**Bacterial multispecies studies and microbiome analysis of a plant disease**

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Although the great majority of bacteria found in nature live in multispecies communities, microbiological studies have focused historically on single species or competition and antagonism experiments between different species. Future directions need to focus much more on microbial communities in order to better understand what is happening in the wild. We are using olive knot disease as a model to study the role and interaction of multispecies bacterial communities in disease establishment/development. In the olive knot, non-pathogenic bacterial species (e.g. *Erwinia toletana*) co-exist with the pathogen (*Pseudomonas savastanoi* pv. *savastanoi*); we have demonstrated cooperation among these two species via quorum sensing (QS) signal sharing. The outcome of this interaction is a more aggressive disease when co-inoculations are made compared with single inoculations. *In planta* experiments show that these two species co-localize in the olive knot, and this close proximity most probably facilitates exchange of QS signals and metabolites. *In silico* recreation of their metabolic pathways showed that they could have complementing pathways also implicating sharing of metabolites. Our microbiome studies of nine olive knot samples have shown that the olive knot community possesses great bacterial diversity; however, the presence of five genera (i.e. *Pseudomonas*, *Pantoea*, *Curtobacterium*, *Pectobacterium* and *Erwinia*) can be found in almost all samples.

**INTRODUCTION**

Most bacterial research in plant pathology thus far, with the exception of crown gall caused by *Agrobacterium tumefaciens*, has focused most commonly on herbaceous plant diseases rather than woody plant diseases (Mansfield *et al.*, 2012). In recent years, the olive plant bacterial pathogen *Pseudomonas savastanoi* pv. *savastanoi* has begun to be studied, developing as a new model for woody plant disease (Rodriguez-Moreno *et al.*, 2008, 2009; Rodriguez-Palenzuela *et al.*, 2010). Olive trees (*Olea europaea* L.) infected by *Pseudomonas savastanoi* pv. *savastanoi* develop overgrowths, referred to as galls, knots or tumours, mainly on the aerial parts of the plants, with their incidence being rare on leaves and fruits. The productivity of olive trees infected with *Pseudomonas savastanoi* pv. *savastanoi* is reduced and no effective treatment is yet in place (Matas *et al.*, 2012; Quesada *et al.*, 2010; Ramos *et al.*, 2012; Schroth *et al.*, 1973; Young, 2004). The few studies of *Pseudomonas savastanoi* pv. *savastanoi* virulence have implicated type III secretion, phytohormones and quorum sensing (QS) as being involved in the disease process (Hosni *et al.*, 2011; Iacobellis *et al.*, 1994; Pérez-Martínez *et al.*, 2010; Surico *et al.*, 1985). A recent signature-tagged mutagenesis screening resulted in the identification of numerous other mechanisms associated with the virulence of *Pseudomonas savastanoi* pv. *savastanoi* (Matas *et al.*, 2012).

Interestingly, several other bacterial species have been isolated within the olive knot that are harmless or non-pathogenic to the olive plant (Moretti *et al.*, 2011; Ouzari *et al.*, 2008; Rojas *et al.*, 2004). The possible role of these *Pseudomonas savastanoi* pv. *savastanoi* co-residents in the olive knot has rarely been addressed; isolates of *Pantoea agglomerans* and *Erwinia toletana* are believed to create multispecies communities with *Pseudomonas savastanoi* pv. *savastanoi*. More specifically, our laboratory has reported...
previously that disease progression and knot volume was increased significantly by co-inoculation of \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} with \textit{E. toletana}. In addition, it was shown that \textit{E. toletana} and \textit{Pantoaea agglomerans} could rescue \textit{in planta} a QS mutant of \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} by exogenously providing \textit{N}-acylhomoserine lactone (AHL) signals to \textit{Pseudomonas savastanoi} pv. \textit{savastanoi}. The main conclusions drawn from that work were the exchange/sharing of QS AHL signals between \textit{E. toletana}, \textit{Pantoaea agglomerans} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi}, and the synergistic effect on the disease when \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} were co-inoculated in olive plants. Cell numbers of both \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} increase when co-inoculated, providing further evidence of mutualism and interspecies interactions (Hosni et al., 2011). \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} is believed to grow as a biofilm inside the olive knot (Rodríguez-Moreno et al., 2009), and questions that therefore arise are how are \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} distributed spatially inside the olive knot, and how are they organized with respect to each other? Multispecies interactions within bacterial consortia are now beginning to be studied, and factors such as metabolic sharing, biofilm formation and chemical signalling are thought to play important roles (Hosni et al., 2011; Kolenbrander et al., 2010; Kuramitsu et al., 2007; Ramsey et al., 2011).

