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**Pseudomonas syringae pv. actinidiae Draft Genomes Comparison Reveal Strain-Specific Features Involved in Adaptation and Virulence to Actinidia Species**

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**Abstract**

A recent re-emerging bacterial canker disease incited by *Pseudomonas syringae pv. actinidiae* (Psa) is causing severe economic losses to *Actinidia chinensis* and *A. delicosa* cultivations in southern Europe, New Zealand, Chile and South Korea. Little is known about the genetic features of this pathovar. We generated genome-wide illumina sequence data from two Psa strains causing outbreaks of bacterial canker on the *A. delicosa* cv. Hayward in Japan (J-Psa, type-strain of the pathovar) and in Italy (I-Psa) in 1984 and 1992, respectively as well as from a Psa strain (I2-Psa) isolated at the beginning of the recent epidemic on *A. chinensis* cv. Hort16A in Italy. All strains were isolated from typical leaf spot symptoms. The phylogenetic relationships revealed that Psa is more closely related to *P. syringae* *theae* than to *P. avellanae* within genomospecies 8. Comparative genomic analyses revealed both relevant intrapathovar variations and putative pathovar-specific genomic regions in *Psa*. The genomic sequences of J-Psa and I-Psa were very similar. Conversely, the I2-Psa genome encodes four additional effector protein genes, lacks a 50 kb plasmid and the phaseolotoxin gene cluster, *argK-tox* but has acquired a 160 kb plasmid and putative prophage sequences. Several lines of evidence from the analysis of the genome sequences support the hypothesis that this strain did not evolve from the *Psa* population that caused the epidemics in 1984–1992 in Japan and Italy but rather is the product of a recent independent evolution of the pathovar *actinidiae* for infecting *Actinidia* spp. All Psa strains share the genetic potential for copper resistance, antibiotic detoxification, high affinity iron acquisition and detoxification of nitric oxide of plant origin. Similar to other sequenced phytopathogenic pseudomonads associated with woody plant species, the Psa strains isolated from leaves also display a set of genes involved in the catabolism of plant-derived aromatic compounds.

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**Introduction**

*Pseudomonas syringae* is a worldwide phytopathogenic microorganism mainly adapted to plant species, both monocotyledon and dicotyledon, and either cultivated or grown in wild habitats. In addition to its well-known dispersal and colonization of cultivated crops by avenues such as seeds, bulbs, bud grafting, rain and wind, there is also evidences that strains of *P. syringae* strains can be disseminated in various environments through the water cycle and in Antarctic areas [4].

The most common symptoms of *P. syringae* include leaf spots and necrosis, fruit specks and scabs, flower wilting, twig die-back, branch and trunk cankers and, in particular circumstances, plant death [5]. On the basis of visually assessed symptoms and host range tests and with the aid of biochemical, physiological and nutritional tests and molecular typing, *P. syringae* (i.e. the *P. syringae* species complex) is divided into 57 pathovars [6]. To genetically circumscribe 48 *P. syringae* pathovars and some related species of phytopathogenic pseudomonads, Gardan et al., [7] performed DNA-DNA hybridisation and ribotyping analyses and pointed out nine discrete genomospecies. In this study *P. s. pv. actinidiae* (Psa) was not included. By performing repetitive-sequence PCR, ARDRA and AFLP analyses, this pathovar was subsequently placed into genomospecies 8 together with *P. avellanae* and *P. s. pv. theae* [8,9].

Psa is the causal agent of bacterial canker of kiwigreen (*Actinidia deliciosa*), and was first reported in Japan [10]. It was then subsequently isolated in South Korea [11] and Italy [12]. In the Asian countries the pathogen caused relevant economic losses [13], whereas in Italy it has incited occasional leaf spot, twig die-back and bark canker over the past 15 years but never destructive outbreaks [14]. A bacterial canker outbreak on *A. delicosa* in central China (Shaanxi province) was observed during 1990–1991 and reported ten years later [15]. Subsequently, another record of this disease on *A. delicosa* was reported also in the Anhui province (Southeast China) [16]. Recently, the pathogen has been found in Portugal [17] and Chile [18]. During 2008-2011, Psa suddenly and very rapidly incited severe epidemics of bacterial canker in central Italy. During these epidemics the kiwigold (*A. chinensis*) was first affected and, afterwards, *A. delicosa* [19]. Psa was first isolated from *A. chinensis* in southwest China (Sichuan province) in 1989.
and MLST, has revealed that there are currently clonal outbreaks of bacterial canker to both Actinidia species irrespective of the geographical areas of origin of the isolates and that the strains of the present epidemics are distinct from those causing bacterial canker on A. delicosa in the past [19,25]. As a virulence factor, some Psa strains produce phaseolotoxin [28,29], which is encoded by a mobile gene cluster representing one of the first examples of horizontal gene transfer among phytopathogenic bacteria [30,31].

