Characterization of Chilean *Pseudomonas syringae* pv *actinidiae* strains isolated from infected orchards

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Pseudomonas syringae pv. *actinidiae* (PSA) strain is a major problem for the kiwifruit industry worldwide. So far, 5 biovars of PSA have been identified, of which the most virulent form is biovar 3. This is the only biovar that has been detected in Chile, which is the third kiwifruit exporter country and is currently suffering from canker disease produced by PSA. Single nucleotide polymorphisms (SNPs) analyses have classified the biovar 3 strain into three groups: the European, the New Zealander and the Chilean groups, which have evolved from a common Chinese PSA ancestor. Although Chilean strains have been used in phylogenetic analysis, there is no information about genomic diversity within this group or whether they present microbiological characteristics that could affect its virulence. In this work we studied 15 Chilean bacterial isolates collected from orchards with canker disease symptoms, and classified them as PSA using a different PCR techniques. To gain more information on the relationship between the isolates we sequenced part of three conserved genes widely used to classify bacterial strains: *gtl (*Citrate Synthase*)*, *rpo*D (Sigma subunit of RNA polymerase II) and *gyr*B (Gyrase B). Using these sequences we performed a phylogenetic analysis that included some PSA reference sequences. Fourteen PSA Chilean isolates were grouped with PSA reference strains and three of them formed a subgroup within the PSA clade, suggesting clear differences at the genomic level among the isolates. We evaluated three microbiological traits in all the isolates: motility (swimming and swarming), and ability to induce a hypersensitive response in tobacco plants. All the isolates were able to induce the hypersensitive response in tobacco plants and were also able to perform both types of movements in appropriated growing conditions.

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ABSTRACT

Pseudomonas syringae pv. *actinidiae* (PSA) strain is a major problem for the kiwifruit industry worldwide. So far, 5 biovars of PSA have been identified, of which the most virulent form is biovar 3. This is the only biovar that has been detected in Chile, which is the third kiwifruit exporter country and is currently suffering from canker disease produced by PSA. Single nucleotide polymorphisms (SNPs) analyses have classified the biovar 3 strain into three groups: the European, the New Zealander and the Chilean groups, which have evolved from a common Chinese PSA ancestor. Although Chilean strains have been used in phylogenetic analysis, there is no information about genomic diversity within this group or whether they present microbiological characteristics that could affect its virulence. In this work we studied 15 Chilean bacterial isolates collected from orchards with canker disease symptoms, and classified them as PSA using a different PCR techniques. To gain more information on the relationship between the isolates we sequenced part of three conserved genes widely used to classify bacterial strains: *gtl (*Citrate Synthase*)*, *rpo*D (Sigma subunit of RNA polymerase II) and *gyr*B (Gyrase B). Using these sequences we performed a phylogenetic analysis that included some PSA reference sequences. Fourteen PSA Chilean isolates were grouped with PSA reference strains and three of them formed a subgroup within the PSA clade, suggesting clear differences at the genomic level among the isolates. We evaluated three microbiological traits in all the isolates: motility (swimming and swarming), and ability to induce a hypersensitive response in tobacco plants. All the isolates were able to induce the hypersensitive response in tobacco plants and were also able to perform both types of movements in appropriated growing conditions.

INTRODUCTION

The plant disease produced by the bacterium *Pseudomonas syringae* pv. *actinidiae* (PSA) is the most important threat to the kiwifruit industry worldwide (Cameron & Sarojini 2014; Vanneste et al. 2013). This phytopathogenic bacterium is able to enter the plant through natural openings such as stomata and lenticels or wounded tissues. Once inside the plant, it replicates and moves systemically causing the well-characterized canker symptoms (Balestra et al. 2009; Renzi et al. 2012).

PSA was first reported in Japan (1984) (Takikawa et al. 1989); Korea (1989) (Koh et al. 1994) and; in the early 90's, in Italy (Scortichini 1994), although at that time the disease was highly destructive in Japan and Korea, the consequences for the kiwifruit plants in Italy were no more than leaf spot and twig dieback in some orchards (Cameron & Sarojini 2014). Since 2008, PSA has spread to almost all kiwifruit producer-regions, causing devastation of entire orchards (Vanneste et al. 2013). Genomic studies of PSA have demonstrated that the strains first detected in Italy, Japan and Korea, and the strains causing the canker disease outbreak worldwide have different origins, but both of them originated in China (Baltrus et al. 2014; Marcelletti et al. 2011; McCann et al. 2013). So far, 5 biovars have been described; biovars 1 and 2, also known as the Japanese and Korean variants, are able to produce the toxins phaseolotoxin and coronatine, respectively (Chapman et al. 2012; Vanneste et al. 2013). Biovar 3 is responsible for the big outbreak, ongoing since 2008 and has been found in Italy (outbreak of 2008), in most kiwifruit producer European countries, and also in regions as far away as New Zealand and Chile (Balestra et al. 2009; Chapman et al. 2012; Everett et al. 2011; Mazzaglia et al. 2012; Scortichini et al. 2012; Vanneste et al. 2011b). PSA biovar 4, previously described as a low virulent variant

of PSA, has recently been reclassified as *Pseudomonas syringae* pv *actinidifoliorum* (Cunty A. et al. 2015). Biovar 5 is restricted to a local area of Japan (Saga prefecture) and, by comparison of genomic sequences, is closely related to biovar 2 although it does not produce the coronatine toxin (Cameron & Sarojini 2014; Fujikawa & Sawada 2016). Finally, a new PSA biovar 6 has been reported in a small region of Japan. This strain is able to produce both phaseolotoxin and coronatine toxins (Cameron & Sarojini 2014; Chapman et al. 2012; Ferrante & Scortichini 2010; Sawada et al. 2016).