Recent research efforts are beginning to highlight the importance of social and molecular behaviour in multispecies bacterial communities (Duan et al., 2003; Hosni et al., 2011; Kim et al., 2008; Kolenbrander et al., 2010; Kogaonkar et al., 2013; Kuramitsu et al., 2007; Maldonado-González et al., 2013; Mitri et al., 2011; Ramsey et al., 2011; Stolyar et al., 2007). For example, in human oral bacteria, synergistic interactions occur via a mechanism of metabolite cross-feeding where the commensal \textit{Streptococcus gordonii} degrades glucose generating l-lactate, which is then used by the pathogen \textit{Aggregatibacter actinomycetemcomitans} (Ramsey et al., 2011). Another interesting finding demonstrated the effect of the resident oropharyngeal microflora on the incoming human fibrocytic opportunistic pathogen \textit{Pseudomonas aeruginosa}, where it was shown that the presence of the resident bacteria enhances pathogenicity most probably by chemical signalling (Duan et al., 2003). More recently, it was also reported that recognition of peptidoglycan of Gram-positive bacteria is a cue and results in increased expression of virulence factors in \textit{Pseudomonas aeruginosa} (Kogaonkar et al., 2013). These kinds of examples of multispecies interactions among bacteria are likely to increase dramatically in the future.

Research on interactions that involve more than two species will be challenging using current microbiology methods. However, with the decreasing cost of techniques such as metagenomics, metatranscriptomics and metabolomics, these are rapidly becoming the preferred tools for addressing which bacteria are present in a niche, which genes are expressed when, and how this gene expression reflects on the metabolites present. Environments such as the ocean and soils are currently being characterized extensively with regard to their bacterial composition, and other complex niches like plants (Sessitsch et al., 2012) and humans (Human Microbiome Project Consortium, 2012; Maurice et al., 2013; Wang et al., 2011) are now also being studied.

Olive knot disease caused by \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} provides a niche for studying bacterial multispecies interaction in disease as we have shown that \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} interacts with resident bacteria such as \textit{E. toletana}. In this study, we investigated bacterial localization during the course of \textit{in vitro} generated olive knots caused by co-inoculations of \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} and \textit{E. toletana}. The study of a possible metabolic interaction between these two species was evaluated \textit{in silico}. Finally, the microbiome of nine naturally occurring olive knots from different regions of Italy was analysed to determine the composition of their bacterial community.

**METHODS**

**Bacterial strains and growth conditions.** \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} DAPP-PG 722 and \textit{E. toletana} DAPP-PG 735, and their derivatives DAPP-PG 722–GFP and DAPP-PG 735–DsRedExpress, were routinely grown at 28 °C in Luria–Bertani (LB) broth. When required, antibiotics were added in the following concentrations: nitrofurantoin, 50 µg ml⁻¹, and kanamycin, 100 µg ml⁻¹. \textit{Escherichia coli} DH5α was grown at 37 °C in LB broth and when appropriate antibiotics were added in the following concentrations: ampicillin, 100 µg ml⁻¹, and kanamycin, 50 µg ml⁻¹.

\textit{Pseudomonas savastanoi} pv. \textit{savastanoi} and \textit{E. toletana} were tested for growth on minimal M9 medium with sole carbon and nitrogen sources. Co-inoculations of \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} were performed on M9 minimal medium containing a sole carbon source as follows: \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} were grown overnight in rich media, and cells were then pelleted and washed twice in M9 medium, and these cultures were used as inocula into an M9 medium with a unique carbon source. \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} were then resuspended in M9 medium, and these cultures were used as inocula into an M9 medium with a unique carbon source. Amounts of \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} and \textit{E. toletana} that were inoculated resulted in OD600 0.05 for each isolate, meaning that the mixed culture had OD600 0.1. Bacterial growth was monitored constantly by measuring OD600 and by plating on rich media.

The biodegradation of aromatic acids by \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} was analysed by reverse-phase HPLC, using a Varian 9010 solvent delivery system equipped with a Varian 9050 UV/vis detector. \textit{E. toletana} and \textit{Pseudomonas savastanoi} pv. \textit{savastanoi} were grown in M9 minimal medium supplemented with 0.1 % of the aromatic acid. Samples were withdrawn from cultures after growth and centrifuged at 12 000 g. The supernatant was diluted 100-fold into methanol and filtered through 0.2 µm filters; 10 µl samples were loaded on a 5 µm spherical C18 reverse-phase column (Supelcosil LC18 150 x 4.6 mm; Supelco), and eluted with 35 % methanol and 65 % water with 0.1 % acetic acid at a flow rate of 0.8 ml min⁻¹. The eluted metabolites were detected at 280 nm.