These re-emerging, sudden and destructive worldwide cases of bacterial canker on highly-prized crops such as kiwigréen and kiwigold prompted us to an in-depth investigation of the genomic structure of Psa. Comparative genomics can provide insights into the host-pathogen interaction pathways, differential virulence factors and the chronological evolution of pathogens [32]. In recent years, complete or draft genome analyses have been performed for important phytopathogenic pseudomonads such as P. s. pv. tomato [33,34], P. s. pv. phaselicola [35], P. s. pv. syringae [36], P. s. pv. oryzae [37], P. s. pv. tabaci [38], P. s. pv. aesuli [39], P. savastanoi pv. savastanoi [40] and P. savastanoi pv. glycinea [41].

For the sequencing, we selected three representative Psa strains: NCPPB3739 (= KW 11), the type-strain of the pathovar, which was isolated in 1984 in Japan from A. delicosa, cv. Hayward [10]; NCPPB3871, which was isolated in 1992 in Italy from A. delicosa, cv. Hayward [12]; and CRA-FRU 8.43, which was isolated in 2008 in Italy from A. chinensis, cv. Hort16A [19,42]. All strains were isolated from leaf spot symptoms. These strains, which were isolated from the same organ and represent the initial outbreaks of bacterial canker on A. delicosa in Japan (1984) and Italy (1992) as well as the current severe epidemics on A. chinensis in Italy, are good candidates for elucidating the host-pathogen relationships and the evolutionary adaptation of Psa towards two Actinidia species. The aim of this study was to investigate the biology and evolution of Psa strains that cause bacterial canker to different Actinidia species in many areas of world. We achieved this aim by performing a comparison of the genes found in the draft genomes of the Psa strains with other P. syringae pathovars and by determining the genomic variation among the Psa strains. We demonstrate that Psa shows relevant intrapathovar variations which are probably due to the gain and loss of variable genomic regions. Similar to the other sequenced phytopathogenic pseudomonads associated with woody plant species, the Psa strains isolated from Actinidia spp. leaves also display a set of genes involved in the catabolism of plant-derived aromatic compounds.

**Results**

**Genome-wide sequence data**

We generated genome-wide Illumina IIx sequence data from one strain of Psa isolated in Japan, NCPPB3739 (= KW 11) which is the type-strain of the pathovar and here referred to as J-Psa, and two Psa strains from Italy, NCPPB3871 and CRA-FRU 8.43, which were isolated during two different outbreaks of bacterial canker on Actinidia species and here referred to as I-Psa and I2-Psa, respectively. The Illumina sequencing provided nearly 10 millions of 100 nt reads that passed the quality checking. Sequencing of the J-Psa library provided 1,672,966 reads which were assembled into 833 contigs (N50 = 14,838; largest contig: 67,329) for a total of 3,931,199 nts (a coverage of 27.7 x). Sequencing of the I-Psa library provided 4,083,706 reads which were assembled into 466 contigs (N50 = 27,730; largest contig: 122,209) for a total of

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**Figure 1. Disease symptoms of Psa on Actinidia spp. leaves and main leader.** The sequenced I-Psa and I2-Psa strains from Italy were isolated from the leaves herein showed. A) Leaf symptoms on Actinidia delicosa cv. Hayward (June, 1992); b) leaf symptom on A. chinensis cv. Hort16A (June, 2008). Note the red-rusty colour of the spots and the chlorotic halo on A. delicosa and the brownish spot without halo on A. chinensis; c) large canker in deep winter, induced by Psa on the main leader of an adult A. chinensis cv. Hort16A plant, in central Italy (February, 2009). Note the complete destruction of all the external woody tissues of the plant.

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5,938,909 nt (a coverage 67.6 x). Finally, sequencing of the 12-Psa library resulted in 3,823,264 reads which were assembled into 590 contigs (N50 = 22,372; largest contig: 85,902) for a total of 6,144,044 nt (a coverage 61.2 x). Based on the previous sequenced genomes of *P. syringae* pathovars, the obtained 6 Mb genome of Psa was of the expected size. The G + C content of the three strains ranges from 58.5 and 58.8% (Table 1). The sequences of the assemblies have been deposited in DDBJ/EMBL/GenBank under the following accessions: AFT000000000 (I-Psa), AFTG000000000 (I2-Psa), AFTH000000000 (J-Psa).

Pairwise alignment between the draft genomes of *Psa* and the complete genome of *Pto* DC3000 and occurrence of variable regions

To investigate differences between the genomes of the three *Psa* strains and *Pto* DC3000, the closest pathovar of genomospecies 8 according to Gardan et al. [7], the draft genomes were aligned and compared using MAUVE 2.3.1 software (Figure 2). The percent similarities between the three *Psa* and *Pto* DC3000 are of 81.99, 82.03 and 79.48 for J-Psa, I-Psa and I2-Psa, respectively (Table 1). The alignment of the three *Psa* draft genomes with the complete genome of *Pto* DC3000 is shown in Figure 2. An *ad hoc* PERL script was used to establish the similarity of the three *Psa* genomes. The genomes of J-Psa and I-Psa resulted 99.75% and display only about 14,000 nt of differences, whereas the I2-Psa genome displays a similarity of 88.20% with those of the other two *Psa* strains. For each *Psa* strain, the presence of variable regions along the genomes, which are good candidates for horizontal gene transfer, were identified as regions larger than 10 kb in a contig and appeared as a gap in the genome alignment or as regions characterised by a different G+C content with respect to the average content of the three *Psa* strains. The variable regions found by comparison of the three *Psa* strains among themselves and with *Pto* DC3000 are shown in Tables 2, 3 and S1. The highest content of variable regions, namely 12, was found when I2-Psa was compared with J-Psa and I-Psa (Table 4). Variable region 7 was characterised by the presence of a prophage PSPPHO6, which has also been previously found in *Pb* 1448A. Other evidence for the presence of mobile genetic elements has been found in variable region 8 (plasmid-partitioning protein and transposase) and 11 (phage and prophage). Variable region 2 of J-Psa and I-Psa includes the phaselotoxin cluster (Table 3). An example of the occurrence of variable regions in the *Psa* genomes is shown in Figure 3.

