

Evidence for an Opportunistic and Endophytic Lifestyle of the *Bursaphelenchus xylophilus*-Associated Bacteria *Serratia marcescens* PWN146 Isolated from Wilting *Pinus pinaster*

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Abstract Pine wilt disease (PWD) results from the interaction of three elements: the pathogenic nematode, *Bursaphelenchus xylophilus*; the insect-vector, *Monochamus* sp.; and the host tree, mostly *Pinus* species. Bacteria isolated from *B. xylophilus* may be a fourth element in this complex disease. However, the precise role of bacteria in this interaction is unclear as both plant-beneficial and as plant-pathogenic bacteria may be associated with PWD. Using whole genome sequencing and phenotypic characterization, we were able to investigate in more detail the genetic repertoire of *Serratia marcescens* PWN146, a bacterium associated with *B. xylophilus*. We show clear evidence that *S. marcescens* PWN146 is able to withstand and colonize the plant environment, without having any deleterious effects towards a susceptible host (*Pinus thunbergii*), *B. xylophilus* nor to the nematode model *C. elegans*. This bacterium is able to tolerate growth in presence of xenobiotic/organic compounds, and use phenylacetic acid as carbon source. Furthermore, we pres-

ent a detailed list of *S. marcescens* PWN146 potentials to interfere with plant metabolism via hormonal pathways and/or nutritional acquisition, and to be competitive against other bacteria and/or fungi in terms of resource acquisition or production of antimicrobial compounds. Further investigation is required to understand the role of bacteria in PWD. We have now reinforced the theory that *B. xylophilus*-associated bacteria may have a plant origin.

Keywords *Bursaphelenchus xylophilus* · Endophyte · Nematode · *Serratia marcescens* · Pine wilt disease

Introduction

Pine wilt disease (PWD) is considered to be the most devastating disease of Eurasian coniferous forests [1]. At least three major elements are involved in the development of PWD: the

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plant parasitic nematode *Bursaphelenchus xylophilus* (PWN, pine wood nematode) which is the causal agent of the disease; the wood-boring beetle *Monochamus* sp. (Cerambycidae) which is the main insect-vector of the PWN; and the host tree which are most *Pinus* species [1, 2]. Other organisms have also been shown to be involved in this complex disease, including fungi and bacteria [3, 4]. In the particular case of bacteria, several studies have reported their dual role in PWD development, as mutualistically associated [5, 6] and even putatively synergetic with PWN infection [1, 7, 8], or as potential host defenders against PWN [9, 10]. The bacterial communities associated with *B. xylophilus* are predominantly dominated by β - and γ -proteobacteria, of which *Pseudomonas*, *Burkholderia*, *Serratia* are major representatives [11, 12]. Vicente and co-workers [11] compared the culturable bacterial communities of PWN obtained from long-term lab cultures and from symptomatic *Pinus pinaster* and found that *Serratia* was highly abundant in both. In the characterization of bacterial communities of *Monochamus galloprovincialis*, the key insect-vector of PWN in European forests, a high abundance of *Serratia* sp. was also reported [7].

The genus *Serratia* is known for its environmental adaptability, and is easily found as a free-living or host-opportunistic microorganisms in water, soil, animals, plants, and even insects [13]. Eighteen species and four subspecies of *Serratia* have been described to date [14], and almost all of these have been, or in the process of, being genome sequenced. The type species of this genus is *S. marcescens* and is commonly identified as a multidrug-resistant nosocomial pathogen [15]. This species also has been isolated as a pathogen from insects, *S. marcescens* subspecies *marcescens* Db11 [16], from plants, *S. marcescens* AGPim1A [17], and as a non-pathogenic associated bacterium from PWN, formerly referred as *Serratia* sp. PWN146 [7, 18]. Phenotypically, *S. marcescens* PWN146 is characterized by: multidrug-resistance (Amp₅₀, Ery₅₀, Kan₅₀, Tet₁₅, Rif₅₀, $\mu\text{g}/\text{mL}$); cellulase activity; formation of biofilm; production of siderophores and by its ability to induce hypersensitive reactions (HR) in *Nicotiana tabacum* [18]. Moreover, *S. marcescens* PWN146 has no ACC deaminase activity, phosphate solubilization, and does not significantly promote root elongation of *Brassica campestris*, but tested positively for indole acetic acid (IAA) and exopolysaccharides (EPS) production [18]. Vicente et al. [7] showed that *Serratia marcescens* PWN146, and two more *Serratia* sp. (LCN4 and LCN16), could assist PWN in severe oxidative stress conditions, suggesting a facultative association with the nematode during PWD progression. Hence, in this study, we aimed to: (1) obtain the whole genome sequence of *S. marcescens* PWN146 in order to gain new insights into the genetic content that could explain PWN146 behavior in a plant-associated lifestyle and ultimately in interaction with the PWN; (2) prove *S. marcescens* PWN146 ability to colonize plants; and (3) to complete phenotypic characterization with

biochemical description, nematicidal activity against *B. xylophilus* and the model *C. elegans*, and pathogenicity in a susceptible host. Understanding the host-microbe interactions can be exploited for the development of new strategies of management and control of PWD.

Experimental Procedures

Bacteria Growth and Biochemical Characterization

Non-pigmented *S. marcescens* PWN146 was originally isolated from the cuticle of PWN extracted from PWD-symptomatic *P. pinaster* [12]. Unless otherwise stated, *S. marcescens* PWN146 was grown in Luria Broth (LB) medium at 28 °C, washed with 1× PBS (phosphate-buffered saline), and OD₆₀₀ adjusted to 0.5–0.8. The biochemical characterization of this isolate was conducted using the VITEK 2 system with GN (Gram-negative) cards. Tolerance and degradation of terpenes and aromatic compounds were tested by inoculation of 20 μL of PWN146 overnight culture in, respectively, trypticase soy broth (TSB) and minimal medium M9 (without C source), and incubation at 28 °C during 5 days. The following compounds were analyzed: phenol (50; 100; 200 mg/L), benzoic acid (10; 50; 100 mg/L), xylol (0.1, 0.5, 1 %), toluene (0.1, 0.5, 1 %), (+)- α -pinene (0.1, 0.5, 1 %), (–)- α -pinene (0.1, 0.5, 1 %), α -pinene (isomer mix) (0.1, 0.5, 1 %), (+)- β -pinene (0.1, 0.5, 1 %) (–)- β -pinene (0.1, 0.5, 1 %), β -pinene (isomer mix) (0.1, 0.5, 1 %), (+)-3-carene (0.1, 0.5, 1 %), 3-carene (0.1, 0.5, 1 %) and R-(+)-limonene (0.1, 0.5, 1 %).

