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ORIGINAL ARTICLE

Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease

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Pathogenic bacteria interact not only with the host organism but most probably also with the resident microbial flora. In the knot disease of the olive tree (*Olea europaea*), the causative agent is the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*). Two bacterial species, namely *Pantoea agglomerans* and *Erwinia toletana*, which are not pathogenic and are olive plant epiphytes and endophytes, have been found very often to be associated with the olive knot. We identified the chemical signals that are produced by strains of the three species isolated from olive knot and found that they belong to the *N*-acyl-homoserine lactone family of QS signals. The *luxI/R* family genes responsible for the production and response to these signals in all three bacterial species have been identified and characterized. Genomic knockout mutagenesis and *in planta* experiments showed that virulence of *Psv* critically depends on QS; however, the lack of signal production can be complemented by wild-type *E. toletana* or *P. agglomerans*. It is also apparent that the disease caused by *Psv* is aggravated by the presence of the two other bacterial species. In this paper we discuss the potential role of QS in establishing a stable consortia leading to a poly-bacterial disease.
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Introduction

Bacterial diseases result from the ability of the pathogens to colonize and regulate gene expression in response to the host environment. Consequently, most studies thus far have been centered on the molecular interactions that take place between host and bacteria. Are there any interactions between the pathogen and other bacteria, which occupy the same niche ('residents')? For instance Sibley *et al.* (2008) have shown recently that there are interspecies bacterial interactions between the human pathogen *Pseudomonas aeruginosa* and the indigenous non-pathogenic bacterial residents present in the host (Duan *et al.*, 2003; Sibley *et al.*, 2008). With this work we intend to initiate a systematic study into the role of interspecies signaling in plant bacterial pathogenesis by using as model the olive knot disease caused by the bacterial pathogen *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*).

P. savastanoi is closely related to *Pseudomonas syringae*, which is a plant pathogen able to cause many different symptoms in various plants (Hirano and Upper, 2000; Hofte and De Vos, 2006). In recent years, considerable progress has been made in understanding the virulence mechanisms caused by *P. syringae* on herbaceous plants and several strains have also been sequenced (Feil *et al.*, 2005; Joardar *et al.*, 2005). By contrast, progress in the understanding of the pathogenicity of *P. savastanoi* on woody plants has been very slow. *P. savastanoi* strains can infect various woody host species such as olive, ash and oleander, often inducing an overgrowth of the infected tissues, causing knots, cankers and wart-like excrescences (Hirano and Upper, 2000; Kennelly *et al.*, 2007). Various pathovars are distinguished within this species, including pv. *savastanoi*, pv. *fraxini* and pv. *nerii* causing knots and galls on members of the Oleaceae family and oleander (Gardan *et al.*, 1992; Vivian and Mansfield, 1993).

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Psv is the causative agent of the olive knot disease in the olive plant (*Olea europaea* L.) (Gardan *et al.*, 1992; Vivian and Mansfield, 1993; Hirano and Upper, 2000). Surprisingly, very few molecular studies on the *Psv* virulence determinants have thus far been performed, and these initial investigations have established that a type-III secretion system, and the phytohormones indole-3-acetic acid (IAA) and cytokinins are involved in knot development (Surico *et al.*, 1985; Glass and Kosuge, 1988; Sisto *et al.*, 2004; Rodriguez-Moreno *et al.*, 2008).

Interestingly, apart from *Psv*, two other bacterial species have been found very often to be associated with the olive knot, namely *Pantoea agglomerans* (Gavini *et al.*, 1989; Fernandes and Marcelo, 2002; Marchi *et al.*, 2006) and *Erwinia toletana* (Rojas *et al.*, 2004). To date little is known about the possible synergistic and community effects of these *Enterobacteriaceae* on knot development. *P. agglomerans* is widespread in many diverse natural and agricultural habitats; in particular, it is associated with many plants as a common epiphyte and endophyte (Lindow and Brandl, 2003). It can either depress the growth of *Psv* in olive plants probably through antibiotic production or in some cases it can also increase the knot size (Marchi *et al.*, 2006). Their frequent presence and isolation from the same environment, where they coexist as common endophytic residents of olive knots, hints that interactions, community formation and synergisms might take place. The olive knot bacterial community therefore provides a special niche to study the role of interspecies communication and community interplay between the pathogen and resident bacteria in the development of disease.

A cell–cell signaling mechanism, which is known to have an important role in virulence in plant pathogenic bacteria, is the quorum sensing (QS) intercellular communication system (Von Bodman *et al.*, 2003). QS regulates gene expression in response to cell density through the production and detection of signal molecules (for reviews, see references Bassler (2002) and Fuqua and Greenberg (2002)). In Gram-negative bacteria, the most common signal molecules are the *N*-acyl homoserine lactones (AHLs), which are produced by an acyl homoserine lactone synthase belonging in most cases to the LuxI-protein family. A transcriptional sensor/regulator belonging to the LuxR family then forms a complex with the cognate AHL at threshold ('quorum') concentrations thereby affecting the transcription of target genes (Fuqua *et al.*, 2001). Bacteria in nature mostly grow as poly-microbial consortia, which most likely involve interspecies signaling through the action of diffusible signal molecules (Ryan and Dow, 2008; Duan *et al.*, 2009). Understanding the signaling taking place in poly-bacterial communities will be a challenge for future studies as most investigations on QS thus far have involved mono-culture set-ups.

The role of AHLs in interspecies signaling and community formation is not clear and, at least in our view, not studied sufficiently. This work is meant as the beginning of a systematic study of this issue using the olive knot community between *Psv*, *P. agglomerans* and *E. toletana* as a model system. Do all three species produce AHL signals and are they involved in inter-species signaling? AHL QS thus far is regarded as being species/strain-specific, however it is not known if AHL QS has a major role in niches involving stable cooperation between different bacterial members. Results have shown that all three species possess AHL QS systems producing similar AHLs and in two cases producing the same AHLs. AHL QS has been shown here for the first time to be pivotal for *Psv* pathogenicity as virulence AHL QS-knockout mutants is strongly reduced. Pairwise co-inoculations of the olive plant with wild-type residents and *Psv* AHL synthase mutants restore full *Psv* virulence. This shows that the different species can form stable microbial consortia where AHL QS has a major role. Co-inoculation studies *in planta* have also shown that synergisms among the different bacterial species take place, indicating that the knots of the olive tree can be caused by a poly-bacterial disease.

Materials and methods

Bacterial strains, plasmids and media

The *Psv*, *P. agglomerans* and *E. toletana* strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown at 28 °C in M9 minimal medium supplemented with glucose (Sambrook *et al.*, 1989), in King's B medium (King *et al.*, 1954) or in Luria–Bertani. Six AHL bacterial biosensors were used for AHL detection: *Chromobacterium violaceum* strain CVO26 (McClean *et al.*, 1997), *Agrobacterium tumefaciens* NTL4/pZLR4 (Shaw *et al.*, 1997), *Escherichia coli* MT102/pJBA132 (Andersen *et al.*, 2001), *Pseudomonas putida* F117/pASC8 and *P. putida* F117/pKRC12 (Riedel *et al.*, 2001). The *Chromobacterium, Agrobacterium* and *Pseudomonas* AHL detector strains were grown at 30 °C as recommended, whereas the *E. coli* detector strains were grown at 37 °C. Antibiotics at the following final concentrations were added when required: ampicillin, 100 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; tetracycline, 15 μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Pseudomonas*); gentamicin, 10 μg ml^{-1} (*E. coli*), 30 μg ml^{-1} (*Agrobacterium*) and 40 μg ml^{-1} (*Pseudomonas*); kanamycin, 50 μg ml^{-1} (*E. coli* and *C. violaceum*) or 100 μg ml^{-1} (*Pseudomonas*, *Pantoea* and *Erwinia*); nitrofurantoin, 50 μg ml^{-1}.

