

Plant holobiont interactions mediated by the type VI secretion system and the membrane vesicles: promising tools for a greener agriculture

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Summary

A deeper understanding of the complex relationship between plants and their microbiota is allowing researchers to appreciate a plethora of possibilities to improve crops using chemical-free alternatives based on beneficial microorganisms. An increase in crop yield from the promotion of plant growth or even simultaneous protection of the plants from the attack of phytopathogens can be achieved in the presence of different plant-associated microorganisms known as plant-growth-promoting rhizobacteria (PGPR) and biocontrol agents (BCAs), respectively. Thus, the study of the great diversity of plant-microbe and microbe-microbe interactions is an attention-grabbing topic covering studies of interactions since the plant seed and through all developmental stages, from root to shoot. The intricate communication systems that plant holobionts co-evolved has resulted in many different strategies and interplays between these organisms shaping the bacterial communities and the plant fitness simultaneously. Herein, we emphasize two understudied delivery systems existing in plant-associated bacteria: the type VI secretion system (T6SS) and the membrane vesicles with a huge potential to boost a highly demanded and necessary green agriculture.

Introduction

We live in an overpopulated world of 7.7 billion persons that continue growing in a worryingly exponential trend and without deceleration. The latest estimations from the United Nations indicate that the world's population will reach 9.7 billion by 2050, that is a 2 billion increase in the upcoming 30 years (United Nations, 2019). This overpopulation exposes many challenges including finding a formula to cover the nutritional needs of billions with a sustainable approach to preserve our planet. Extensive agriculture has made possible to feed the enormous number of people inhabiting the Earth in the last decades. The agricultural practices managed in extensive farming is supported by the use of fertilizers that allow high yield in short periods of times and pesticides/insecticides that avoid losing crops due to plagues and diseases (infections induced by bacteria, fungi or small animals). However, fertilizers and pesticides have a critical drawback, they both are detrimental to the environment being responsible for the hazardous contamination of soil and water. The dangerous effects are uncountable including human and animal poisoning and degradation of soils (Sharma and Singhvi, 2017). An alternative line of action is the so-called "green agriculture" or "sustainable agriculture" that refers to an agricultural production system able to sustain the need of the growing population and parallelly the urge to protect and preserve our planet. To cover these two key points, it will be necessary to continue using fertilizers and pesticides to maintain the necessary crop yield to feed the global population but those should not be a source of contamination. Fertilizers and pesticides based on beneficial microorganisms are a clever solution to the current dilemma avoiding the use of toxic chemical compounds. Organisms with the capacity to promote plant growth (PGPR) including nitrogen-fixing bacteria, and control pests (BCAs) have been used in the past and widely demonstrated their worthiness (Lucke *et al.*, 2020). A deeper understanding of the physiology of these microorganisms and the molecular mechanisms responsible for the positive effects on crops will allow extensive and efficient use of green fertilizers and pesticides that will become a real and clean alternative for the agriculture of the future. Here, we are focusing on two delivery systems used by beneficial bacteria to assist

plants: the type VI secretion system (T6SS) and the membrane vesicles. These systems have been discovered fairly recently and current investigations in the fields are showing encouraging data regarding the value of these systems to become important tools in agricultural biotechnology applications.