### Table 1. General features for *Pseudomonas syringae* pv. *actinidiae* draft genomes.

|              | J-Psa | I-Psa | I2-Psa |
|--------------|-------|-------|--------|
| No. reads    | 1,672,966 | 4,083,706 | 3,823,264 |
| No. contigs  | 833   | 466   | 590    |
| N50          | 14,838 | 27,730 | 22,372 |
| Largest contig size | 67,329 | 122,209 | 85,982 |
| Total size (bp) | 5,931,199 | 5,938,909 | 6,144,044 |
| G+C content (%) | 58.8%  | 58.8%  | 58.5%  |
| Calculated genome coverage | 27.7%  | 67.6%  | 61.2%  |
| Genome similarity with *Pto*DC3000 (%) | 81.99% | 82.03%  | 79.48%  |

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*Psa* is phylogenetically closer to *P. s. pv. theae* than to *P. avellanae*

To establish phylogenetic relationships between *Psa* and the other pathovars or species of the *P. syringae* complex, we used MultiLocus Sequence Type (MLST) analysis. The relationships within the nine genomospecies, which were determined by Gardan et al. [7], were assessed by considering as many strains of each genomospecies for which the complete or partial sequence of orthologous housekeeping genes was deposited in the NCBI databank as possible. For members of eight out of nine genomospecies, we constructed a concatenated dendrogram based on the neighbour-joining (NJ) algorithm using the *gyrB*, *rpoB* and *rpoD* gene fragments for a total of 1,646 nucleotides. For genomospecies 7 (*P. s. pv. tagetis* and *P. s. pv. helianthi*), there were not enough gene sequences in the databank, and consequently, it was not included in the analysis. The phylogenetic tree is shown in Figure 4. *Psa* appears closely related to *P. s. pv. theae*-type strain and slightly distant from *P. avellanae*-type strain, which are the other two members of genomospecies 8. Genomospecies 3 (*P. s. pv. tomato*) is the most related to the genomospecies 8, as has already established by Gardan et al., [7]. Furthermore, we analysed the phylogenetic position of the three *Psa* strains among the *P. syringae* pathovars using more orthologous genes. In addition, a concatenated tree, based on the maximum likelihood (ML) algorithm and the *acnB*, *fukA*, *glaA*, *gig*, *rpoB* and *rpoD* gene fragments for a total of 2,926 nucleotides, was also built (Figure S1). Again, the *Psa* strains are more closely related to *P. s. pv. theae* than to *P. avellanae*. In addition, I2-Psa appears to not be identical to the other two *Psa* strains sequenced here. The high genetic similarity between *Psa* and *P. s. pv. theae* observed in the present and previous studies, the records of bacterial canker incited by *Psa* from both cultivated and wild *Actinidia* species in eastern Asia countries [10,11,13,15,16,20–24] and the fact that *P. s. pv. theae* has, so far, been solely reported solely in Japan, led us to postulate that these two closely-related *P. syringae* pathovars also have their origin in eastern Asia, as has already been hypothesised by Ushiyama et al. [23]. The consideration that the *Actinidiaceae* and *Theaceae* families, which are genetically closely related, both originated in eastern Asia would support such an hypothesis.

*Psa* harbors putative pathovar-specific mobile genetic elements of potential importance in adaptation to *Actinidia* spp.

With the total protein complement of the *Psa* strains, we focused on putative proteins encoded by the genome of each strain that showed no significant homologies with proteins encoded by previously sequenced genomes of phytopathogenic pseudomonads. In particular, all the *Psa* strains display putative phage integrases, integrase family proteins, and transposases. Interestingly, all the *Psa* strains have putative homologues to the cAMP protein *Fic* which induces filamentation in the bacterial cells. Such proteins have been found in the *Dickeya zeae* strain ECH11591, which causes soft rot diseases, but have never been reported in phytopathogenic pseudomonads.

Comparison of the protein complement of *Psa*

Because figures calculated by MUMMER may be severely overestimated due to the misassembly of the short Illumina reads that may occur in correspondence to repeated sequences, we used the predicted protein complements to estimate the differences among the three *Psa* strains. An ORF search using GLIMMER predicted 5,670 genes in the J-Psa genome draft which included 20.57% hypothetical proteins and 3.54% conserved hypothetical.
Figure 2. Pairwise alignment between the draft genomes of J-Psa, I-Psa and I2-Psa and the complete genome of P. syringae pv. tomato DC3000 using the MAUVE software. Colored blocks outline genome sequence that align to part of another genome, and is presumably homologous and internally free from genomic rearrangement (Locally Colinear Blocks or LCBs). Areas that are completely white were not aligned and probably contain sequence elements specific to a particular genome. Blocks below the centre line indicate regions that align in the reverse complement (inverse) orientation. A profile is drawn within each LCB with the height of the color corresponding to the average degree of sequence conservation.