Nematicidal Activity Against *C. elegans* and *B. xylophilus* Ka4

Nematicidal activity of PWN146 was tested against *Caenorhabditis elegans* and *B. xylophilus*. *C. elegans* N2 Bristol strain culturing and handling were carried out at 20 °C as described by Brenner [19]. Synchronized L1 stage *C. elegans* were obtained by treating egg-containing/or gravid adults with NaOCl and allow to grow on NGM plates seeded with *E. coli* OP50 (control) or *S. marcescens* PWN146 at 20 °C, for 24, 48, and 72 h. At each time-point, *C. elegans* were removed from the plate, washed with M9 buffer (three times) to remove bacterial cells, and picked for microscope slide preparation. Agar (5 %, w/v) pads were prepared containing 20–25 nematodes. Images were taken using a Nikon SMZ1000 binocular microscope equipped with a Visualix VTCH-1.4CICE CCD camera. The deleterious effects of *S. marcescens* PWN146 in *C. elegans* N2 development was assessed by measuring the nematode size at each time-point ($n = 20\text{--}25$). Image processing was conducted using Image J

[20]. Data was statistically analyzed using STATISTICA software version 7.0. Homogeneity of variances was checked by Levene's test. Data was subjected to ANOVA analysis to statistical differences between treatments (OP50 and PWN146) each time (24, 48, and 72 h). A post-hoc Tukey's test at 95 % confidence level was used for multiple means comparison after significant ANOVA.

B. xylophilus Ka4, previously grown in *B. cinerea* on barley seeds, were extracted overnight in Baermann funnels, surface-sterilized with L-lactic acid (3 %, v/v) [21] and suspended in sterile 1× PBS at a concentration of 1.5 nematodes per μL . Two treatments were established: (1) 100 μL 1× PBS + 100 μL Ka4 suspension (approximately 150 nematodes) as null treatment; and (2) 100 μL of Ka4 suspension (approximately 150 nematodes) and 100 μL of bacterial suspension. This experiment was established in a sterile 96-well plate whereas each treatment was repeated three independent times with five repetitions. The plate was incubated at 25 °C, and nematode mortality was checked 24 h later as described by Barbosa et al. [22].

Pathogenicity in *Pinus thunbergii*

The pathogenicity of *S. marcescens* PWN146, alone or in association with *B. xylophilus* Ka4, was tested in 3.5-year-old greenhouse grown *P. thunbergii* saplings. The saplings were grafts obtained from a single pine tree at the Forest Products Research Institute, Forest Tree Breeding Center in Ibaraki, Japan in 2012. Four treatments, each with four biological replicates totally randomized, were established: (1) *P. thunbergii* (inoculation with sterile ddH₂O); (2) *P. thunbergii* inoculated with *B. xylophilus* Ka4; (3) *P. thunbergii* inoculated with *S. marcescens* PWN146; and (4) *P. thunbergii* inoculated with *B. xylophilus* Ka4 in association with *S. marcescens* PWN146. Both nematodes and bacteria were prepared as mentioned above. Nematode inoculum was adjusted to 1000 nematodes (mixed-stages) per 100 μL of ddH₂O. The inoculation procedure was conducted according to Futai and Furuno [23]. Two small-wounds (1 cm) were made in the middle of stem in the first and second nodes using a sterile blade. A sterilized piece of cotton was placed in the wound site and fixed with parafilmTM (Bemis Company, Inc.). Nematodes suspension was directly injected into the cotton (inoculation point). The trial was maintained in the greenhouse conditions for 10 weeks (between 16th July 2015 till 28th August 2015). Observations were conducted every week, and watering was conducted twice a week. Symptomology was scored as follows: 0, no needle discoloration; 1, 25 % needle discoloration (brown yellowish); 2, 50 % needle discoloration (yellowish to brown); 3, 75 % needle discoloration (needles browning); and 4, 100 % needle discoloration

(complete wilted tree). The disease incidence was calculated according to Fang [24] (Equation 1):

$$\begin{aligned} \text{Disease incidence (\%)} &= \frac{\sum \text{number of disease plants} \times \text{symptom stage}}{\text{total number of plants} \times \text{highest symptom stage}} \\ &\times 100 \end{aligned}$$

Genome Sequencing, Annotation, and Comparative Analysis

A single-colony culture of *S. marcescens* PWN146 in 10 mL of LB was incubated overnight shaking at 27 °C. Genomic DNA was then extracted using the QIAGEN Genomic DNA Purification kit (first and second sequencing round) and QIAGEN Genomic-tip 500/G kit (third sequencing round), following the manufacturer's instructions.

The DNA was first sequenced on the Roche Titanium 454 platform at the Centre for Genomic Research, University of Liverpool, with large-insert 3-kb paired-end libraries. This gave a total 329,241 sequences, mean trimmed length 425.6 bp, totalling 140Mbp. Initial assemblies were performed with Roche "Newbler" gsAssembler [25], and MIRA v4.0.2 [26]. This data was supplemented with two runs from Illumina MiSeq commissioning test runs at the James Hutton Institute, as one of 11 and 4 barcoded samples, yielding 1.3 and 5.7 million paired reads respectively, totalling 1.8Gbp. Again, multiple assemblies were evaluated, and while improved, a closed chromosome was not achieved. In the third and final round of sequencing, one 20-kb insert SMRTbell library was generated with size selection on the BluePippin (Sage Science). The *S. marcescens* PWN146 genome was sequenced in 2 SMRT P6-C4 chemistry cells on the PacBio RS II platform (Pacific Biosciences), generating 66,159 reads (N50 16,506 bp) totaling 844.9 Mb. The Pacbio sequences were assembled using the Celera assembler PBcR hierarchical pipeline (with default parameters) [27] and corrected using Quiver algorithm [28], giving a closed chromosome and two closed plasmids.

Nucleotide sequence position 1 of *S. marcescens* PWN146 genome was ordered according to the published *S. marcescens* Db11 and SM39 [15], and checked using MAUVE 2.4 (Supplemental Fig. 1) [29]. Automatic genome annotation was performed using PROKKA 1.11 [30], further supported by BLAST2GO [31] and KAAS (KEGG Automatic Annotation Server) [32]. Genome visualization and manual review was performed in ARTEMIS 16.0 [33].

BLAST Ring Image Generator (BRIG) [34] was used for genome comparison between *S. marcescens* PWN146 and other complete genomes of *S. marcescens* (Table 1), and

Table 1 List of complete genomes of *S. marcescens* from environmental and clinical sources

Strain	Origin	Size (Mb)	GC%	Genes	CDS	Replicons	Reference
<i>Serratia marcescens</i> PWN146	Environmental	5.4	59.28	5405	5068	Chromosome 2 plasmids	This study
<i>Serratia marcescens</i> subsp. <i>marcescens</i> Db11	Clinical	5.1	59.50	4743	4607	NZ_HG326223.1	[35]
<i>Serratia marcescens</i> FGI94	Environmental	4.8	58.90	4436	4290	NC_020064.1	[36]
<i>Serratia marcescens</i> WW4	Environmental	5.2	59.59	4871	4744	NC_020211.1	[37]
<i>Serratia marcescens</i> SM39	Clinical	5.3	59.73	4950	4806	NZ_AP013063.1 1 plasmid	[15]
<i>Serratia marcescens</i> CAV1492	Clinical	5.8	58.63	5460	5274	NZ_CP011642.1 2 plasmids	[35]
<i>Serratia marcescens</i> RSC-14	Environmental	5.1	59.60	4745	4593	CP012639.1 6 plasmids	[35]
<i>Serratia marcescens</i> SmUNAM836	Clinical	5.2	59.82	4944	4774	CP012685.1 1 plasmid	[38]