Type VI secretion system

The T6SS is a molecular weapon present in gram-negative bacteria and used to outcompete other microorganisms encountered in close-contact within their ecological niche. The T6SS is structurally related to bacteriophages although they are located inside the producer bacterium and partially ejected outward. The spring-like nanomachine is loaded with effectors that are directly secreted inside target cells inhibiting their growth, provoking cell death or manipulating the host. Most T6SS effectors have anti-bacterial activity and have been proven quite efficient against other gram-negative bacteria although anti-eukaryotic effectors against animal and fungi cells have been also described (Allsopp *et al.*, 2020). Antimicrobial effectors target nucleic acids (nucleases) or other essential cytoplasmic elements (ADP-ribosyl transferases (Tre1), glycohydrolases (Tne), and (p)ppApp synthases (Tas1) or disrupt the cell wall (amidases, muramidases) or the cytoplasmic membrane (lipases, pore-forming bacteriocins) (Hernandez *et al.*, 2020). The arsenal of T6SS effectors has continued to grow since the first identified T6 effector (Hood *et al.*, 2010) and nowadays about ten to twenty has been described for *Pseudomonas aeruginosa* and *Vibrio cholerae*. More likely, we are just spotting the tip of the iceberg since most T6 effectors lack a hallmark to be recognized as such and unbiased approach are experimental-setting dependent and might not reveal a complete landscape. When comparing with the average number of effectors in other secretion systems that have been studied for many decades such as T3SS (ranging from 15 to 50 per bacteria), we could think that the number of T6 effectors will continue to increase too. The studies of T6SS in plant-associated bacteria are under-represented in the literature (Bernal

et al., 2018) and they exemplify an excellent source for identification of new effectors with the potential to target plant pathogens and interestingly to directly interact with plant cells (beneficial and deleterious effects).

T6SS has been extensively studied for the last 14 years and although the structure and the general mechanism of action are known (Bernal *et al.*, 2021), there are still many different aspects of this secretion machine to be further explored and discovered. Among the main mysteries of T6SSs are the signals that trigger expression, assembling and firing of this effective but otherwise energetically-costing killing machine. In some species such as *P. aeruginosa*, the system is surface-contact-dependent and touching a solid surface is a signal that activates the system (Silverman *et al.*, 2011). In these bacteria, the presence of sister cell lysis has been described as a stimulus for T6SS expression (LeRoux *et al.*, 2015) and membrane stress, in general, has been seen to activate T6SS (Kamal *et al.*, 2020; Stolle *et al.*, 2021). In other cases, specific environmental signals such as components of a host cell (i.e. chitin) are responsible for T6SS activation (Borgeaud *et al.*, 2015). Since most of the studies have been done with human pathogens including *P. aeruginosa* and *V. cholerae*, little is known of the signals triggering T6SS activation in the context of plants. Most T6SSs described in plant-associated bacteria are involved in inter-bacterial competition whether they are pathogens or beneficial microorganisms (Bernal *et al.*, 2018). One of the best-understood systems in this context is that of the plant pathogen *Agrobacterium tumefaciens*. The system is induced in low environmental pH, a condition found around wounds in plants (Wu *et al.*, 2012) where outcompeting foes can be key to pathogenesis. In beneficial microbes as *P. putida*, the T6SS has been described as a major mechanism of biocontrol (Bernal *et al.*, 2017) and in nitrogen-fixing bacteria, it seems to play a role in symbiosis (Salinero-Lanzarote *et al.*, 2019). The systems are commonly active in the stationary phase of growth but most environmental cues further inducing the systems are still unknown.

Studies expanding our understanding of the regulation of T6SSs in plant-associated bacteria and those uncovering new effectors involved in modulating the plant microbiota or plant-microbe

interactions will be pivotal to advance in this field. Specific examples and future perspectives of the different uses of T6SS to improve crop production will be detailed in a section below.