**Construction of pBRR2GFP and pBRR2DsRedExpress plasmids.** Digestion of pBK-miniTn7-gfp1 (Koch et al., 2001) and
miniTn7(Km, Sm) P$_{1904}$-DsRedExpress-a (Lambertsen et al., 2004) with NolI yielded fragments of 2 kb that were blunt-ended by treatment with DNA polymerase I. Fragments were cloned into pBBR1MCS5 (Kovach et al., 1995) digested with SmaI. To generate pBBR2-GFP and pBBR2-DsRedExpress, the cassette was then transferred to pBBR1MCS2 using Clal/SpeI restriction enzymes. The resulting plasmids were maintained in E. coli DH5α and transferred to Pseudomonas savastanoi pv. savastanoi or E. toletana by triparental conjugation using helper strain E. coli DH5α (pRK2013).

**Plant infection and isolation of bacteria from olive knots.** *O. europaea* plants derived from seeds germinated in vitro (collected originally from an ‘Arbequina’ plant) were micropropagated and rooted as described previously (Rodríguez-Moreno et al., 2008) in Driver Kuniiyuki Walnut (DKW) medium (Driver & Kuniiyuki, 1984). Rooted explants were transferred to DKW medium without hormones and kept for at least 2 weeks in a growth chamber at 25 °C with a 16 h photoperiod prior to infection. The olive plants used for in vitro studies were 60–80 mm long (stem diameter 1–2 mm) and contained three to five internodal fragments. Micropropagated olive plants were wounded by excision of an intermediate leaf and infected in the stem wound with a bacterial suspension under sterile conditions. For this purpose, bacterial lawns were grown for 48 h on LB plates and resuspended in 10 mM MgCl₂. The concentration of the bacterial cells was adjusted to OD₆₀₀ 0.1 for single inoculations, corresponding to $10^8$ c.f.u. ml⁻¹. For co-inoculations, a mixed bacterial suspension containing $10^6$ c.f.u. ml⁻¹ of each species was prepared. The plant wounds were infected with 2 μl of the resulting cell suspension. The plants were then incubated in a growth chamber at 25 °C with a 16 h photoperiod and a light intensity of 35 μmol m⁻² s⁻¹. At different time points, Pseudomonas savastanoi pv. savastanoi and E. toletana cells were recovered from the infected explants and spotted onto LB plates as described previously (Maldonado-González et al., 2013). Population densities were calculated from at least three replicates. The morphology of the olive plants infected with bacteria was visualized using a stereoscopic microscope (Leica MZ FLIII).

**Real-time monitoring of bacterial infection by epifluorescence microscopy and CLSM.** To visualize bacterial infection within knots in real-time, whole knots were examined directly with a 4× objective (Leica MZ FLIII) equipped with a 100 W mercury lamp, a GFP2 filter (excitation 480/40 nm; emission 510LP nm) and a red fluorescent protein (RFP) filter (excitation 546/10 nm; emission 570LP nm). Images were captured using a high-resolution digital camera (Nikon DVM1200). To visualize bacterial infection within the knots of the olive plants with CLSM, the knots were sampled 28 days p.i. at 1 cm above and below the inoculation point. These samples were fixed and embedded in agarose as described previously (Rodríguez-Moreno et al., 2009). Samples were fixed overnight at 4 °C in 2.5% paraformaldehyde prepared in 0.1 M phosphate buffer, pH 7.4. The fixed samples were then transferred into 2.5% paraformaldehyde with an ascending gradient of 10, 20 and 30% sucrose for 10, 20 and 30 min, respectively. Finally, samples were embedded in 7% low-melting-point agarose and cooled to 4 °C. Sections (40 and 60 μm) were cut from the knot samples using a vibrating microtome (Leica CM1325). Fluorescence of the bacterial cells within knot sections was visualized by epifluorescence microscopy using a Nikon Microphot FXA microscope. For confocal microscopy, we used an inverted CLSM (TCS-NT; Leica) equipped with detectors and filters that detect simultaneously green and red fluorescence. Images of green fluorescence were acquired at an excitation wavelength of 488 nm and an emission wavelength of 500–550 nm, whilst red fluorescence emission was recorded in the interval between 575 and 625 nm. The images were acquired by sequential scan analysis and processed using Leica LAS AF Lite software.

**SavCyc, TolCyc and SavtolCyc creation and availability.** Metabolic pathways were recreated using the software Pathway Tools (Karp et al., 2010). The Pathological input file was prepared according to the instructions in the Pathway Tools (version 16.5) user’s guide by feeding the software with the latest annotated files of *Pseudomonas savastanoi* pv. *savastanoi* and *E. toletana* (Passos da Silva et al., 2013; Rodríguez-Palenzuela et al., 2010) draft genomes, and merged files of the *Pseudomonas savastanoi* pv. *savastanoi* and *E. toletana* draft genomes. Initial builds of the *Pseudomonas savastanoi* pv. *savastanoi*, *E. toletana* and joined *Pseudomonas savastanoi* pv. *savastanoi*/*E. toletana* metabolic pathways were named SavCyc, TolCyc and SavtolCyc, respectively, using the default reference database, MetaCyc. MetaCyc pathways were included as the reference pathways (Caspi et al., 2012). No manual curation was performed on the created databases. Created databases are available via Pathway Tools software and at http://ftp.icgeb.org/pub/tmp/Passos.

