentially function as PGPR under saline conditions, the tolerance of all the isolates to increasing concentrations of NaCl was tested by spotting serial dilutions of overnight cultures grown in LB medium onto plates of LB-agar with100 mM or 500 mM NaCl added. Most of the isolates grew normally at the lower concentration (data not shown), but only one third of them showed high tolerance in the presence of 500 mM NaCl (Table 2). Additionally, biochemical properties that have been usually associated with plant growth promotion were tested: total indole and siderophore production and phosphate solubilization. As shown in Table 2, indole and siderophore production were detected in most isolates, albeit to different levels, whereas the ability to solubilize inorganic phosphate was only relevant in a few cases. Based on these characteristics, four isolates were selected for further analysis: two *Pseudomonas* strains (isolates 5 and 19), one corresponding to *Halomonas* sp. (isolate 6) and one to *Cronobacter*sp.(isolate 24). Growth of the isolates in liquid medium in the presence of 500 mM NaCl confirmed their halotolerant nature (Figure 1).

Next, the ability of these four isolates to colonize soybean seeds and roots was tested, as a requirement for their efficiency as PGPR. Bacterial adhesion to seeds was determined after 10 minutes of incubation with each bacterial suspension (≈$3.5\times10^8$cfu/ml), as described in Experimental procedures, whereby the number of attached bacteria was calculated. As shown in Figure 2A, isolates 19 and 24 showed significantly higher seed colonization capacity than the other two strains. Similar results were obtained when root colonization was analyzed 5 days after seed inoculation, germination and growth in sterile sand (Figure 2B). In both experiments, isolate 19 showed the best performance, the number of recovered cells being 1-2 orders of magnitude higher than those corresponding to isolate 24. This and the fact that some *Cronobacter* species are responsible for food-associated opportunistic infections, made us select isolate 19 for detailed characterization. Amplification of a 625 bp fragment of its 16S rDNA with specific primers for *P. stutzeri* (Bennasar *et al.* 1998) supported the species level assignment, which was further confirmed by complete genome sequencing (see below). Consequently, we here ~~on~~ name isolate 19 as *Pseudomonas stutzeri* MJL19.

**Sequencing and genome analysis of *Pseudomonas stutzeri* MJL19**

A draft genome sequence of *P. stutzeri* MJL19 was obtained by Illumina sequencing. The assembled genome data (36 contigs, with a total length of 4,557,416 bp and an average G+C content of 64.26%) were submitted to the comprehensive genome analysis service at PATRIC (www.patricbrc.org; Davis *et al.*2016), and annotated using RAST tool kit (Brettin*et al.* 2015). The genome of MJL19 has 4,295 protein-coding sequences, 54 tRNA genes, and 7 rRNA genes. Phylogenetic analysis with reference and representative genomes (Figures S2 and S3), and in silico DNA-DNA hybridization analysis (Table 3) indicate that MJL19 is closely related to *Pseudomonas stutzeri* A1501, a nitrogen-fixing strain isolated from rice and considered an endophyte of this plant (Desnoues *et al.*, 2003). A preliminary survey of the automated annotation of MJL19 did not allow identification of any homologs of the *nif* genes, suggesting that this strain does not have nitrogen-fixing activity. Additional elements with a potential role in the interaction with plants and plant growth promotion include genes predicted to encode ACC deaminase (59% identical to this protein from *P. fluorescens* CHA0), inorganic phosphatases, auxin carrier family proteins, and siderophore (desferrioxamine/alcaligin-like) synthesis proteins. A gene encoding trehalose synthase is also present in the genome of MJL19,

suggesting that this microorganism could take advantage of trehalose as osmoprotectant under saline stress, as is the case for other plant growth promoting *Pseudomonas* species (Orozco-Mosqueda *et al.* 2019). Genes of relevance for bacterial colonization of biotic and abiotic surfaces could also be identified, such as those involved in the synthesis of flagella, type IV pili, curli, and at least three exopolysaccharides: Psl-like, cellulose and alginate.

**MJL19 shows positive chemotaxis response towards soybean root exudates**

Different reports have shown that bacterial motility and chemotaxis towards exudates are relevant traits for root colonization (de Weert *et al.* 2002; Capdevila *et al*. 2004; Martinez-Granero *et al*. 2006). Thus, the chemotactic response of MJL19 to soybean seed and root exudates was analyzed. Initially, qualitative assays were done on semi-solid agar plates (Figure S4). MJL19 showed positive chemotactic response to soybean seed exudates, as well as to casaminoacids, which are commonly used as chemoattractants (Yang *et al*. 2015). Glucose, on the other hand, failed to trigger a chemotactic response. Quantitative capillary assays (Martín-Mora *et al.*2016) were also carried out using root exudates of plants grown under non-saline and saline conditions. As shown in Figure 3, both types of exudates caused a similar positive chemotactic response, which was stronger than that resulting from casamino acids.

**Saline conditions stimulate attachment and biofilm formation by MJL19**

Besides chemotactic motility, bacterial surface colonization and biofilm formation are also relevant for the interaction with plants and efficient persistence in the root system (Yousef-Coronado *et al.* 2008; Martínez-Gil *et al.* 2010; 2013). We therefore analyzed biofilm formation during growth in borosilicate glass tubes and the influence of increasing NaCl concentrations on this process. Results are shown in Figures 4 A and B. Under these conditions, strain MJL19 showed significant attachment and biofilm formation, which increased with NaCl supplementation in a concentration-dependent manner, to the point that at 0.5 M NaCl most of the culture was either attached to the surface or forming large aggregates (Figure 4A).