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Origin and evolutionary relationships among Psa strains

Further analysis of these I2-Psa specific ORFs was performed to determine their origin. We searched the 35 Pseudomonas spp. and pathovar genomes available as draft or as complete genome sequences from NCBI as well as the draft genomes of P. avellanae BCIP613 and P. syringae pv. theae NCPPB2598, type-strains of these pathogens, whose draft genomes are available (Marcelletti, Ferrao, Scortichini, unpublished data in these labs) for comparison using BLASTn. The results of this search showed that among the 398 I2-Psa-specific ORFs, there were 238 ORFs for which no homolog could be found in P. avellanae BCIP 613 or P. s. pv. theae NCPPB2598. It was also found that 49% (i.e. 196) of the 398 proteins that do not have homologous in J-Psa or I-Psa matched sequences in at least one of the genomes of Pto strains assessed, specifically 158, 144, 138, 114 and 116 matches for Pto strains K10, Max13, NCPPB1108, T1 and DC3000, respectively. These evidence as well as the annotation of several of the deduced proteins as phage or prophage proteins strongly suggests that a large part of this I2-Psa-specific DNA has been acquired by horizontal gene transfer from a strain of P. syringae genomospecies 3. However, within the group of 398 I2-Psa specific DNA, we also detected eight ORF that had significant BLASTn hits in the P. avellanae BCIP613 or P. s. pv. theae NCPPB2598 draft genome sequences but no hit in any of the 35 Pseudomonas spp. and pathovars genome sequences available from NCBI (Table 4). The most obvious explanation for this result is that these eight sequences shared by I2-Psa and other strains of genomospecies 8 were lost by I-Psa and J-Psa during their evolution.

To gain access to further information about the origin of I2-Psa, we analysed the DNA sequences of all the genes that were polymorphic in all three Psa strains and compared them with their orthologous in the P. avellanae BCIP613 or P. s. pv. theae NCPPB2598 draft genome sequences. As reported in the Table 4, although the pattern displayed by I2-Psa was more
Table 2. Variable regions (VR) found in the draft genomes of J-Psa (NCPPB 3739), I-Psa (NCPPB 3871) and I2-Psa (CRA-FRU 8.43) compared with the complete genome of Pto DC3000.

| VR | Strains       | Contigs     | Coordinate        | % GC | VR-encoded genes                                                                 |
|----|--------------|-------------|-------------------|------|----------------------------------------------------------------------------------|
| 1  | CRA-FRU8.43A | Contig234   | from start to 11429 | 56.1 | Glycosyl transferase, group 1 family protein                                     |
|    |              | Contig729   | from start to 11429 | 56.2 | HlyD family secretion protein                                                    |
|    |              | Contig248   | from 18 to 11447   | 56.2 | Mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase            |
|    |              |             |                   |      | Outer membrane efflux protein                                                   |
|    |              |             |                   |      | Type I secretion system ATPase, PrtD                                             |
| 2  | CRA-FRU8.43A | Contig65    | from 16690 to 32252 | 62   | 11 putative type III secretion system component                                  |
|    |              | Contig226   | from 17561 to 33132 | 62   | 3 hypothetical protein                                                           |
|    |              | Contig60    | from 80641 to 96212 | 62   | LuxR family transcriptional regulator                                           |
|    |              |             |                   |      | Myosin heavy chain B (MHC B)                                                    |
|    |              |             |                   |      | No database match                                                                |
|    |              |             |                   |      | TPR domain-containing protein                                                    |
|    |              |             |                   |      | Type III secretion system protein                                                |
| 3  | CRA-FRU8.43A | Contig50    | from 15151 to 25647 | 57.3 | 2 Prepilin                                                                        |
|    |              | Contig515   | from 1455 to 11968 | 57.2 | 2 Type II secretion system protein E                                              |
|    |              | Contig262   | from 11911 to 22424 | 57.2 | No database match                                                                |
|    |              |             |                   |      | Type II and III secretion system protein                                          |
|    |              |             |                   |      | Type II secretion system protein                                                  |
|    |              |             |                   |      | Type IV pilus protein                                                            |
| 4  | CRA-FRU8.43A | Contig130   | from 150 to 13257  | 57.9 | 2 No database match                                                               |
|    |              | Contig771   | from 194 to 13301  | 57.9 | Filamentous hemagglutinin                                                        |
|    |              | Contig401   | from 195 to 13302  | 57.9 | Hemolysin activator protein precursor                                            |
| 5  | CRA-FRU8.43A | Contig175   | from start to 15590 | 60   | ABC transporter, periplasmic oligopeptide-binding protein                         |
|    |              | Contig46    | from start to 15590 | 60   | ABC transporter, permease protein                                                |
|    |              | Contig35    | from start to 15590 | 60   | Achromobactin biosynthetic protein AcSD                                           |
|    |              |             |                   |      | Conserved hypothetical protein                                                   |
|    |              |             |                   |      | Diaminobutyrate–2-oxoglutarate aminotransferase                                  |
|    |              |             |                   |      | Dipeptide ABC transporter, ATP binding protein                                   |
|    |              |             |                   |      | Dipeptide transporter dppD-like protein                                           |
|    |              |             |                   |      | Hypothetical protein RL0789                                                      |
|    |              |             |                   |      | No database match; (similar to of nd with eval = nd)                             |
|    |              |             |                   |      | PupR protein                                                                      |
|    |              |             |                   |      | Putative transporter, permease protein                                           |
|    |              |             |                   |      | RNA polymerase, sigma-24 subunit, ECF subfamily                                  |
|    |              |             |                   |      | Sigma-70 region 2                                                                |
|    |              |             |                   |      | TonB-dependent siderophore receptor                                              |
| 6  | CRA-FRU8.43A | Contig175   | from 15597 to 27012 | 62.1 | Achromobactin biosynthetic protein AcSB                                           |
|    |              | Contig96    | from start to 11415 | 61.8 | Achromobactin biosynthetic protein AcSC                                           |
|    |              | Contig35    | from 15592 to 27007 | 61.9 | Achromobactin-binding periplasmic protein precursor                             |
|    |              |             |                   |      | Achromobactin transport ATP-binding protein CbrD                                 |
|    |              |             |                   |      | Achromobactin transport system permease protein CbrB                              |
|    |              |             |                   |      | Achromobactin transport system permease protein CbrC                              |
|    |              |             |                   |      | Dimethylmenaquinone methyltransferase                                            |
|    |              |             |                   |      | Drug resistance transporter EmrB/QacA subfamily                                  |
|    |              |             |                   |      | Hypothetical protein                                                             |
|    |              |             |                   |      | Om/DAP/Arg decarboxylase 2:Om/DAP/Arg decarboxylase 2                            |
|    |              |             |                   |      | LucA/lucC                                                                        |

