Lysobacter enzymogenes antagonizes soilborne bacteria using the type IV secretion system

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Originality-Significance Statement

Bacterial Type IV Secretion System (T4SS) is a multiprotein complex involved in plasmid conjugation and interactions with eukaryotic hosts. Here, we show that T4SS in *Lysobacter enzymogenes*, a soilborne biocontrol bacterial species, acts as a contact-dependent inter-bacterial killing system. *L. enzymogenes* uses T4SS to kill ecologically relevant plant beneficial (*Pseudomonas* spp.) and pathogenic (*Pectobacterium carotovorum*) bacterial species. Consequently, *L. enzymogenes* reduces their ability to inhibit plant pathogens and infect the plant respectively. These findings reveal that the contact-dependent T4SS might mediate the cell-cell interactions among bacteria occurring in the soil microbiome, having a multiple ecological impact, which extends the functional range and ecological significance of bacterial T4SS.

Summary

Soil microbiome comprises numerous microbial species that continuously interact with each other. Among the modes of diverse interactions, cell-cell killing may play a key role in shaping the microbiome composition. Bacteria deploy various secretion systems to fend off other microorganisms and Type IV Secretion System (T4SS) in pathogenic bacteria was shown to function as a contact-dependent, inter-bacterial killing system only recently. The present study investigated the role played by T4SS in the killing behaviour of the soilborne biocontrol bacterium *Lysobacter enzymogenes* OH11. Results showed that *L. enzymogenes* OH11 genome encompasses genes encoding all the components of T4SS and effectors potentially involved in inter-bacterial killing system. Generation of knock-out mutants revealed that *L. enzymogenes* OH11 uses T4SS as the main contact-dependent weapon against other soilborne bacteria. The T4SS-mediated killing behaviour of *L. enzymogenes* OH11 decreased the antibacterial and antifungal activity of two *Pseudomonas* spp. but at the same time, protected carrot from infection by *Pectobacterium carotovorum*. Overall, this study showed for the first time the involvement of T4SS in the killing behaviour of *L. enzymogenes* and its impact on the multiple interactions occurring in the soil microbiome.

Introduction

A relevant number of plant beneficial bacteria are effective biocontrol agents of plant pathogenic microorganisms and defined as “green” biopesticides. They may play key roles in making crop production more sustainable by protecting crop plants from pathogen infection reducing the chemical input in agriculture (Hayat *et al*., 2010). In particular, soilborne bacteria establish various interactions with other microorganisms and, as a consequence, soil and plant health depend on these interactions (Wei *et al*., 2019). Among the modes of interactions, bacterial killing may play a major role in shaping and controlling the microbial communities in the environment (Chen *et al*., 2011; Wang *et al*., 2020).

Bacteria able to kill/prey upon other bacteria are included in different phyla (Jurkevitch, 2007) and they are characterised by different modes of predation, namely

epibiotic predation, endobiotic predation and group attack (Pérez *et al*., 2016). In the case of the endobiotic predation, typical for members of the genera *Bdellovibrio* and *Daptobacter*, predator cells are able to invade the periplasm and/or cytoplasm of prey bacteria using lytic enzymes (Guerrero *et al*., 1986; Rotem, 2014). Differently, predator bacteria acting through the epibiotic predation establish a contact with the prey bacterial cells and start to feed on them, a strategy extensively studied in *Myxococcus xanthus* (Thiery and Kaimer, 2020). Group attack indicates a cooperative predation where predator bacterial cells hunt in group releasing diffusible metabolites (Velicer and Mendes-Soares, 2009).

Within the soil microbiome, bacterial species belonging to the genus *Lysobacter* are acquiring a higher importance for their plant beneficial activities (Puopolo *et al*., 2018). For instance, *L. enzymogenes* C3 and OH11 are reported to effectively control Bipolaris leaf-spot of tall fescue and anthracnose of pear fruit, caused by the fungal pathogens, *Bipolaris sorokiniana* and *Colletotrichum fructicola*, respectively (Kilic-Ekici and Yuen, 2003; Zhao *et al*., 2021). *Lysobacter antibioticus* 13-1 could protect rice against the infection by *Xanthomonas oryzae* pv. *oryzae* causing bacterial rice blight (Ji et al., 2008). The prophylactic application of *L. capsici* AZ78 to grapevine leaves leads to a remarkable reduction of grapevine downy mildew caused by the plant pathogenic oomycete *Plasmopara viticola* (Puopolo *et al*. 2014). Since the establishment of the genus, *Lysobacter* members have been indicated as facultative predadors able to lyse several microorganisms (Christensen, 2015; Hungate *et al*., 2021) and the mechanisms involved in the killing of plant pathogenic oomycetes have been characterised in *L. capsici* and *L. enzymogenes* (Tofazzal Islam, 2010; Tomada *et al*., 2017; Zhao *et al*., 2017). Regarding the ability to prey upon bacteria, *Lysobacter* spp. are reported to act similarly to *Myxococcus* spp. through epibiotic predation and group attack (Pérez *et al*., 2016) and the cell-cell contact has been proven to be fundamental in the predatory behaviour of type strains of *L. capsici*, *L. enzymogenes* and *L. oryzae* species (Seccareccia *et al*., 2015). As facultative predators, *Lysobacte*r spp. may kill and feed on other bacterial species residing in soils (Hungate et al., 2021; Lueders et al., 2006). However, the molecular mechanisms involved in the establishment of cell-cell contact and the killing behaviour of *Lysobacter* spp. are not clear yet.

It is now widely accepted that secreted proteins, named effectors, play an important role in bacterial interactions with host cells or among themselves (Granato *et al*., 2019) and bacteria have evolved several types of specialized secretion systems to release effectors (Denise *et al*., 2020). For instance, bacterial cells inject effectors that are toxic particularly against bacteria using Type VI Secretion System (T6SS; Mougous, 2006; Galán and Waksman, 2018; Liang *et al*., 2019) as in the case of the plant beneficial bacterium *Pseudomonas* (*Ps.*) *putida* that uses its T6SS as a plant warden against bacterial phytopathogens (Bernal *et al*., 2017). Type IV Secretion System (T4SS) is a multiprotein complex that may deliver DNA, effectors and protein-DNA complex to the extracellular milieu or into the eukaryotic and prokaryotic target cells (Backert and Meyer, 2006; Alvarez-Martinez and Christie, 2009; Sgro *et al*., 2019). Two T4SS systems are most studied in pathogenic bacteria,

including the VirB/D4 T4SS from the plant pathogen *Agrobacterium tumefaciens* and the Dot/Icm T4SS from the human pathogenic *Legionella* (*Le.*) *pneumophila* (Alvarez-Martinez and Christie, 2009). The *Le. pneumophila* T4SS is responsible for translocating hundreds of effectors into the host cell to modulate diverse cellular processes promoting pathogen survival (Luo and Isberg, 2004; Qiu and Luo, 2017). The T4SS of *A. tumefaciens* is required for delivery of the Transfer-DNA (T-DNA) from the tumour-inducing plasmid into host plant cells (Vergunst, 2000).