Several molecular studies have been carried out in order to understand the origin and evolution of PSA. Although all PSA strains belong to a monophyletic clade when they are compared with other *Pseudomonas* strains, it is clear that the Japanese and Italian strains (first detection) belong to the same linage, which is different from the recent outbreak (included the Italian 2008 outbreak) (Ferrante & Scortichini 2010; Marcelletti et al. 2011). Moreover, the analyses of fully assembled and draft PSA genomes from strains isolated in Japan, Italy, China, New Zealand and Chile, indicate that PSA biovar 3 originated in China, where there is a more diverse PSA population according to SNPs analysis (Butler et al. 2013; McCann et al. 2013). From there, this biovar reached other kiwifruit producer countries through independent transmission events of the primal Chinese population. Once in the new niche, each strain continues its independent evolution (Butler et al. 2013; McCann et al. 2013). In fact, although Italian, New Zealander and Chilean PSA share a recent common ancestor, idiosyncratic SNPs define 3 clear groups: the Italian group, the New Zealander group and the Chilean group (Butler et al. 2013). In agreement with these data, Ciarroni et. al.(2015), using MLVA (*Multiple loci Variable number of Tandem Repeats Analysis*), were able to classify PSA biovar 3 into 4 subgroups associated to their

isolation origins: the Chinese, European, Chilean and New Zealander groups (Ciarroni et al. 2015).

PSA biovar 3 is the hyper-virulent form of PSA, which must have developed and selected or acquired factors that makes it more efficient in infecting kiwifruit. Concerning the factors involved in the virulence of a bacterium, one of the most important is the repertoire of effector proteins that are successfully injected into the plant cells (Alfano & Collmer 2004; Büttner 2016; Cunnac et al. 2011). Although PSA contains sequences with homology to many effectors genes described in *Pseudomonas spp*. (Marcelletti et al. 2011; McCann et al. 2013; Vanneste et al. 2011a), to our knowledge there is no experimental evidence showing a direct role of any of these effectors in PSA virulence. Other important factors involved in a successful infection process are the behavior of the bacteria before reaching the entry site and the strategy used to move through the plant (Jones et al. 2004; Pehl et al. 2012; Verstraeten et al. 2008). Mobile bacterium can move toward a more favorable environment through swimming, which is the individual movement of one bacterium, mainly driven by its flagella (Sokolov et al. 2007). This movement allows them to find and associate to more complex bacterial structures, forming multicellular communities (Sokolov et al. 2007; Verstraeten et al. 2008). These surface-associated multicellular communities are often used by bacteria because they give them several advantages over the individual-cell mode, particularly regarding their resistance to stressful environmental conditions or to chemical compounds (Verstraeten et al. 2008). It has been shown that PSA is able to form biofilms, sessile bacterial communities embedded within and attached to a surface (Ghods et al. 2015; Renzi et al. 2012). This association allows the bacterial community to be protected from several stress conditions in both, epiphytic and endophytic colonization phases. The swarming movement is another example of bacterial multicellular association, but in this

105 case the final purpose is the collective movement over surfaces. This bacterial motility also
106 requires the flagella, and in some cases the development of secondary structures and changes in
107 cell morphologies (Verstraeten et al. 2008). The swarming process is the fastest way for a
108 bacterial community to move over colonized surfaces (Eberl et al. 1999; Kearns 2010). Italian
109 PSA strains were able to perform swarming and swimming movements (Patel et al. 2014).

110 Biofilm formation and swarming motility are controlled in opposite ways (Caiazza et al. 2007;
111 de la Fuente-Núñez et al. 2012; Kearns 2010). Thus, the bacteria have to make a decision
112 whether to form a biofilm and attach strongly to surfaces or to become swarmer cells to move
113 and colonize other niches (Verstraeten et al. 2008).

114 In this work we studied 15 bacterial isolates from Chilean kiwifruit orchards that show canker
115 disease symptoms. We showed that all Chilean isolates are efficiently identified as PSA by the
116 Duplex PCR technique, ruling out closely related strains. Furthermore, the comparison of three
117 conserved gene sequences allowed us to group the fourteen PSA isolates into a PSA clade, and to
118 distinguish at least three Chilean isolates that form a subgroup within the clade. Furthermore, we
119 evaluated the ability of the isolates to trigger the hypersensitive response (HR) in tobacco plants
120 (Ferrante & Scortichini 2010; Vanneste 2013; Vanneste et al. 2011a), verifying that all the
121 Chilean isolates are able to activate this defense response albeit with different strength levels.
122 This suggests a differential degree of recognition of PSA strains. Finally, we demonstrated that
123 all the Chilean isolates are able to perform swarming and swimming bacterial movements. We
124 showed evidence of morphological changes in the bacterial surface when they commit to
125 swarming motility, suggesting that these bacterial changes are needed for the secretion of
126 compounds that facilitate the swarming movement.

MATERIALS & METHODS

Bacterial isolates

Fifteen bacterial samples were collected from kiwifruit orchards by the Chilean Agricultural and Livestock Service (SAG). The locations of each isolate are detailed in Table S1. The bacteria were grown in King's B medium plates supplemented with 20 µg/ml nitrofurantoin.

Bacterial motility assays

Samples from bacteria isolates were taken from fresh King's B plates and resuspended in 0.85% NaCl to a final OD_{600}=2. For swimming assays, 2 µl of the suspensions were inoculated onto the center of Moka medium-bacto-agar 0.3% plates (Cunty A. et al. 2015) supplemented with 0.05% triphenyl tetrazolium chloride previously poured and dried for 20 min. The inoculation was performed by puncturing inoculates halfway through the depth of the agar. For swarming assays, 2 µl of the bacterial suspension were inoculated onto the center of PG-0.5% agar plates as described previously (Matilla et al. 2007). The plates were incubated at 28°C and monitored at 24, 48 and 72 h for pink halos in the case of the swimming assays.

Identification of PSA by PCR

Duplex PCR was performed as described (Gallelli et al. 2011). The primer sequences were KN-F/R:CACGGATACATGGGCTTATGC/CTTTTCATCCACACACTCCG; AvrD-F/R: TTTCGGTGGTAACGTTGGCA/ TTCCGCTAGGTGAAAAATGGG. RG PCR was performed according to (Rees-George et al. 2010). The primer's sequences were F1: TTTTGCTTTGCACACCCGATTTT; R2: CAGGCACCCTTCAATCAGGATG. PCR to

identify biovar 1 and 2 were performed as described in (Lee et al. 2016); the primer sequences to identify biovar 1 were PsaJ-F: GACGTCGACGACAAGGTGAT and PsaJ-R AGTAAACCGTGCCGTCATCTC and for biovar 2 were PsaJ-R: AGTAAACCGTGCCGTCATCTC and PsaK-F GACAAAGCCAAAAAGGCGA.