Membrane vesicles

Two types of membrane vesicles are encountered in nature depending on their cellular localization: extracellular and intracellular vesicles. Extracellular vesicles (EVs) constitute membranous vehicles that endow intra-species, inter-species and inter-kingdom molecular trafficking of sRNAs, DNA, proteins and metabolites (Toyofuku *et al.*, 2019). Dependent on the encased cargo they might fulfil various biological functions such as virulence, horizontal gene transfer, a trade-off of metabolites, interception of bacteriophages and cell-to-cell communication (Caruana and Walper, 2020). To date, three universal mechanisms have been postulated for EV generation. In gram-negative bacteria, the main routes encompass i) outer membrane blebbing originating outer membrane vesicles (OMVs) which harbour exclusively periplasmic elements and ii) explosive cell lysis giving rise to outer-inner membrane vesicles (OIMVs) and explosive outer membrane vesicles (EOMVs) which can both harbour cytoplasmic material i.e. genomic DNA, RNA and cytosolic enzymes. In gram-positive bacteria, the major vesiculation events occur through iii) bubbling cell death from which cytoplasmic membrane vesicles (CMVs) stem (Toyofuku *et al.*, 2019). Although membrane vesicles formation is nowadays deemed a common trait for most bacteria, importantly found in plant-related microbiota such as rhizobia species, the mechanisms for cargo selection and machinery assembly remain largely elusive. Conversely, substantial evidence has been gathered regarding the plethora of stimuli that trigger the EV biogenesis routes. The general factor generating EV is membrane mechanochemical stress, e.g. in the case of OMV blebbing, the disruption of the crosslinks between peptidoglycan and the outer membrane promotes the dissociation between these layers, which in turn leads to membrane protrusion and ultimate EV release (Mozaheb and Mingeot-Leclercq, 2020). Recognized

determinants that elicit outer membrane blebbing are iron limitation, membrane intercalation of signalling molecules (i.e. quorum sensing molecules) and hydrophobic compounds, accumulation of misfolded proteins and peptidoglycan fragments in the periplasmic space, imbalanced synthesis of the cell wall components or phospholipid accumulation in the outer leaflet (Toyofuku *et al.*, 2019). In contrast, the formation of OIMVs/EOMVs and CMVs via explosive cell lysis or bubbling, respectively, appears to be commonly caused by the action of phage-derived endolysins, which are produced upon activation of the bacterial SOS response under stress conditions (Turnbull *et al.*, 2016; Toyofuku *et al.*, 2017).

In addition to EVs, the existence of bacterial intracellular vesicles (IVs), also known as organelles, with a devoted function, was initially postulated upon the discovery of chromatophores in photosynthetic bacteria (López-Marqués *et al.*, 2004). Ever since that seminal study, several cytosolically invaginated microbial organelles have been shown to fulfil different functions (i.e. magnetosome or anammoxosome) (Greening and Lithgow, 2020). Naturally occurring bacterial organelles are employed by bacteria to enhance the yield of biochemical processes as they increase the local concentration of metabolites and enzymes thereby channelling chain reactions (Greening and Lithgow, 2020). Despite a general lack of knowledge regarding the molecular players involved in the construction of bacterial organelles, the potential is clear and future studies shedding light on IVs will pave the way to increase the efficiency of metabolic pathways using engineered intracellular organelles. We deepen into the biotechnological applications of EVs and IVs in the following section.

Green agriculture strategies based on T6SS and membrane vesicles

In the field of agriculture, the domestication of T6SS and membrane vesicles will allow to develop strategies to benefit crops by engineering bacterial strains with enhanced capacity to promote plant growth (PGPR) and/or to protect plants from phytopathogens (BCAs) (Fig. 1). T6SS and

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membrane vesicles might work complementary, for instance, the T6SS can protect plants by killing phytopathogens in a contact-dependent manner (Bernal *et al.*, 2017), while secreted vesicles may navigate large distances (Bos *et al.*, 2021) and physical contact is not required. These complementary strategies could be met in a beneficial microbe and be specifically used as convenient, e.g. using the T6SS to outcompete the surrounding pathogens in the niche and the EVs to reach pathogens that are not found in close contact with the biocontrol agent (Fig. 1A). Both systems may be wired and concurrently activated upon command since a large number of environmental signals, especially mechanical stress and membrane perturbations are a shared cue for the activation of T6SS and EVs (Toyofuku *et al.*, 2019; Kamal *et al.*, 2020). A deeper understanding of the cues activating both systems in nature, the mechanisms involved in their assemblies and the molecules to be trafficked through them will grant a spectrum of possibilities to be exploited in the field. The following section delves into these opportunities especially focusing on the enhancement of BCAs and PGPR (i.e. nitrogen-fixing bacteria) using the T6SS and membrane vesicles as main tools.