In many bacteria, attachment and biofilm formation correlate with elevated levels of the intracellular second messenger cyclic diguanylate (c-di-GMP; Römling and Simm 2009; Boyd and O'Toole 2012). The above results would be compatible with MJL19 having physiologically high levels of c-di-GMP, that could also increase in response to NaCl. To test if this was the case, the c-di-GMP biosensor plasmid pCdrA::*gfp* (Rybtke *et al.*2012) was introduced in *P. stutzeri* MJL19 and fluorescence was examined after growth on LB-agarplates with 0, 100 and 500 mM NaCl. As a control, *Pseudomonas putida* KT2440 harboring the biosensor plasmid was also included, since this strain has physiological low levels of c-di-GMP that increase in response to NaCl (Ramos-González *et al.* 2016; Tagua *et al.*; submitted). As shown in Figure 5, high fluorescence was detected in MJL19 harboring pCdrA::gfp, with a significant intensification in response to NaCl consistent with the increased biofilm formation observed in saline conditions. In these experiments, we observed changes in colony size and morphology due to NaCl (Figure 5), suggesting that extracellular matrix components (exopolysaccharides and/or proteins) could be influenced by salinity.

Taking advantage of the pCdrA::*gfp* biosensor, we also evaluated biofilm formation and c-di-GMP contents on soybean roots. Figures 6Band C show that *P. stutzeri* MJL19 grows forming thick biofilms on the root surface both in absence and presence of NaCl, respectively. As observed with bacteria growing in solid medium, fluorescence intensity is seemingly higher for bacteria colonizing roots in saline conditions (Figure 6C).As a control, Figure 6A shows that there is no fluorescence in the soybean roots without inoculation.

**_P. stutzeri_ MJL19 promotes seed germination and plant growth under saline conditions**

To further explore the potential use of *P. stutzeri* MJL19 in agrobiotechnology, we evaluated its impact on soybean germination and plant growth under saline and non-saline conditions. Figure 7A shows that meanwhile NaCl (100 mM) reduced germination rate by 10% when seeds were not inoculated; inoculation with *P. stutzeri*MJL19 increased the germination rate by 10% in non-saline conditions and 20 % in saline conditions. Even though saline conditions did not affect root length; bacterial inoculation increased it in both saline and non-saline conditions (Figure. 7B). For hypocotyl length salinity negatively affected this parameter, but this effect was compensated when seeds were

inoculated with *P. stutzeri*MJL19 (Figure. 7C).Bacterial inoculation also favored hypocotyl grow thin non saline conditions. Accordingly, analysis of the vigor index (VI) showed a higher value for seeds inoculated with *P. stutzeri* MJL19 in both saline and non-saline conditions (Figure 7D). Finally, seed inoculation positively impacted on fresh and dry weight parameters in both saline and non-saline conditions (Figure 7E and F).

Further experiments were done in greenhouse conditions using non-saline and saline soil (Table S1)and plant growth was monitored over a period of 5 weeks. Results are presented in Figure 8. Interestingly, bacterial inoculation had no effect on stem length of plants grown in non-saline soil. In contrast, *P. stutzeri* MJL19 significantly increased this parameter in saline soil from week 3 until the end of the experiment (Figure 8A). Consistently, dry weight of roots and aerial part of 5 weeks-old plants showed higher values when they were inoculated with *P. stutzeri* MJL19 and grown in saline soil (Figure 8B).In order to address the general health status of plants, relative chlorophyll content index (SPAD) was measured as reported by Chang and Robison 2003. In non-saline soil, non-inoculated and inoculated plants showed no differences in chlorophyll content along the 5 weeks duration of the experiment (Figure 8C). However, comparison of inoculated plants with non-inoculated ones grown in saline soil, showed an increasing trend of SPAD index from week 3 and on, becoming significant in week 5 (Figure 8C). These results are indicative of higher chlorophyll content and better nutritional status of the plants in the presence of the bacterium. All these results support the plant growth promoting properties of MJL19 under saline stress conditions.

**DISCUSSION**

Soil salinity is a major concern for crop production worldwide, but of particular relevance in certain areas. The rapid and expansive land-use changes occurring in semiarid regions of Latin America are prime examples of this problem (Grau *et al.* 2005; Gasparri and Grau 2009). Currently, Argentina, Brazil, China and the United States are responsible for more than 95% of soybean production worldwide. In Argentina, expansion of soybean cultivation and its overexploitation have resulted in negative effects on soils such as desertification and salinization, leading to increasing needs for fertile lands that in turn have caused extensive forest clearing. Attempts to develop a more