Data from *Serratia marcescens* strains CAV1492, Db11, WW4, SM39, FGI94, RSC-14, and SmUNAM836 retrieved from NCBI, Genome Assembly and Annotation Report (<http://www.ncbi.nlm.nih.gov/genome/genomes/1112?>)

genomic islands were annotated by Island Viewer 3.0 using the available genomes as reference: *S. marcescens* Db11, *S. marcescens* CAV1492, *S. marcescens* WW4, *S. marcescens* SM39, and *S. marcescens* FGI94 [39]. The average nucleotide identity (ANI) and tetranucleotide signature was calculated using JSpecies 1.2.1 software [40]. Orthologous genes analysis between *S. marcescens* genomes (Table 1) and *S. marcescens* PWN146 was conducted using OrthoFinder 0.4 [41]. For phylogenetic analysis of PWN146, four housekeeping genes (*atpD*, *dnaJ*, *gyrB*, and *rpoB*) of *Serratia*-type strains were concatenated using Seaview 4.0 [42], and maximum likelihood (ML) tree was constructed using K2 (Kimura 2-parameters) in MEGA6 [43]. Phylogenetic robustness was inferred by bootstrap analysis using 1000 iterations.

Scanning Electron and Confocal Laser Scanning Microscopy

To observe *S. marcescens* PWN146 adhesion to *B. xylophilus* Ka4 cuticle after 1 and 48 h contact, nematodes were firstly surface-cleaned with L-lactic acid (3 %, v/v) and suspended in sterile ddH₂O. After 1 and 48 h contact, nematodes were removed from bacterial suspension and prepared for scanning electron microscope (SEM) observation as described in Shinya et al. [44]. SEM images were taken using a JSM-6510LA (JEOL, Tokyo, Japan).

The ability of *S. marcescens* PWN146 to infect and colonize plants was tested on fast-growing vigor tomato plants (*Lycopersicon esculentum* cv “Momotaro”). GFP-labeled *S. marcescens* PWN146 made previously [7] was prepared as follows. Bacteria were grown overnight in LB broth supplemented with 30 µg mL⁻¹ gentamicin and 50 µg mL⁻¹ rifampicin, washed three times with 1× PBS, and the OD₆₀₀

adjusted to 0.5. Tomato seeds were surface-sterilized with 70 % EtOH (w/v) for 1 min, and 1 % NaOCl (w/v) for 10 min, followed by several rinses with sterile ddH₂O. Seeds (15–20) were treated with two treatments: (1) ddH₂O only, considered the null treatment; and (2) bacteria for 1 h. Afterwards, seeds were transferred to fresh agar plates. Ten days after inoculation (DAI), seedling colonization was observed with a confocal laser scanning microscope LSM710 (Carl Zeiss, Germany) equipped with an Argon laser (458, 477, 488, 514 nm) and detectors for GFP (FITC dye, 495–590 nm) and plant auto fluorescence (492–510 nm). Images were obtained in a z-series with 20–30 optical sections, and processed in ZEN Image 2.0 software (Carl Zeiss, Germany).

Results and Discussion

Genome Structure and Comparative Analysis

The complete genome of *S. marcescens* PWN146 is contained in one single chromosome of 5,485,668 bp with an overall G+C content of 59.28 %, and two F-like plasmids pPWN146.1 (139,402 bp with G+C of 55.8 %) and pPWN146.2 (61,142 bp with G+C of 56.5 %). A total of 5405 genes were predicted in the PWN146 genome (chromosome and plasmids), from which 5288 (97.8 %) were considered coding sequences (CDS) with 1076 (20.3 %) hypothetical proteins of unknown function, and 117 (2.1 %) RNA genes (22 rRNA, 94 tRNA, and 1 tmRNA). Furthermore, 4820 (91.1 %) CDS were annotated with InterPro signatures, 3448 (65.2 %) and 3159 (59.7 %) were assigned, respectively, to Gene Ontology (GOs) and KO terms (Supplementary Tables S1 and Fig. S2). Both plasmids, pPWN146.1 and pPWN146.2 have type IV pili (conjugal transfer pilus assembly proteins) which allow their motility [45]. In fact,

pPWN146.1 has two sets of type IV pili proteins suggesting co-integration of plasmids [46]. Plasmid pPWN146.1 also has toxin-antitoxin systems integrated (*vapCB*, and incomplete *relE/stbE-relB/stbD*), which are involved in plasmid transfer fitness [47]. As in pPWN146.1, pPWN146.2 is mostly composed by hypothetical proteins of unknown function, which limits the interpretation of their role.

Bacterial genomes with low number of MGEs (mobile genetic elements), such as phages, transposons (Tn) or inserted elements (IS), are usually found in bacteria adapted to restricted environments, such as obligate intracellular symbionts [48]. In the PWN146 chromosome, two Tn10 transposon sequences (Tn10-Tet, tetracycline resistance), 20 transposases/IS and 89 phage-like proteins were found, mostly surrounding hypothetical proteins. PWN146 chromosome also contains Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Type I (universal *cas1*, PWN146_04951; *cas3*, PWN146_04952), subtype I-F (*csy1234*, PWN146_04953-56), flanked by two repeat regions. CRISPR_Cas systems are known to provide adaptive and hereditary immunity against previously encountered bacteriophages and plasmids, and can be located in specialized genome regions encoding defense or stress proteins [49]. PWN146 CRISPR_Cas is located upstream of a phosphonate *phn* operon (*phnPONMLKJIHGF*) reported to be involved in the acquisition of phosphorus from the natural environment under phosphate limiting condition, and also linked with antibiotic activities or components of cellular macromolecules (i.e., glycoproteins or phospholipids) [50].

Comparative genome analysis between all *S. marcescens* strains (Table 1) is presented in Fig. 1a. The closest *Serratia* strains to PWN146 are the host-pathogen *S. marcescens* CAV1492, SmUNAM836 and SM39 (Figs. 1a and 2a). The ANI between PWN146 and CAV1492 and SmUNAM836 was 98 %, followed by SM39 and Db11 (96 %), and environmental strains WW4 and RSC-14 (95 %). All strains belong to the same species according to the 95–96 % range limits for species definition [40]. The tetranucleotide signature between PWN146 and CAV1492 was 0.9994 (Fig. 2a). These results are further corroborated by the phylogenetic analysis of housekeeping genes (*atpD*, *dnaJ*, *gvrB*, and *rpoB*) (Fig. 2b), which cluster PWN146 jointly with CAV1492 within the *S. marcescens* clade. The environmental strain *S. marcescens* FG194, an insect isolate, was considered the most different strain to PWN146 with an ANI of 86 %, actually suggesting this is a different *Serratia* species. A total of 16 genomic islands (GIs), ranging between 4 and 26 kb, were predicted in the PWN146 genome by at least one method (SIGI-HMM, IslandPath-DIMOB, IslandPick) (Fig. 1b, Supplemental Table S2) [39]. These GIs include 258 genes, which are mostly annotated as hypothetical proteins and MGEs, and genes involved in the biosynthesis of antibiotics (gramicidin, *igrB*, PWN146_00556, PWN146_00573 and PWN146_00251;