Biocontrol

Biocontrol agents can exert their functions by directly killing or inhibiting the growth of plant pathogens using different mechanisms (i.e. T6SS, siderophores and small toxic compounds as antibiotics) or by inducing the plant immune system in a way the plant is prompted to defend itself (Berlanga-Clavero *et al.*, 2020). A novel approach is an interference, at the expression level, of phytopathogens virulence factors via RNA molecules (Cai *et al.*, 2018).

The most classic among the inter-bacterial competition mechanism is probably the secretion of siderophores (i.e. pyoverdine). Siderophores are molecules with the capacity to sequester iron, a limiting element for microbial growth; and bacteria with the capacity to secrete them and internalize them back once they are iron-associated, present considerable fitness advantages. Thus, an enhanced capacity to secrete siderophores will improve the biocontrol capacities of a bacterium,

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and this could be reached by inducing the release or uptake of siderophores-associated EVs (Fig. 1A). In fact, siderophores and other iron-affine molecules like the *Pseudomonas* quinolone signal (PQS) have been detected in OMVs and assisted by a T6SS effector for internalization (Lin *et al.*, 2017). Besides siderophores, a plethora of small toxic molecules are known to interfere with basic biological functions of bacteria including cell growth, division or destabilize membrane including antibiotics, bacteriocins, other ions metal sequestering molecules (phenazines and cyanide), surfactants, volatile compounds and slimy-polymers (Berlanga-Clavero *et al.*, 2020). Biocontrol agents with an enhanced capacity to secrete these small toxic compounds will be excellent products to be used for efficient production in sustainable agriculture (Fig. 1A).

On the other hand, the newest described mechanism of biocontrol is the T6SS. This secretion system is present in many plant-related beneficial microorganisms and its role in the control of crop pests and diseases is still underestimated because of unawareness of its importance. Very little is known about effectors targeting phytopathogens (bacteria, fungi or even small animals) or the cues that activate the system in the rhizosphere or the phyllosphere. Thus, BCAs harbouring T6SSs are, in fact, an immense source of potentially novel antimicrobial compounds. The discovery of new plant-associated T6SS effectors will allow identifying novel targets for antimicrobial therapeutic drugs to combat the rising problem of antimicrobial resistance (AMR) bacteria not only for agriculture purpose but also for clinical use. These antimicrobial molecules could be secreted directly inside the phytopathogen by the T6SS or could potentially be targeted to be secreted by EVs to reach further distances (Fig. 1A).

Moreover, biocontrol agents can trigger the plant immune system as an indirect mechanism of protection. Interestingly, several volatile compounds and surfactants stimulate the plant immune system by mimicking plant hormones activating the salicylate, jasmonic acid and ethylene signalling pathways (Berlanga-Clavero *et al.*, 2020). Again, EVs and T6SS come forth as excellent delivery systems for this type of molecules (Fig. 1B). Lastly, blocking the expression of phytopathogens virulence factors using RNA has been proven efficient for plants (Middleton *et al.*,

2020). Plants can use small (interference) RNA molecules to block the translation of virulence factors using EVs (Middleton *et al.*, 2020). This EV-based strategy could be adopted by biotechnologically improved BCAs to efficiently deliver silencing miRNAs into phytopathogen (Fig. 1C). An additional application of small RNAs could be to directly target the plant to foster the activation of the immune response to suppress phytopathogen entry and avoid infection (Fig. 1B).

Therefore, T6SS/EV enhanced BCAs will present improved capacity in three fronts: outcompeting the phytopathogens, blocking virulence factors and inducing the plant immunity system (Fig. 1ABC).