Transmission electronic microscopy

PSA 7 isolate was grown in two conditions: PG-0.5% agar plates to promote swarming movement, and King´s B-1% agar plates used as a control where the swarming is not allowed. Samples from the plates were taken, centrifuged and the pellet fixed using a solution of 2.5% glutaraldehyde in 0.1M cacodylate buffer pH: 7.2 during 48 h. Afterwards, the pellets were washed for 1h using cocadylate buffer and post-fixed for 1.5 h with a solution of 1% osmium tetraoxide. The samples were washed with distilled water and then stained with 2% uranyl acetate for 60 min. An acetone series of 50, 70, 95 and 100% (30 min each), were used to dehydrate the samples and the pre-embedded was done in epon/acetone 1:1 overnight. The samples were embedded in epon pure, incubated at 60°C for 48 h to allow polymerization. An Ultramicrotome Leica Ultracut R was used to obtain sections between 20-80 nm from each sample; the slices were place on 300 mesh copper grids, stained with 4% uranyl acetate in methanol for 2 min and in Reynold's lead citrate solution for 5 min. The samples were observed using a Phillips Tecnia 12 Biotwin transmission electron microscope at 80 kV.

Phylogenetic analysis

Partially amplified sequences from *glt*, *gyrB* and *RpoD* genes were obtained by PCR according to (Sarkar & Guttman 2004). The primers used were cts-Fp

AGTTGATCATCGAGGGCGCWGCC, cts-RP pAGTTGATCATCGAGGGCGCWGCC; gyrB-FPs AGTTGATCATCGAGGGCGCWGCC, gyrB TRATBKCAGTCARACCTTCRCGSGC; RpoD-FpAAGGCGARATCGAAATCGCCAAGCG RpoD-RpGGAACWKGCGCAGGAAGTCGGCACG. The sequences were aligned using Clustal Omega y ClustalX, Multiple Sequence Alignment software from de *EMBL-EBI* (http://www.ebi.ac.uk/Tools/msa/clustalo/) along with the genes from reference PSA genomes: MAFF 302091(biovar 1), KACC 10594 (biovar 2) and CRA-FRU 8.43, PSA 1B T10_06115 (biovar 3), *Pseudomonas viridiflava* CC1582*, Pseudomonas fluorescens* SBW25 and *Pst* DC3000. The sequences were concatenated and used to build the phylogenetic tree using the *MrBayes* v3.2.6 sofware and the Neighbor-Joining (NJ) method, with 1.000 *bootstrapping*. The *FigTree* version 1.4.2 software was used to obtain the phylogenetic tree image.

HR assays

Inoculums of PSA isolates were grown from fresh King's B plates in 3 ml of King's B medium supplemented with nitrofurantoine 20µg/ml. The bacterial suspensions were centrifuged and resuspended in 0.85% NaCl until OD_{600}= 0.01 (10^7 cfu/ml) and infiltrated into *Nicotiana tabacum* plants. The plants were maintained in a growth chamber with a photoperiod of 16/8 h at 24±2°C during 7 days. Samples from the mock side of the infiltrated leaf were taken and homogenized in 200 µl of NaCl 0.85%; 1/10 dilutions were prepared and plated into King´s B/Nitrofurantoine agar plates. The plates were incubated for 48 h and the colony forming units were counted.

RESULTS

Identification of Chilean PSA strains from kiwifruit orchards

Different research groups have been trying to find the fastest and most accurate method for the identification of PSA from infected orchards. This is important in order to control or prevent the disease in healthy orchards. The most common technique used is PCR, and several specific PCR primers for PSA detection have been published. One of the first set of primers designed were reported by Koh and Nou (2002), based on the RAPD technique (*Random Amplified Polymorphic DNA*) (Koh & Nou 2002). However, these primers also amplify fragments from other *Pseudomonas spp*. (Rees-George et al. 2010). Lately, Rees-George et al, (2010) developed a set of primers that amplify the internal transcribed spacer (ITS) between 16S and 23S RNA ribosomal PSA genes (RG-PCR), but this set of primers were not specific for PSA (Vanneste et al. 2013). Duplex PCR considers two sets of primers in the same reaction (Gallelli et al. 2011; Koh & Nou 2002), giving specific amplification patters for *Pseudomonas* species. This approach gives a fairly specific pattern for PSA consisting of two bands of 492 bp and 226 pb corresponding to *ompP1* and *avrD1* respectively (Gallelli et al. 2011; Vanneste 2013).

The bacterial samples used in this study were collected from 15 different kiwifruit orchards that showed canker disease symptoms. All of them were isolated from a very narrow region in the center of Chile but in different seasons (Table S1). We first used 3 reported methods to identify the bacterial isolates as PSA. Genomic DNA from the isolates were used to perform Duplex PCR (Gallelli et al. 2011), RG-PCR (Rees-George et al. 2010) and nested PCR. In our hands, nested PCR showed inconsistency between replicates, thus we show results only for Duplex and RG PCRs **(**Figure 1). Duplex PCR amplification from the samples showed a two-band pattern, as

previously reported for PSA, in contrast with our negative control *Pst*DC3000 strain which shows just one band of around 490 pb (Fig. 1A). The same result was obtained using RG-PCR, where specific PSA primers were used to amplify the ITS region **(**Fig. 1B). In this case, a PCR product of the same size as that of PSA was observed in a *Pst*DC3000 DNA sample, used as a negative control of the technique. According to these results, 14 bacterial isolates correspond to PSA, while sample 8 does not. Furthermore, we evaluated the biovar of the PSA Chilean isolates using specific primers to identify biovars 1 and 2 (Lee et al. 2016) (Fig. 1C). None of the PSA isolates nor the 10627 strain biovar 3 used as a control (b3) showed the reported PCR products for biovar 1 and 2, except for the positive controls (genomic DNA from 9617 (biovar 1) and KACC 10754 (biovar 2). This result suggests that the populations analyzed do not correspond to PSA biovar 1 or biovar 2.