Recently, the VirB/D4 T4SS in *Stenotrophomonas maltophilia* and *Xanthomonas citri* was found to be involved in the transfer of effectors lethal to bacterial competitors, providing advantages in the colonization of their hosts and the environment (Souza *et al*., 2015; Bayer-Santos *et al*., 2019). Similarly to the mode of action of the T6SS toxin-immunity protein pair (Yang *et al*., 2018), toxicity of the T4SS effectors from *X. citri* cells is neutralised by binding to the neighbouring immunity proteins (Alvarez-Martinez and Christie, 2009; Russell *et al*., 2011; Russell *et al*., 2013; Souza *et al*., 2015). The effectors translocated by the bacterial-killing T4SS of *S. maltophilia* and *X. citri* have a conserved C-terminal signature, known as the *Xanthomonas* VirD4-interacting protein conserved domain (XVIPCD) (Alegria *et al*., 2005; Souza *et al*., 2015; Bayer-Santos *et al*., 2019). This domain is required for the physical interaction between those T4SS effectors and VirD4, a T4SS specific ATPase indispensable for effector translocation (Souza *et al*., 2015; Sgro *et al*., 2019).

As several genomes of *Lysobacter* spp. are predicted to encode a *X. citri*-like T4SS system (Sgro *et al*., 2019), we investigated the role played by T4SS in the predatory behaviour of the model strain *L. enzymogenes* OH11 (OH11). Results showed that OH11 employs an *X. citri*-like T4SS and effectors as the main contact-dependent weapon against other soilborne bacteria. The killing behaviour of OH11, dependent on T4SS, decreased the biocontrol activities of two *Pseudomonas* (*Ps.*) spp. strains and, at the same time, showed to be essential to control the plant pathogenic bacterium *Pectobacterium* (*P.*) *carotovorum*. These new findings represent the first demonstration of the involvement of the bacterial-killing T4SS in the killing behaviour of *L. enzymogenes* species and, as a consequence, in the soil bacterial interactions.

Results

L. enzymogenes OH11 genome encodes a T4SS and toxic effectors similar to the plant pathogen *X. citri*

Mining the OH11 genome allowed us to find a VirB/D4 T4SS gene cluster that includes all T4SS homologous components of the phylogenetically related species, *X. citri* (Fig. 1A, 1B; Table S1). We further identified 16 putative T4SS effectors possessing a conserved C-terminal XVIPCD domain (Fig. 1C; Table S1). Amoeg these 16 genes, four genes encoding the predicted effector proteins (Le4235, Le4230, Le4232 and Le4236) were located closer to T4SS locus whereas the remaining 12 genes were located in other genome regions far from the T4SS locus (Fig. 1B and 1C). Release of toxin effectors containing the XVIPCD domain is one of the key features of the bacterial-killing T4SS in *X. citri* (Souza *et al*., 2015). To test whether the

predicted T4SS effectors in OH11 have such antibacterial activity, six effector genes (*Le4232*, *Le0989*, *Le0908*, *Le4230*, *Le1288* and *Le3316*; Fig. 1C), were randomly selected and each of them was expressed in the periplasm or cytoplasm of the *Escherichia coli* BL21 cells driven by an IPTG or arabinose inducible promoter. Induced toxicity assays revealed that the expression of three effector genes, namely *Le0989*, *Le0908* and *Le1288*, in the *E. coli* BL21 periplasm had a toxic effect on the tested bacterial strain (Fig. 2A), as well as the positive control, the T6SS effector gene, *pldB* from *Pseudomonas* (*Ps.*) *aeruginosa* PA01 (Jiang *et al*., 2014). In contrast, none of the six effector genes showed toxic activity when expressed in the *E. coli* BL21 cytoplasm (Fig. 2B), whereas the expression of the type III secretion system (T3SS) effector AvrRox1 from *X. oryzae* pv. *oryzae* RS105 (Triplett *et al*., 2016), used as positive control, had a toxic effect. These results are consistent with the bioinformatics analysis indicating that both Le0989 and Le0908 contain one or two N-terminal peptidoglycan-binding domains.

Toxicity of the *X. citri* T4SS effectors could be neutralised by forming a toxin-immunity protein pair in *X. citri*, as reported earlier (Souza *et al*., 2015). We verified the neutralization of effector toxicity in OH11 by focusing on Le0908 only due to the following considerations. Among the three identified toxic effectors, Le1288 seems to have strong leakage expression evidenced by its visible toxicity to *E. coli* BL21 even without IPTG induction (Fig. 2A). The reason for this unusual phenomenon remains unknown. However, it is probable that Le1288 is possibly more toxic compared to the other effector proteins, i.e. Le0908. This Le1288 property limited us to use this effector to design the following protein expression and pull-down assays (see below). Thus, between the remaining Le0908 and Le0989, we chose the shorter-length protein, Le0908 (Fig. 1C) for the following assays. Co-expression of *Le0908* with the adjacent *Le0909* (a hypothetical protein) gene, but not the *Le0907* (IS30 family transposase) gene, partially neutralised the Le0908-induced toxicity in *E. coli* BL21 (Fig. 2C, 2D), suggesting that Le0909 is a potential immunity protein of Le0908. Accordingly, the pull-down assays showed that the full-length Le0908 fused with a C-terminal His tag, Le0908-His directly bound with the truncated Le0909 fused with a C-terminal FLAG tag, Le0909T-FLAG lacking the signal peptide (Fig. 2E, 2F).

The direct binding of effectors to the T4SS ATPase VirD4 is another important feature characterising the bacterial-killing T4SS (Alegria *et al*., 2005; Souza *et al*., 2015). To test whether the OH11 T4SS also possesses this key feature, we selected Le0908 to investigate its potential binding with VirD4 by carrying out pull-down assays. Notably, we found that the full-length VirD4-His or Le0909-His was insoluble in *E. coli*. Due to this technical aspect, we decided to use the soluble and truncated version, VirD4 T or Le0909T to carry out pull-down assays. Experiments showed that the full-length Le0908-His bound to the truncated VirD4 fused with a C-terminal FLAG tag, VirD4T-FLAG lacking both signal peptide and transmembrane domains, and this binding is dependent on the presence of the XVIPCD domain of Le0908 (Fig. 2E, 2G, 2H). These findings collectively suggested that OH11 might assemble a *X. citri*-like T4SS involved in the release of toxic effectors potentially involved in a

contact-dependent killing activity. As the truncated version of VirD4 and Le909 were used, we could not exclude the *in vivo* importance of the signal peptide and/or transmembrane domain for VirD4 or Le0909 activity or function. However, the truncation-based pull-down data provided an *in vitro* evidence indicating the presence of a bacterial-killing T4SS in *L. enzymogenes*.