Plant Growth Promoting Rhizobacteria

Plant Growth Promoting Rhizobacteria are bacteria with the capacity to promote the growth of the plant they are associated with. Several mechanisms have been described for this set of microorganisms including solubilization of inorganic phosphorous, mineralization of organic phosphorous and/or the ability to fix nitrogen (Perez-Montaña, 2014). In this specific context, we are focusing on the capacity of rhizobia to fix nitrogen for the leguminous plants. After seed germination, an intricate molecular dialogue is established between rhizobia and leguminous plants. This symbiotic interaction begins with the legume roots release of phenolic compounds (i.e. flavonoids) into the rhizosphere. These signal molecules induce the synthesis of nodulation (Nod) factors by the rhizobia species. The plant, in turn, recognizes these signals and initiate the nodulation process (i.e. nodule development, occupation by bacteria and transformation of the rhizobia into the nitrogen-fixing form: the bacteroid) (Oldroyd, 2013). A few studies suggest that the T6SS might play a role in rhizobia nodulation (Fig. 1D) and, as in the case of the T3SS, this role could be positive or negative depending on the way the effectors are recognized by the host (Salinero-Lanzarote *et al.*, 2019). A wide set of Nod factors -proteins and metabolites- have been largely studied. A fraction of them is known to be naturally packed and conveyed by EVs in the presence of flavonoids such as naringenin i.e. oligosaccharides (Taboada *et al.*, 2019) implying a

differential cargo selection upon sensing specific plant molecules. While the means of transport of others -cellulases, homoserine lactones, phytohormones, siderophores, indoleacetic acid- remains to be clarified, genetic engineering of rhizobia species may enable EV-mediated traffic (Fig. 1E).

In the opportune line of action of enhancing rhizobia-nitrogen fixation, IVs emerge as a potent tool to fulfil this purpose. IVs are recognized bacterial bioreactors that improve the efficiency of enzymatic pathways by local reallocation of metabolic and enzymatic resources (Greening and Lithgow, 2020). The introduction of nitrogen-fixing enzymes into these membranous microcompartments promises to boost their activity (Fig. 1F).

The increasing knowledge regarding bacterial vesicles and T6SS will enhance the capabilities of BCAs and PGPRs to be used as biotechnological applications with great benefits in sustainable agriculture. A critical trait of these systems is the ubiquity across multiple microbial species present in the different plant niches and through all growth stages. This opens the gates to the manipulation of diverse integrants of the microbiota expanding the reach of these nanomachines by allowing control over the different plant functional units (i.e. the rhizosphere, the phyllosphere and the endosphere).

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References

- Allsopp, L.P., Bernal, P., Nolan, L.M., and Filloux, A. (2020). Causalities of war: The connection between type VI secretion system and microbiota. *Cellular microbiology* 22, e13153.
- Berlanga-Clavero, M.V., Molina-Santiago, C., De Vicente, A., and Romero, D. (2020). More than words: the chemistry behind the interactions in the plant holobiont. *Environmental microbiology* 22, 4532-4544.
- Bernal, P., Allsopp, L.P., Filloux, A., and Llamas, M.A. (2017). The *Pseudomonas putida* T6SS is a plant warden against phytopathogens. *The ISME journal* 11, 972-987.
- Bernal, P., Furniss, R.C.D., Fecht, S., Leung, R.C., Spiga, L., Mavridou, D.A., and Filloux, A. (2021). A novel stabilization mechanism for the type VI secretion system sheath. *Proceedings of the National Academy of Sciences* 118.
- Bernal, P., Llamas, M.A., and Filloux, A. (2018). Type VI secretion systems in plant-associated bacteria. *Environmental microbiology* 20, 1-15.
- Borgeaud, S., Metzger, L.C., Scignari, T., and Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* 347, 63-67.
- Bos, J., Cisneros, L.H., and Mazel, D. (2021). Real-time tracking of bacterial membrane vesicles reveals enhanced membrane traffic upon antibiotic exposure. *Science advances* 7, eabd1033.
- Cai, Q., Qiao, L., Wang, M., He, B., Lin, F.-M., Palmquist, J., Huang, S.-D., and Jin, H. (2018). Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science* 360, 1126-1129.
- Caruana, J.C., and Walper, S.A. (2020). Bacterial membrane vesicles as mediators of microbe–microbe and microbe–host community interactions. *Frontiers in microbiology* 11, 432.