sustainable agriculture comprise the use of microorganisms that impact plant physiology in a positive way (Glick 2014;Mishra *et al.* 2016) including increased resistance to soil salinity (Smith *et al.* 2017;Yang *et al.* 2009). Among all bacteria associated with plants, those residing in the rhizosphere are the ones with the greatest effect. In this work, we isolated bacteria from the rhizosphere of succulent plants (*Sesuvium portulacastrum*) growing in the Salinas Grandes salt flat. We postulated that plants and associated microbes constitute a holobiont that allows growth in such aggressive saline conditions (Qin *et al.* 2016). In fact, isolated strains from native desert plants conferred salinity stress tolerance to the experimental model plant Arabidopsis thaliana (Eida *et al.* 2018). In our work, plants sampled were located in an area in close proximity to soybean fields, which at the moment form part of the crop frontier. This border is established by the harsh agricultural conditions and surpassing it requires dealing with saline stress. As expected, we isolated several bacteria with high salinity resistance, belonging to typical genera isolated from bulk soil and rhizosphere. Among them, *P.stutzeri* MJL19 has shown promising potential as PGPR for soybean under saline conditions. The efficiency of root colonization by *P. stutzeri*MJL19 correlates with the observation that soybean exudates have a strong positive chemoattracting effect on this bacterium. Chemotaxis towards root exudates is considered a relevant rhizosphere fitness determinant (Joseph *et al.* 2007; Molina *et al.* 2004; Van Bastelaere *et al.* 1999). The chemical nature of root exudates varies greatly among different plant species (Vives‐Peris *et al.* 2019) and the microbial ability to perceive and respond to these molecules is also quite variable (Bacilio-Jiménez *et al.* 2003; de Weert *et al.* 2002; Hardoim*et al.* 2008;Vílche*et al.* 2000). Interestingly, root exudates obtained from plants grown on non-saline or saline conditions caused the same response (Figure 3), suggesting that the amount of chemotactically active compounds does not vary significantly between the two conditions. It is of particular interest to define which molecule(s) are involved in the observed response. Another feature generally used for predicting PGPR potential is biofilm formation (~~Lugtenberg and Kamilova 2009)~~ (Seneviratne *et al* 2010)~~.~~ Consequently, in this work we evaluated *P. stutzeri*MJL19 ability and dynamics of biofilm formation. Previous work has shown that in other *Pseudomonas* strains, such as *P. putida* KT2440, the dynamics of biofilm formation in rich medium include a fast attachment and biomass accumulation early during growth, followed by detachment

after reaching stationary phase (Yousef-Coronado *et al.* 2008). In contrast, in *P. stutzeri* MJL19 the sessile population was retained in borosilicate tubes even after 48h (Figure 4). Interestingly, addition of NaCl increased biofilm formation in *P. stutzeri* MJL19, to a point that resulted in a nearly complete loss of turbidity of the liquid culture. Biofilm formation is associated with a raise in c-di-GMP levels (Römling and Simm2009). We observed in this work that *P. stutzeri* MJL19 has naturally high c-di-GMP levels which remarkably further increase with salinity both *in vitro* and in soybean roots (Figures 5 and 6).Analysis of the genome of *P. stutzeri* MJL19 indicates the presence of at least 11 genes encoding proteins with potential diguanylatecyclase activity, based on the presence of GGDEF domains. The precise contribution of these and other elements to biofilm formation and response to high NaCl concentrations will be pursued in the future. In any case, the combination of positive chemotaxis response and strong biofilm formation, also observed on root surfaces, are likely key to the success of *P. stutzeri*MJL19 in the colonization of soybean roots. These features are actually expected given that bacteria selection was partially based on seed adhesion and root colonization (Figure 2 A and B).

In addition, *P. stutzeri* MJL19 increased seed germination, particularly in saline conditions (Figure 7A). Finally, results obtained in greenhouse experiments with saline soil confirm the PGPR activity of this strain in these conditions, while the effect is less evident under non-saline conditions. Given its native habitat and the observation that *P. stutzeri* MJL19 grows slightly better in liquid cultures supplemented with NaCl (Figure 1), it seems reasonable to think that *P. stutzeri* MJL19 is highly adapted to salinity and therefore its physiological performance is enhanced on those conditions. Microbes can achieve plant protection by reprogramming plant physiological changes induced by salinity stress (Ilangumaran and Smith 2017; Radhakrishnan and Baek 2017). One of the possible mechanisms involved in restoring plant ionic homeostasis implies the production of exopolysaccharides (EPS) that trap ions in the rhizosphere (Arora *et al.* 2010; Ashraf *et al.* 2004). Consistent with this hypothesis, we evidenced EPS production by this strain (data not shown) and~~we~~ observed colony morphology changes upon NaCl addition(Figure 5). These changes are likely associated with variations of matrix components such as various types of EPS (Martínez-Gil *et al*. 2013; Zhu *et al*. 2014). Nevertheless, a combination of mechanisms is more likely. In fact, it was

recently described that a strain of *Pseudomonas stutzeri* mitigates salt stress in *Brassica napus* L. by activating the antioxidant defense system of plants and by triggering the rearrangement of cell walls, which, consequently, promotes plant growth (Szymańska*et al* 2019).Hence, a thorough mechanistic study revealing the role of each component in our system is yet to be done.

The positive results obtained with *P. stutzeri* MJL19 make of this strain a good candidate for the development of a biofertilizer specific for crops in saline soils. Evaluation of this strain under field conditions is currently ongoing. Our expectation is to contribute with a new resource for turning saline soils into adequate soils for agriculture and thus reduce the negative impact of forest clearing.

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**CONFLICT OF INTEREST**

Authors declare no conflict of interest

**AUTHOR CONTRIBUTION**

CA, PAV, MJL,MEU, REDC Designed experiments and analyzed results

MJL, AMZ,CCDS Performed experiments

CA, PAV, MEU Wrote the manuscript

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**FIGURE LEGENDS**

**Figure 1.** Growth of selected isolates in M9 minimal medium with glucose as carbon source, without (open circles) or with (closed circles) 0.5 M NaCl. Data correspond to averages and standard deviations from four replicas. A representative experiment is shown.

**Figure 2.** Adhesion on soybean seeds (A) and root colonization (B) by the four selected isolates. Experiments were carried out as described in material and methods section. Data correspond to averages and standard deviations from 10 seeds/plants.

**Figure 3.** Quantitative capillary chemotaxis assay of *P. stutzeri* MJL19 towards soybean root exudates obtained from plants growing without (RE) and with 100 mM NaCl (RE_NaCl). CAA 0.1% corresponds to 0.1% of casamino acids. The control corresponds to buffer. Data are the averages and standard deviations of three independent experiments with triplicate samples. Different letters indicate statistically significant differences ($P \leq 0.05$).