Recombinant DNA techniques

Recombinant DNA techniques, including digestion using restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, end filling using the Klenow enzyme and transformation of *E. coli* were performed as described by Sambrook *et al.* (1989). Plasmids were

Table 1 Bacterial strains isolated from olive or oleander knots caused by *Pss*, the plasmids and the primers used in this study

Strain, plasmid or primer	*Relevant characteristics*	*Reference or source*
P. savastanoi pv. *savastanoi*		
LMG 2209[T]	Olive knots (Ex-Yugoslavia)	—
DAPP-PG 536	Olive (Italy)	Moretti *et al.* (2008)
DAPP-PG 722	Olive (Italy)	This study
DAPP-PG 723	Oleander knots (Italy)	This study
DAPP-PG 724	Oleander knots (Italy)	This study
DAPP-PG 725	Olive knots (Italy)	This study
DAPP-PG 726	Oleander knots (Malta)	This study
DAPP-PG 727	Oleander knots (Italy)	This study
DAPP-PG 728	Olive knots (Italy)	This study
DAPP-PG 685	pssI: Km of *Pss* DAPP-PG 722	This study
DAPP-PG 686	pssR: Km of *Pss* DAPP-PG 722	This study
Pantoea agglomerans		
CFBP 3845[T]	—	—
DAPP-PG 729	Olive knots (Italy)	This study
DAPP-PG 730	Oleander knots (Italy)	This study
DAPP-PG 731	Olive knots (Italy)	This study
DAPP-PG 732	Oleander knots (Malta)	This study
DAPP-PG 733	Oleander knots (Italy)	This study
DAPP-PG 734	Olive knots (Italy)	This study
DAPP-PG 691	pagI: Km of *P. agglomerans* DAPP-PG 734	This study
DAPP-PG 692	pagR: Km of *P. agglomerans* DAPP-PG 734	This study
E. toletana		
CFBP 6631[T]	Olive knots (Spain)	Rojas *et al.* (2004)
DAPP-PG 735	Olive knots (Italy)	This study
DAPP-PG 736	*tolI*: Km of *E. toletana* DAPP-PG 735	This study
DAPP-PG 737	*tolR*: Km of *E. toletana* DAPP-PG 735	This study
Plasmid		
pLAFR3	Broad-host-range cloning vector, IncP1; Tet^R	Staskawicz *et al.* (1987)
pMOSBlue	*E. coli* cloning vector; Amp^R	GE Healthcare, Milan, Italy
pJD100	pLAFR3 containing *P. savastanoi* DNA	This study
pTH100	pLAFR3 containing *E. toletana* DNA	This study
pBBR mcs-5	Broad-host-range vector; Gm^R	Kovach *et al.* (1995)
pMOSPSSIR	pMOSBlue with a 5.8-kb fragment containing the QS genes of *Psv*; Amp^R	This study
pBBRtolIR	pBBR mcs-5 with an 8-kb *Hin*dIII fragment containing the QS genes of *E. toletana*; Gm^R	This study
pKNOCK-Km	Conjugative suicide vector; Km^R	Alexeyev (1999)
pKNOCK-pssI	Internal PCR *Eco*RV *pssI* fragment of *P. savastanoi* cloned in pKNOCK-Km	This study
pKNOCK-pssR	Internal PCR *Eco*RV *pssR* fragment of *P. savastanoi* cloned in pKNOCK-Km	This study
pKNOCK-pagI	Internal PCR *Eco*RV *pagI* fragment of *P. agglomerans* cloned in pKNOCK-Km	This study
pKNOCK-pagR	Internal PCR *Eco*RV *pagR* fragment of *P. agglomerans* cloned in pKNOCK-Km	This study
pKNOCK-tolI	Internal PCR *Eco*RV *tolI* fragment of *E. toletana* cloned in pKNOCK-Km	This study
pKNOCK-tolR	Internal PCR *Eco*RV *tolR* fragment of *E. toletana* cloned in pKNOCK-Km	This study
Primers		
PssI For	5′-CCTGTGATCCCTACATGC-3′	This study
PssI Rev	5′-AGACTTGCGCACGATCCG-3′	This study
pssR For	5′-AGCGATTTCGCTGTACGACT-3′	This study
pssR Rev	5′-ACCACATTGGCGATTTCACT-3′	This study
PagI For	5′-ACTGGCTTTGTCCCTGGTAA-3′	This study
PagI Rev	5′-AACGGGAAGTTGCTATGTGG-3′	This study
pagR For	5′-TGCCTACGCCATTATGAACA-3′	This study
pagR Rev	5′-GCATGTGTGGCGTTATTCAC-3′	This study
TolI For	5′-TTAAATGTACGGGTGAGATG-3′	This study
TolI Rev	5′-TTTTAATATGGTGAGCATCG-3′	This study
TolR For	5′-TCCAATTACCCGAAGGAGTG-3′	This study
TolR Rev	5′-CCCCAGAATCACAGCAATTT-3′	This study

Abbreviation: *Psv*, *Pseudomonas* pv. *savastanoi*.
[T]Indicates type of strain: CFBP, Collection Française des Bactéries Phytopathogènes; DAPP-PG, Dipartimento di Scienze Agrarie e Ambientali, sezione di Arboricoltura e Protezione delle Piante, Perugia (Italy); LMG, Belgian Coordinated Collections of Microorganisms.

purified using Jet star columns (Genomed, Löhne, Germany); total DNA from *Psv*, *P. agglomerans* and *E. toletana* was isolated by Sarkosyl–Pronase lysis as described previously by Better *et al*. (1983). Triparental matings between *E. coli* and *Psv*, *P. agglomerans* or *E. toletana* were performed using the helper strain *E. coli* DH5 (pRK2013). DNA sequence homology searches were performed using the National Center for Biotechnology Information BLAST.

Cloning and inactivation of QS genes in P. savastanoi, P. agglomerans *and* E. toletana

For *Psv* DAPP-PG 722 and *E. toletana* DAPP-PG 735, two cosmid libraries were constructed by using the cosmid pLAFR3 (Staskawicz *et al*., 1987) as vector. DNA inserts were prepared by partial *Eco*RI digestion of the two genomic DNAs and then each was ligated in the corresponding site in pLAFR3. The ligated DNA was then packaged into λ-phage heads using the Gigapack III Gold packaging extract (Agilent Technologies, Santa Clara, CA, USA) and the phage particles were transduced to *E. coli* HB101 as recommended by the supplier. The two sets of *E. coli* HB101, each harboring a cosmid library, were conjugated *en masse* into the AHL biosensor *C. violaceum* CVO26 as acceptor. Each conjugation gave rise to one transconjugant: cosmid pTH100 originating from the cosmid bank of *E. toletana* DAPP-PG 735 and cosmid pJD100 originating from *Psv* DAPP-PG 722, which allowed strain CVO26 to produce violacein and turn purple. The AHL QS system of *E. toletana* DAPP-PG 735 was designated *etoI/R* and was localized in an 8-kb *Hin*dIII fragment, which was cloned into pBBRMCS-1, producing pBBRtolIR. The AHL QS system of *Psv* DAPP-PG 722 was designated *pssI/R* and was localized in a 5800-bp *Nru*I fragment, which was cloned into pMOSBlue producing pMOSPSSIR.