tyrocidine, *tycC*, PWN146_00554 and PWN146_00571; plipastatin, *ppsDE*, PWN146_00557 and PWN146_00574; and surfactin, *urfAD*, PWN146_00575); drug resistance proteins (bicyclomycin resistance protein, *bsr*, PWN146_03395); cell wall-degrading enzymes (chitinase class I, PWN146_02145); and insecticidal toxin (type-1Aa cytolytic delta-endotoxin, *cyt1Aa*, PWN146_05139). Curiously, *cyt1Aa* is surrounded by IS66 (PWN146_05136-38) and IS2 (PWN146_05140), which may suggest a horizontal gene-transfer (HGT) event, perhaps from the animal and human pathogen *Aeromonas salmonicida* (BlastP: 49 % identity, *e* value $5e^{-60}$) [51]. *Aeromonas* sp. were already isolated from *B. xylophilus* extracted from PWD-symptomatic pines as well as *B. mucronatus* [10, 52]. Orthologous analysis predicted 276 CDS unique to PWN146 in comparison with the other *S. marcescens* strains (Table 1), most of which were found in the PWN146 predicted GIs (Supplemental Table S2). Furthermore, the same analysis performed between PWN146 and the closest *S. marcescens* CAV1492, SmUNAM836 and SM39 (Fig. 2c) showed that the number of conserved genes in PWN146 ranged between 4302 and 4327 and the number of unique genes varies between 740 and 763 genes. Among the unique genes of PWN146 in relation to CAV1492, we identified genes predicted in plant-associated bacteria, such as: plant cell wall-degrading enzymes, cellulase (PWN146_01039) and alpha-beta hydrolases (PWN146_02529); and xenobiotic efflux pumps (*aaeAB*, PWN146_00522, PWN146_00524; PWN146_05144 and PWN146_05146) [53, 54].

Nematode-Bacteria Interaction

The nematocidal activity of *S. marcescens* PWN146 was tested against *C. elegans* (Fig. 3) and the PWN *B. xylophilus* Ka4. The development of *C. elegans* was slightly delayed in the presence of PWN146 after 48 h and 72 h incubation compared with *E. coli* OP50. *C. elegans* is a simple model to test virulence mechanisms of diverse bacteria, from pathogens to symbionts [55, 56]. In the case of *B. xylophilus* Ka4, PWN146 showed no nematocidal effect after 24 h (Supplemental Table S3). These results indicate that *S. marcescens* PWN146 has no nematocidal effect towards nematodes, and are consistent with previous observations that suggested a beneficial effect towards different *B. xylophilus* strains under specific conditions [7]. In contrast, Paiva et al. [9] reported that another *B. xylophilus*-associated bacteria *Serratia* sp. A88copa13 was highly toxic to the nematode and attributed this activity to the presence of secreted serine proteases and other bacteriocins. The methodology used in the present study was different from Paiva et al. [9], making it difficult to compare directly with this study. However, we could also find three serine proteases from subtilisins family (MEROPS S08.094) (PWN146_01829, PWN146_04255, and

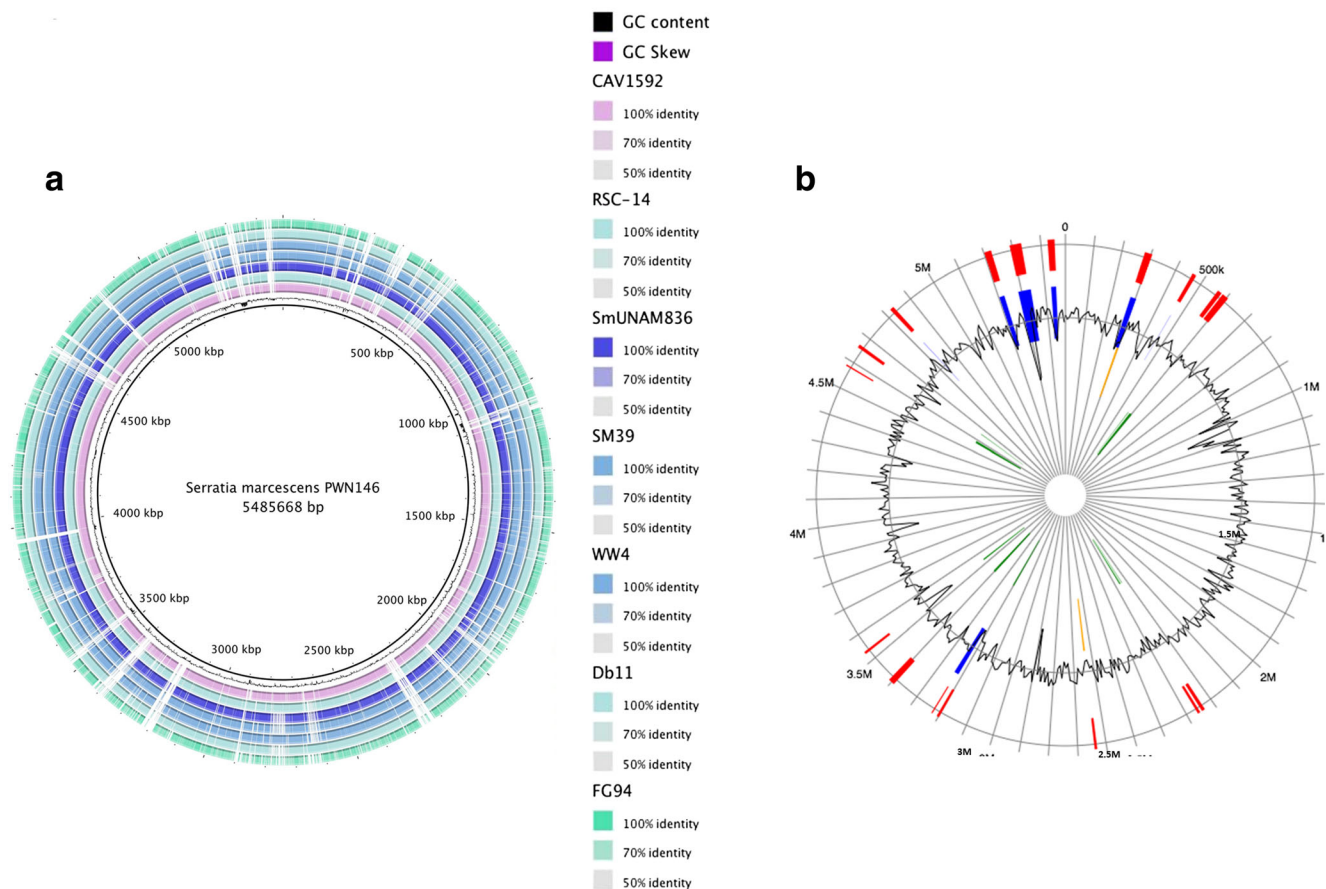


Fig. 1 Circular visualization of comparative genome analysis of *S. marcescens* strains. This analysis was performed in BRIG (BLAST Ring Image Generator) [34] (a); and in IslandViewer 3.0 [39] for genomic Islands (GIs) prediction (b). GIs in blue, orange and green

indicate, respectively, prediction by IslandPath-DIMOB, SIGI-HMM, and IslandPick approaches. GIs in red were predicted by at least one of the three above described approaches