**Sample collection and processing for metagenomics.** Young knots were collected from diseased ‘Frantoio’, ‘Cima di Mola’ and ‘Oliva Rossa’ olive trees grown in Umbria (Central Italy) and Apulia (South Italy). Genomic DNA was isolated from 1 g fresh-weight knots using a cetyltrimethylammonium bromide (CTAB) plant DNA extraction protocol. Briefly, knots (these were not sterilized as they are porous and *Pseudomonas savastanoi* pv. *savastanoi* is known to be localized mainly near the surface) were ground to a fine powder in liquid nitrogen within a cooled mortar. The ground tissue was mixed with 6 ml ice-cold extraction buffer (100 mM Tris/HCl, pH 8.0, 500 mM NaCl, 50 mM EDTA, 10 mM β-mercaptoethanol) and transferred to a 15 ml Falcon tube. After addition of 0.8 ml 10% SDS and incubation at 65 °C for 30 min, 2 ml ice-cold 5/3 K3 solution (5 M acetic potassium; glacial acetic acid) were added. The suspension was centrifuged for 10 min at 5000 g at 4 °C and the supernatant was filtered through a paper filter (S&K 95S). After 2-propanol precipitation and centrifugation for 10 min at 10000 g at room temperature, the pellet was dissolved in 400 μl TE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and RNase (20 mg ml⁻¹) treatment was performed for 20 min at room temperature. After incubation for 15 min at 65 °C with CTAB buffer [0.2 M Tris/HCl, pH 7.5, 2 M NaCl, 0.05 M EDTA, 2% (w/v) CTAB], two chloroform/isoamyl alcohol (24:1) extractions were performed. Nucleic acid was precipitated from the aqueous layer by the addition of an equal volume of ethanol (96%). After centrifugation, the pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 110 μl nuclease-free water.

**Bacterial 16S rRNA PCR amplification and pyrosequencing.** The PCR strategy was carried out by GATC Biotech (Konstanz) as follows. First 16S rRNA PCR for the V1/V3 region using primers 27F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGCTGCT- TGG) was performed. The PCR conditions used were: denaturation at 98 °C for 30 s, followed by 25 cycles at 98 °C (10 s), 56 °C (30 s) and 72 °C (10 s) and a final extension at 72 °C per 1 min. Next, PCR products were column purified, and a second PCR was performed to add the sequencing adaptors and multiplex identifiers. The PCR conditions for the second PCR were the same as above, except only five amplification cycles were performed. The library was sequenced on a Roche GS FLX following standard protocols from Roche.

**Metagenomics data analysis.** Unassembled reads were uploaded to the open-access MG-RAST server (http://metagenomics.anl.gov/) (Meyer et al., 2008). Analyses were performed using the MG-RAST pipeline applying the RDP dataset. Results were imported into an Excel (Microsoft) spreadsheet curated manually for graphical presentation and table creation. Alpha diversity was estimated using the MG-RAST metagenomics analysis server using the same sequencing reads for classification. Results are based on RDP using a maximum E-value of 1e–2, a minimum identity of 80%, and a minimum alignment length of 50 bp applying Bray–Curtis distance.
and ward clustering. Data were normalized based on MG-RAST version 3.0. Sequences obtained during this study were deposited for public access in the MG-RAST server under the accession numbers 4516653.3, 4516654.3, 4516655.3, 4516656.3, 4516657.3, 4516658.3, 4516659.3, 4516660.3 and 4516661.3.

RESULTS

_Pseudomonas savastanoi pv. savastanoi_ and _E. toletana_ cells co-localized in the olive knot

Our laboratory has reported previously that the _Pseudomonas savastanoi_ pv. _savastanoi_ bacterial pathogen and the _E. toletana_ bacterial resident share AHL QS signals in the olive knot, and when co-inoculated lead to a more aggressive disease as seen in a larger knot-size and larger population sizes in hyperplastic tissue for both bacterial species (Hosni et al., 2011). In order to obtain an insight into this interspecies interaction between a pathogen and a harmless resident bacteria, _Pseudomonas savastanoi pv. savastanoi_ and _E. toletana_ cells were co-inoculated in _in vitro_ micropropagated olive plants. Localization of bacteria was followed in real-time throughout knot development using stereoscopic epifluorescence microscopy combined with fluorescent tagging of _Pseudomonas savastanoi pv. savastanoi_ DAPP-PG 722 and _E. toletana_ DAPP-PG 735 with GFP and DsRedExpress, respectively (Figs 1, S1 and S2, available in the online Supplementary Material).