Different genomic null mutants were created in the AHL QS system of *Psv* DAPP-PG 722, *P. agglomerans* DAPP-PG 734 and *E. toletana* DAPP-PG 735 as follows. For *Psv* (i) an internal 246-bp fragment of *pssI* was amplified from strain DAPP-PG 728 genomic DNA using the primers PssI For and PssI Rev (Table 1), and (ii) a 518-bp fragment of *pssR* was amplified using the primers pssR For and pssR Rev (Table 1). Similarly, (i) an internal 387-bp fragment of *pagI* was amplified from strain DAPP-PG 734 genomic DNA using the primers PagI For and PagI Rev (Table 1), which were designed using the published *pagI/R* genes of *P. agglomerans* pv. *gypsophilae* (Chalupowicz *et al*., 2009), and (ii) a 561-bp fragment of *pagR* was amplified using the primers pagR For and pagR Rev (Table 1). For *E. toletana* (i) an internal 350-bp fragment of *tolI* was amplified from strain DAPP-PG 735 genomic DNA using the primers TolI For and TolI Rev (Table 1), and (ii) a 474-bp fragment of *etoR* was amplified using the primers TolR For and TolR Rev (Table 1). All the above-mentioned PCR products were cloned into pKNOCK-Km digested using *Eco*RV, generating pKNOCK-pssI, pKNOCK-pssR, pKNOCK-pagI, pKNOCK-pagR, pKNOCK-etoI and pKNOCK-etoR (Table 1). These plasmids were then used as a suicide delivery system in order to create knockout mutants of *Psv* strain DAPP-PG 722, *P. agglomerans* DAPP-PG 734 and *E. toletana* DAPP-PG 735 by homologous recombination as described previously Alexeyev (1999). These experiments allowed the creation of the following genomic mutants: DAPP-PG 722PSSI, DAPP-PG 722PSSR, DAPP-PG 734PAGI, DAPP-PG 734PAGR, DAPP-PG 735ETOI and DAPP-PG 735ETOR (Table 1). All the mutants were verified by PCR using primers specific to the pKNOCK-Km vector and to the genomic DNA sequences upstream and downstream from the targeted genes.

AHL extraction, visualization and quantification

Psv, *P. agglomerans* and *E. toletana* strains were first tested for the production of AHLs using a 'T-streak' analysis on solid medium as described previously by Piper *et al*. (1993), using the AHL biosensors (all reviewed by Steindler and Venturi, 2007) *A. tumefaciens* NTL4 (pZLR4), *C. violaceum* CVO26, *E. coli* MT102 (pJBA132), *Pseudomonas* F117 (pKRC12) and *Pseudomonas* F117 (pASC8) on Luria–Bertani agar plates.

AHLs were purified from spent supernatant and separated using a C_{18} reverse-phase chromatography thin-layer chromatography (TLC) plate as described previously by Shaw *et al*. (1997). For visualization on TLC, the plate was overlaid with a thin layer of AB top agar seeded with *A. tumefaciens* NTL4 (pZLR4) in the presence of 100 μg ml^{-1} X-gal as previously described by Shaw *et al*. (1997), or with Luria–Bertani top agar seeded with *C. violaceum* CVO26 (McClean *et al*., 1997).

AHL detection and identification by high-performance liquid chromatography and MS

The AHLs produced by *Psv*, *E. toletana* and *P. agglomerans* were identified by LC/MS/mass spectrometry (MS) in a multiple reaction monitoring experiment as described previously by Gould *et al*. (2006). Monitoring was performed on the transition from the parent ion to both the acyl and the lactone moiety peaks. The peaks were compared to known standards and evaluated by chromatographic retention time analysis as well. A 200-ml volume of cell-free culture supernatants were extracted by using the same volume of ethyl acetate, after addition of 0.1% acetic acid. The organic phases were separated and dried under a chemical hood. The extracted samples for LC/MS/MS were resuspended in 100 μl of acetonitrile, filtered through a 0.2-μm filter (Millex LCR4; Millipore, Billerica, MA, USA) and diluted to 300 μl with MilliQ water containing 0.1% trifluoroacetic acid. A 100-μl volume of this solution was injected onto a 2.0 mm-by-150 mm Gemini C_{18}

column (Phenomenex, Torrance, CA, USA) operated at a flow rate of 200 μl min^{-1}, with the effluent flowing directly into the mass spectrometer. Solvent-A consisted of water containing 0.05% trifluoroacetic acid and Solvent-B comprised acetonitrile containing 0.05% trifluoroacetic acid. The column was equilibrated in 20% B for 15 min, the sample injected and the column was washed for additional 15 min. A gradient elution method from 20% B to 95% B in 40 min was then used for the separation of the AHLs and the column finally washed in 95% B for an additional 10 min before re-equilibration.

Motility assays, biofilm formation, EPS, IAA and siderophore production, lipase and protease activities

Proteolytic and lipolytic activities, swarming and swimming were determined as reported previously Huber *et al*. (2001). The method used to detect siderophores was adapted from the universal chemical assay on chrome azurol-S (CAS) agar plates (Schwyn and Neilands, 1987), as described previously by Caballero-Mellado *et al*. (2007). IAA production by bacteria was measured using a colorimetric assay as described previously by Gordon and Weber (1951) and Vasanthakumar and McManus (2004).

Analysis of exoploysaccahride (EPS) production of *P. agglomerans* and *E. toletana* was tested on Kings medium B (KB) solid medium, whereas *Psv* was tested on minimal mannitol (MM) solid medium (0.2% yeast extract, 2% mannitol, 1.5% agar). Bacterial strains were grown in Luria–Bertani plates, streaked to yield individual colonies and grown at 28 °C for 24 h. Single colonies were then streaked on KB or MM agar and grown for 48 h at 28 °C. Colonies producing EPS have a fluidal, mucoid appearance, whereas those deficient in EPS have a distinct non-mucoidal, creamy colony morphology.

In planta *experiments*

All *in planta* experiments were performed using 1-year-old olive (cv. Frantoio) plants. To prepare the inocula, bacteria were grown onto NA (Nutrient Agar) at 28 °C for 48 h, suspended in sterile deionized water and adjusted spectrophotometrically to approximately 2×10^8 c.f.u. ml^{-1}. For the inoculations, 10 μl of the bacterial suspension containing 10^8 c.f.u. ml^{-1} or water (for control plants) were placed in wounds (3–5 per plant) made in the bark of olive stems using a sterile scalpel as described previously by Moretti *et al*. (2008). Wounds in inoculated and control plants were protected with Parafilm M (American National Can, Chicago, IL, USA). The plants were maintained in transparent polycarbonate boxes to reach high RH values (90–100%) and kept in a growth chamber at 22–24 °C, with illumination at 70 μE m^{-2} s^{-1} and a 12-h light period.

In the first *in planta* experiment, it was verified whether the PssI/R AHL QS system of *Psv* was involved in pathogenicity and virulence. The *Psv* DAPP-PG 722 parental strain and the *ppsI* and *ppsR* derivative knockout mutants were inoculated in olive plants and disease severity was evaluated after 60 days by measuring the depth of the tissue overgrowths using a Vernier caliper.

In the second experiment, the effect of co-inoculation of olive plants with *Psv* or the respective AHL QS mutants and *E. toletana* or *P. agglomerans*, and their respective AHL QS mutants, on disease severity and bacterial growth *in planta* was evaluated 60 days after the inoculation. For co-inoculation, the two bacterial suspensions were mixed (ratio of 1:1) to obtain a final concentration of 10^8 c.f.u. ml^{-1}. Disease severity was recorded by determining the volume of knots, which was calculated by measuring the length, width and depth (subtracting the stem diameter measured above the knot from that measured below the knot) of the knot using a Vernier caliper (Moretti *et al.*, 2008). For bacterial growth determination, the tissue in correspondence of the inoculation site was excised and homogenized by mechanical disruption. Serial dilutions of the resulting bacterial suspensions were plated onto NA plates and incubated at 27 ± 1 °C. Colony counts were made after 24 and 48 h of incubation. *Psv* colonies were easily distinguishable from those of *E. toletana* and *P. agglomerans*. *Psv* colonies, which grew slower with respect to those of *E. toletana* and *P. agglomerans*, were small, white, with a slightly raised matt centre and a flat transparent waved edge. *E. toletana* colonies were non-pigmented, circular, convex, with entire margins, translucent fluidal and highly mucoid (only wild type). *P. agglomerans* colonies were yellow, circular, slightly convex, with an irregular margin, a more or less wrinkled surface, translucent fluidal and highly mucoid (only wild type).