Phylogenetic analysis of PSA Chilean isolates.

To confirm the previous results and to gain more insight into the phylogenetic relationships between PSA Chilean isolates, we amplified the sequences from three different genes that are conserved in bacterial species (Butler et al. 2013; Sarkar & Guttman 2004). PCR products from *glt* (Citrate Synthase)*, rpoD (*Sigma Factor 70) and *gyrB* (DNA gyrase B) were obtained, sequenced, concatenated and aligned to build a phylogenetic tree using the Neirbour Joinig method (*MrBayes* v3.2.6 software). Sequences of the analyzed genes from KACC10594, MAFF30209, T10_6115, CRA-FRU 8.43 and PSA1B reference genomes were added to the analysis as well. As shown in Figure 2, 14 out of the 15 bacteria isolates form a cluster with the PSA reference strains. Although three genes are not enough to distinguish between biovars 1, 2 and 3, they were sufficient to detect a subgroup among the Chilean isolates (Figure 2). The

branch containing PSA 1, 5, 6, 9 and 13, shows a subclade containing PSA 1, 5 and 13. This data indicates that at the molecular level the Chilean PSA strains show molecular differences among them, even when only three conserved genes are used for comparison. The analysis of the sequences shows that PSA 8 is closely related to the *Pseudomonas fluorescens* strain, confirming the results obtained using the Duplex and RG PCRs techniques.

Microbiological parameter of PSA Chilean strains

We evaluated several microbiological parameters in three of the Chilean PSA isolates to compare their behavior in terms of growth rate, ability to incorporate foreign DNA and motility features. The generation time for all the isolates was around 2 hours, without significant differences among them or compared to our control *Pst*DC3000. Similar results were obtained when we assayed their capability to accept foreign DNA. The maintenance of a *Pseudomonas* replicative plasmid (pBBRMCS-5) (Kovach et al. 1995; Perez-Martinez et al. 2007) or a plasmid containing the miniTn5 elements that transfers to the bacterial genome (pUTKm) (de Lorenzo et al. 1990; Perez-Martinez et al. 2007), were the same for all the samples (Table 1).

Evaluation of the SA Chilean isolates in their ability to trigger the HR in tobacco plants

Other feature reported for PSA is their ability to generate HR in tobacco plants (Scortichini et al. 2012; Vanneste et al. 2011a). The HR is triggered by recognition of a pathogenic microorganism by the plant, generally associated to the induced defense response or effector triggered immunity (ETI), but it has also been observed in the basal response or PTI (Katagiri & Tsuda 2010; Wu et al. 2014). Thus, we evaluated this plant-pathogen interaction in all PSA Chilean isolates (Figure 3). Suspension of PSA isolates prepared to OD_{600}=0.01, corresponding to 10^7 cfu/ml, were

infiltrated in half of tobacco leaves and the remaining halves were mock inoculated. In parallel, the same suspensions were plated on King's B-agar plates to confirm the initial bacterial inoculum. After 7 days, the HR was very strong in the *Pst* DC3000 strain, while the PSA isolates showed a wide range of HR, being the strongest one that generated by PSA isolates 9 and 13 (Figure 3). To confirm the successful containment of the bacteria, we took tissue from the mock inoculated side of each leaf and performed the quantification of PSA in King's B-agar plates as previously described (Greenberg et al. 2000). We did not observe growth of PSA on the plates, confirming the development of a successful HR in tobacco leaves for all PSA isolates.

Swimming and swarming movements of PSA Chilean isolates

Bacterial movement can be an important factor in the interaction with the host (Scharf et al. 2016). Thereby, we evaluated swimming and swarming movements in the 14 PSA isolates. These movements depend of the presence of at least one functional flagellum (Kearns 2010). As shown in Figure 4 all the isolates are able to perform the swimming movement in a PG-0.3% medium (individual bacterium movement powered by the rotation flagella), in accordance with the pink halo around of the bacteria inoculation sites (Fig. 4A). The 14 PSA isolates also show the swarming movement, most of them displaying a classical dendritic pattern **(**Fig. 4B**)**.

The swarming movement depends mostly on the main bacterial flagellum along with the interaction between cells and the surface they are in contact with. Nevertheless, some species require the development of secondary flagella around the bacterial surface, surfactant secretion, or even the type IV pilus (Caiazza et al. 2007; Ghods et al. 2015; Jones et al. 2004; Kearns 2010). Thus, we asked whether PSA Chilean isolates show morphological changes when they have performed the swarming movement. Therefore, we examined the shape of samples of one

PSA isolate taken from swarming plates (Moka-0.5% agar medium plates) and from normal plates (no swarming observed) as a control, since we do not have PSA mutants in the swarming motility. These samples were prepared and analyzed by Transmission Electronic Microscopy (TEM). Fig. 4C (upper panel) shows bacterial growth in normal King's B plates. The lower panel shows the bacterial preparation from the swarming plates. Unfortunately, as it was previously reported (Ghods et al. 2015; Scortichini et al. 2012), we did not observe flagellar structures neither in control bacteria nor in bacteria that had swarmed. However, the bacteria with swarming motility have a protrusion of the outer membrane covering the bacterium surface. These structures are no present in PSA that are not swarming. The structure observed has the appearance of secretion vesicles, but further analyses are necessary to confirm this result.

DISCUSION

The bacterial canker disease produced by *Pseudomonas syringae* pv *actinidiae* infection has provoked extensive damage in kiwifruit producer countries, and Chile has not been the exception. In this work we studied 15 bacterial isolates collected from orchards showing canker disease symptoms in the region of Maule, Chile, in 2012 and 2013. The bacterial isolates were studied at the molecular and microbiological levels, identifying them as PSA biovar 3 (Fig 1) with differences in their conserved genes *glt, rpo*D and *gyr*B, that allowed us to identify subgroups in the populations studied (Fig. 2). The microbiological characteristics analyzed in this study show that all the PSA isolates behave similarly in their ability to trigger HR in tobacco plants (Fig. 3), and also in their swimming and swarming motility (Fig. 4). TEM analysis indicates that PSA Chilean isolates show changes in the morphology of the bacteria plasma membrane (Fig. 4C), suggesting an active secretion system.