PWN146_04256) and other bacteriocins (i.e., colicin V), which are widely present in Enterobacteriaceae, in the PWN146 genome. Subtilisins are the second largest family of serine proteases with a broad biological function (e.g., cellular nutrition, host invasion, maturation of polypeptides, and extracellular adhesins) [57]. Using SEM, Paiva et al. [9] showed an increased number of bacteria in the cuticle of *B. xylophilus* after 24 and 48 h of incubation, which led to the nematode cuticle degradation. In our study, we could also observe the adhesion of PWN146 in *B. xylophilus* Ka4 cuticle after 1 and 48 h incubation (Fig. 4a, b). PWN146 population density increased along the 48 h, covering the nematode's cuticle without any deleterious effect (Fig. 4b). Any visible damage to nematode cuticle in these images is due to the SEM preparation techniques, as shown in Fig. S3.

Plant-Associated Lifestyle

Recent research on the role of bacteria in PWD has suggested that *B. xylophilus*-associated bacteria may be pine endophytic

bacteria that show phenotypic plasticity, that are able to survive and colonize the plant environment and, under certain circumstances, may be nematode-synergetic [7, 10, 58]. GFP-tagged *S. marcescens* PWN146 was inoculated in tomato seeds and observed for their ability to colonize and invade the root system. After 10 DAI, we were able to detect GFP-PWN146 attached to the root hairs (Fig. 4c) and within, occupying the intercellular spaces (Fig. 4d). Moreover, the inoculation of *S. marcescens* PWN146 alone has not induced PWN-symptoms in 4-year-old *P. thunbergii* during 10 WAI (weeks after inoculation) (Supplemental Fig. 4). This result is in contradiction with previous results [18]. *S. marcescens* PWN146 have induced disease symptoms in 1-year-old *P. pinaster* even though not as *B. xylophilus* [18]. This result may suggest the different hosts and development age can be accounted for the different behaviors of *S. marcescens* PWN146. In this trial, the first PWD symptoms were seen in the treatment *B. xylophilus* Ka4 around the second and third WAI, while in the treatments *B. xylophilus* Ka4 + *S. marcescens* PWN146 the symptoms appeared the third and fourth WAI. Pine trees from both treatments were wilted between the ninth and tenth WAI.

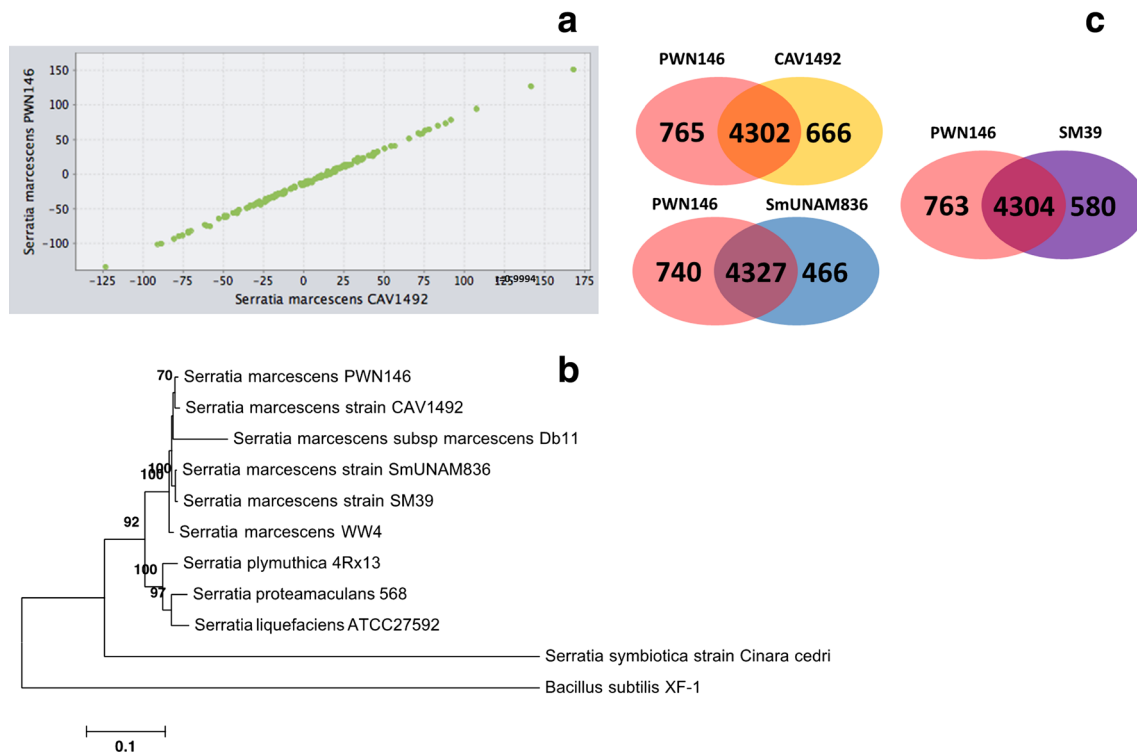


Fig. 2 Taxonomic inference of *S. marcescens* PWN146 and orthologous analysis between the closest *S. marcescens* (Table 1). The tetranucleotide signature between PWN146 and CAV1492 is shown in **a**. The

phylogenetic analysis of housekeeping genes is presented in **b**. Venn diagrams showing the orthologous between PWN146 and CAV1492, SmUNAM836, SM39 are indicated in **c**

In view of the previous results, PWN146 genome analysis was focused on understanding its genetic repertoire which could facilitate a plant-associated lifestyle (Supplemental Table S3). Using comparative genomics, Mitter et al. [48] reported the existence of typical endophytic traits among plant-associated bacteria; these traits were not exclusively linked to the endophytic behavior, but based on different interaction strategies with the host plant. Successful endophytes, facultative or obligate, express colonization traits that allow their entrance in the plant habitat. These steps involve: recognition,

adherence, invasion, colonization, and growth [59]. The *S. marcescens* PWN146 genome encode genes potentially involved in these first steps of plant-bacteria interaction. PWN146 is motile, with a complete flagella biosynthesis set (PWN146_02410-53) and chemotaxis proteins such as methyl-accepting chemotaxis proteins (i.e., *mcp*, PWN146_00924; *tsr*, PWN146_01795, PWN146_02459, PWN146_04878; *tar*, PWN146_04546; *tap*, PWN146_02458, PWN146_04818), chemotaxis protein MotAB (PWN146_02462-63), and the two-component system *cheZYBRWA* (PWN146_02454-57, PWN146_02460-61). Surface adhesion in PWN146 may be accomplished by means of fimbria adhesins (*mrkD*, PWN146_00970, PWN146_02613), surfactin (PWN146_00575), filamentous hemagglutinins (*fhaB*, PWN146_03175), and the *bcsAZC* operon for cellulose biosynthesis (PWN146_04615-18), which was shown to be related to root adhesion [60]. Its ability to enter the host plant may be passive, through wood cracks, or active by secreting plant cell wall-degrading enzymes [61]. In PWN146, we found one gene that could encode a cellulase (PWN146_01039), four genes coding for alpha-beta hydrolases (PWN146_02229, PWN146_02529, PWN146_02579, PWN146_03420), cupin (PWN146_02588) and an alternative pathway, although incomplete, for degradation of galacturonate via *uxaAB* (PWN146_02810-11, PWN146_03205) [60].