Greening, C., and Lithgow, T. (2020). Formation and function of bacterial organelles. *Nature Reviews Microbiology* 18, 677-689.

Hernandez, R.E., Gallegos-Monterrosa, R., and Coulthurst, S.J. (2020). Type VI secretion system effector proteins: Effective weapons for bacterial competitiveness. *Cellular Microbiology* 22, e13241.

Hood, R.D., Singh, P., Hsu, F., Güvener, T., Carl, M.A., Trinidad, R.R., Silverman, J.M., Ohlson, B.B., Hicks, K.G., and Plemel, R.L. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell host & microbe* 7, 25-37.

Kamal, F., Liang, X., Manera, K., Pei, T.-T., Kim, H., Lam, L.G., Pun, A., Hersch, S.J., and Dong, T.G. (2020). Differential cellular response to translocated toxic effectors and physical penetration by the type VI secretion system. *Cell reports* 31, 107766.

Leroux, M., Kirkpatrick, R.L., Montauti, E.I., Tran, B.Q., Peterson, S.B., Harding, B.N., Whitney, J.C., Russell, A.B., Traxler, B., and Goo, Y.A. (2015). Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*. *Elife* 4, e05701.

Lin, J., Zhang, W., Cheng, J., Yang, X., Zhu, K., Wang, Y., Wei, G., Qian, P.-Y., Luo, Z.-Q., and Shen, X. (2017). A *Pseudomonas* T6SS effector recruits PQS-containing outer membrane vesicles for iron acquisition. *Nature communications* 8, 1-12.

López-Marqués, R.L., Pérez-Castiñeira, J.R., Losada, M., and Serrano, A. (2004). Differential regulation of soluble and membrane-bound inorganic pyrophosphatases in the photosynthetic bacterium *Rhodospirillum rubrum* provides insights into pyrophosphate-based stress bioenergetics. *Journal of bacteriology* 186, 5418-5426.

Lucke, M., Correa, M.G., and Levy, A. (2020). The Role of Secretion Systems, Effectors, and Secondary Metabolites of Beneficial Rhizobacteria in Interactions With Plants and Microbes. *Front Plant Sci* 11, 589416.

Middleton, H., Yergeau, É., Monard, C., Combiér, J.-P., and El Amrani, A. (2020). Rhizospheric Plant–Microbe Interactions: miRNAs as a Key Mediator. *Trends in Plant Science*.

- Mozaheb, N., and Mingeot-Leclercq, M.-P. (2020). Membrane Vesicle Production as a Bacterial Defense Against Stress. *Frontiers in microbiology* 11, 3120.
- Oldroyd, G.E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat Rev Microbiol* 11, 252-263.
- Perez-Montaña, F., Alias-Villegas, C., Bellogin, R.A., Del Cerro, P., Espuny, M.R., Jimenez-Guerrero, I., Lopez-Baena, F.J., Ollero, F.J., and Cubo, T. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. *Microbiol Res* 169, 325-336.
- Salinero-Lanzarote, A., Pacheco-Moreno, A., Domingo-Serrano, L., Durán, D., Ormeño-Orrillo, E., Martínez-Romero, E., Albareda, M., Palacios, J.M., and Rey, L. (2019). The Type VI secretion system of *Rhizobium etli* Mim1 has a positive effect in symbiosis. *FEMS microbiology ecology* 95, fiz054.
- Sharma, N., and Singhvi, R. (2017). Effects of chemical fertilizers and pesticides on human health and environment: a review. *International journal of agriculture, environment and biotechnology* 10, 675-679.
- Silverman, J.M., Austin, L.S., Hsu, F., Hicks, K.G., Hood, R.D., and Mougous, J.D. (2011). Separate inputs modulate phosphorylation-dependent and-independent type VI secretion activation. *Molecular microbiology* 82, 1277-1290.
- Stolle, A.-S., Meader, B.T., Toska, J., and Mekalanos, J.J. (2021). Endogenous membrane stress induces T6SS activity in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* 118.
- Taboada, H., Dunn, M.F., Meneses, N., Vargas-Lagunas, C., Buchs, N., Andrade-Dominguez, A., and Encarnacion, S. (2019). Qualitative changes in proteins contained in outer membrane vesicles produced by *Rhizobium etli* grown in the presence of the nod gene inducer naringenin. *Arch Microbiol* 201, 1173-1194.