298 **Identification of PSA through PCR methods**

299 The identification of PSA from infected orchards is an issue that has been addressed by different
300 research groups trying to find the fastest and more accurate method. A rapid method for PSA
301 detection is essential for preventing and controlling PSA infections.
302 In order to study the Chilean isolates we used three PCR methods based on primers specifically
303 designed to detect PSA. The most consistent results were obtained with the Duplex PCR (Gallelli
304 et al. 2011), which indicated that fourteen of the fifteen isolates were PSA, and allowed us to
305 eliminate the bacterial sample called PSA8 as PSA (Fig. 1). These results were confirmed by the
306 sequencing of the three conserved genes used to build the phylogenetic tree. The primers for RG-
307 PCR were designed to amplify the internal transcribed region between the ribosomal genes 16S-
308 23S (Rees-George et al. 2010). Amplification products of the right sizes were observed in all the
309 PSA samples. However, there was also amplification from DNA of the negative control
310 *Pst*DC3000, indicating that this method has to be complemented by sequence analysis of the
311 amplicon. Nested PCR is considered to be a highly sensitive technique because of the use of a
312 primary PCR product as template for a second and more specific amplification (Biondi et al.
313 2013). However, it frequently shows inconsistencies between different replicates, and was
314 therefore no used in this study. In our hands the most efficient method to detect PSA was the
315 Duplex PCR. There are a number of other methods we did not test in this study, included qPCR
316 analysis that could be more sensitive in detecting PSA, however, this is a complex technique that
317 may be difficult to use as a fast diagnostic tests.

318 **Genetic differences among the Chilean isolates of PSA**

A comparison of whole genome sequences of PSA has shown how closely related the strains collected around the world are. However, they are different enough that can be grouped according to their origin and isolation date (Butler et al. 2013; Chapman et al. 2012; Ciarroni et al. 2015; Vanneste et al. 2013). The sequences of the hypervirulent PSA variant that caused the pandemic, allows its classification according to idiosyncratic SNPs and MVLA analyses into four groups: New Zealander, European, Chinese and Chilean groups.

We analyzed a small PSA Chilean population using the partial sequences of three conserved genes *glt*, *rpo*D and *gyr*B, and compared them to the sequences of PSA reference strains (Fig. 2). As previously reported, the number of genes we used to build the phylogenetic tree were not enough to distinguish among biovars, but it was sufficient to detect subgroups among the Chilean PSA isolates. In the MVLA study we also found different groups within the Chilean population. In fact, when we analyze 13 VNTR loci, the most variable group is the Chilean one. This can be seen in a Neighbor-Joining dendogram (Ciarroni et al. 2015). Both results suggest a high divergence within the PSA biovar 3 of the Chilean population, which could be explained by the introduction of more than one biovar 3 strain or by the introduction of one strain that is in constant change in order to better adapt to new environmental conditions. Further genomic analyses are needed to distinguish between these hypotheses.

Microbiological features of PSA Chilean isolates

HR is triggered by PSA Chilean isolates

Due to the differences at the genomic level, we asked whether these differences could also be observed in the phenotypes. One of the characteristics of PSA strains is the ability to trigger HR

in tobacco. We observed that all Chilean PSA isolates produce this response in tobacco plants, albeit with different strength (Figure 3). It has been shown that *Pseudomonas syringae* pv tabaci and *Pseudomonas syringae* pv *macuolicola* trigger morphologically different programmed cell death processes (Krzymowska et al. 2007).

Therefore, the plant might not equally recognize PSA isolates. In some cases the response is very fast and strong and in others it is barely noticeable. Nevertheless, the HR triggered by PSA isolates in tobacco are efficient in containing the spread of the pathogens. Moreover, all the isolates are efficient in infecting kiwifruit. On the other hand, the fact that PSA provokes HR in tobacco plants means that this pathogen is recognized as such by the plant. Therefore this system could be an important source of information on the proteins involved in the generation of induced immunity, which could be used in the future in kiwifruit plants.

PSA Chilean isolates show swarming and swimming motility

PSA is a Gram negative bacillus with a polar flagellum (Scortichini et al. 2012). The ability to move over surfaces gives the bacteria a big advantage in finding natural openings on plant surfaces for colonization and infection of plant tissues. The swimming movement is used by a single bacterium to move and associate with other individuals or to directly associate with an already established bacterial community (Kearns 2010; Sokolov et al. 2007). The swarming motility has advantages over swimming, since it allows the bacterial community to move faster and in a more coordinated manner than individual cells. Both movements depend on the development of active flagella. However, if a bacterium has swimming motility it does not imply that it can perform swarming movement (Attmannspacher et al. 2008). Besides the flagella, some strains require the development of other structures to be able to swarm, such as high number of

peritrichous flagella (Verstraeten et al. 2008), multinucleated and elongated cells, etc. (Rather 2005). We showed that all the Chilean PSA isolates have the swimming and swarming motility, characteristics previously described in Italian strains (Patel et al. 2014). The swarming movement was not identical in all the strains, but they all show a dendritic pattern already described for other strains. Using TEM we could not observe the flagella or flagellum structures neither in the swarmers nor in the non-swarmer cells, probably due to the technique used to prepare the samples. Nevertheless, we did find morphological changes in the swarmer cells (Fig. 4C). In 90% of the bacterial population we observed protrusion of the plasma membrane, which could correspond to an active secretion process. It was reported that swarming motility requires a viscous slime layer composed of carbohydrates, proteins, glycolipids, peptides, surfactants, etc; which allow them to keep the humidity they need to facilitate movement (Daniels et al. 2006; Eberl et al. 1999). We speculate that the formation of vesicles on the membrane surfaces might be an indicator of the formation of the slim layer that improves bacterial movement. Nevertheless, more experimental data is necessary to identify the nature of these structures.

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CONFLIC OF INTERES

The authors have no conflict of interest to declare.

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542

Table 1(on next page)

Representative data for PSA Chilean strains.