T4SS is required for the contact-dependent killing activity of *L. enzymogenes* against soilborne bacteria

To validate the involvement of T4SS in the contact-dependent killing activity, we investigated whether OH11 has the ability to kill the model competitor *E. coli* in a contact-dependent manner, similarly to *X. citri* (Dong *et al*., 2013; Grohmann *et al*., 2018; Klein *et al*., 2020). As expected, the T6SS-producing *A. citrulli* xjl12 (Tian *et al*., 2015), used as a positive control, was effective in killing the LacZ-labelled *E. coli* BL21 in a contact-dependent manner. Similarly, OH11 was also effective in killing the same strain when their cells were mixed at different ratios and co-inoculated (Fig. 3A). Differently, inoculation of OH11 into the surface of LB agar plates containing *E. coli* did not form any inhibitory zone, suggesting that *L. enzymogenes* is unable to kill *E. coli* through secreting diffusible antibacterial factors (Fig. S1).

To validate the involvement of T4SS in the OH11 killing behaviour, we generated a T4SS-defective mutant (Δ*virD4*) through the in-frame deletion of *virD4*, which is essential for the T4SS assembly and activity in *X. citri*, as reported previously (Souza *et al*., 2015). The Δ*virD4* strain exhibited a growth curve similar to the wild-type OH11 in liquid LB broth (Fig. S2). Under the co-culture conditions on LB agar dishes, the killing efficiency of the Δ*virD4* mutant against the LacZ-labelled *E. coli* BL21 was visibly reduced compared to the wild-type OH11 and the Δ*virD4* complemented strain (Fig. 3B). At the same time, OH11 mutants defective in secondary-metabolite biosynthesis, T1SS, T2SS and T6SS, strongly inhibited the growth of *E. coli* BL21, similar to the wild-type OH11 (Table S2). The contact-killing activity of OH11 against *E. coli* was also confirmed via fluorescent microscopy. We individually grew the mCherry-labelled Δ*virD4* and wild-type OH11 and observed that both strains displayed similar fluorescent signal intensity (Fig. S3). Using the mCherry-labelled Δ*virD4* and wild-type OH11 as killers, we found that the wild-type OH11 efficiently inhibited the growth of the GFP-labelled *E. coli* BL21 when mixed in a 1:1 ratio. Such contact-dependent killing activity was severely impaired in the Δ*virD4* mutant (Fig. 3C, 3D). Similarly, the contact-dependent killing activity was impaired when *E. coli* and wild-type OH11 were separated by a 0.22-μm membrane, or only co-culture with the cell-free supernatant of wild-type OH11 on agar plates was used (Fig. 3E, S4).

Next, soilborne biocontrol bacterial strains *Ps. fluorescens* 2P24 and *Ps. protegens* Pf-5 and the plant pathogenic bacterial strain *P. carotovorum* PccS1, sharing the same ecological niche of OH11, were tested. We found that wild-type OH11 was unable to inhibit the growth of *Ps. fluorescens* 2P24, *Ps. protegens* Pf-5 and *P. carotovorum* PccS1 through secreting diffusible antibacterial factors (Fig. S5). Similarly, no killing activity was observed when wild-type OH11 and the tested bacterial strains were separated by a 0.22-μm filter membrane or the tested bacterial

strains were grown in the presence of wild-type OH11 cell-free supernatant on agar plates (Fig. S6, S7). In contrast, the mCherry-labelled, wild-type OH11 was always effective in killing all GFP-labelled soilborne bacterial strains tested when co-inoculated at a 1:1 ratio. At the same time, the contact-dependent killing activity was attenuated significantly in the case of the Δ*virD4* mutant (Fig. 4A-F). Notably, the low mCherry fluorescence observed when the Δ*virD4* strain was co-cultured with the tested bacterial strains (Fig. 4) might be possibly related to the counterattack of these bacterial strains via T6SS (Decoin *et al*., 2014; Vacheron *et al*., 2019; Wang *et al*., 2018). Indeed, the mCherry fluorescence in the Δ*virD4* strain was visibly increased when it was co-cultured with the GFP-labelled a T6SS-inactive mutant of *Ps. protegens* Pf-5 (Δ*tssA*) (Fig. S8). These results collectively suggested that T4SS might mediate the contact-dependent OH11 ability to antagonize other soilborne bacterial competitors.

T4SS contact-dependent killing activity of *L. enzymogenes* decreases the antagonism of plant beneficial *Pseudomonas* spp.

OH11 and *Ps. fluorescens* 2P24 both act as antagonists of soil fungi through the production of the antifungal secondary metabolites Heat Stable Antifungal Factor (HSAF) and 2,4-diacetylphloroglucinol respectively (Parret *et al*., 2005; Li *et al*., 2006). Thus, we investigated whether the T4SS-mediated killing of 2P24 by OH11 provides *L. enzymogenes* advantages to "privately" kill fungi as nutrients when OH11 and 2P24 are both close to fungal pathogen. To test this hypothesis, the plant pathogenic fungus *Valsa piri* was selected. Results from fungal inhibition assays in Petri dishes showed that *Ps. fluorescens* 2P24 inhibited the mycelial growth *V. pyri* SXYL134 when inoculated individually on the growth medium, whereas Δ*lafB*, an OH11 derivative unable to produce HSAF did not show any inhibition (Fig. 5A). Co-inoculation (1:1) of Δ*lafB* and *Ps. fluorescens* 2P24 reduced significantly the antifungal activity of the latter strain. The antifungal activity of *Ps. fluorescens* 2P24 occurred when it was co-inoculated with the OH11 double mutant Δ*lafB*Δ*virD4* (Fig. 5A, 5B). Fluorescent microscopy and CFU quantification showed that wild-type OH11 and Δ*lafB*, were able to kill *Ps. fluorescens* 2P24 when co-inoculated at 1:1 ratio, whereas such contact-dependent killing activity was significantly reduced in Δ*lafB*Δ*virD4* (Fig. 4C, 5C). Noteworthy, the Δ*lafB*Δ*virD4* strain showed a growth curve and a mCherry-derived fluorescent signal intensity similar to wild-type OH11 (Fig. S2, S9).