In the third and fourth experiments, the effect of co-inoculation of olive plants with *Psv* and *E. toletana* on bacterial growths was evaluated 3, 8, 15, 30 and 60 days post inoculation (dpi) as described in the second experiment. Three plant replicates for each treatment level ((1) *Psv*, (2) *E. toletana*, (3) *Psv* + *E. toletana*) were included in each experiment. For each plant, inocula were placed in three wounds made at three different positions: basal (about 20 cm above the soil level), intermediate and apical.

Statistical analyses

The data of the first and second *in planta* experiments were subjected to analysis of variance and the means were compared by Duncan's multiple-range test, by using the software DSAASTAT v. 1.1 (Onofri, 2007).

The data of the third and fourth *in planta* experiments were transformed into base 10 logarithms (to correct for heteroscedasticity) and were

used to parameterize a linear mixed model (Garrett *et al*., 2004; Onofri, 2010) describing the relationship between the number of bacterial cells and the square root of time (for each strain/group) by way of second-order polynomial functions. Preliminary analyses showed that the effect of inoculation position (basal, intermediate and apical) was not significant and, therefore, plant and inoculation position within plant were added as random effects to the model, to account for grouped data. The estimation of parameters was performed by maximum likelihood, as implemented in the nlme package in the R statistical environment (Venables and Ripley, 2002). The differences among strains/groups in terms of growth kinetics were assessed by using likelihood ratio tests.

DNA sequencing and nucleotide sequence accession numbers

All DNA sequences were performed either at the CRIBI center (University of Padova, Italy) or at Macrogen (www.macrogen.com) and the nucleotide sequences were deposited in GenBank/EMBL/DDBJ. The *etoI/R* QS locus of *E. toletana* DAPP-PG 735 is deposited under accession number FN870373, whereas the *pssI/R* locus of *Psv* is deposited under accession number FN870374.

Results

AHL production and the QS systems of the P. savastanoi*,* P. agglomerans *and* E. toletana *strains*

First, it was of interest to establish if the *Psv* pathogen and olive knot residents produce AHL QS signaling molecules. All strains that we tested (Table 1) produced AHL compounds as detected initially by plate T-streak assays of *C. violaceum* CV026 and *A. tumefaciens* NTL4 (pZLR4), and *E. coli* MT102 (pJBA132), biosensors. By TLC analysis we initially assigned tentatively the type of the AHLs produced by *Psv*, *P. agglomerans* and *E. toletana* (Figure 1 and Table 2). The results showed that all *Psv* and *E. toletana* strains produced two AHL molecules tentatively identified as C6-3-oxo-HSL and C8-3oxo-HSL (Figure 1). *P. agglomerans* on the other hand most probably produced C4-HSL and C6-HSL. In order to unequivocally confirm the AHLs produced by the three stains, we used C_{18} reverse-phase high-performance liquid

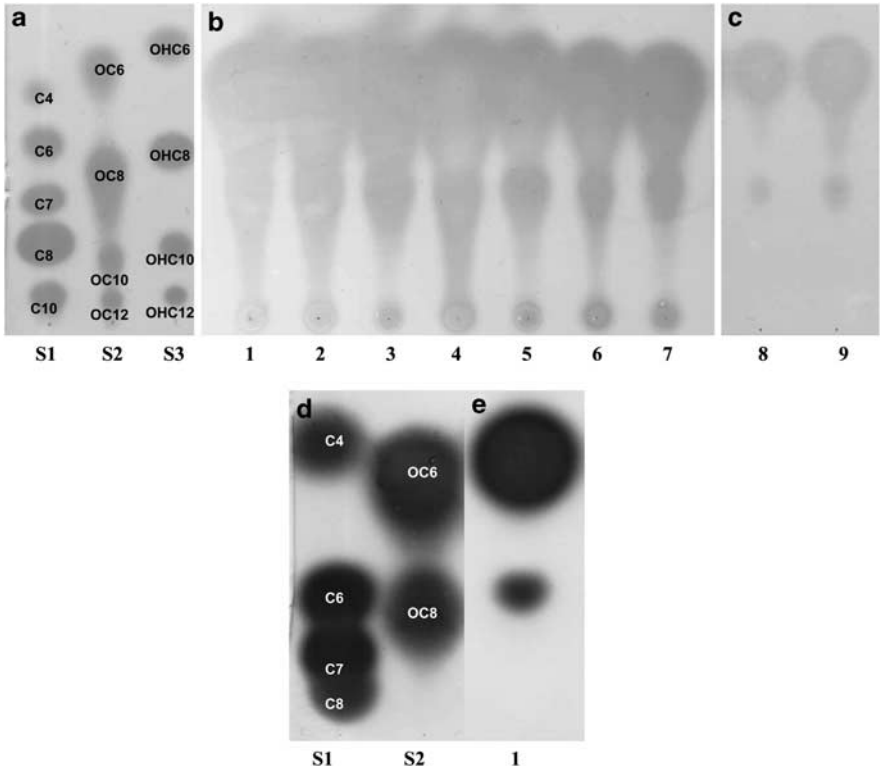

Figure 1 AHL production of *Psv* isolated from olive and oleander knots, *E. toletana* and *P. agglomerans*. (**a**) TLC analysis of the synthetic AHL standards using the *A. tumefaciens* pNTL4 biosensor as overlay. (**b**) TLC analysis of the AHLs produced by seven *Psv* strains using the *A. tumefaciens* pNTL4 biosensor as overlay. Lanes: (1) Strain LMG 2209^T; (2) DAPP-PG 536; (3) DAPP-PG 722; (4) DAPP-PG 723; (5) DAPP-PG 725; (6) DAPP-PG 726; (7) DAPP-PG 728. (**c**) TLC analysis of the AHLs produced by two *E. toletana* strains using the *A. tumefaciens* pNTL4 biosensor as overlay. Lanes: (8) Strain CFBP 6631^T; (9) DAPP-PG 735. (**d**) TLC analysis of the synthetic AHL standards using the C. *violaceum* CV026 biosensor as overlay. (**e**) TLC analysis of the AHLs produced by *P. agglomerans* DAPP-PG 734 (Lane 1) using the C. *violaceum* CV026 biosensor as overlay. AHL, acyl homoserine lactone; *Psv*, *Pseudomonas* pv. *savastanoi*; TLC, thin-layer chromatography.

Table 2 Phenotypes tested for AHL QS regulation in *Psv* DAPP-PG 722, *P. agglomerans* DAPP-PG 734 and *E. toletana* DAPP-PG 735

	Psv *DAPP-PG 722*			P. agglomerans *DAPP-PG 734*			E. toletana *DAPP-PG 735*		
	WT	*pssI*$^-$	*pssR*$^-$	*WT*	*pagI*$^-$	*pagR*$^-$	*WT*	*etoI*$^-$	*etoR*$^-$
Phenotype									
AHL	+	−	+	+	−	+	+	−	+
IAA	+	+	+	+	+	+	+	+	+
Siderophore	+	+	+	+	+	+	+	+	+
Protease	-	−	−	−	−	−	−	−	−
Lipase	+	+	+	+	+	+	+	+	+
Swarming	+	+	+	-	−	−	−	−	−
Swimming	+	+	+	+	+	+	+	+	+
EPS	+	−	−	+	−	+	+	−	+++

Abbreviations: AHL, acyl homoserine lactone; EPS, exoploysaccahride; IAA, indole-3-acetic acid; *Psv*, *Pseudomonas* pv. *savastanoi*; QS, quorum sensing; WT, wild type.