The data represent the average of at least three independent replicates ± the standard deviation.

1

PSA isolates	Generation time (h)	Transposition frequency (CFU/µg pUTKm)	Transformation frequency (CFU/µg pBBRMCS-5)
1	2.8 ±0.6	$4.4 \times 10^3 \pm 2.4 \times 10^3$	$4.0 \times 10^2 \pm 2 \times 10^2$
5	3.1±0.6	$1.85 \times 10^3 \pm 0.8 \times 10^3$	$4.4 \times 10^2 \pm 3 \times 10^2$
13	2.3±0.7	$3.7 \times 10^3 \pm 2 \times 10^3$	$1.1 \times 10^2 \pm 0.6 \times 10^2$

2 The data represent the average of at least three independent replicates ± the standard deviation

Figure 1

Identification of PSA Chilean isolates

Agarose electrophoresis of DNA showing the amplification products from DNA of the 15 Chilean isolates indicated on top of each panel. **A)** Duplex PCR. The amplification of genes *ompP1* and *AvrD1* are indicated on the right hand side of the panel. **B)** RG-PCR. ITS: Interspace region of 16S-23S rDNA. Amplification of the *rpo*D gene was used as control of DNA integrity in both cases **C)** Amplification of specific markers for detection of PSA biovar 1 (*Psaj/F/R*) and biovar 2 (*PsakF/R*). b1: DNA from strain 9617 (biovar 1). b2: DNA from strain KACC 10754 (biovar 2). b3: DNA from strain. b3: DNA from strain 10627 (biovar 3). Amplification of the gene coding for *HopAA1.2* was used as a control of DNA integrity. DC: *Pst* DC3000 DNA used as control. These experiments were repeated three times with the same results.

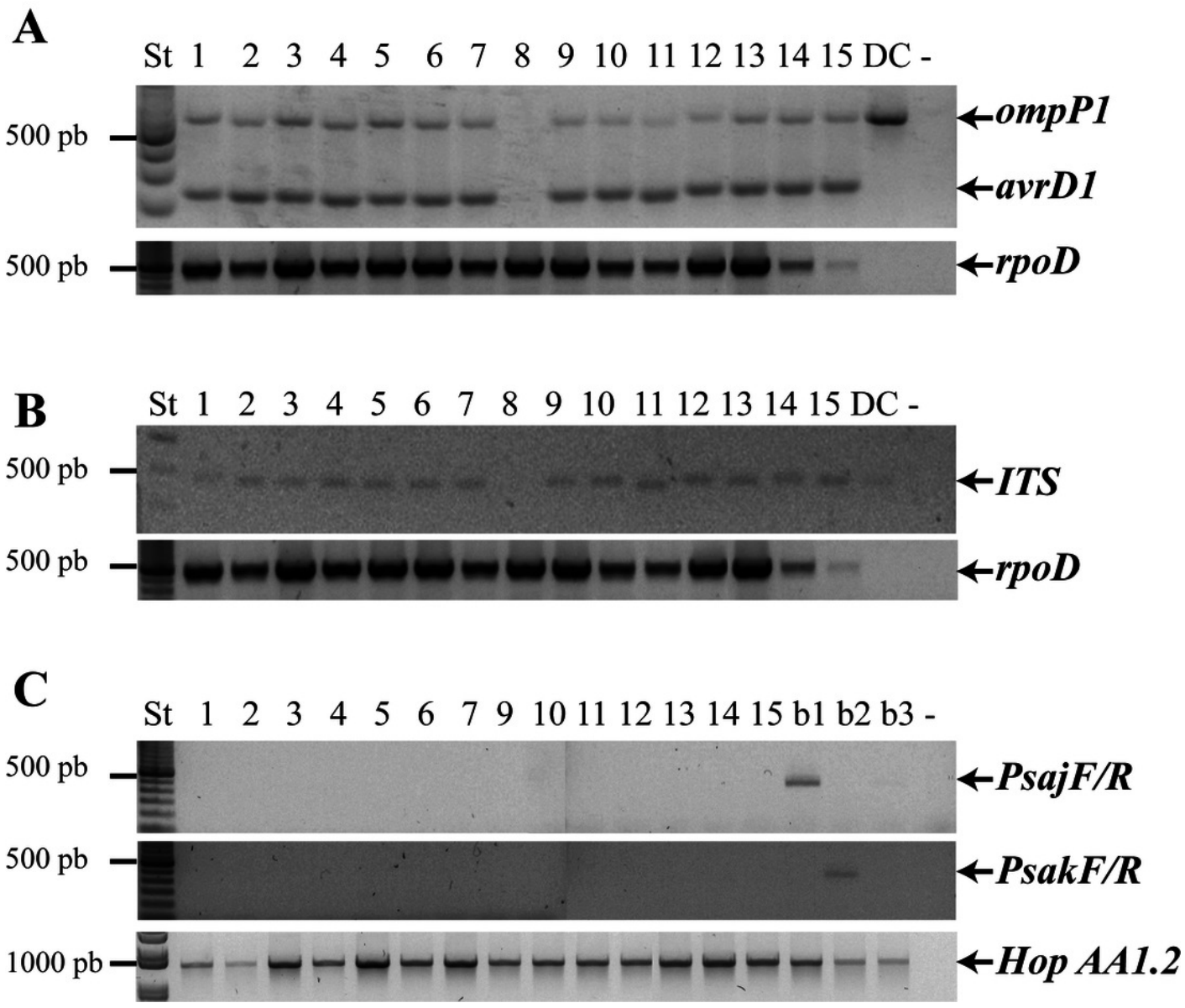

Figure 2

Phylogenetic tree of PSA Chilean strains.

Partial sequences of *glt*, *rpoD* and *gyrB* genes were amplified from all PSA Chilean isolates, sequenced and concatenated. The same regions were taken from the reference sequences of *Pseudomonas viridiflava* CC1582, *Pseudomonas syringae* pv. *tomato* DC3000, *Pseudomonas syringae* pv. *actinidiae* MAFF302091 (biovar 1), KACC10594 (biovar 2) and CRA-FRU 8.43, PSA1B, T10_06115 (biovar 3), from the database. The sequences were aligned and the phylogenetic tree was constructed using the Neighbor-Joining method. The percentage of bootstrap value of 1000 iterations are shown in each node. *Pseudomonas fluorescens* SBW25 was used as *outgroup.* The bar 0.03 indicates phylogenetic distance.