Similarly, we found that co-inoculation (1:1) of OH11 with the biocontrol strain *Ps. protegens* Pf-5 remarkably reduced the antibacterial activity of the latter strain against *P. carotovorum* PccS1, and this reduction was significantly rescued when the Δ*virD4* mutant was used in the co-inoculation (Fig. 5D, 5E). As controls, the wild-type OH11 and the Δ*virD4* mutant failed to inhibit the growth of *P. carotovorum* PccS1 through diffusible antibacterial factors (Fig. S5). These results collectively revealed that the bacterial-killing T4SS of OH11 might impair the antagonism activity of bacteria belonging to the genus *Pseudomonas*.

L. enzymogenes attenuates the *P. carotovorum* virulence on carrot through contact-dependent killing behaviour mediated by T4SS

As OH11 could kill *P. carotovorum* PccS1 through the contact-dependent T4SS, we explored whether this behaviour may help the biocontrol *L. enzymogenes* species to protect plants against the infection by *P. carotovorum* PccS1. We observed that single inoculation of *P. carotovorum* PccS1 on carrot (*Daucus carota* L.) slices caused tissue rot, while the wild-type OH11 and its derivative Δ*virD4* did not cause any symptom similarly to the negative control (H_2O) (Fig. 6A). To guarantee the infection of carrot slices when *P. carotovorum* PccS1 was inoculated in mixed community with *L. enzymogenes* strains, we co-inoculated the two bacterial strain at a 5:1 ratio (*P. carotovorum* : *L. enzymogenes*) in all the combinations. We found that co-inoculation of *P. carotovorum* PccS1 with wild-type OH11 significantly reduced the virulence of *P. carotovorum* PccS1. Differently, co-inoculation of *P. carotovorum* PccS1 with Δ*virD4* caused a level of disease similar to the one caused when *P. carotovorum* PccS1 was inoculated singularly (Fig. 6A, 6B). Accordingly, the mCherry-labelled wild-type OH11 efficiently killed the GFP-labelled *P. carotovorum* PccS1, whereas such contact-dependent killing activity was reduced significantly in the Δ*virD4* mutant (Fig. S10). These results suggested that OH11 might employ T4SS as a contact-dependent weapon against *P. carotovorum* PccS1 to reduce its virulence and hence protects host plant.

Discussion

Among the soil microbiome, soil bacteria co-habit with a vast array of microbial competitors and they evolved different strategies to persist efficiently in the environment (Faust and Raes, 2012). Bacterial predation/killing is one of these strategies (Pérez *et al*., 2016) and it is shared by several bacterial genera, such as *Lysobacter* (Tofazzal Islam, 2010; Christensen, 2015; Tomada *et al*., 2017; Zhao *et al*., 2017; Puopolo *et al*., 2018). *Lysobacter enzymogenes*, the best characterised species of the genus, is a natural predator of fungi and oomycetes through the secretion of lytic enzymes and HSAF, a broad-spectrum toxin (Li *et al*., 2006; Palumbo *et al*., 2005; Yu *et al*., 2007). Moreover, members of this species are able to kill other bacterial species (Lueders *et al*., 2006; Seccareccia *et al*., 2015).

In the present study, we discovered a functional T4SS killing system in the model strain *L. enzymogenes* OH11 and investigated its role in the antagonistic behaviour of *L. enzymogenes* species. Our results showed that *L. enzymogenes* deploys T4SS as the main contact-dependent weapon against other Gram-negative bacteria. This *L. enzymogenes* ability is likely achieved by translocating toxic effectors possessing the conserved XVIPCD domain into the competitor bacterial cells, similar to the plant pathogen *X. citri* (Souza *et al*., 2015). The *L. enzymogenes* bacterial-killing T4SS likely prefers to attack the ecological-relevant, Gram-negative bacteria. Indeed, we found that the mCherry-labelled *L. enzymogenes* OH11 was unable to kill the GFP-labelled *Bacillus subtilis* NCD-2, a soilborne, Gram-positive, biocontrol agent (Guo *et al*., 2019) when both strains were co-cultured on agar plates in a 1:1 ratio (Fig. S11). Interestingly, the best-characterised, widespread

contact-dependent killing machinery, T6SS is mainly reported to attack Gram-negative bacteria (Coulthurst, 2019). Moreover, involvement of T4SS in the killing behaviour of bacterial predators has been already reported in the cyanobacterium *Vampirovibrio chlorellavorus*, an obligate parasite of the green algae *Chlorella* spp. (Soo *et al*., 2015). In this case, it was proposed that *V. chlorellavorus* might transfer hydrolytic enzymes and a T-DNA from its plasmids into the *Chlorella* cells. Once inside the prey cells, lytic enzymes will proceed with the degradation of cell macromolecules and the out products will be transported outside the prey cells through transporter encoded on the T-DNA (Soo *et al*., 2015).It is highly probable that the killing behaviour characterising *L. enzymogenes* species is less sophisticated than the behaviour of *V. cholorevallavorus*. Based on our results, we may propose that *L. enzymogenes* cells kill other bacteria by establishing a contact using T4SS. Once established, *L. enzymogenes* cells most likely transfer effectors with toxic activity in the competitor bacterial cells through T4SS leading to their death. The dead cells of competitor bacterial species will represent a source of nutrients for the *L. enzymogenes* cells endowed with a vast number of lytic enzymes that will degrade the main components of the dead cells. Similarly, *L. capsici* cells induce cell death in the plant pathogenic oomycete *Phytophthora infestans* and up-regulates a relevant number of genes deputed to the degradation of the main components of microbial cell walls (Tomada *et al*., 2017).

As *L. enzymogenes* may kill other bacterial cells using T4SS, we questioned ourselves on what might be the impact of this bacterial species on the activity of other soilborne bacteria. To answer this ecological question, we investigated the interaction of our model strain with plant beneficial and pathogenic bacteria. Firstly, we found that the contact-dependent killing of *L. enzymogenes* against plant beneficial *Pseudomonas* spp. might determine a reduction of their inhibitory activity against plant pathogenic bacteria and fungi (Fig. 7A, 7B). However, we may not exclude that killing *Pseudomonas* spp. may give to *L. enzymogenes* access to plant pathogenic bacteria and fungi and use them as source of nutrients. These evidences are also important from a practical point of view. Indeed, both *Lysobacter* and *Pseudomonas* genera encompass several species that might be exploited for the development of commercial biopesticides (Haas and Défago, 2005; Puopolo *et al*., 2018) and the production of microbial consortia has been proposed as a solution to make biopesticides more efficient when applied in the field (Pertot *et al*., 2017; De Vrieze *et al*., 2018). According to our results, the combination of biocontrol *L. enzymogenes* and *Pseudomonas* strains might not lead to an increase of plant protection efficacy due to the contact-dependent killing behaviour of *L. enzymogenes* cells against plant-beneficial *Pseudomonas* spp. The use of biocontrol *L. enzymogenes* strains having the T4SS inactivated might be a possible solution to this problem. Indeed, the viability of both strains will allow the implementation of both biocontrol activities, rather than bacterial competition that might impair reduce overall biocontrol activity.