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| VR | Strains | Contigs | Coordinate | % GC | VR-encoded genes |
|---|---------|---------|------------|------|-----------------|
| 1 | NCPPB3739 | Contig 367 | from 3002 to 14705 | 59.5 | 2 No database match |
|   | NCPPB3871 | Contig 71 | from start to 11703 | 59.5 | Dak phosphatase |
|   |           |          |             |      | Glycerone kinase |
|   |           |          |             |      | Iron-sulfur cluster-binding protein, Rieske family |
|   |           |          |             |      | Periplasmic binding protein/LacI transcriptional regulator |
|   |           |          |             |      | Putative sugar-binding region |
|   |           |          |             |      | Quinoprotein |
|   |           |          |             |      | Ribose ABC transporter, ATP-binding protein |
|   |           |          |             |      | Ribose/galactose isomerase |
|   |           |          |             |      | Short chain dehydrogenase |
|   |           |          |             |      | Sorbitol dehydrogenase, putative |
|   |           |          |             |      | Sugar ABC transporter, ATP-binding protein |
| 2 | NCPPB3739 | Contig 717 | from 11215 to 49219 | 52.4 | 13 Hypothetical protein |
|   | NCPPB3871 | Contig 151 | from 18056 to 56060 | 52.4 | 2 Deoxycytidine triphosphate deaminase |
|   |           |          |             |      | 2 Fatty acid desaturase |
|   |           |          |             |      | 2 ISPsy25, transposase |
|   |           |          |             |      | 2 Phage integrase family site specific recombinase |
|   |           |          |             |      | HAD superfamily hydrolase |
|   |           |          |             |      | L-arginine/lysine amidontransferase, putative |
|   |           |          |             |      | Ornithine aminotransferase |
|   |           |          |             |      | Phaseolotoxin-insensitive ornithine carbamoyltransferase |
|   |           |          |             |      | Pyruvate phosphate dikinase PEP/pyruvate binding subunit |
|   |           |          |             |      | RtrR protein |
| 3 | NCPPB3739 | Contig 248 | from 1965 to 37060 | 57.8 | 13 Hypothetical protein |
|   | NCPPB3871 | Contig 169 | from 1965 to end | 57.8 | 3 Conserved hypothetical protein |
|   |           |          |             |      | 3 No database match |
|   |           |          |             |      | Bacteriophage-related protein |
|   |           |          |             |      | Baseplate assembly protein J |
|   |           |          |             |      | Baseplate assembly protein W |
|   |           |          |             |      | Baseplate assembly protein V |
|   |           |          |             |      | Bifunctional DNA primase/polymersase |
|   |           |          |             |      | Deoxynucleotide monophosphate kinase |
|   |           |          |             |      | Holin |
|   |           |          |             |      | Lysozyme |
|   |           |          |             |      | Major capsid protein E |
|   |           |          |             |      | Major tail sheath protein |
|   |           |          |             |      | Major tail tube protein |
|   |           |          |             |      | P2-like prophage tail protein X |
|   |           |          |             |      | Phage DNA packaging protein, Nu1 subunit of terminase |
|   |           |          |             |      | Phage late control gene D protein |
|   |           |          |             |      | Phage protein U |
|   |           |          |             |      | Portal protein, lambda family |
|   |           |          |             |      | Prophage PSPPH06, adenine modification methyltransferase |
|   |           |          |             |      | Prophage PSPPH06, site-specific recombinase, phage integrase family |
|   |           |          |             |      | Prophage PSPPH01, transcriptional regulator |
|   |           |          |             |      | Tail fiber protein H, putative |
|   |           |          |             |      | Tail protein I |
|   |           |          |             |      | Tail tape measure protein |
|   |           |          |             |      | Terminase, large subunit |
hypothesis testing. Based on the results, as shown in the tree in which consisted of 166,160 nucleotide positions was used to determine the genealogy with maximum likelihood and perform which was the draft genomes of 