- Toyofuku, M., Cárcamo-Oyarce, G., Yamamoto, T., Eisenstein, F., Hsiao, C.-C., Kurosawa, M., Gademann, K., Pilhofer, M., Nomura, N., and Eberl, L. (2017). Prophage-triggered membrane vesicle formation through peptidoglycan damage in *Bacillus subtilis*. *Nature communications* 8, 1-10.
- Toyofuku, M., Nomura, N., and Eberl, L. (2019). Types and origins of bacterial membrane vesicles. *Nature Reviews Microbiology* 17, 13-24.
- Turnbull, L., Toyofuku, M., Hynen, A.L., Kurosawa, M., Pessi, G., Petty, N.K., Osvath, S.R., Cárcamo-Oyarce, G., Gloag, E.S., and Shimoni, R. (2016). Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature communications* 7, 1-13.
- United Nations (2019). Department of Economic and Social Affairs, Population Division. World Population Prospects 2019.
- Wu, C.F., Lin, J.S., Shaw, G.C., and Lai, E.M. (2012). Acid-induced type VI secretion system is regulated by ExoR-ChvG/ChvI signaling cascade in *Agrobacterium tumefaciens*. *PLoS Pathog* 8, e1002938.

Figure Legends:

Fig. 1. Proposed biotechnological strategies for biocontrol (A, B & C) and plant-growth promotion (D, E & F). The scheme focuses on the interspecies and inter-kingdom interactions mediated by T6SS and EV/IVs with the potential to be harnessed for the benefit of the plant. The EVs showed display a single membrane bilayer for the sake of simplicity. Nevertheless, EVs should be considered to be generically illustrated and might encompass all types of vesicles: OMVs, CMVs and OIMVs. BCA = Biocontrol agent; PGPR = Plant growth-promoting rhizobacteria; T6SS = Type IV secretion system; IS = Plant immune system. Plant cells lacking photosynthetic capacities due to their rhizospheric location are featured with a fainter and brownish appearance. **A)** Engineering of the T6SS and EVs systems of plant-inhabiting bacteria to target bacterial or fungal pathogens resulting in pathogen inhibition; **B)** manipulation of the EVs to convey plant immune system-activating molecules -such as the represented biosurfactants- leading to pathogen growth suppression; **C)** introduction of small RNAs (interference RNAs) into the EV cargo to target pathogenic fungi and bacteria to silence virulence factors genes; **D)** tailoring of the T6SS to internalize effectors into the host by contact-dependent or –independent interactions to promote nodulation; **E)** genetic manipulation of rhizobia species to encase nodulation factors (a generic lipochitooligosaccharide is represented) into the EVs to target the host cells for the activation of the nodulation process; **F)** engineering intracellular membrane vesicles in the bacteroid to allocate nitrogen-fixing enzymes (Nif) and metabolites in closer confinement enhancing thereby the pathway efficiency. Interactions in A, B and C may occur in different parts of the holobiont (rhizosphere, phyllosphere, below-aboveground), whereas those in D, E, and F take place in the rhizosphere. This figure was created with BioRender.com.