Acquired images showed that when inoculated alone, _E. toletana_ was not visible (the red observed was mostly autofluorescence of the plant) (Fig. 1c). In contrast, when _E. toletana_ was co-inoculated with _Pseudomonas savastanoi pv. savastanoi_, _E. toletana_ cells were abundant and visible at the inoculation site (Fig. 1a). In addition, in co-inoculation, epifluorescence revealed that _E. toletana_ was distributed throughout the knot and the distribution of _E. toletana_ matched the position of _Pseudomonas savastanoi pv. savastanoi_. Pathogen distribution as observed with epifluorescence did not show any significant alteration in the knot when inoculated alone compared with when co-inoculated with _E. toletana_ (Fig. 1a, b). To further study the localization and distribution of the two species expressing autofluorescent proteins in the knot, transversal transections made via vibratome sectioning of the knot were analysed by CLSM (Fig. 2). This allowed exploration of the inner localization and distribution of _Pseudomonas savastanoi pv. savastanoi_ and _E. toletana_ within the knot without the necessity of further manipulation or staining of the samples. It was observed that _E. toletana–DsRedExpress_ bacterial cells were localized in the vicinity of _Pseudomonas savastanoi pv. savastanoi–GFP_ cells, suggesting that _E. toletana_ strictly requires the close presence of _Pseudomonas savastanoi pv. savastanoi_ for growth and persistence in the olive knot (Fig. 2a, b). In both single and co-inoculations (Fig. 2c, a, respectively), _Pseudomonas savastanoi pv. savastanoi_ was present mainly on the surface or in the outer regions of the knots, as was reported to be its common location by Maldonado-González et al. (2013).

This indicated that the presence of _E. toletana_ does not interfere with the preferred positioning within the knot of _Pseudomonas savastanoi pv. savastanoi_. The number of c.f.u. was measured at 0 and 28 days p.i. (Fig. 3). These results further corroborated the observations reported by Hosni et al. (2011) that in the presence of _Pseudomonas savastanoi pv. savastanoi_, c.f.u. numbers of _E. toletana_ increased dramatically compared with single inoculations in adult 1-year-old olive plants, suggesting that observations in micropropagated olive plants can be correlated with what occurs in adult plants.

_Pseudomonas savastanoi pv. savastanoi_ and _E. toletana: in silico_ analysis of possible metabolic complementarity/exchanges

The previous report of AHL signal sharing and mutualistic behaviour as well as results shown here on strict co-localization of _Pseudomonas savastanoi pv. savastanoi_ and _E. toletana_ prompted us to analyse the recently published genomes of _Pseudomonas savastanoi pv. savastanoi_ and _E. toletana_ for possible metabolic complementarity and/or exchange. This is in view of the fact that both species grow more in the olive knot when both are present, indicating probable metabolic benefits in the model used here using plantlets, only _E. toletana_ grows more, whereas when using
Identification of olive knot bacterial communities via metagenomics

Several bacterial species have been isolated from olive knots via metagenomics (Hosni et al., 2011). We have performed an analysis on the metabolic potential of the two genomes via the reconstruction of metabolic pathways. This was achieved by feeding the annotated draft genome sequences of the two species separately (Pseudomonas savastanoi pv. savastanoi and E. toletana) and merged into Pathological (version 16.5) to predict their metabolic pathways. Reconstruction of predicted pathways from Pseudomonas savastanoi pv. savastanoi (SavCyc), E. toletana (TolCyc) and merged genomes (SavtolCyc) was made by matching all fed annotated enzymes with a database of enzymes with known function in order to predict possible single reactions that could be assembled into pathways to be tested experimentally (Karp et al., 2010).

After creating the predicted pathways, the merged SavtolCyc was used to highlight all the new pathways that could emerge by joining the enzymes present in the two separate genomes. By evidencing all the reactions not shared between SavtolCyc/SavCyc and SavtolCyc/TolCyc on the cellular overview panel, an interactive visual method to track all singular contributions given by the two single bacterial species to the pathways was possible. Interestingly, several plant-related compounds, including shikimate, sucrose and salicylate (Fig. 4), were evidenced as their predicted degradation pathways were only complete when the genomes of the two species were combined. The degradation benefits of these aromatic compounds by bacterial consortia could be relevant since these phenolics are found commonly in plants. The presence of both E. toletana and Pseudomonas savastanoi pv. savastanoi could allow them to more efficiently degrade these compounds and utilize them as carbon sources or possibly to detoxify them as they could be toxic compounds if found at high concentrations. In addition, as salicylates and phenols are involved in the plant defence response in plant/pathogen interactions (Loake & Grant, 2007), olive/Pseudomonas savastanoi pv. savastanoi included (Roussos et al., 2002), it is possible to hypothesize that the increased knot size we documented when Pseudomonas savastanoi pv. savastanoi and E. toletana were co-inoculated in olive plants (Hosni et al., 2011) is due to the collaborative bacterial degradation of these plant defence compounds.