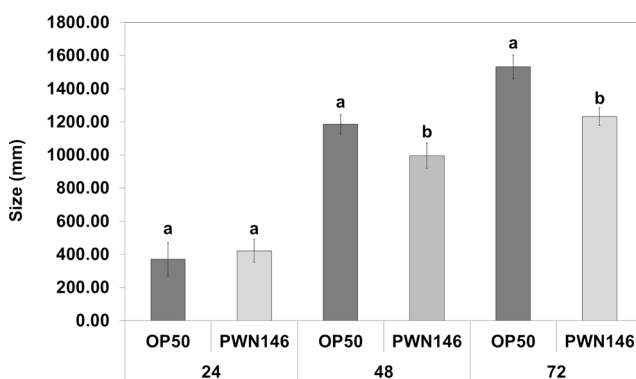


Fig. 3 Growth effect of *S. marcescens* PWN146 in the nematode model *C. elegans* during 24, 48 and 72 h. The *E. coli* strain OP50, routinely used for *C. elegans* growth, was used to infer the normal nematode growth. Error bars indicate standard deviation. Different letters indicate statistical differences at 95 % confidence level (post-hoc Tukey's Test)

Several characteristics are considered competitive factors for a plant environment, namely detoxification of reactive

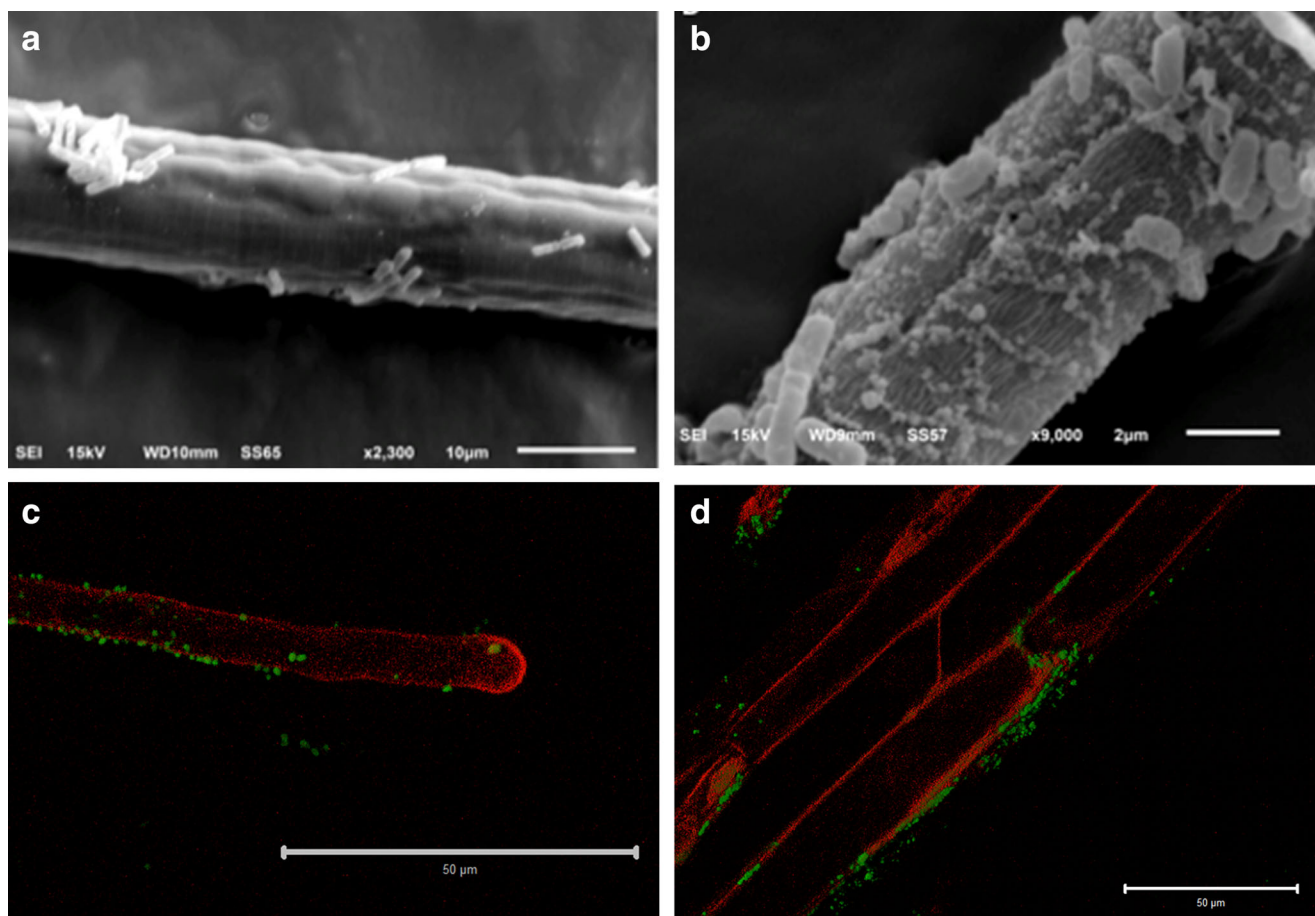


Fig. 4 Microscopic observations of *S. marcescens* PWN146 in interaction with nematode and plant. Images **a** and **b** show *S. marcescens* PWN146 attachment to *B. xylophilus* Ka4 cuticle, after 48 h association, using SEM microscopy. Images **c** and **d** show,

respectively, the colonization of tomato root hairs and internal localization by GFP-labeled *S. marcescens* PWN146, after 10 DAI (days after inoculation)