It is also worth noting that deployment of T4SS enabled *L. enzymogenes* to protect the plant carrot from infection by the plant pathogenic bacterium, *P. carotovorum* through contact-dependent killing activity (Fig. 7C). The ability of

Lysobacter spp. biocontrol strains to control plant pathogenic bacteria have received scarce attention so far (Ji *et al*., 2008). Thus, our results might stimulate the evaluation of biocontrol *L. enzymogenes* strains also for their ability to control plant disease caused by plant pathogenic bacteria.

Our observations collectively demonstrate that the killing behaviour of *L. enzymogenes* based on the contact-dependent T4SS killing system in soil microbiome might have diverse ecological impacts on microbial population and agro-ecosystem. This might be sustained by evidence on the correlation between the abundance of *Lysobacter* spp. in agricultural soils and the occurrence of plant diseases caused by plant pathogenic bacteria. Indeed, a low abundance of *Lysobacter* spp. was associated with an increase of tobacco plants attacked by *Ralstonia solanacearum*, the causal agent of tobacco bacterial wilt disease (She *et al*., 2017). Differently, the abundance of *Lysobacter* 16S rRNA gene sequences correlated positively with suppression of potato common scab caused by *Streptomyces* spp. (Rosenzweig *et al*., 2012). Based on these evidences, it would be interesting to investigate in future how the introduction of the facultative predator *Lysobacter* spp. in agricultural soils might modify the microbial populations.

Based on our results, we may propose that *L. enzymogenes* may establish multiple interactions in the soil microbiome using "long-distance" weapons as diffusible antibiotics (HSAF) and "short-distance" weapons (i.e. T4SS) as the contact-dependent killing behaviour. Overall, we feel confident that our results might contribute to understand better the role played by *L. enzymogenes* in the complexity of the soil microbiome.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this work are listed in supplemental Table S3. Unless otherwise stated, the *L. enzymogenes* strain OH11 (CGMCC No. 1978) and its derivatives were grown in Luria-Bertani (LB) at 28 °C. Kanamycin (Km, 25 μg/mL) was added in the media for mutant generation, and gentamicin (Gm, 150 μg/mL) for plasmid maintenance. *Escherichia coli* strains were grown in LB medium at 37 °C whereas, *P. carotovorum* PccS1, *Ps. fluorescens* 2P24 and *Ps. protegens* Pf-5, were grown in the same medium at 28 °C.

Genetic methods

Double-crossover homologous recombination approaches were used to generate gene in-frame deletion mutants in OH11 as described previously (Qian *et al*., 2013). In brief, 300- to 500-bp DNA fragments cloned from two flanking regions of genes were amplified by PCR with specific primers (Table S4) and cloned into the broad-host suicide vector pEX18Gm (Table S3). The recombinant vectors were transformed into OH11 cells by electroporation. The LB medium containing 1.2% agar (LA) supplemented with Km (25 μg/mL) and Gm (150 μg/mL) were used to select single-crossover recombinants. Next, the transformants were selected for double crossovers by growing them in LA dishes containing 10% (w/v) sucrose and

Km (100 μg/mL). The mutants were verified by PCR using specific primers (Table S4).

To construct gene complementation strains, full-length genes and their predicted promoters were amplified by PCR using specific primer pairs (Table S4) and cloned into the chromosomally-integrated suicide vector, pEX18GM-ChiA (Xu *et al*., 2015). The generated vector carrying the target genes were transformed into the OH11 wild-type or mutant strains by electroporation. The complementation strains were selected on the LA plates containing Km (100 μg/mL) and Gm (150 μg/mL). All constructs were verified by PCR and sequencing.

Bioinformatics analyses

The T4SS structural proteins from the phylogenetically related bacterial strain *X. citri* 306 (NC_003919.1) were used as queries to run local BLASTp to identify the respective homologs in the OH11 genome. A protein was considered present when the E-value was lower than 10^{-5} and a percentage of similarity with the corresponding *X. citri* 306 homologous protein was higher than 35%. For prediction of the presence of XVIPCD-domain proteins in OH11, the XVIPCD domain sequences of 13 *X. citri* XVIPCD proteins derived from *X. citri* 306 (Souza *et al*., 2015) were aligned by the MUSCLE tool first, and then were used to construct the profile of Hidden Markov Model (HMM), followed by the HMM search against OH11 proteins using the hmmsearch program implemented in HMMER (Finn *et al*., 2011). A XVIPCD domain was considered present when the HMM search E-value was lower than 10^{-5}.

Effector-induced toxicity assay in *Escherichia coli*

For cytoplasmic expression, each effector gene (Table S1) was cloned into the arabinose inducible plasmid pBAD/Myc-HisA (Table S3). The empty vector and the *avrRxo1* gene with known antibacterial activity from *X. oryzae* pv. *oryzae* RS105 (Triplett *et al*., 2016) were used as the negative and positive control, respectively. For artificial expression in periplasm, each effector gene was cloned into the isopropyl β-D-thiogalactoside (IPTG) inducible plasmid pET22 b (+)-PldB (Table S3), which contains the periplasm-localized signal peptide of the antibacterial T6SS effector PldB from *Ps. aeruginosa* PA01 (Jiang *et al*., 2014). The empty vector and *pldB* gene were used as the negative and positive control, respectively.

All recombinant constructs were individually transformed into *E. coli* BL21 (DE3) by electroporation. All transformed strains were grown in LB medium without arabinose or IPTG. The exponentially growing cells were collected and adjusted to optical density at 600 nm (OD_{600}) of 0.5, and serially diluted (10 fold) by fresh LB. Finally, 5 μL of each bacterial dilution culture was spot-inoculated onto the surface of LA dishes amended with and without 2% arabinose (for cytoplasmic expression) or 0.1 mM ITPG (for periplasmic expression). LA dishes were incubated at 37 °C for 24 h and photographed using the Nikon camera (D7100, Japan). The growth test of *E. coli* strains containing both effector and immunity protein genes were performed in a similar way.