**Figure 4.** Biofilm formation on abiotic surface. The formation was analyzed for bacteria growing 4, 24 and 48 h in LB medium only or with100 mM and 500 mM NaCl, at 40 rpm in an orbital shaker (A). Crystal Violet measurement ($DO_{540}$) (B). Data represent mean values ± standard error. Different letters indicate statistically significant differences ($P \leq 0.05$).

**Figure 5.** Influence of salinity on colony morphology and c-di-GMP content in *P. stutzeri*MJL19.IP, pixel intensity per colony diameter for *P. stutzeri*MJL19 pCdrA and *P. putida*KT2440 pCdrAgrowing on LB plates, LB with 100 and 500 mM NaCl. Data represent mean values ± standard error. Different letters indicate statistically significant differences ($P \leq 0.05$).Photographs of colonies of *P. stutzeri*MJL19 pCdrA growing in LB (19LB), LB with 100 mM NaCl (19LB100)or LB with 500 mM NaCl (19LB500) and *P. putida* KT2440 pCdrA growing in LB (KTLB), LB with 100 mM NaCl (KTLB100) or LB with 500 mM NaCl (KTLB500).

**Figure 6.** *P.stuzeri* MJL19 pCdrA growth on the surface of soybean roots without (B) and with 100 mM NaCl (C). Fluorescence intensity is indicative of biofilm formation. (A) control with no inoculation.

**Figure 7**. *P.stutzeri*MJL19 inoculation effect on soybean germination(A), root length (B), hypocotyl length (C), vigor index (D), fresh weight (E) and dry weight (F) in non-saline (H2O) and saline conditions (100 mM NaCl). Soybean seeds were inoculated with *P.stuzeri*MJL19 as described in material and methods section and not inoculated (C-negative control). Data represent mean values± standard error. Different letters indicate statistically significant differences ($P \leq 0.05$).

**Figure 8.** Plant growth promotion in saline soil. Soybean seeds inoculated with *P.stuzeri*MJL19 or without inoculation (c) were cultivated in pots containing non-sterile saline soil. Plants were kept under greenhouse conditions for 5 weeks and stems length was measured weekly (A).After that time, plants were removed and dry weight was evaluated (B).SPAD index was evaluated weekly during the 5 weeks period of the experiment (C). Data represent mean values ± standard error. Different letters indicate statistically significant differences ($P \leq 0.05$).

**Table 1.**Identification of culturable bacterial isolates from the rhizosphere of *Sesuvium portulacastrum*, based on 16S rDNA sequences and database comparison using Targeted Loci Nucleotide BLAST.

| Isolate | Best match | Alignment identities and % |
|---|---|---|
| **1** | *Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28 | 1406/1407 (99.9%) |
| **2** | *Bacillus licheniformis* DSM 13 | 1389/1403 (99%) |
| **3** | *Pseudomonas zhaodongensis* NEAU-ST5-21 | 1388/1400 (99.1%) |
| **4** | *Pseudomonas xanthomarina* KMM 1447 | 1378/1395 (98.8%) |
| **5** | *Pseudomonas knackmussii* B13 | 1399/1410 (99.2%) |
| **6** | *Halomonasandesensis*LC6 | 1367/1389 (98.4%) |
| **7** | *Enterobacter cloacae* subsp. *dissolvens* LMG 2683 | 1393/1412 (98.7%) |
| **8** | *Bacillus cereus* ATCC 14579 | 1403/1412 (99.4%) |
| **9** | *Cronobacterdublinensis* subsp.*lausannensis* E515 | 1396/1402 (99.6%) |
| **10** | *Pseudomonas stutzeri* ATCC 17588 | 1391/1391 (100%) |
| **11** | *Pseudomonas alcaliphila* NBRC 102411 | 1380/1393 (99%) |
| **12** | *Pseudomonas zhaodongensis* NEAU-ST5-21 | 1384/1399 (98.9%) |
| **13** | *Pseudomonas chloritidismutans* AW-1 | 1383/1393 (99.3%) |
| **14** | *Bacillus aryabhattai* B8W22 | 1410/1411 (99.9%) |
| **15** | *Bacillus aryabhattai* B8W22 | 1409/1410 (99.9%) |
| **16** | *Pseudomonas taiwanensis* BCRC 17751 | 1362/1366 (99.7%) |
| **17** | *Bacillus tequilensis* 10b | 1402/1402 (100%) |
| **18** | *Enterobacterxiangfangensis* 10-17 | 1368/1389 (98.5%) |
| **19** | *Pseudomonas stutzeri* ATCC 17588 | 1377/1377 (100%) |
| **20** | *Siccibactercolletis* 1383 | 1388/1396 (99.4%) |
| **21** | *Pseudomonas zhaodongensis* NEAU-ST5-21 | 1364/1379 (98.9%) |
| **22** | *Acinetobacter gyllenbergii* RUH 422 | 1375/1401 (98.1%) |
| **23** | *Pseudomonas stutzeri* ATCC 17588 | 1395/1397 (99.8%) |
| **24** | *Cronobactercondimenti* 1330 | 1367/1367 (100%) |