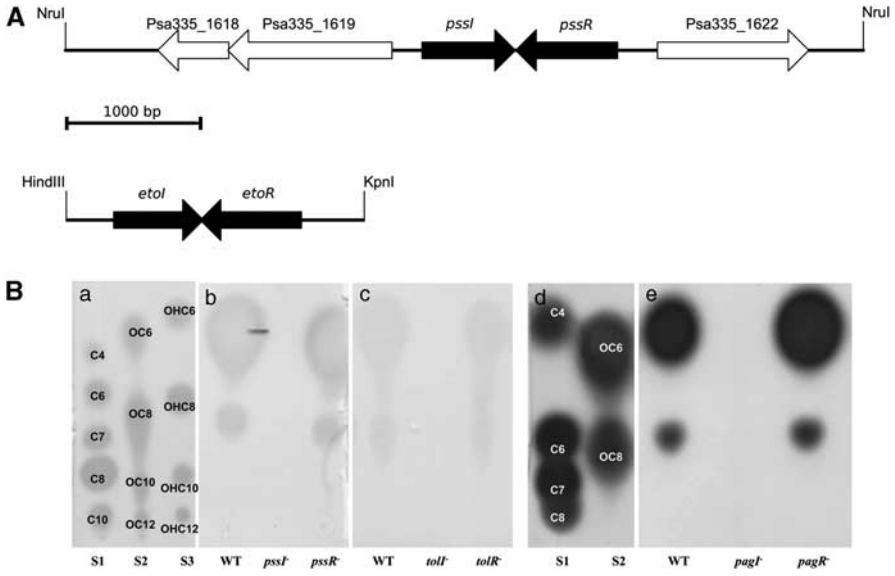

Figure 2 Gene maps and AHL production by AHL QS-knockout mutants of the olive knot isolates of *Psv*, *E. toletana* and *P. agglomerans*. (**A**) Gene maps of the cloned *ppsI/R* and *etoI/R* loci of *Psv* and *E. toletana*, respectively. Neighboring the *ppsI/R* system, genes are shown that are orthologs in sequenced *Psv* NCPPB 3335. In fact the region sequenced here in strain DAPP-PG 722 is orthologous to the one present in strain NCPPB 3335. (**B**) (a) TLC analysis of the synthetic AHL standards using the *A. tumefaciens* pNTL4 biosensor as overlay. (b) TLC analysis of the AHLs produced by the parent *Psv* DAPP-PG 722 strain and mutant derivatives using the *A. tumefaciens* pNTL4 biosensor as overlay. *pssI*$^-$ is DAPP-PG 722PSSI; *pssR*$^-$ is DAPP-PG 722PSSI. (c) TLC analysis of the AHLs produced by the parent strain *E. toletana* DAPP-PG 735 and mutant derivatives using the *A. tumefaciens* pNTL4 biosensor as overlay. *tolI*- is DAPP-PG 735TOLI; *tolR*- is DAPP-PG 735TOLR. (d) TLC analysis of the synthetic AHL standards using the *C. violaceum* CV026 biosensor as overlay. (e) TLC analysis of the AHLs produced by the parent *P. agglomerans* DAPP-PG 734 strain and mutant derivatives using the *C. violaceum* CV026 biosensor as overlay. *pagI*$^-$ is DAPP-PG 734PAGI; *pagR*$^-$ is DAPP-PG 734PAGR. AHL, acyl homoserine lactone; *Psv*, *Pseudomonas* pv. *savastanoi*; QS, quorum sensing; *savastanoi*; TLC, thin-layer chromatography.

chromatography and mass spectrometry, and were able to verify the production of 3-oxo-C6-HSL and 3-oxo-C8-HSL by *Psv* and *E. toletana*, and C6-HSL and C4-HSL by *P. agglomerans* (Supplementary Figure 1).

The AHL QS system of *Psv* DAPP-PG 722 was identified and cloned, as explained under Materials and methods, consisting of a *luxI* homolog designated *pssI* and a *luxR* homolog designated *pssR* (Figure 2a). The PssI/R system shows a very high degree of homology (over 90%) to the AhlI/R AHL QS system of *P. syringae*, which responds to C6-3-oxo-HSL and is involved in virulence (Quinones *et al.*, 2005). Similarly, we identified and cloned the AHL QS system of *E. toletana* DAPP-PG 735 (see section Materials and methods) consisting of a *luxI* homolog designated *etoI* and a *luxR* homolog designated *etoR* (Figure 2a). The EtoI/R system shows significant homology (approximately 60%) to the ExpI/R system of *Erwinia chrysanthemi* pv. *zeae* (=*Dickeya zeae*), which is responsible for not only producing but also responding to C6-3-oxo-HSL (Nasser *et al.*, 1998; Hussain *et al.*, 2008). It was

established by PCR, cloning and sequencing that the *P. agglomerans* AHL QS system of strain DAPP-PG 734 was orthologous (over 95% identity) to the PagI/R system of *P. agglomerans* pv. *gypsophilae* (Chalupowicz *et al.*, 2009).

Psv, *P. agglomerans* and *E. toletana* knockout mutants were generated in both the *luxI*- and the *luxR*-family QS genes as described under Materials and methods. None of the *luxI*-family mutants of the three species (DAPP-PG 722PSSI, DAPP-PG 734PAGI and DAPP-PG 738ETOI; Table 1) produced detectable levels of AHLs when purified from spent supernatants (Figure 2b). This result indicated that all three species most probably possess only one AHL QS system; for *Psv*, further support for this conclusion is provided by the fact that only one *luxI/R* family pair is present in its genome, which has been sequenced recently (Rodriguez-Palenzuela *et al.*, 2010). The *luxR*-family mutants on the other hand (DAPP-PG 722PSSR, DAPP-PG 734PAGR and DAPP-PG 738ETOR; Table 1) produced amounts of AHLs similar to the amounts produced by the wild type (Figure 2b), indicating that for all three systems there was no signal amplification loop through the regulation of the *luxI*-family gene. These conclusions were based on the TLC analysis of spent supernatant extracts purified from equal amounts of culture at the same growth phase. This is a very good indication, however measurements throughout the growth phase would probably confirm this conclusion further.

QS in Psv *regulates virulence* in planta

To examine whether the PssI/R AHL QS system of *Psv* was involved in pathogenicity and virulence, the *Psv* DAPP-PG 722 parental strain and the *ppsI* and *ppsR* derivative knockout mutants were inoculated on 1-year-old olive plants and disease severity was evaluated after 60 days. The results showed clearly that mutations in *pssI* and *pssR* drastically reduced knot size and therefore the virulence of the mutants (Figure 3a). Knot depth was reduced significantly ($P \leqslant 0.01$) in the stems inoculated with the mutants DAPP-PG 722PSSI and DAPP-PG 722PSSR as compared with those inoculated with