We performed growth tests on sucrose and salicylic acid of Pseudomonas savastanoi pv. savastanoi and E. toletana both as single and mixed cultures in minimal medium as described in Methods. It was established that Pseudomonas savastanoi pv. savastanoi could not grow on salicylic acid and sucrose as unique carbon sources (data not shown), confirming the in silico data described above. Similarly, E. toletana also could not grow in either sucrose or salicylic acid; this was partly in accordance with in silico metabolic profiles as E. toletana was predicted to utilize sucrose as a unique carbon source. We also performed mixed inoculations of E. toletana and Pseudomonas savastanoi pv. savastanoi with either sucrose or salicylic acid as the sole energy source as described in Methods. With salicylic acid, we did not observe any growth or transformation (as determined by HPLC analysis of spent supernatants) of the aromatic acid when both species were co-inoculated (data not shown). In minimal medium with sucrose, we observed similar results, i.e. no growth when using a mixed inoculum of E. toletana and Pseudomonas savastanoi pv. savastanoi. These initial binary growth tests did not evidence metabolic complementarity as indicated by the in silico analysis; the reason for this is unknown currently.

Fig. 2. CLSM images of transversal vibratome sections from (a) olive knots co-inoculated with Pseudomonas savastanoi pv. savastanoi–GFP and E. toletana–DsRedExpress, (c) inoculated with Pseudomonas savastanoi pv. savastanoi–GFP alone and (e) with E. toletana–DsRedExpress alone at 28 days p.i. Images were acquired with a ×63 objective. (b, d, f) Magnifications of highlighted area from (a), (c) and (e), respectively. Whilst Pseudomonas savastanoi pv. savastanoi–GFP could be found at different regions of the olive knot, E. toletana–DsRedExpress could only be found mixed with Pseudomonas savastanoi pv. savastanoi–GFP cells.

1-year-old olive plants in 60-day-old olive knots, they both grow more as reported by Hosni et al. (2011)]. We have performed an analysis on the metabolic potential of the two genomes via the reconstruction of metabolic pathways. This was achieved by feeding the annotated draft genome sequences of the two species separately (Pseudomonas savastanoi pv. savastanoi and E. toletana) and merged into Pathological (version 16.5) to predict their metabolic pathways. Reconstruction of predicted pathways from Pseudomonas savastanoi pv. savastanoi (SavCyc), E. toletana (TolCyc) and merged genomes (SavtolCyc) was made by matching all fed annotated enzymes with a database of enzymes with known function in order to predict possible single reactions that could be assembled into pathways to be tested experimentally (Karp et al., 2010).
The presence of a multispecies bacterial community within the olive knot (Moretti et al., 2011; Ouzari et al., 2008; Rojas et al., 2004); many of these isolates belong to the genera Erwinia and Pantoea. It was of interest here to perform an exhaustive high-throughput analysis of the bacterial population inside olive knots since to our knowledge it has never been performed. Nine olive knots belonging to three different varieties of olive trees were collected from five regions of Italy, and a metagenomics approach based on the amplification and sequencing of the hypervariable 16S rRNA regions V1/V3 was used as described in Methods.

The data indicated rich and diverse bacterial life in this niche. We wish to note that the olive knot material was not sterilized prior to DNA purification as knots are porous and Pseudomonas savastanoi pv. savastanoi is known to also colonize intercellular spaces very near the surface. It is therefore possible that some of the bacterial species identified could be living on the surface of the olive knot as epiphytes. Among all bacterial 16S rRNA sequences retrieved, the gammaproteobacteria class was by far the most represented, accounting for up to 90% of the total bacterial population (Table S1). Unsurprisingly, Pseudomonas savastanoi pv. savastanoi makes up almost 50% of the bacterial load; the other most abundant bacteria belong to the Pantoea genera (Fig. 5) and, interestingly, Pantoea agglomerans has long been known to be commonly found in or associated with olive knots (Hosni et al., 2011; Ouzari et al., 2008; Quesada et al., 2007; Savastano, 1886). The abundance of other genera showed some distinct features in every knot; however, among the different samples, a common core of bacterial genera was evident composed of Clavibacter, Curtobacterium, Enterobacter, Erwinia, Hymenobacter, Kineococcus, Pectobacterium and Sphingomonas (Fig. 5). The geographical position of extraction seems to have little effect on the common core; however, for genera that are underrepresented such as Vibrio, their abundance is greater in samples from the south of Italy.