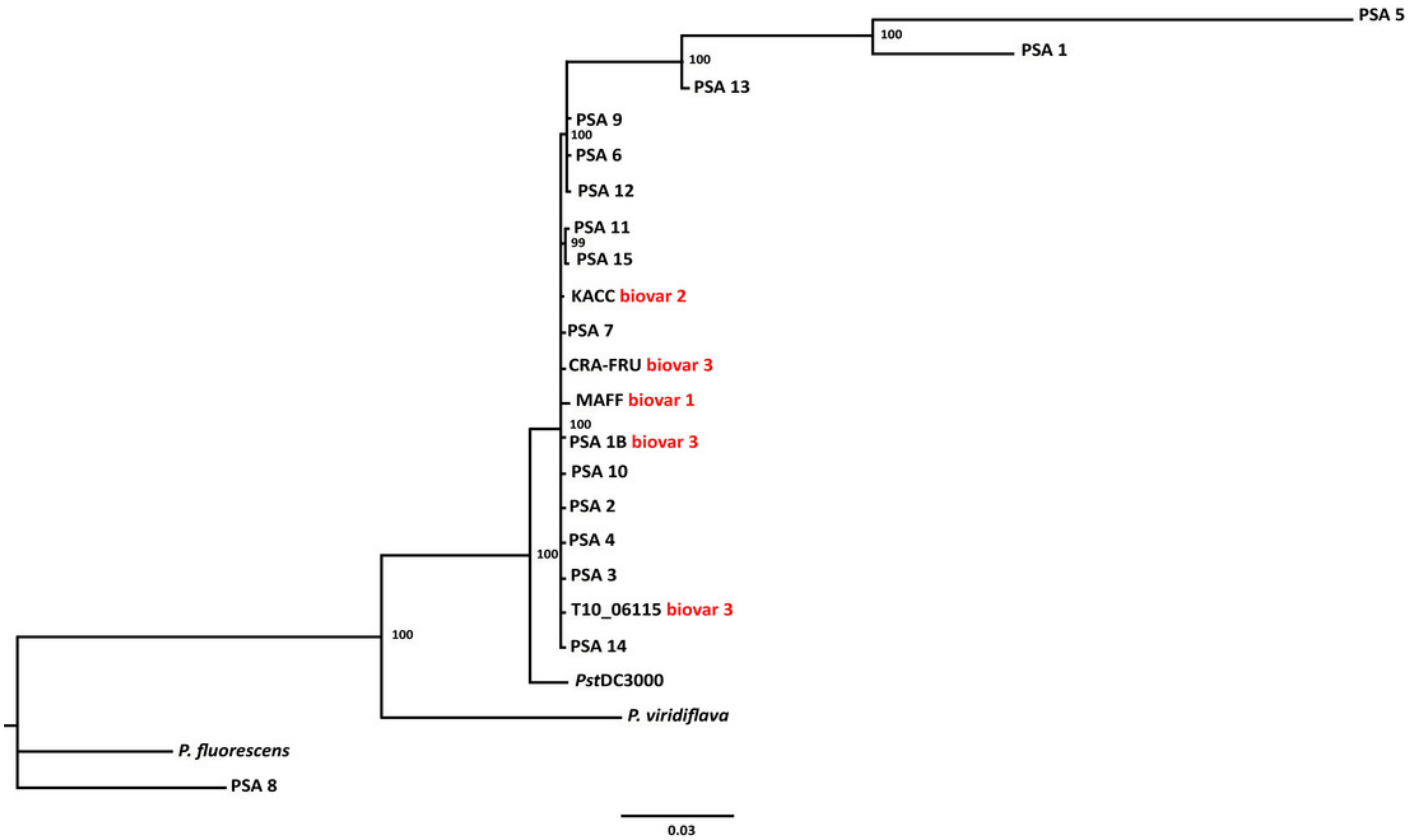

Figure 3

Hypersensitive response induced by PSA Chilean Isolates in tobacco plants.

Pictures of representative leaves 7 dpi of PSA isolates indicated on each picture. The white * indicates the side of each leaf inoculated with PSA, the other side were mock inoculated. The arrow head shows the HR response in the leaves. *Pst*DC3000: positive control. This experiment was repeated three times with the same result.