Pull-down assays

The plasmids, pUT18C and pKT25 of the bacterial two-hybrid system (Table S3) were modified to carry out pull-down experiments. In brief, pUT18C containing Le0909-FLAG and pKT25 containing Le0908-His were co-transformed to *E. coli* BL21 (DE3) by electroporation. The transformed *E. coli* BL21 (DE3) was grown in LB (20 mL) until OD_{600} reached 1.0. Cells were subsequently collected by centrifugation (12,000 rpm for 10 min at 4 °C). The collected cells were re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH, 7.4), followed by sonication (Sonifier 250; Branson Digital Sonifier, Danbury, USA). After centrifugation (12,000 rpm for 10 min at 4 °C), 1 mL of soluble protein was mixed with 15 µL of anti-FLAG magnetic beads (Bimake, Shanghai, China) according to the manufacturer's instructions. After an overnight incubation at 4 °C, the beads were washed five times for 6 min with 1 mL of 10 mM PBS buffer (pH, 7.4) containing 1% Triton X-100. Proteins bound to the beads were eluted with 45 µL elution buffer (0.2 M glycine, pH 0.3), followed by eluent neutralization with 5 µL of neutralization buffer (1.5 M Tris, pH 10). To evaluate their potential binding, plasmid pUT18C containing VirD4-FLAG and pKT25 containing Le0908-His were used following the procedure described above. The eluted protein samples were identified by Western blots using the specific anti-His (No. M30111L, Abmart, Shanghai, China) and anti-FLAG (No. M20008S, Abmart, Shanghai, China) monoclonal antibodies.

Contact-dependent killing assay

The LacZ-labelled strain *E. coli* BL21 (Wang *et al*., 2020) was used as one of the competitor strains and was grown in LB at 37 °C overnight. The wild-type OH11, Δ*virD4* and the respective complemented strain were used as killer strains and were cultivated in LB medium overnight at 28 °C with constant shaking at 200 rpm. The T6SS-active, plant pathogenic bacterium *A. citrulli* xjl12 was chosen as a positive control, as reported earlier (Tian *et al*., 2015). Cells of all test bacterial strains were collected by centrifugation (6,000 rpm for 3 min at room temperature) and suspended in fresh LB and the absorbance was adjusted to OD_{600} 1.0. The resultant cells of killer and competitor strains were mixed at various ratios and co-inoculated on LA dishes supplemented with or without 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside (X-gal, 100 µg/mL) and incubated at 28 °C. After 24 h, the colonies with and without blue appearance were photographed using the Nikon camera (D7100, Japan).

For fluorescent microscopy, the plasmid pYC12, carrying a mCherry gene driven by the plasmid-carrying constitutive promoter (P_{tac}), was introduced into the killer strains (OH11 and Δ*virD4*). The plasmid pMSC21, containing a constitutively expressed GFP gene, was transferred into *E. coli* BL21, the competitor strain. Similarly, the plasmid pBBR1-MCS5 with a constitutively expressed GFP gene was transformed into the competitor strains - *P. carotovorum* PccS1 (Wang *et al*., 2018), *Ps. fluorescens* 2P24 (Yin *et al*., 2013) and *Ps. protegens* Pf-5 (Parret *et al*., 2005). After an overnight incubation at 28 °C in LB medium in an orbital shaker (200 rpm), all the bacterial cells were collected by centrifugation (6,000 rpm for 3 min at room temperature) and suspended in fresh LB to reach a final OD_{600}, 1.0. A volume of 750

μL of the resultant cell suspension of the killer strains (*L. enzymogenes* strains) was mixed with competitor strains (*E. coli* BL21, *P. carotovorum* PccS1, *Ps. fluorescens* 2P24 and *Ps. protegens* Pf-5) in two ratios (1:1 and 5:1). Then, a volume of 5 μL of the mixture culture was spot-inoculated on LA dishes, followed by 24 h incubation at 28 °C. A 0.22-μm filter membrane was used to separate the growth of killer and tested competitor strains. The stereoscopic fluorescence microscope (Nikon SMZ25, Nikon, Japan) was used to observe the fluorescence signal. The GFP and mCherry fluorescence was excited at 488 nm and 561 nm, respectively. Subsequently, each co-inoculated colony was picked out and suspended in fresh LB (1 mL). The suspended bacterial cells (150 μL) were grown in LA plates without antibiotics for 3 days at 28 °C, followed by calculation of competitor colonies with GFP fluorescence observed by stereoscopic fluorescence machine (Nikon SMZ25, Nikon, Japan). After that, the number of recovered CFU of competitor cells was determined and the mean $\log_{10}$ CFU was calculated. All the experiments were carried out three times and three replicates were used for each treatment. Comparisons of mean values were carried out using Student's T-test (α=0.05) implemented in the SPSS 14.0 package (SPSS Inc., Chicago, IL, USA).

Antifungal and antibacterial assays

In fungal inhibition assay, a plug (2 mm diameter) cut from the border of a 5-day old colony of the soilborne fungal pathogen *V. pyri* SXYL134 (Table S3) was transferred from Potato Dextrose Agar on the centre of dishes containing 1/10 Trytpic Soy Broth agar amended with glucose 2% (w/v). Subsequently, 2 μL of OH11 (killer) and *Ps. fluorescens* 2P24 (competitor) cell suspension (OD_{600}, 1.0) was inoculated individually or in combination (1:1 mixture) on the edge dishes previously inoculated with *V. piri*. The antagonistic activity was indicated by the inhibition zones around the colonies after 3-day incubation at 28 °C. In bacterial inhibition assay, 1 mL of overnight culture of the competitor strains *E. coli* BL21, *P. carotovorum* PccS1, *Ps. fluorescens* 2P24 and *Ps. protegens* Pf-5 was mixed with melted 25-mL LA medium and poured into Petri dishes. Once solidified, 2 μL of the *L. enzymogenes* strain OH11 cell suspension (OD_{600}, 1.0) was spot-inoculated on the surface of LA dishes containing each competitor bacterial strain described above. A volume of 1 μL of the antibiotic gentamycin (50 μg/mL) was spot-inoculated in the centre of each dish and used as a positive control. After 3-day incubation at 28 °C, the inhibition zones were photographed using the Nikon camera (D7100, Japan) in both antifungal and antibacterial assays. The area of the inhibition zone was calculated according to the following formula - area= $\pi \times (\text{radius})^2$, in which the radius is the average radius of the longest axis and the shortest axis of the inhibition zones as described previously (Yang et al., 2020). All the experiments were carried out three times and three replicates were used for each treatment. Comparisons of mean values were carried out using Student's T-test (α=0.05) implemented in the SPSS 14.0 package (SPSS Inc., Chicago, IL, USA).