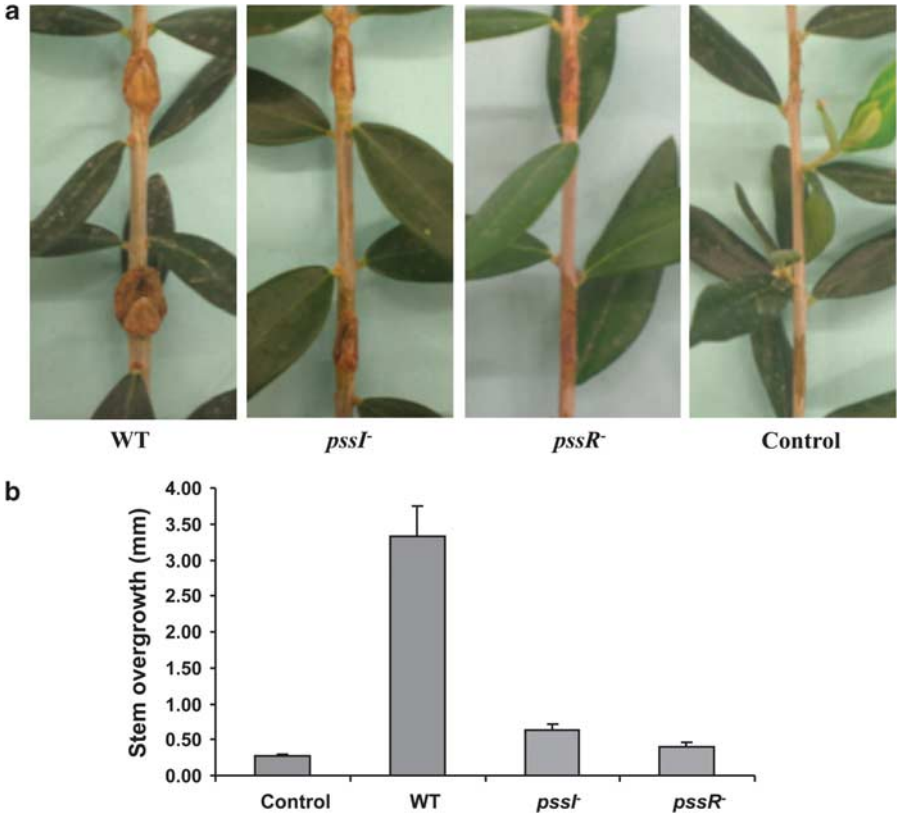

Figure 3 Role of AHL QS on olive knot formation induced by *Psv*. (**a**) The stems of 1-year-old olive plants (cv. Frantoio) were inoculated with 10^8 c.f.u. ml^{-1} *Psv* DAPP-PG 722 (wild type: WT), *pssI*$^-$ and *pssR*$^-$ derivative mutants, and with control plants. Disease symptoms were observed 60 days after the inoculation. Large raised knots were observed in stems inoculated with DAPP-PG 722 (wild type). The AHL QS deficiency mutants *pssI*$^-$ and *pssR*$^-$ induced only limited cell proliferation, which remained restricted to the wound margins. (**b**) Knot size was estimated by measuring the depth of the tissue overgrowths in five plants per each treatment, and for three knots per plant. Statistically significant differences in knot size between the wild type and the QS mutants were determined by analysis of variance. Each value is the mean of five replicates ± s.d. The means are not significantly different ($P = 0.01$) according to Duncan's multiple-range test. AHL, acyl homoserine lactone; *Psv*, *Pseudomonas* pv. *savastanoi*; QS, quorum sensing; *savastanoi*.

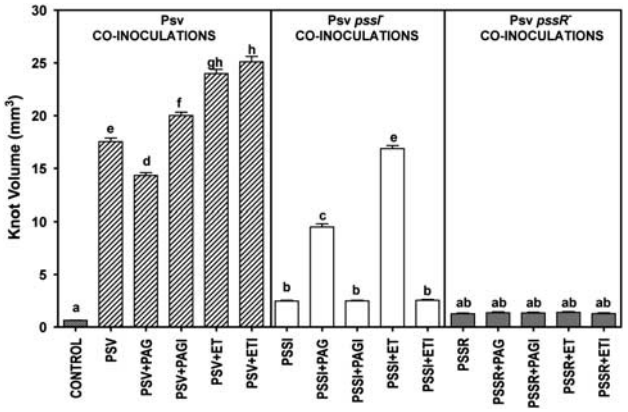

Figure 4 Effect of co-inoculation of olive plants (cv. Frantoio) with *Psv* and *E. toletana*, *P. agglomerans* and the respective AHL QS mutants. *Psv*: The columns indicate effect on disease severity, expressed as knot volume (60 days after the inoculation), of inoculation of the WT Psv (hatched columns), of QS-impaired mutants of *Psv* (white columns represent PSSI mutant and black columns PSSR mutants) alone or in combination with *P. agglomerans* (Pag) or *E. toletana* (Et) and their relative QS-impaired mutants, PagI, PagR, EtoI and EtoR. Olive control plants were inoculated with distilled water (gray column). Each value is the mean of five replications ± s.e. Mean followed by the same letters are not statistically different at $P = 0.05$ (Duncan's multiple-range test). AHL, acyl homoserine lactone; *Psv*, *Pseudomonas* pv. *savastanoi*; QS, quorum sensing.

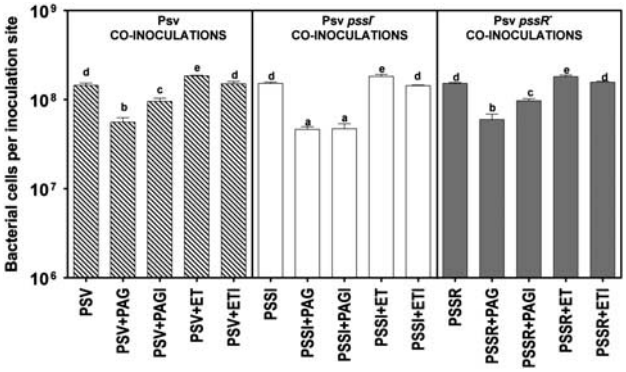

Figure 5 Effect on *Psv* levels of co-inoculation of olive plants (cv. Frantoio) with *Psv* and *E. toletana*, *P. agglomerans* and the respective AHL QS mutants. *Psv*: The black columns indicate effect on bacterial growth *in planta* (60 days after the inoculation) of inoculation of the QS-impaired mutants of *Psv* of the same bacterium PssI (white columns) or PssR (hatched columns) alone or in combination with *P. agglomerans* (Pag) or *E. toletana* (Et) and their relative QS-impaired mutants, PagI, PagR, EtI and EtR. Each value is the mean of five replications ± s.e. Mean followed by the same letters are not statistically different at $P = 0.05$ (Duncan's multiple-range test). *Psv*, *Pseudomonas* pv. *savastanoi*.

the wild type, showing reductions of approximately 80% and 88%, respectively (Figure 3b). Importantly, it was therefore concluded, to our knowledge, that that AHL QS, for the first time, had been observed to have a central role in the virulence of *Psv*.

Psv *AHL synthase mutants can be rescued by the presence of* E. toletana *and in part by* P. agglomerans

The presence of *E. toletana* and *P. agglomerans* in olive knots could result in the formation of a poly-microbial community with the *Psv* pathogen. As *E. toletana* and *P. agglomerans* both produce AHLs and as *Psv* AHL-negative mutants have very low virulence (see above), this allows an elegant setup in order to establish if AHL signals are shared and if interspecies communities form in the olive knot.

As *E. toletana* produces structurally the same AHLs as *Psv* (see above), an *in planta* co-inoculation was initially set up, where the *pssI* AHL synthase mutant was used for co-infection of olive plants together with the *E. toletana* DAPP-PG 735 wild-type strain. This co-inoculation resulted in the *Psv ppsI* mutant being able to induce knot formation just like the wild-type strain (Figure 4). This can most probably be attributed to the two strains making a consortium, where *E. toletana* provides the AHLs to DAPP-PG 722PSSI, which is then able to induce AHL QS target gene expression resulting in olive knot formation. Similar pairwise inoculations were also performed with the *P. agglomerans* DAPP-PG 734 wild-type strain and here only partial restoration of knot formation was observed (Figure 4). This could be because of *P. agglomerans* producing different AHLs than *Psv*, which the PssI/R system, most probably responded to with less efficiency. Importantly, pairwise *in planta* inoculations with either the *E. toletana* or the *P. agglomerans* AHL synthase mutant, *etoI* or *pagI*, respectively, did not restore olive knot formation. This clearly indicates that interspecies communication through sharing of AHLs was key in the previous co-inoculation experiments where the *Psv* AHL synthase mutants were co-inoculated independently with the two wild-type strains (Figure 4).

The *Psv pssR* mutant could not be complemented for olive knot formation by *E. toletana* and *P. agglomerans*; the most likely reason being that the the *ppsR Psv* mutant cannot respond to AHLs. These results also indicate that *E. toletana* and *P. agglomerans* wild-type strains cannot cause disease alone in the absence of wild-type *Psv*. Importantly, in all co-inoculation experiments, comparable amounts of *Psv* colony-forming units (c.f.u.) were present at the inoculation site (Figure 5).

It was concluded that both *E. toletana* and *P. agglomerans* can form stable interspecies communities with *Psv* and that communication through sharing of AHL signals was taking place *in planta*.