**Table 2.** NaCl tolerance and PGPR biochemical indicators of the 24 isolates.

| Isolate | 500 mM NaCl[a] | Total indole production[b] | Siderophore production[c] | Phosphate solubilization[d] |
|---|---|---|---|---|
| **1** | ++ | + | ++ | + |
| **2** | + | + | + | + |
| **3** | + | + | +++ | + |
| **4** | + | ++ | +++ | - |
| **5** | ++ | ++ | +++ | + |
| **6** | ++ | +++ | +++ | + |
| **7** | +++ | + | ++ | + |
| **8** | + | + | + | - |
| **9** | +++ | ++ | ++ | + |
| **10** | + | + | ++ | - |
| **11** | +++ | + | +++ | + |
| **12** | + | + | ++ | - |
| **13** | ++ | + | +++ | + |
| **14** | + | + | + | +++ |
| **15** | +++ | + | + | + |
| **16** | + | + | + | + |
| **17** | +++ | + | + | + |
| **18** | ++ | ++ | +++ | ++ |
| **19** | +++ | +++ | +++ | ++ |
| **20** | +++ | ++ | ++ | + |
| **21** | ++ | ++ | +++ | ++ |
| **22** | ++ | + | + | + |
| **23** | - | + | ++ | - |
| **24** | +++ | ++ | +++ | ++ |

[a]Isolates were classified as follows,based on growth after 24 h on LB plates with 500 mM NaCl: highly resistant (+++), growth observed in $10^{-3}$ dilution spots; moderately resistant (++), growth observed in $10^{-2}$ dilution spots; with low resistance (+), growth observed in $10^{-1}$ dilution spots; salt-sensitive (-), no growth observed in $10^{-1}$ dilution spots.

[b]Total indol production: ≤10 µg/ml IAA (+), 10-20 µg/ml IAA (++),≥20 µg/ml IAA (+++).

[c]Siderophore production (SP=HD/CD): SP = 1 (-), SP between 1.0 and 1.2 (+), SP between 1.2 and 1.5 (++) and SP ≥ 1.5 (+++).

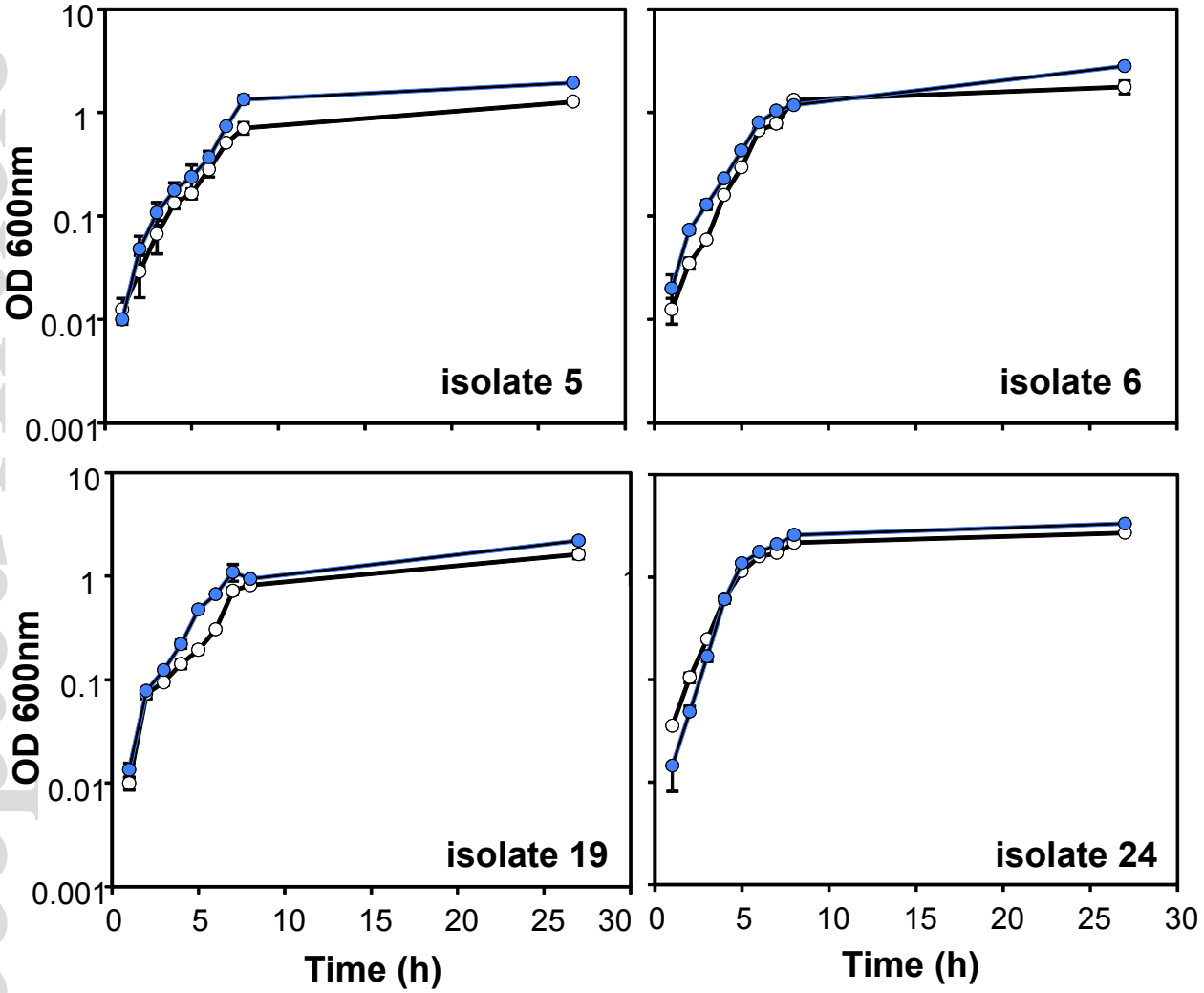
[d]Phosphate solubilization index (PSI=HD/CD): PSI = 1 (-), PSI between 1.0 and 1. 2 (+), PSI between 1.2 and 1.5 (++) and PSI ≥ 1.5 (+++).