Furthermore, we randomly selected 171 ORFs from the list of genes that were found to be orthologues among the three strains and in the draft genomes of P. avellanae BCIP613 or P. s. pv. theae NCPPB2598. The alignment of the concatenation of these genes, which consisted of 166,160 nucleotide positions was used to determine the genealogy with maximum likelihood and perform hypothesis testing. Based on the results, as shown in the tree in Figure 5a, the I2-Psa strains originated from a common ancestor of the I-Psa and J-Psa strains and was not a derivative of either of these strains. To provide statistical support to this presumptive genealogy, we estimated the likelihood of an alternative genealogy with the constraint that I2-Psa and I-Psa were monophyletic (Figure 5b), and performed a test for monophyly to evaluate whether such a null hypothesis could be rejected with statistical significance. The aim of the monophyly test is the evaluation, by means of parametric bootstrap, of the significance of a likelihood ratio calculated comparing a null hypothesis with the unconstrained maximum likelihood tree. The log likelihoods of the null hypothesis and the unconstrained tree were -263874.12 and -263011.44, respectively, and their ratio (A = 1725.36) was compared with the delta distribution in a set of simulations of simulated sequences evolved in silico using the unconstrained tree as guidance. The comparison showed that the null hypothesis was to be rejected (P = 0.016). In summary, the analysis of ancestral state residue conservation, the maximum likelihood analysis of genealogies and the evidence of eight ORFs that are genomospecies β-specific and are present only in I2-Psa strongly suggest that these strains did not evolve from the organisms that caused the epidemic in 1984–1992 but rather from a common ancestor.

Secrecion systems associated with pathogenicity and virulence

The three Psa strains sequenced display structural genes involved in the biosynthesis of the type I, II, III, IV and VI secretion systems (TSS). The T1SS encodes for orthologues of the HlyD family secretion protein involved in the transportation of the Escherichia coli α-haemolysin toxin. Both the general secretion Sec-pathway and the Tat-pathway of the T2SS are present in the three Psa strains. These two secretion systems are also present in the other sequenced plant pathogenic pseudomonads, even though the T1SS is not present in Pto DC3000 [42]. In Pto DC3000, the twin-arginine translocation (Tat) system of the T2SS appears to be an important virulence determinant [43]. A complete T3SS, similar to those found in the P. syringae complex [44] has also been found in the three Psa strains. The T3SS contains the hop/bhc cluster and the transcriptional regulatory HrpL, HrpR and HrpS proteins. TrbC and VirK protein orthologues for the T4SS are present in the three Psa strains. In addition, I2-Psa also displays other orthologous proteins for the T4SS, namely TraG and VirB8, which are also present in other P. syringae pathovars. Noteworthy the GC contents of these genes are lower than the flanking genomic regions, this indicating the occurrence of possible genomic islands. Finally, the three Psa strains also display two clusters of orthologous proteins of the T6SS: the Vgr family protein, which is identical to that of P. putida GB-1 and the OmpA family protein which is required for the T6SS functionality [45]. ImpA, an inner membrane protein of the T6SS is also present. The precise functions of these secretion systems in Psa have yet to be investigated.

Type III secretion system effectors

A comparison of the effector repertoire of the three Psa strains based on the complete effector repertoire of P. s. pv. tomato DC3000, other P. syringae strains and Psa MAFF302091 reveals a “core” set of 33 hop and 6 avr putative effector genes that are conserved in all strains (Figure 6). In general, in these putative effectors, the amino acid identity is very high (i.e. >90%) to the most similar orthologue of the P. syringae pathovars found in GenBank (Table S3). However, for hopAC1, hopAE1, hopH1, hopM1, and hopZ3, the amino acid identity with respect to their most similar orthologues ranged from

| Table 4. Single Nucleotide Polymorphisms (SNP) found among the three Psa strains, P. avellanae BPIC631 and P. s. pv. theae NCPPB2598 draft genomes in the genes that were found polymorphic between I-Psa and J-Psa. ORF names refer to the I-Psa genome draft. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| ORF             | Position in ORF | I-Psa           | J-Psa           | I2-Psa          | Psth            | Pav              |
| orf00015-contig244 | 761             | T               | G               | T               | T               | T                |
| orf00040-contig34 | 3138            | G               | T               | G               | T               | T                |
| orf00018-contig32 | 24              | A               | A               | A               | C               | G                |
| orf00020-contig429 | 639             | C               | C               | C               | C               | C                |
| orf00001-contig216 | 1628            | C               | T               | C               | C               | C                |
| orf00007-contig330 | 92              | T               | G               | T               | G               | G                |

Ancestral residues (identified as the residues displayed by P. avellanae and P. s. pv. theae) are highlighted. Pav = P. avellanae; Psth = P. s. pv. theae. § = orthologous not found.