Carrot slice inoculation and virulence tests

The plant pathogen *P. carotovorum* PccS1, *L. enzymogenes* strain OH11 and Δ*virD4* were cultivated in LB medium at 28 °C overnight with constant shaking at 200 rpm. Cells of all bacterial cultures were collected by centrifugation (6,000 rpm for 3 min at room temperature) and suspended in fresh LB, and adjusted to OD_{600}, 1.0. The resultant cells of killer (OH11 and Δ*virD4*) and competitor (*P. carotovorum* PccS1) strains were inoculated onto the surface of carrot slices individually or in combination (competitor/killer: 5:1). Sterilized H_2O was used as a negative control. The inoculated carrot slices were incubated in a greenhouse (28 °C, 80% relative humidity). After 24 h, the symptoms corresponding to area of carrot slices showing soft rot of were quantified by measuring the average radius of the longest axis and the shortest axis of the area. The area of carrot slices showing symptoms was subsequently calculated according to $\pi \times (\text{radius})^2$. The experiment was repeated three times and three replicates were used for each treatment. Each replicate consisted in a group of three carrot slices. Comparisons of mean values were carried out using Student's T-test (α=0.05) implemented in the SPSS 14.0 package (SPSS Inc., Chicago, IL, USA).

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Author contributions

G.Q, B.N. S.C. and G.P. conceived the project and designed experiments. X.S., B.W., N.Y., L.Z., H.W., Y.D., B.N. and J.F. carried out experiments. X.S., B.W., D.S., G.Q. analysed data. G.Q. and G.P. wrote and revised the manuscript. All the authors read and approved the submission for publication.

Data availability statement

The sequence data of the present work have been submitted to the NCBI GenBank. The accession numbers are presented in supplemental Table S1.

Conflict of Interest

The authors have no conflict to declare.

Figure caption

Figure 1. *Lysobacter enzymogenes* OH11 encodes a bacterial-killing T4SS system. **(A)** Schematic illustration of T4SS structure. The structural components of T4SS are indicated by different colours. **(B)** Genomic arrangement of the T4SS gene cluster in *L. enzymogenes* OH11 (OH11) genome. The gene encoding each T4SS structural component is indicated by number listed at the bottom of the cluster with the same colour described in Fig. 1A. Protein similarity and identity are listed in Table S1 with comparison to the respective component of the bacterial-killing T4SS system in *Xanthomonas citri* 306 (Souza *et al*., 2015). **(C)** Prediction of T4SS effector candidates containing a conserved, C-terminal XVIPCD domain in OH11 genome. Six effectors with asterisks were randomly selected for toxicity investigation.

Figure 2. Characterisation of T4SS effector toxicity and toxin-immunity pair in *Lysobacter enzymogenes* OH11. (A) IPTG-induced expression of six T4SS effector genes in the periplasm of *Escherichia coli* BL21. The empty vector and *pldB*, a *Pseudomonas aeruginosa* gene encoding a T6SS toxic effector, were used as negative and positive control, respectively. **(B)** Arabinose (Ara)-induced expression of six T4SS effector genes in the cytoplasm of *E. coli* BL21. The empty vector and *AvrRxo1*, a *Xanthomonas oryzae* RS105 gene encoding a T3SS toxin effector, were used as negative and positive control, respectively. **(C)** Genomic organisation of the toxic T4SS effector gene, *Le0908* highlighted with green. **(D)** Le0908 and Le0909 formed a toxin-immunity pair. The toxicity of Le0908 to *E. coli* BL21 was neutralised by co-expression of *Le0909*, but not *Le0907* in the *E. coli* periplasm. **(E)** Schematic map of the full-length Le0908 and Le0909 and their truncations (Le0908T and Le0909T). **(F)** Pull-down assay reveals the full-length Le0908-His bound to the truncated Le0909 (Le0909T-FLAG) without signal peptide Flag fused proteins were pulled down and reacted against antibodies. **(G)** Schematic map of the full-length VirD4 and its truncation (VirD4T). **(H)** Pull-down assay reveals the full-length Le0908-His directly interacted with the truncated VirD4 (VirD4T-FLAG) without the signal peptide and transmembrane domain, and the XVIPCD domain of Le0908 is required for this protein-protein interaction. Flag fused proteins were pulled down and reacted against antibodies.

Figure 3. T4SS is required for the contact-dependent killing activity of *Lysobacter enzymogenes* against *Escherichia coli.* (A) Contact-dependent killing activity. *L. enzymogenes* OH11 (OH11) was used as the killer strain, while the LacZ-labelled *E. coli* BL21 was used as the competitor. The T6SS-active *Acidovorax citrulli* xjl12 and the LB broth were used as a positive and a negative control, respectively. Dash-line boxes indicate mixed various ratios shown in the plates of *E. coli* with OH11, Xjl12 or LB. **(B)** Contribution of T4SS to the contact-dependent killing activity of OH11 against the LacZ-labelled *E. coli*. Δ*virD4*, the OH11 T4SS-defective mutant with in-frame deletion of *virD4*, a T4SS specific ATPase. Δ*virD4* (*virD4*), the complemented strain. **(C)** Fluorescence microscopy validated the contact-dependent

killing activity of OH11 against *E. coli*. The mCherry-labelled OH11 or Δ*virD4* was mixed with the GFP labelled *E. coli* BL21 in a 1:3 ratio and co-inoculated on agar plates for 24 h, followed by observation of the GFP and mCheery fluorescence signals. Bars indicate 2 mm. **(D)** Recovered *E. coli* BL21 living cells (competitor) after co-culture (24 h) from Fig. 3C. Mean values ± standard deviation of three replicates for each treatment are represented by the columns. Asterisks indicate values that differ significantly according to Student's t-test (α=0.01). (E) Membrane separation blocked the cell-cell contact-dependent killing of the GFP-labelled *E. coli* by the mCherry-labelled *L. enzymogenes* OH11. The fluorescence signals were observed after 24-hour incubation. Bars indicate 2 mm.