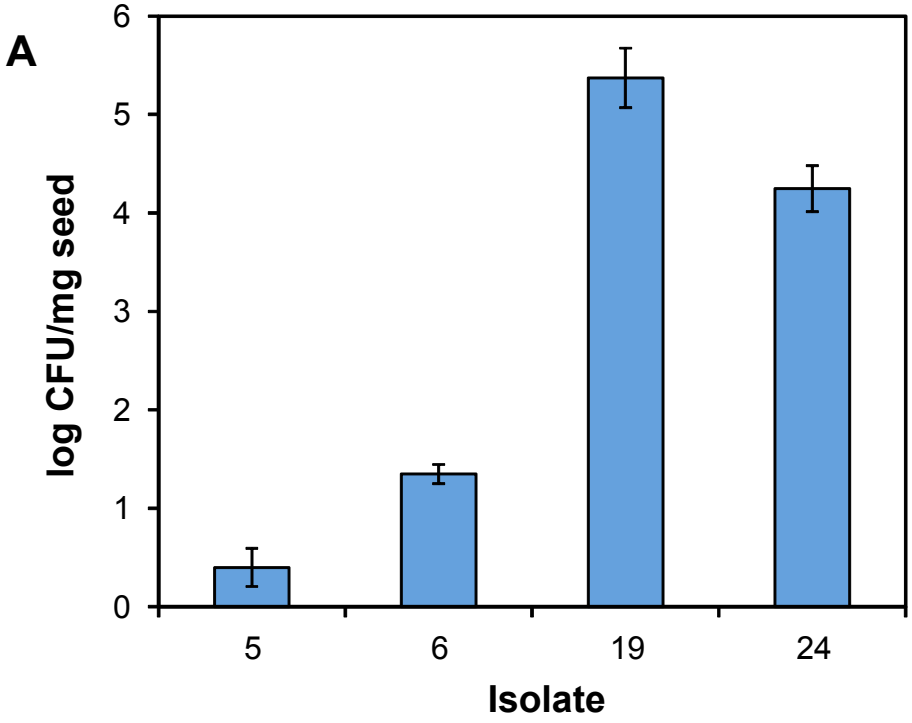
HD: halo diameter, CD: colony diameter.

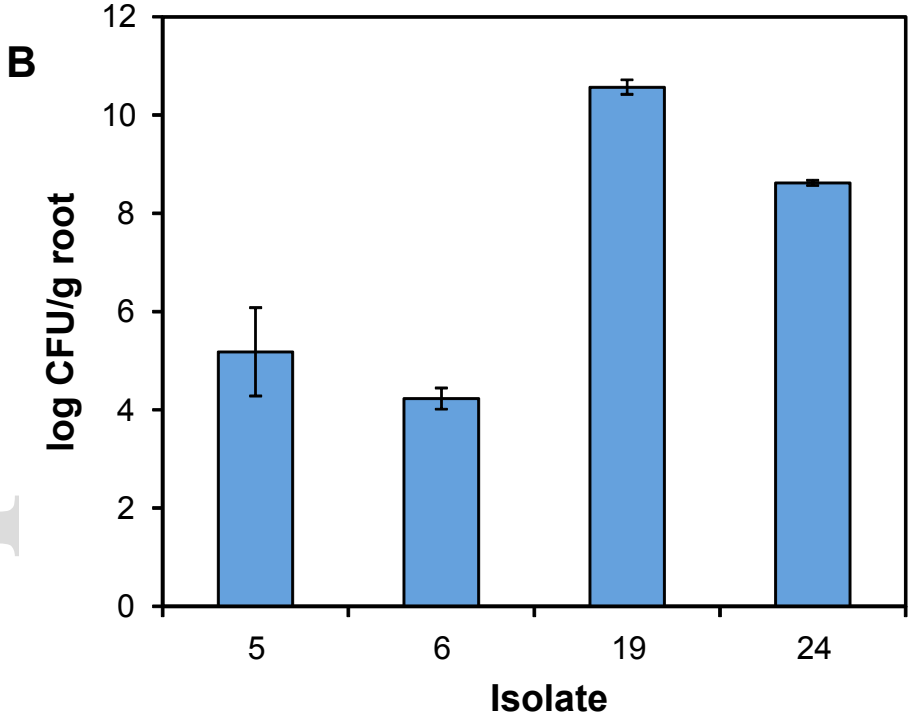
**Table 3.** In silico DNA-DNA hybridization(dDDH) analysis of the genome of MJL19