**DISCUSSION**

Olive knot disease has thus far been studied poorly, mainly focusing on the causal agent Pseudomonas savastanoi pv. savastanoi (Marchi et al., 2009; Matas et al., 2009; Pérez-Martínez et al., 2008, 2010; Ramos et al., 2012; Rodriguez-Moreno et al., 2009; Scortichini et al., 2004; Surico et al., 1985). The description of several virulence factors (Hosni et al., 2011; Iacobellis et al., 1994; Matas et al., 2009; Matas et al., 2012; Pérez-Martínez et al., 2010; Ramos et al., 2012; Rodriguez-Moreno et al., 2008, 2009; Rodriguez-Palenzuela et al., 2010; Rojas et al., 2004; Surico et al., 1985) and the normal localization on the intercellular space of the parenchymal tissue as a biofilm structure are, to our knowledge, the main recent discoveries (Rodriguez-Moreno et al., 2009).

We reported previously that harmless *E. toletana* can colonize the knot in the presence of *Pseudomonas savastanoi* pv. *savastanoi* and demonstrated sharing of AHL QS signals as one of the interactions on the knot microenvironment (Hosni et al., 2011). In addition, co-inoculation of *Pseudomonas savastanoi* pv. *savastanoi* with *E. toletana* resulted in increased knot size. Interestingly, this synergism was also observed when the AHL QS *luxI* AHL

![Fig. 3. Counts (c.f.u.) per inoculation site from (a) single and (b) co-inoculations using Pseudomonas savastanoi pv. savastanoi (PSV)–GFP and E. toletana (ET)–DsRedExpress. Gray bars represent number of cells used for inoculations and grey stripped bars represent c.f.u. retrieved from the inoculation site at 28 days p.i. The number of c.f.u. from the output (28 days p.i.) of E. toletana–DsRedExpress when co-inoculated with Pseudomonas savastanoi pv. savastanoi–GFP was more than two orders of magnitude higher than in single inoculations. Bars indicate means ± SD (n=3). Statistical significance was calculated using Student’s t-test (*P ≤ 0.05).*](http://mic.sgmjournals.org)
synthase mutant of *E. toletana* was used, indicating that most likely signal sharing was not the only interaction taking place between *E. toletana* and *Pseudomonas savastanoi pv. savastanoi* (Hosni et al., 2011). The localization of *E. toletana* in relation to *Pseudomonas savastanoi pv. savastanoi* during knot development could provide insights on possible mechanisms that play a role in the interaction. By using epifluorescence stereoscopic microscopy and CLSM, we observed that *E. toletana* was consistently in the vicinity of *Pseudomonas savastanoi pv. savastanoi*, possibly meaning that *E. toletana* requires this close proximity with *Pseudomonas savastanoi pv. savastanoi* for its persistence and growth in the olive knot. This closeness is in line with AHL signal sharing observed previously to be taking place between *Pseudomonas savastanoi pv. savastanoi* and *E. toletana* in the olive knot (Hosni et al., 2011); AHLs of *E. toletana* can therefore easily diffuse into *Pseudomonas savastanoi pv. savastanoi*. In addition, close proximity can result in community signalling by cell contact and/or facilitated sharing of metabolites. *Pseudomonas savastanoi pv. savastanoi* cells actually form biofilm structures in the olive knot.

**Fig. 4.** Graphical representations of complementary metabolic pathways between *Pseudomonas savastanoi pv. savastanoi* and *E. toletana*. Codes for genes encoded by *Pseudomonas savastanoi pv. savastanoi* possess the prefix PSA, whilst codes for genes encoded by *E. toletana* possess the prefix GDRG. In the pathways presented for the degradation of shikimate (a), sucrose (b) and salicylate (c), there are reactions that could only be catalysed by one of the species, meaning that the absence of one of the two species would impair the complete mineralization of these compounds.

**Fig. 5.** Graphical representation of bacterial abundance determined from the metagenomics studies of nine olive knots. Different colours are assigned to each of the nine samples and the organization of the key follows the same order as the samples on the graph (from bottom to top). Genera *Pseudomonas* and *Pantoea* are clearly the main constituents of these communities. *Pectobacterium*, *Curitobacterium*, *Kineococcus*, *Sphingomonas*, *Clavibacter* and *Hymenobacter* compose a group that is less abundant if compared with *Pseudomonas* and *Pantoea*, but these genera are found consistently in all samples. Although *Erwinia*, *Proteus*, *Enterobacter*, *Acinetobacter*, *Klebsiella*, *Kyntococcus*, *Methylcobacterium*, *Xanthomonas* and *Leifsonia* are not present in all samples, the abundance of these genera in some samples is significant. A considerable number of sequences could not be assigned to any genera with accuracy. Data were normalized according to the MG-RAST manual.
(Rodríguez-Moreno et al., 2009), thus this close proximity of E. toletana during knot formation suggests that mixed biofilms most probably occur, and this could be more advantageous for both species via facilitating diffusion of metabolites and signals, possibly resulting in a more stable biofilm.