Figure 4. T4SS determines the contact-dependent killing activity of *Lysobacter enzymogenes* OH11 against soilborne bacteria. (A-C) T4SS-mediated, contact-dependent killing activity of the mCherry-labelled OH11 against the GFP-labelled *Pectobacterium carotovorum* PccS1 **(A)**, *Pseudomonas* (*Ps.*) *fluorescens* 2P24 **(B)**, *Ps. protegens* Pf-5 **(C)**. The mCherry-labelled killer strains (OH11 or Δ*virD4*) were mixed with the GFP-labelled competitor strains in a 1:1 ratio and co-inoculated on agar plates for 24 h, followed by observation of the GFP and mCheery fluorescence signals. Bars indicate 2 mm. **(D-F)** Recovered living cells of competitor - *P. carotovorum* PccS1 **(D)**, *Ps. fluorescens* 2P24 **(E)** and *Ps. protegens* Pf-5 **(F)** from Fig. 4A-C. Mean values ± standard deviation of three replicates for each treatment are represented by the columns. Asterisks indicate values that differ significantly according to Student's t-test (α=0.01).

Figure 5. The contact-dependent killing activity of *Lysobacter enzymogenes* OH11 impairs the antibacterial and antifungal activity of plant beneficial *Pseudomonas* bacteria. (A) Plate antifungal assay against the soil pathogenic fungus, *Valsa pyri*. **1**, *Pseudomonas* (*Ps.*) *fluorescens* 2P24; **2**, Δ*lafB* - the antifungal-deficient mutant of *L. enzymogenes* OH11 (OH11), carrying an in-frame deletion in the *lafB* gene of the HSAF biosynthetic operon; **3**, Δ*lafB*Δ*virD4* – an OH11 derivative with double mutations in *lafB* and *virD4*; **4**, 2P24: Δ*lafB* (1:1); **5**, 2P24: Δ*lafB*Δ*virD4* (1:1). (**B**) Statistical analysis of antifungal zones. Length of the longest axis and shortest axis of the inhibition zones were determined and averaged as the radius, R, according to an earlier report (Yang *et al*., 2020). The inhibition zones were calculated by using the formula $\pi \times R^2$. Mean values ± standard deviation of three replicates for each treatment are represented by the columns. Asterisks indicate values that differ significantly according to Student's t-test (α=0.01). **(C)** Fluorescent observation of the contact-dependent killing of the mCherry-labelled Δ*lafB* or Δ*lafB*Δ*virD4* against the GFP-labelled Pf-5 that was co-inoculated on agar plates in a 1:1 ration and co-inoculated. The fluorescence signals were observed after 24-hour incubation. Bars indicate 2 mm. **(D)** Plate antibacterial assay against the soil pathogenic *Pectobacterium carotovorum* PccS1. **1#**, OH11; **2#**, Δ*virD4* - the *virD4* mutant; **3#**, Pf-5 – *Ps. protegens*; **4#**, Pf-5: OH11 (1:1); **5#**, Pf-5:Δ*virD4* (1:1). Bars indicate the radius of the zones. **(E)** Statistical analysis of antibacterial zones. The antibacterial zones were determined by $\pi \times R^2$. Mean values ± standard deviation of three replicates for each treatment are represented by the columns. Asterisks indicate values that differ significantly according to Student's t-test (α=0.01).

Figure 6. T4SS-dependent killing activity of *Lysobacter enzymogenes* OH11

against *Pectobacterium carotovorum* impairs pathogen virulence on host carrot. (A-B) Plant inoculation assay (**A**) and statistical analysis of maceration zones (**B**). Black dash-lined triangles indicate the initial inoculation, while the red dash-line triangles indicate the maceration symptom. PccS1: OH11 and PccS1: Δ*virD4*, co-inoculation of PccS1 and OH11 or Δ*virD4* in a 5:1 ratio. Maceration zones were determined similar to Fig. 5B. Mean values ± standard deviation of three replicates for each treatment are represented by the columns. Asterisks indicate values that differ significantly according to Student's t-test (α=0.01). Bar indicates 1 cm.

Figure 7. The *Lysobacter enzymogenes* OH11 T4SS-mediated, contact-dependent competition model with soil microbiome bacteria. According to the findings of the present study, we proposed that *L. enzymogenes* OH11 (OH11), a soilborne biocontrol bacterium, deploys T4SS as the main contact-dependent weapon against beneficial and pathogenic bacteria occurring in the soil microbiome. Killing of soil biocontrol bacteria not only gives access of *L. enzymogenes* to the nutrients released by their competitor **(A)**, but also decreases the antibacterial and antifungal activity of the plant beneficial *Pseudomonas* bacteria, providing *L. enzymogenes* advantages to "privately" kill fungal or bacterial pathogens as additional nutrient sources **(B)**. T4SS also favours OH11 to protect plant from the infection of the pathogenic bacterium, *Pectobacterium carotovorum* by the contact-dependent killing **(C)**.

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Supporting information

Table S1. GenBank accession numbers of T4SS components and effectors.
Table S2. Contact-dependent killing activity of *L. enzymogenes* strains against the LacZ-labelled *E. coli.*
Table S3. Strains and plasmids used in this study.
Table S4. Primers used in this study.
Figure S1. *L. enzymogenes* OH11 failed to kill *E. coli* through secreting diffusible factors.
Figure S2. The Δ*virD4* and Δ*virD4lafB* strains exhibited a growth curve similar to the

wild-type OH11 of *L. enzymogenes* in liquid LB broth.

Figure S3. The Δ*virD4* strain displayed a fluorescent signal intensity similar to the wild-type OH11.

Figure S4. The cell-free supernatant of *L. enzymogenes* OH11 failed to kill *E. coli* when co-culture on LB agar plates in a 1:1 ratio.

Figure S5. *L. enzymogenes* OH11 failed to kill three soilborne bacteria through secreting diffusible factors.

Figure S6. Membrane separation blocked the cell-cell contact-dependent killing of three soilborne bacteria by *L. enzymogenes* OH11.

Figure S7. The cell-free supernatant of *L. enzymogenes* OH11 failed to kill three soilborne bacteria when co-culture on LB agar plates in a 1:1 ratio.

Figure S8. Evidence of the counterattack of *Pseudomonas protegens* Pf-5 against *L. enzymogenes* OH11 via type VI secretion system (T6SS).

Figure S9. The Δ*lafB*Δ*virD4* strain displayed a fluorescent signal intensity similar to Δ*virD4*.

Figure S10. Fluorescent observation of the contact-dependent killing of the mCherry-labelled *L. enzymogenes* OH11 or Δ*virD4* against the GFP-labelled PccS1 that was co-inoculated on agar plates in a 1:5 ratio.

Figure S11. The mCherry-labelled *L. enzymogenes* OH11 was unable to kill the GFP-labelled *Bacillus subtilis* NCD-2 when both strains were co-cultured on agar plates in a 1:1 ratio.