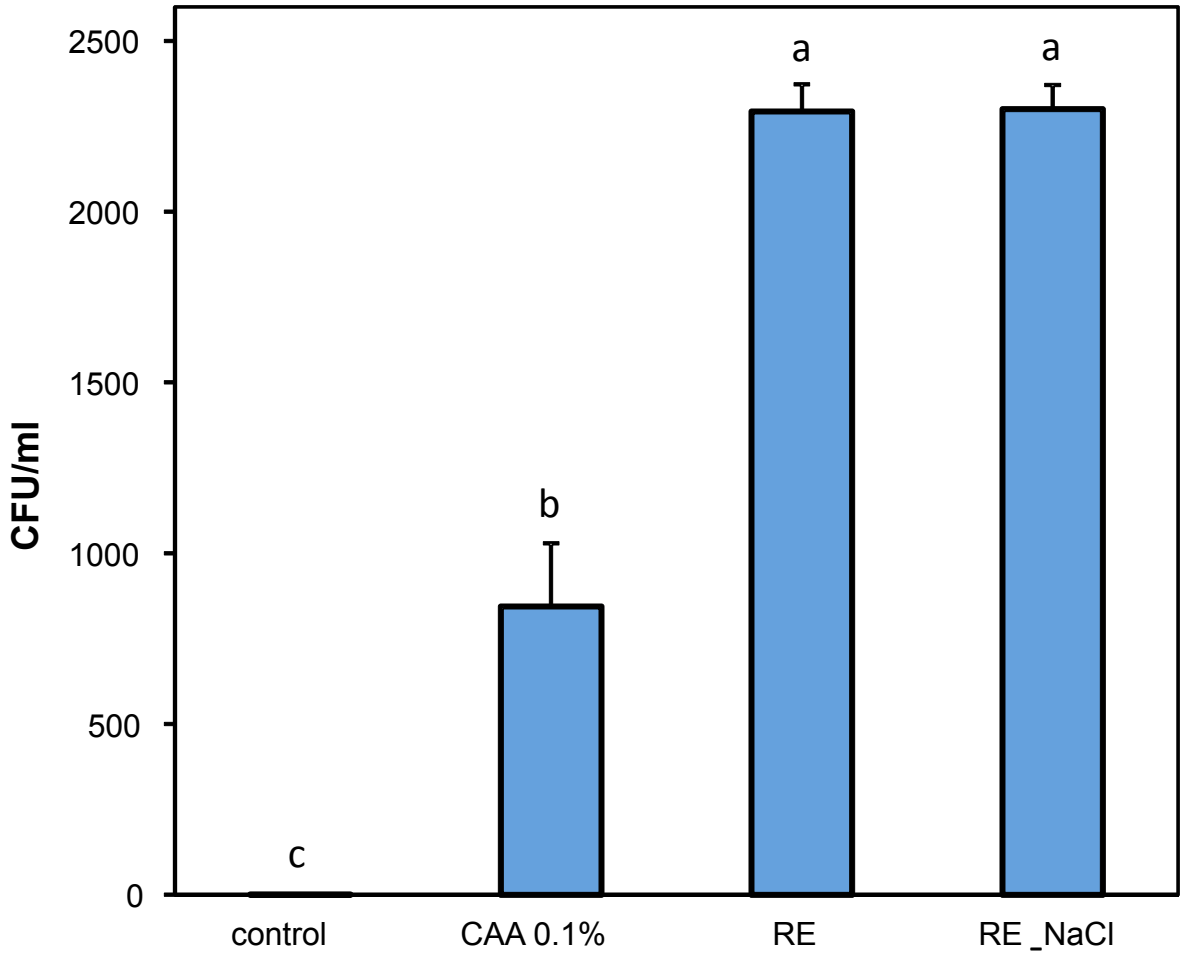
| Subject strain | dDDH d0, % | C.I. (d0, %) | dDDH d4, % | C.I. (d4, %) | dDDH d6, % | C.I. (d6, %) | G+C content difference (%) |
|---|---|---|---|---|---|---|---|
| *Pseudomonas stutzeri* ATCC 17588 | 80.6 | [76.7–84.0] | 81.9 | [79.0–84.4] | 83.8 | [80.5–86.6] | 0.33 |
| *Pseudomonas songnenensis* NEAU-ST5-5T | 80.2 | [76.3–83.6] | 34.1 | [31.7–36.7] | 68.2 | [64.8–71.4] | 1.01 |
| *Pseudomonas chloritidismutans* DSM 13592 | 60.6 | [56.9–64.1] | 31.3 | [28.9–33.8] | 52.3 | [49.2–55.4] | 1.76 |
| *Pseudomonas kunmingensis* DSM 25974 | 68.8 | [64.9–72.5] | 31.1 | [28.7–33.6] | 58 | [54.8–61.2] | 1.64 |
| *Pseudomonas perfectomarina* CCUG 16156 | 68.5 | [64.6–72.2] | 29.6 | [27.2–32.1] | 56.8 | [53.7–60.0] | 2.87 |
| *Pseudomonas nitrititolerans* GL14 | 34.6 | [31.3–38.2] | 24.1 | [21.8–26.5] | 30.9 | [28.0–34.0] | 1.21 |
| *Pseudomonas balearica* DSM 6083 | 42.6 | [39.2–46.0] | 24 | [21.7–26.5] | 36.4 | [33.4–39.4] | 0.44 |
| *Pseudomonas saudiphocaensis* 20 BN | 29 | [25.6–32.6] | 23.1 | [20.8–25.5] | 26.5 | [23.7–29.7] | 3.02 |
| *Pseudomonas nosocomialis* A31/70 | 32.9 | [29.5–36.5] | 23.1 | [20.8–25.6] | 29.4 | [26.5–32.5] | 1.3 |
| *Pseudomonas guguanensis* JCM 18416 | 22.7 | [19.4–26.3] | 22.5 | [20.2–24.9] | 21.7 | [18.9–24.7] | 0.04 |
| *Pseudomonas pseudoalcaligenes* NBRC14167 | 20 | [16.8–23.6] | 22.4 | [20.1–24.9] | 19.4 | [16.7–22.5] | 2.01 |
| *Pseudomonas toyotomiensis* JCM 15604 | 20.3 | [17.2–24.0] | 22 | [19.8–24.5] | 19.7 | [17.0–22.7] | 1.65 |
| *Pseudomonas zhaodongensis* NEAU-ST5-21 | 27.2 | [23.9–30.9] | 21.5 | [19.3–23.9] | 24.9 | [22.1–28.0] | 4.67 |
| *Pseudomonas guariconensis* LMG 27394 | 16.9 | [13.8–20.4] | 21.1 | [18.8–23.5] | 16.7 | [14.2–19.7] | 1.66 |