oxygen species (ROS) and secondary compounds, hormonal stimulation, nutrient acquisition, and production of antimicrobial compounds [48, 59, 62, 63]. The oxidative burst is one of the primary defense mechanisms of plants used against abiotic (i.e., climatic conditions) or biotic (i.e., microorganism infection) stresses, and results in the massive production of ROS (O_2^- , H_2O_2 , OH^-) as a means of barring invasion and network signaling for internal regulation [64]. *In planta*, endophytic bacteria are well equipped with scavenging enzymes for ROS neutralization, including superoxide dismutases (SODs), catalases (CATs), and peroxiredoxins (PRXs), as well as with extracellular polysaccharides and intracellular polyesters [65, 66]. Previously, Vicente et al. [7] showed that *S. marcescens* PWN146 was able to tolerate high concentrations of H_2O_2 (up to 100 mM), thus indicating its resilience under oxidative stress conditions. In *S. marcescens* PWN146 genome, we found a total of 3 SODs (Mn-SOD encoded by *sodA*, PWN146_04547; Fe-SOD encoded by *sodB*, PWN146_01640; and Cu-Zn-SOD encoded by *sodC*, PWN146_01653); 2 CATs (*kata*, PWN146_02860; catalase-peroxidase *katG*, PWN146_02711); 1 PRX (α -hydroperoxide

reductase *aphD*, PWN146_03228) and more 4 putative PRXs (*bcp_1*, PWN146_03146; *bcp_2*, PWN146_03147; PWN146_04298; *tsa*, PWN146_00357); 1 chloroperoxidases (*cpo*, PWN146_02709); 1 thiol peroxidases (*tpx*, PWN146_02051); and 1 glutathione peroxidase (*gxp*, PWN146_01611). In addition, we also found 5 glutathione S-transferases (GSTs) (*gstB1*, PWN146_00870; *gstB2*, PWN146_01664; *gstB3*, PWN146_02581; *gstB4*, PWN146_03977; *yfcF*, PWN146_03851); and 5 glutaredoxin (*grxA*, PWN146_01113; *grxD*, PWN146_01642; *grxB*, PWN146_02296; *grxC*, PWN146_03676; *nrdH*, PWN146_03429). We also identified organic hydroperoxide resistance proteins (*ohrRB*, PWN146_04597-98), and two genes encoding paraquat-inducible proteins (*pqiAB*, PWN146_01204-05). The major oxidative stress and general stress regulons, respectively, OxyR/SoxR and RpoS [67], were found in *S. marcescens* PWN146 (*oxyR*, PWN146_04297; *soxR*, PWN146_01236; and *rpoS*, PWN146_00136). PWN146 also seems capable of detoxifying nitric oxide [68] through nitric oxide dioxygenase (*hmp*, PWN146_03325), possibly under regulation by *nrsR*

(PWN146_04859), nitric oxide-sensitive transcriptional repressor. Several studies have indicated a relationship between ROS tolerance and siderophore synthesis and iron uptake systems [54, 69]. Wu et al. [54] reported that the regulation of ROS-responsive genes in *Pseudomonas putida* may be iron-dependent, since antioxidant enzymes such as catalase and hydroperoxide reductase were surrounded with iron-binding proteins (i.e., bacterioferritins). Similarly, in PWN146, *kata* is located downstream an ABC iron complex transport system (PWN146_02857-58) and *gpx* is surrounded by ABC iron complex proteins (PWN146_01605-07). The putative phytoalexin export system AcrAB multidrug efflux pump (*acrBA*, PWN146_00495-00496) [60], coupled with *acrAB* operon repressor (*acrR*, PWN146_00497), was also found in PWN146.

The ability of *S. marcescens* PWN146 to grow in the presence of organic compounds, mostly plant-derived aromatic compounds, is shown in Table 2. Although PWN146 could withstand increasing concentrations of almost all compounds tested (except citral, carvacrol, geraniol, 3-eugenol, and linolool), it could only use phenylacetic acid as a carbon source. In the PWN146 genome, we could find the complete pathways for phenylacetic acid and 4-aminobutyrate (GABA), as seen in other endophytic bacteria [70], but could not find the complete metabolic pathways for limonene and pinene degradation, nor the *dit* gene cluster involved in diterpenoids degradation [71], which may explain why *Serratia* sp. PWN146 is unable to use these compounds as its sole carbon source. We were able to identify the most abundant enzymes found in metagenomes of pine beetles [72] involved in the catabolism of limonene and pinene, namely, 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase (PWN146_01252, PWN146_02955, PWN146_04724), two putative genes for limonene 1,2-monooxygenase (*limB*, PWN146_01979; PWN146_04915), 2S-(hydroxymethyl) glutathione dehydrogenase (*frmA*, PWN146_01004, PWN146_05142) and 1 alcohol dehydrogenase (*adhE*, PWN146_02193).

Vicente et al. [18] have shown that PWN146 is able to produce the plant auxin IAA and siderophores, but failed to show ACC deaminase activity. Corroborating this, we were not able to find ACC deaminase genes, only *S*-adenosylmethionine synthetase (*metK*, PWN146_03699) which is involved in the biosynthesis of ethylene [73]. The production of IAA in PWN146 is conducted via indole-3-pyruvate decarboxylase (*ipdC*, PWN146_03001), the main IAA (IPyA) biosynthesis pathway found in plant-beneficial bacteria [54, 60, 61]. The bacterial volatiles acetoin and 2,3-butadineol are also emitted for plant-growth enhancement [74]. Similar to *Enterobacter* sp. 638 [60], PWN146 genome encodes *budABC* (*budA*, acetolactate decarboxylase, PWN146_03021; *budB*, acetolactate synthase, PWN146_03022; and *budC*, (*S,S*)-butanediol dehydrogenase, PWN146_01493), and pyruvate dehydrogenase *poxB* (PWN146_01136), which can also convert small amounts of acetoin. Two genes putatively involved in the

catabolism of acetoin and 2,3-butadinediol were found (*acoR*, PWN146_01820 and PWN146_03820), however the *acoABCX* cluster was not identified [75]. In addition to IAA and volatiles, phytohormone-like substances such as polyamines [63], and pyrroloquinolone quinone [76] have been also considered plant-growth promoting compounds. PWN146 genome encodes a complete pathway for polyamine biosynthesis, *speABDE* (PWN146_03697-98; PWN146_03725-26), spermidine/pustrecine (*potDCBA*, PWN146_01447-50), and putrescine (*potFGHI*, PWN146_01117-20) transport systems, and pyrroloquinone quinone operon *pqqEDCB* (PWN146_01839-42).