It is believed that metabolic sharing/complementarity is one of the features that allows the formation of stable bacterial consortia (Egland et al., 2004; Kim et al., 2008). For example, the diffusion of metabolites can be transformed by one species that can then be further utilized by the neighbouring species, resulting in a mutualistic interaction (Egland et al., 2004; Kim et al., 2008; Ramsey et al., 2011). We have evaluated this possibility between E. toletana and Pseudomonas savastanoi pv. savastanoi using an in silico approach. By predicting all the metabolic pathways that each of the two organisms were able to perform individually, we were able to determine incomplete pathways due to the absence of key enzymes on one genome that could be completed by the presence of these given enzymes on the genome of the second species. Possible degradation pathways of several plant-related aromatic compounds, some of which are associated with plant defence, were only possible in silico when the two genomes were joined. Compounds such as salicylate, shikimate and sucrose are examples that could only be mineralized by the dual-species community. We have tested possible metabolic complementarity using growth tests in liquid and solid media using salicylic acid and sucrose. Under the conditions we tested, we could not detect binary growth of Pseudomonas savastanoi pv. savastanoi and E. toletana, providing evidence for metabolic sharing/complementarity in the mineralization of these compounds. It could be that under the conditions we tested it was not possible to initiate such an interaction that takes place in planta. Future studies need to focus in this direction using all the possible compounds revealed by the in silico analysis that could be transformed using metabolic sharing. It is also possible that the Pathway Tools analysis overpredicts pathways and that this information needs to be validated carefully.

The metagenomics studies proved that apart from Pseudomonas savastanoi pv. savastanoi, there was a dominance of Pantoea throughout all nine samples; the presence of Pantoea agglomerans on olive knots has been extensively described and its role in knot development remains unclear (Hosni et al., 2011; Marchi et al., 2006). These results indicate that the enrichment of Pantoea in the knot environment is an important aspect that merits further attention. It was surprising to see the presence of so many different genera in the olive knot; the consensus of genera present in the different olive knots could indicate a community structure present in the olive knot that could have evolved together with Pseudomonas savastanoi pv. savastanoi. Our studies and observations with E. toletana could be then applicable to other species, and most probably also to multispecies studies involving more than two bacterial species. Clavibacter, Curtobacterium, Hymenobacter, Kineococcus, Proteus and Sphingomonas were present in almost all samples, and to our knowledge they have never been reported in the olive knot. The descriptions of species within these genera being able to tolerate high concentrations of copper (Bagwell et al., 2010), facilitating the mineralization of polycyclic aromatic compounds (Manickam et al., 2012) or even associated with fungi that degrades decaying matter (Kamei et al., 2012) also show diverse biological roles that could aid the maintenance of the disease. Another interesting observation is that alpha diversity seems to be related directly to the geographical location from where the sample was collected (data not shown). Samples from the south of Italy (Clima di Mola, and Oliva Rossa 1 and 2) show a higher diversity when compared with samples from central Italy (Frantoio 1–6). This might be due to the difference of mean temperature and humidity (Teviotdale & Krueger, 2004) or possibly the susceptibility of distinct cultivars of olive trees (Penyalver et al., 2006). Even though a core group of bacterial genera seems to be present in all samples, a large part of the community present in the nine different samples shows considerable variation in abundance. Importantly, however, the phylogenetic variation present among the samples might not necessarily reflect on the genetic pool (Burke et al., 2011). It is possible that major roles in community interactions are played by the genera that are found in all samples (e.g. Pantoea), whilst the other bacteria present in only some or even one of the samples might be recruited in response to some specific traits (e.g. cultivar) or by diverse environmental conditions.

In summary, we shown that Pseudomonas savastanoi pv. savastanoi and E. toletana co-localize during knot development, and the presence of Pseudomonas savastanoi pv. savastanoi is essential for the maintenance of E. toletana cells on olive knots. This is a clear example of harmless and beneficial organisms teaming up in a disease (Venturi & da Silva, 2012). The close proximity of E. toletana and Pseudomonas savastanoi pv. savastanoi in the knot indicates that they are most probably interacting via a variety of mechanism that we have discussed. Using in silico analysis, we found compounds that could be shared in planta, possibly improving the stability of the community. Metagenomics sequencing revealed that some genera are commonly found associated with the olive knot, nevertheless high bacterial diversity occurs among different knots, and this could be related to environmental factors and/or cultivars of O. europaea. This study opens new possibilities on the Pseudomonas savastanoi pv. savastanoi/E. toletana interaction and presents a valid model to study multispecies interactions in a bacterial disease.

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