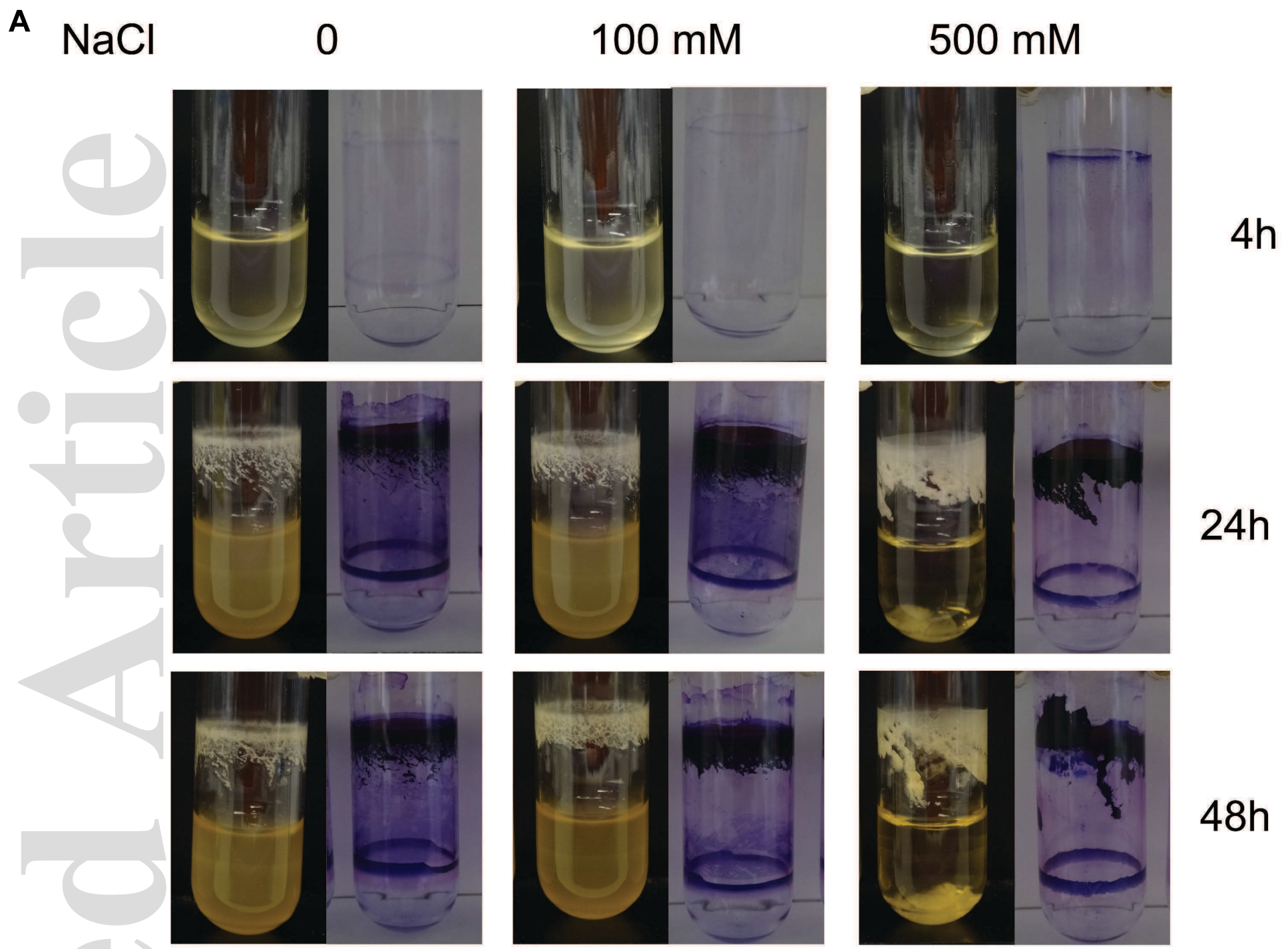
Data obtained from the Type Strain Genome Server (TYGS; https://tygs.dsmz.de) with automatically determined closest type strains. The dDDH % values are provided along with their confidence intervals (C.I.) for the three different formulas used: d0 (length of all HSPs divided by total genome length), d4 (sum of all identities found in HSPs divided by overall HSP length) and d6 (sum of all identities found in HSPs divided by total genome length).

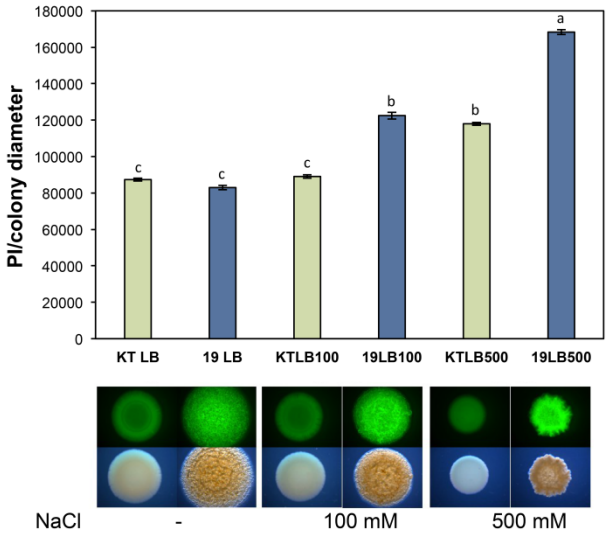








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