Role of interspecies signaling and pairwise consortia in olive knot disease

As evident from the results described above, *Psv* forms bacterial communities with *E. toletana* and *P. agglomerans*. In order to begin to understand the role and outcome of possible interactions among these three bacterial species, the virulence of *Psv* in the presence of the other two olive knot-resident

bacteria was tested *in planta*. One-year-old olive plants were inoculated for each bacterial strain with 10 µl of a bacterial suspension having 10^8 c.f.u. ml^{-1}. As shown in Figure 4, significant enhancement of *Psv* virulence in the presence of *E. toletana* was observed as indicated by a considerable increase (almost 30%) of knot volume. This indicated that *E. toletana* contributes to the pathogenicity of *Psv*; the increase in virulence was not because of changes in the levels of *Psv* in the co-inoculations as there was no significant difference in bacterial load (Figure 5). The same contribution to *Psv* virulence was observed when co-inoculating with the *E. toletana* AHL synthase mutant; one cannot exclude, however, that *Psv* AHL signals are perceived by *E. toletana*. No increase in virulence, on the other hand, was observed when *P. agglomerans* was co-inoculated with *Psv*, and in fact a slight decrease of knot volume was observed. When co-inoculating with the *P. agglomerans* AHL synthase mutant, however, a significant increase in knot volume formation was detected, meaning that QS regulates some factor(s) in *P. agglomerans*, which limit *Psv* growth and/or gene expression.

Following the evident results on the poly-bacterial nature of the disease and of the sharing of AHL signals between *Psv* and *E. toletana*, it was of interest to gain more insight into this bacterial community. The impact of co-inoculation on the growth dynamics of these bacteria was determined in two separate experiments. As the results of these experiments were similar, only data from one of the experiments were reported. Co-inoculations were therefore performed and bacterial populations were determined until 60 dpi. When *Psv* DAPP-PG 722 was inoculated alone, a 10-fold increase could be detected in the bacterial population isolated from inoculated tissue 8 dpi (appreciatively 10^7 c.f.u. per site) (Figure 6a, open circles). The population of *Psv* progressively increased to reach about 1.5×10^8 c.f.u. per site at 60 dpi. When mixed inoculation with *E. toletana* DAPP-PG 735 (Figure 6a, filled circle) was performed, *Psv* growth was significantly increased (likelihood ratio test with $P<0.0001$), whereas when *E. toletana* was inoculated alone in olive plants, a decline was observed in its population sizes (Figure 6b, open triangle). On the other hand, the growth of *E. toletana* could not be described adequately by using a second-order polynomial model, but nonetheless it was stimulated significantly at 30 dpi (the standard error of difference was 0.018) in the presence of *Psv* (Figure 6b, filled triangle), and after 60 dpi, the population size from the mixed infection was approximately 103-fold when compared with results obtained from the single inoculations. These results are a further indication of the intimate and mutualistic relationship between *Psv* and *E. toletana* in the olive knot, leading to enhanced disease symptoms, sharing of AHL QS signals and increasing bacterial growth of both species.

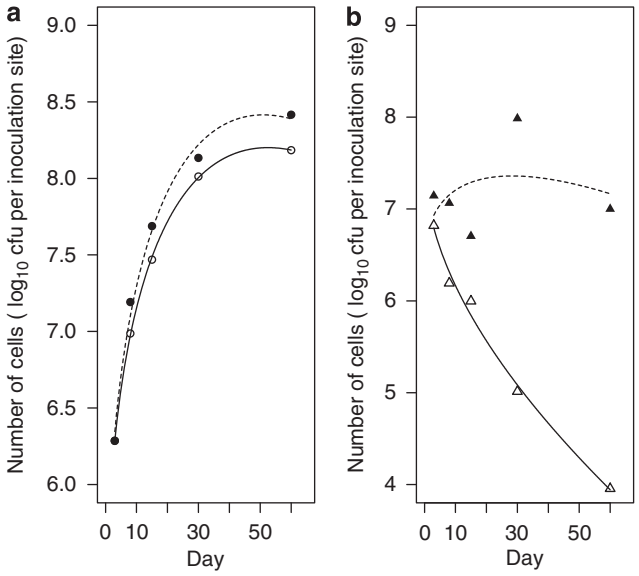

Figure 6 The population dynamics of *Psv* DAPP-PG 722 and *E. toletana* DAPP-PG 735 strains inoculated in olive (cv. Frantoio) stems alone or in combination. (**a**) Growth of *Psv* alone (solid line and open circle) or in combination with *E. toletana* (dotted line and filled circle). (**b**) Growth of *E. toletana* alone (solid line and open triangle) or in combination with *Psv* (dotted line and filled triangle). The symbols indicate observed counts and the lines indicate the linear mixed model fit. The standard error of a mean was 0.013.

Testing the AHL QS-dependent regulation of several phenotypes in P. savastanoi*,* P. agglomerans *and* E. toletana

In order to establish the possible role of the AHL QS systems in the olive knot-associated bacteria, several important phenotypes were tested for AHL QS regulation in all three species. A list of all phenotypes tested for AHL QS regulation is summarized in Table 2.

Interestingly, we determined that *P. agglomerans* DAPP-PG 734 and *E. toletana* DAPP-PG 735 also produce IAA. We tested wild-types *Psv* (DAPP-PG 722), *P. agglomerans* (DAPP-PG 734), *E. toletana* (DAPP-PG 735) and their derivative mutants (DAPP-PG 722PPSI, DAPP-PG 722PSSR, DAPP-PG 734PAGI, DAPP-PG 734PAGR, DAPP-PG 735ETOI and DAPP-PG 735ETOR) for the production of IAA. Similarly, we tested whether AHL QS was involved in siderophore production, EPS production, secreted proteolytic and lipolytic activities, and motility by swimming and swarming. The results of these investigations are summarized in Table 2. Surprisingly, in most tests no significant differences were observed between the parent strains and their QS mutant derivatives under the conditions tested. EPS production was tested qualitatively in a plate assay in all wild-type and mutant strains. Both AHL QS mutants of *Psv* strain DAPP-PG 722 showed a clear reduction in EPS production when compared with the wild type (data not shown); EPS production was restored when mutant DAPP-PG 722PSSI was grown in the presence of exogenous C6-3-oxo- or

C8-3oxo-HSL (data not shown). Similarly, for *P. agglomerans*, inactivation of *pagI* resulted in a reduction of EPS production; however, no reduction was observed in the *pagR* mutant when compared with the wild type. EPS production was restored when mutant DAPP-PG 734PAGI was grown in the presence of C4-HSL or C6-HSL (data not shown). EPS production was also reduced in the *E. toletana* DAPP-PG 735ETOI mutant when compared with that in the wild-type, and complementation through exogenous addition C6-3-oxo-HSL or C8-3oxo-HSL restored EPS production. In *E. toletana*, however, inactivation of the *etoR* significantly increased EPS production (data not shown).

Discussion

Many studies thus far have shown that plant bacterial diseases are a result of complex interactions between the pathogen and host. The interaction of the pathogen with the resident bacterial flora is however much less studied and understood. Do plant pathogenic bacteria form stable bacterial communities with other resident, non-pathogenic bacteria? Are these association networks resulting in a poly-microbial infection? Does intercellular signaling have a role in the establishment of these bacterial consortia? These are the questions we are beginning to address here using the bacterial community present in the olive knot disease. This niche is created by the *Psv* pathogen; however, several other resident, non-pathogenic bacterial species have been isolated in this environment, most commonly *E. toletana* (Rojas *et al*., 2004) and *P. agglomerans* (Marchi *et al*., 2006). The major conclusions of this work are that (i) the three different species isolated from the olive knot produce AHL signals and possess one AHL QS system; notably *E. toletana* and *Psv* produce the same AHLs, (ii) AHL QS in *Psv* is essential for virulence as knockout mutants are basically not virulent, (iii) *Psv* AHL synthase mutants can be rescued by *E. toletana* and in part by *P. agglomerans*, evidencing the sharing of AHL signals and the formation of stable bacterial communities, and (iv) *E. toletana* forms communities *in planta*, acting as an associated pathogen synergistically with the principal pathogen *Psv* as co-inoculations increase the bacterial count of both species and the severity of disease. All the bacterial mutants constructed in this study were produced through homologous recombination events and also involved plasmid insertions. There is, therefore, a possibility that our experiments performed *in planta* without antibiotic selection could lead to mutants reverting to the wild type. As all the various control experiments in our study did not result in a wild-type behavior, we concluded that no significant reversion took place.