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Figure 3. Representative part of the genome alignment between *Psa* strains and *Pto DC3000* showing some variable regions. The violet segments (on the right) point out the variable region 3, present in all three *Psa* strains but not in *Pto DC3000*; the deep blue (on the left) segments point out the variable region 2, present in J-*Psa* and I-*Psa* but absent in I2-*Psa* and *Pto DC3000*. The blue segments indicate another variable region present in *Pto DC3000* and I2-*Psa* but not in J-*Psa* and I2-*Psa*. The figure shows also some others shorter regions (i.e. light green segments) probable examples of horizontal gene transfer.

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Figure 4. Evolutionary relationships of *Psa* strains to other phytopathogenic pseudomonads. Phylogenetic relationships were estimated from concatenated sequences from three housekeeping genes, *gyrB*, *rpoB* and *rpoD* (1,646 bp), using the neighbour-joining (NJ) algorithm. Bootstrap values are reported at each branching. Members of all nine genomospecies (Gardan et al., 1999), except genomospecies 7, are included into the analysis. The letter followed by the number reported in brackets indicates the genomospecies sensu Gardan et al. [7].

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68 to 79%. By contrast, the avrPto1-like effector of the three Psa strains shows an amino acid identity of 44% with the orthologue of *P. s. pv. aesculi* NCPPB3681. All three Psa strains did not show the presence of *hopAB* and *hopAF* which are considered conserved effector genes present in *Pto DC3000*, *Pph 1448A* and *Psy B728a* [46]. Interestingly, I-Psa displays four putative effector genes, namely *hopA1*, *hopAA1-2*, *hopH1*, and *hopZ2*-like, that are not present in the J-Psa and I-Psa strains. The first three putative effector genes display a relative similarity with the orthologues of *Pto DC3000* (91%), *Pto DC3000* (99%) and *P. s. pv. tomato* T1 (97%), whereas *hopZ2*-like shows a similarity of 40% with *hopZ2*. *HopA1* triggers effector-triggered immunity in tobacco, other *Nicotiana* species [47] and in many *Arabidopsis* accessions [48] and is supposedly involved in host range specificity, even though its virulence functions are still unknown [49]. *HopAA1-2* is a paralogue of the *hopAA1-1* effector, which is located in the conserved effector locus (CEL) region of the *hop/hrc* cluster and is considered to be among the ancient *P. syringae* effectors that were acquired before the radiation of *P. syringae* into the current pathovars [50]. *HopAA1-2* is present in *Pto DC3000* but not in *Pto T1*, *Pph 1448A* and *Psy B728a*. Evidence for a strong virulence function for *hopAA1-2* in plants has yet to be fully investigated [51]. Notably, in a wide assessment on the presence/absence of effector genes in 91 strains of *P. syringae* pathovars, Sarkar et al. [52] found that *hopAA1-2* and *hopA1* are among the least distributed effector genes. In fact, these effectors have only been found in eight and 16 out of the 91 strains tested, respectively, and they have only been found present together in three *Pto* strains. If these two effectors can explain the relevant aggressiveness of I2-Psa towards *A. chinensis* and *A. deliciosa* they deserve further in-depth studies. *HopH1* is considered to be a variably distributed effector gene in the *P. syringae* pathovars [53].

**Variation in plasmid content among Psa strains**

Using agarose gel electrophoresis, we compared the number and the size of native plasmids present in the three sequenced *Psa* strains as well as for comparative purposes, in other representative *Psa* strains from Japan (i.e. outbreaks of 1984) and Italy (i.e. outbreaks of 2008–2010 in different Italian regions). We found that all the Japanese strains from the 1984 outbreak as well as I-Psa
harbour a native plasmid of about 50 kb. This plasmid is absent in I2-Psa and in all the other representative Psa strains isolated in Italy during the recent epidemics of bacterial canker on A. chinensis and A. delicosa (Figure 7). Remarkably, I2-Psa as well as all the other Psa strains obtained during the recent epidemics of bacterial canker in Italy displayed the presence of a plasmid of about 160 kb, not present in both J-Psa and I-Psa. These plasmids represent a substantial acquisition of genetic material for I2-Psa. All the three Psa strains have the upfA gene, essential for plasmid replication, of the P. syringae pPT23A-like plasmid family.