Mineral acquisition and supply is also considered a plant-growth promoting feature of competent endophytes and may include traits such as the production of siderophores for iron sequestration and iron transport systems [59]. Furthermore, siderophores are also considered elicitors of induced systemic resistance (ISR), beneficial in the plant-pathogen interaction [63]. PWN146 is well equipped to compete for iron (Supplemental Table S3) containing 2 ferrous-iron transport systems (*feoABC*, PWN146_04162-64; *efeBOU*, PWN146_02401-03), 2 copies of the operon *afuCBA*, iron (III) transport system (PWN146_00750-52; PWN146_03675-77), ABC transporter Mn/Fe (*sitABCD*, PWN146_01567-70), seven genes coding for ferric siderophore transporter (*tonB*), 14 genes coding for iron complex transport system substrate-binding proteins (*fepB*), 12 genes coding for iron complex transport system permease (*fepD*, *fepB*, *fepC*), and 9 genes coding for iron complex transport system ATP-binding (*fluC*). PWN146 also synthesizes enterobactin (*entA*, PWN146_02687; *entF*, PWN146_04788; *entC*, PWN146_02688, PWN146_04790-91; *entE*, PWN146_04792), which is secreted via *entS* (PWN146_01900). Siderophores can also be retrieved by biopolymer transport protein (ExbDB, PWN146_03791-92) and the iron can be recovered via enterochelin esterase (*fes*, PWN146_01903, PWN146_03018, PWN146_04786) [60]. Interestingly, the *S. marcescens* PWN146 genome also encodes several genes related to rhizosphere competence such as heavy metal and drug transporters [60]. We found genes putatively involved in zinc uptake/transport (*znuACB*, PWN146_02267-2269; *zntB*, PWN146_01974 and PWN146_02044); copper resistance (*copA*, PWN146_00520; transcriptional factor *cueR*, PWN146_00526; blue copper oxidase, *cueO*, PWN146_03723; and copper resistance operon *copCD*, PWN146_01382-01383); arsenate resistance (*arsC*, PWN146_03160); lead/cadmium/zinc/mercury-transporting ATPase (*zntA*, PWN146_04676) and nickel transport protein (*nixA*, PWN146_00235). PWN146 was able to grow in TSB medium containing 5 mM CuSO₄ and ZnSO₄ (Table 2). Also, *S. marcescens* PWN146 has the complete operon for urea conversion to ammonia (*ureABCEFGD*, PWN146_00227-33; *utp* urea transporter, PWN146_00234; *ureR* operon transcriptional activator, PWN146_02016), supporting the results from biochemical characterization (Supplemental Table S5).

Table 2 *Serratia marcescens* PWN146 growth in increasing concentrations of xenobiotics compounds

Tested compound	Growth in TSB				Growth in M9 sole carbon source
	0.1 %	0.5 %	1 %	1.5 %	
(+)- α -Pinene	+	+	+	+	-
(-)- α -Pinene	+	+	+	+	-
α -Pinene (isomer mix)	+	+	+	+	-
(+)- β -Pinene	+	+	+	+	-
(-)- β -Pinene	+	+	+	+	-
(+)-3-Carene	+	+	+	+	-
3-Carene (isomer mix)	+	+	+	+	-
R-(+)-limonene	+	+	+	+	-
γ -Terpinene	+	+	+	+	-
<i>p</i> -Cymene	+	+	+	+	-
2-Undecanone	+	+	+	+	-
Mircene	+	+	+	+	-
Toluene	+	+	+/-	-	-
Xylene	+	+	+	-	-
Citral	-	-	-	-	-
Carvacrol	-	-	-	-	-
Geraniol	-	-	-	-	-
3-Eugenol	-	-	-	-	-
Linalool	-	-	-	-	-
	2.5 mM	5 mM	10 mM	20 mM	
CuSO ₄	+	+	-	-	n.a
ZnSO ₄	+	+	-	-	n.a
	0.01 mg/ml	0.05 mg/ml	0.1 mg/ml	0.2 mg/ml	
Benzoic acid	+	+	+	+	-
Phenol	+	+	+	+	-
Phenylacetic acid	+	+	+	+	+

List of aromatic compounds and heavy metals tested in TSB and in minimal medium M9 as sole carbon source. Bacterial growth is indicated by (+)

Generally, high antibiotic resistance is an intrinsic characteristic of *S. marcescens* [15]. This characteristic was previously seen in PWN146 [18], and is corroborated by the presence of genes encoding for tetracycline, macrolide, beta-lactam, aminoglycoside, fluoroquinolone, chloramphenicol resistance, and the existence of multiple multidrug efflux pumps, such as *acrAB* (PWN146_00495-96) (Supplemental Table S4). The PWN146 genome also encodes several genes related to the production of antimicrobial compounds, which jointly with antibiotic resistance may suggest a highly fitness and competitive bacterium in its environment. Similar to the endophytic bacteria *Pseudomonas fluorescens* F113 [77], PWN146 also produces hydrogen cyanide (*hcnABC*, PWN146_01266-68) and pyoverdine (*pvcA*, PWN146_00021), considered as potential antimicrobial compounds. Also, as other endophytic bacteria (*Azoarcus* sp. BH72, *Herbaspirillum seropedicae* SmR1, *Klebsiella pneumoniae* 342, *Stenotrophomonas maltophilia* R551-3, and *Enterobacter* sp. 638), PWN146 encodes chorismate pyruvate lyase gene (*ubiC*, PWN146_04006)

involved in the catabolism of chorismate into the antimicrobial 4-hydroxybenzoate [63]. Additionally, PWN146 may demonstrate fungal antagonism since it encodes several chitinases in its genome (PWN146_00633, PWN146_02145, PWN146_03120, and PWN146_04604), an ability also reported in the poplar endophyte *S. proteamaculans* 568 [78]. Besides the universal two-step secretion systems (SS) *Sec* and *Tat* (Twin-arginine translocation), *S. marcescens* PWN146 encodes type 2 and 6 secretion systems (T2SS and T6SS), mostly characterized for the delivery of toxins and hydrolytic enzymes in both plant-beneficial and plant-pathogenic bacteria [60, 79]. However, it lacks the type three secretion system (T3SS), typically found in plant pathogenic bacteria [24]. We could find several candidate toxins likely to be secreted by all secretion systems, such as: pore-forming toxins, hemolysins (*tlyC*, PWN146_00161; *hlyIII*, PWN146_03621; *shlA*, PWN146_04024); and membrane-damage toxin, phospholipase C (*plcC*, PWN146_03785) (Supplemental Table S4).

Currently, only four *B. xylophilus*-associated bacteria have been sequenced, namely, *Pseudomonas* sp. M47T1 [80], *Serratia* sp. M24T3 [81], *P. fluorescens* GcM5-1A [82], and *Serratia* sp. LCN16 [58]. *Pseudomonas* sp. M47T1, *Serratia* sp. M24T3, and *Serratia* sp. LCN16 were predicted to be plant-beneficial bacteria with endophytic potential [58, 81, 82]. Also, M47T1 and M24T3 showed nematicidal potential against *B. xylophilus* [81, 82]. On the contrary, Feng et al. [82] report that *P. fluorescens* GcM5-1A is potentially plant pathogenic encoding a fully functional T3SS and 79 virulence factors. This study presents the complete genome analysis of the *B. xylophilus*-associated bacterium *S. marcescens* PWN146. Our analysis suggests that PWN146 may have endophytic competence, or at least be considered facultative/opportunistic endophyte, with potential to influence plant metabolism and hormonal pathways besides providing nutritional capacities [62]. We have demonstrated that PWN146 is able to colonize plant tissues, occupying the intercellular spaces, but that it is unable to kill *C. elegans* or *B. xylophilus*. Moreover, *S. marcescens* PWN146 seems to be a highly fitness and competitive bacterium in terms of resources as well as in bacterial and fungal antagonisms. Further investigation continues to be important to understand how bacteria are involved in PWD. Still, we have now more evidence to reinforce the theory that *B. xylophilus*-associated bacteria may have a plant origin [10, 58].

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Sequence Data The nucleotide sequence data reported is available in the EMBL database under the accession number ERS1151563.

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