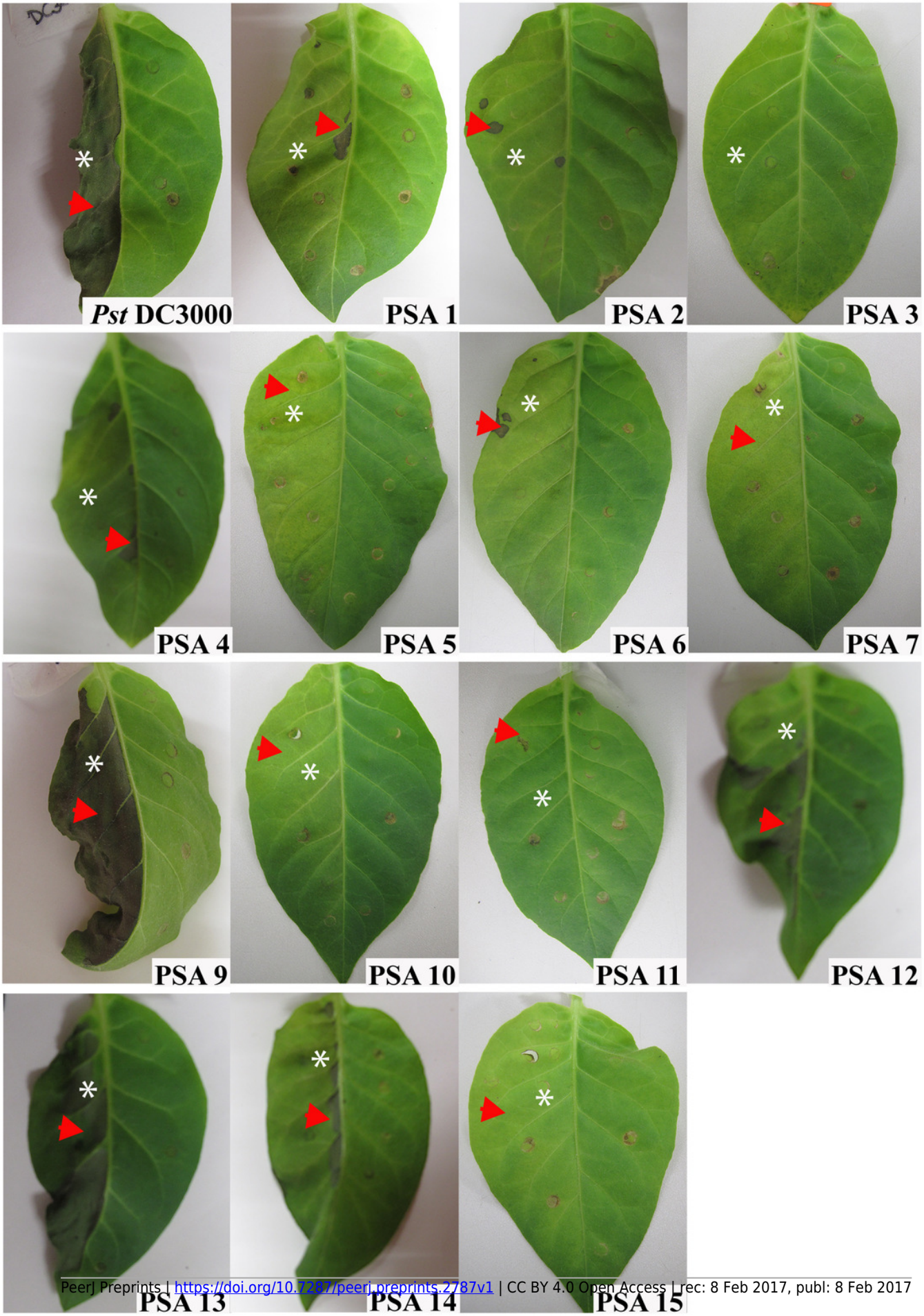

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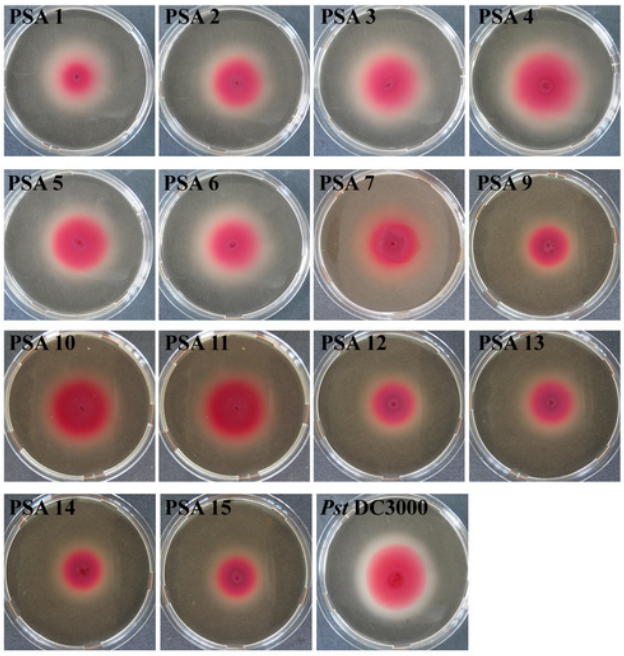
Figure 4

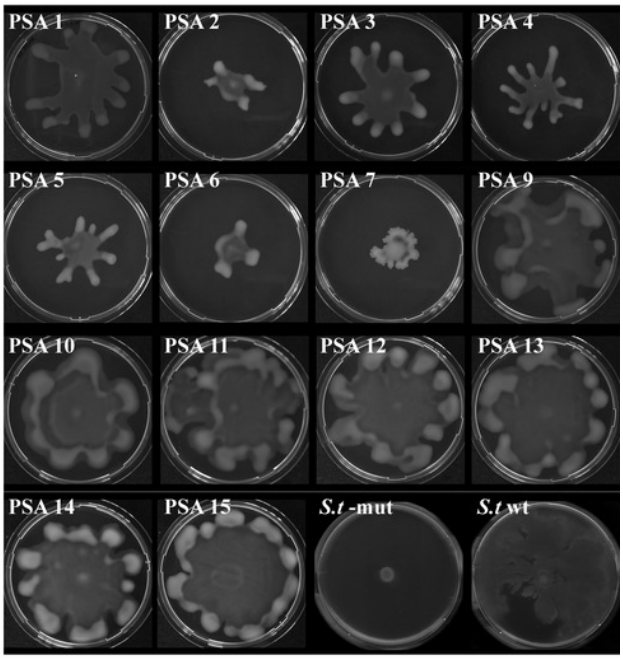
Swimming and swarming motility of the PSA Chilean Isolates.

A) The swimming movement of PSA isolates in PG medium agar plates is seen as a pink halo surrounding the inoculation site. Each bacterial isolate is indicated in the figure. *Pst* DC3000 : positive control for swimming. **B)** Swarming movement of PSA isolates in Moka medium 0.3%agar plates. *S.t mut*: *Salmonella enterica subsp. Enterica* serovar *Thyphymurium* ATCC14028s code 100685 mutant is a negative control, *S.t wt*: *Salmonella enterica subsp. Enterica* serovar *Thyphymurium* ATCC14028s is a positive control. **C)** Transmission electron microscopy pictures of PSA 5. *Upper panel*, PSA 5 grown on king´s B medium (no-swarmer). The black arrow indicates the cell that is shown in more detail on the left panel. *Bottom panel*, PSA 5 grown on PG-medium 0.3% agar (swarmer). The black arrow shows the cell observed with more detail in the left panel. Bar = 0.5 µm. These experiments were repeated three time with the same result.

A



B



C