Currently, the only known molecular determinants of knot development by *Psv* are the type-III secretion system (Sisto *et al*., 2004) and the phytohormones cytokinins and IAA (Surico *et al*., 1985; Glass and Kosuge, 1988; Rodriguez-Moreno *et al*., 2008). To this list, AHL QS can now be added and to our knowledge this is the first regulatory system that has been linked to *Psv* virulence. AHL QS is likely not affecting initial colonization but rather the gene expression of virulence-associated factors at high population densities. It would now be relevant to determine whether AHL QS is involved in the regulation of the three virulence determinants identified thus far. Most likely that AHL QS is also regulating many other virulence factors. The recent sequencing of the *Psv* genome highlighted the presence of many loci, which could be involved in virulence, including five different secretion systems, type-III effectors, catabolic systems of phenolic compounds, chemotaxis, motility, adhesion and toxin genes (Rodriguez-Palenzuela *et al*., 2010). We have reported that EPS production is regulated by the PssI/R system in *Psv*; however, it is not known yet if EPS is involved in the disease process. EPS, for example, is a virulence factor and is regulated by AHL QS is closely related *P. syringae* (Quinones *et al*., 2005).

The life of bacteria inside the knot is unknown at large. A recent study, however, has shown that *Psv* creates the knot by forming bacterial aggregates, microcolonies and multilayer biofilms (Rodriguez-Moreno *et al*., 2009; Perez-Martinez *et al*., 2010). Little is known about the effects of *P. agglomerans* and *E. toletana* on knot development and the possible formation and stability of the multispecies bacterial consortia formed in the olive knot. *P. agglomerans* is widespread in many diverse natural and agricultural habitats; in particular, it is associated with many plants as a common epiphyte and endophyte (Lindow and Brandl, 2003). In addition, *P. agglomerans* can be transformed from a commensal and epiphytic bacterium associated with many plants to a host-specific tumorigenic pathogen, through acquisition of a plasmid-borne pathogenicity island (Barash and Manulis-Sasson, 2007).

The AHLs produced by *E. toletana* (C6-3-oxo-HSL and C8-3oxo-HSL) are the same as the ones produced by *Psv*. This is evidence that there could be interspecies signaling through AHLs taking place between the two species when they occupy the same niche. *P. agglomerans* on the other hand produces C4-HSL and C6-HSL; it cannot be excluded that PssR and/or EtoR are able to respond to these AHLs. Similarly, it is not known if PagR can also respond to the AHLs produced by *E. toletana* and *Psv*. Our studies here involving binary *in planta* co-inoculations have shown that *E. toletana* can rescue the *Psv pssI* AHL synthase mutants for their ability to induce olive knot formation. As the symptoms take at least 25–30 days to develop, this result has the connotation that *E. toletana* and *Psv* are able to form a stable consortium, where the two species undergo

interspecies signaling and AHL sharing. *P. agglomerans* can also rescue, in part, the *Psv ppsI* mutant; the reason for this partial rescue is either that, the AHLs produced by *P. agglomerans* are not recognized well by PssR, or the consortium is not as stable as it is for *E. toletana*. It is possible that in this niche, which allows such a consortium to grow, could then encourage the appearance of bacterial cheaters (for example, signal blind cheaters, which do not respond to AHL signals), which do not contribute to the community, but can benefit from the 'factors' or 'public goods' produced by the QS cooperators (Diggle *et al*., 2007; Venturi *et al*., 2010). Many more bacterial isolates need to be isolated from this niche in order to determine this possibility.

The ability of *Psv* to induce olive knot formation relies on its ability to regulate gene expression in response to the host environment. A question that we also began to address here is, whether the indigenous avirulent microflora contributes to the disease caused by *Psv*. The co-inoculation of *E. toletana* with *Psv* resulted in a significant enhancement of olive knot volume, indicating that this consortium is probably very stable and *Psv* benefits from the presence of *E. toletana* and possibly vice versa. Importantly, in addition, co-inoculation studies determined that growth of *Psv* in the olive knot was significantly stimulated by *E. toletana* and vice versa, indicating a close association, possible metabolic, nutrient and signal sharing, between the two bacterial species. This synergistic effect of *E. toletana* with *P. savastanoi* clearly indicates a mutual benefit for both species when growing in and sharing the same niche. Understanding the mechanisms of cooperation and communication of these two bacteria will shed light on the process of interspecies communication. *E. toletana* is not pathogenic when inoculated with the AHL QS *Psv pssR* mutant, but can act as an accessory pathogen when in consortium with wild-type *Psv*. It is possible that gene expression of *Psv* can be influenced by the presence of bacterial residents, or that the microbial consortium stabilizes *Psv* metabolically. The co-inoculation of *P. agglomerans* with *Psv*, on the other hand, resulted in a reduction of knot volume, which increased when the co-inoculant was the AHL synthase mutant *pagI*. *P. agglomerans* most likely produces and AHL QS regulates the factor, which affects *Psv*, and when the production is relieved, *P. agglomerans* can also act as an accessory pathogen.

There are very few reports on the contribution/role of the host's resident indigenous microbial flora on the action of an incoming pathogen. Interestingly, in 1998 it was reported that in the rhizosphere of wheat, there is an inter-population of bacterial species, which can possibly share AHL molecules (Pierson *et al*., 1998). Recently, two studies have shown that virulence of *P. aeruginosa* is influenced by the indigenous microflora, and have also shown that it affects the gene expression patterns of virulence-associated factors (Duan *et al*., 2003; Sibley *et al*., 2008). It is currently unknown if there is sharing of signals among these bacteria; it has been observed that the AI (autoinducer)-2 signal produced by both Gram-positive and Gram-negative bacteria could be an interspecies signal in this community (Duan *et al*., 2003; Sibley *et al*., 2008). Interestingly however, Dulla and Lindow (2009) have reported very recently that indigenous epiphytic bacteria producing the same AHLs of pathogenic *P. syringae* pv. *syringae* can suppress and interfere with the disease-causing process. This work provided evidence for an AHL-mediated cross-talk in the plant phyllosphere, which resulted in changes of behavior (mainly motility, which meant less invasion) and consequently virulence of *P. syringae* pv. *syringae*. The occurrence of disease in co-inoculations with AHL-producing epiphytic bacteria was reduced by 50%. This is believed to be because of premature and untimely induction of AHL QS affecting the expression of virulence-associated factors. This example shows that interspecies communication with indigenous bacterial flora can also have negative consequences for plant pathogens.

The data presented here further enhance the apparent complexity of poly-microbial infections; our results indicate a role of AHL sharing and interspecies signaling, which provides an excellent working model to further study bacterial community interactions. This study focuses on AHL-mediated interactions in natural habitats; so far the role of QS in plant-associated bacteria has been studied almost exclusively using single strains despite the possibility of interspecies signaling that can be mediated through sharing of AHL signal molecules in mixed microbial communities. The interactions between bacteria in natural settings are complicated by a number of factors, including host response, environmental parameters and the species composition of the community. It is suggested that future studies will need to develop *in vitro* systems for a convenient and detailed understanding of interspecies interactions. We are only just beginning to develop tools for studying microbial interactions in the natural environment.

We will focus our future studies on the stability and the role played by each species in this microbial consortium. *Psv* can be regarded as the niche maker or leader in this consortium, whereas *P. agglomerans* and *E. toletana* as niche occupants or residents, which, however, can become auxiliary pathogens in the presence of a niche maker. At present it is unclear whether or not such pathogenic consortia have a common rationale. We speculate that the olive knot consortium evolved to be stable and cooperative because the augmented gene pool and the combined metabolic repertoire increases the survival chances of the participants under a variety of conditions (Venturi *et al*., 2010). This work suggests that complex interspecies communication processes may be necessary for the formation of such consortia.

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