Presence and absence of the phaseolotoxin gene cluster and other toxins

The phaseolotoxin gene cluster, argK-tox, is located on the chromosome of Pph and Psa. It comprises three tyrosinase-recombinase-encoding genes: tviA, tviB and tviC, which are located at the left end of the cluster; the phdE locus, which contains ORFs showing homologies to genes encoding amino acid transferases and ARAC family and fatty acid desaturases; and the argK gene which encodes the phaseolotoxin-resistant ornithine carbamoyltransferase. The cluster is flanked by two regions, ACT059 and ACT1094 [54]. Thirty-eight kilobases of the argK-tox cluster of the Pph MAFF302282 and Psa NCPPB3739 (i.e. J-Psa) strains are identical [54]. In our draft, we found that J-Psa and I-Psa contain the phaseolotoxin gene cluster, which display homologies of 99.94% and 99.99%, respectively, whereas I2-Psa does not contain this 38 kb region of the cluster and the flanking regions, ACT059 and ACT1094, are contiguous (Figure S2). Some genetic features indicate that the phaseolotoxin cluster, also referred to as the tox-island, has been acquired by P. syringae pvs. phaseolica and actinidiae through horizontal gene transfer from an unknown species [31,55]. The complete absence of the cluster in many highly virulent Psa strains, as found both in Italy and New Zealand [19,27] would confirm such a hypothesis and indicate the remarkable aggressiveness of the pathovar even without this virulence factor. These findings also indicate the separate but convergent evolution of genetically different pseudomonads as phytopathogens of Actinidia spp. Notably, different types of leaf spot lesions on Actinidia spp. have been noticed during the two outbreaks of bacterial canker of kiwifruit in Italy in 1992 and 2008-2011 (Figure 1), even though necrotic spots surrounded by a chlorotic halo have also been frequently observed during these severe epidemics on A. chinensis in Italy. The presence of genes coding for coronatine, syringomycin and syringopeptin toxins was also checked but none of these toxins is present in the draft genomes of the Psa strains of the present study.

Copper resistance and antibiotic detoxification

A thorough search for orthologous genes coding for resistance to copper and antibiotics was performed with the draft genomes of the three Psa strains. All strains contain homologues for the copA and copB genes, which are essential for copper resistance [56]. In P. syringae, copA is located in the periplasmic space, whereas copB is in the outer membrane of the bacterial cell. The other two genes, namely copD and copS, which are required for maximum copper resistance were not found. These results confirm a study by Nakajima et al. [57] who found only copA and copB in the Psa strains, including NCPPB3739, obtained during the initial outbreak of bacterial canker on A. delicosa in Japan when the regular spraying of copper bactericides was not yet applied to the infected orchards. In addition, all the Psa strains display a vast set of orthologous genes involved in antibiotic resistance. Among the five superfamilies of efflux transporters, Psa has genes belonging to the resistance nodulation division (RND), multi antimicrobial resistance (MAR), multidrug endosomal transporter (MET) and major facilitator superfamily (MFS). Recent studies on the antibiotic resistance mechanism in Gram-negative bacteria, have stressed that resistance greatly depends on the constitutive or inducible expression of active efflux systems [58]. As an example, the disruption of MexB, a gene of the MFS superfamily, that is also present in Psa strains, dramatically increased the susceptibility of P. aeruginosa to beta-lactams, tetracyclines, fluoroquinolones and chloramphenicol [58]. In addition, all the Psa strain genomes include genes involved in the enzymatic inactivation of beta-lactams, tellurium and a macrolide ABC efflux protein, mnaAB, which confers resistance to cycloheximide. All the Psa strain genomes contain amprD, a gene coding N-acetyl-anhydromuramyl-L-salanine amidase which cleaves the amide bond between N-acetylmuramyl and L-amino acids in the bacterial cell wall. Finally, I2-Psa also contains a putative lantibiotic dehydratase domain that has never been found in other phytopathogenic pseudomonads.

Iron acquisition, nitric oxide and sucrose metabolism and quorum sensing

The three Psa strain genomes encoded a number of genes involved in iron acquisition, such as the siderophore pyoverdine

![Figure 7. Plasmid profiles of Psa. Agarose gel electrophoresis to compare the number and size of native plasmids in the genome of Psa strains. The gels show also other representative Psa strains from the outbreak of bacterial canker in Japan (i.e. 1984) and from the current severe epidemics in Italy. See also Materials and Methods. Note that as the ca. 50 kb plasmid present in J-Psa and I-Psa is not contained in all the Psa strains isolated from the current epidemic in Italy. By contrast, I2-Psa and other strains obtained from the recent epidemics of bacterial canker in Italy contain a plasmid of about 160 kb.](doi:10.1371/journal.pone.0027297.g007)
and enterobactin involved in the isochorismate synthase and yersiniabactin, a siderophore with a very high affinity for iron. In addition, all the genomes contain the TonB protein. This protein spans the periplasm and is anchored to the cytoplasmic membrane interacting with receptors in the outer membrane to facilitate the uptake of iron-siderophore complexes. In human bacterial pathogens, namely *Hemophilus influenzae* and *H. parainfluenzae*, the inactivation of TonB decreased the ability to cause disease [59].

The three *Psa* strains contain two genes involved in the nitric oxide metabolism, namely nitric oxide dioxygenase and anaerobic nitric oxide reductase, with 100% homology to the same genes in *P. s. pv. aesculi* [39]. These genes might protect *Psa* from the host defence responses incited by nitric oxide. The three *Psa* strains do not have the 8 kb cluster coding for sucrose utilization that is present in the phloem infecting *P. s. pv. aesculi* strain 2250 isolated in Great Britain [39]. The quorum sensing system of *Psa* appears to differ from the classic LuxR/LuxI of other *P. syringae* strains. In fact, the genes of LuxI family are absent from these three strains, which display putative LuxR family genes. The representer genes rsaM and rsaL are also absent in the three *Psa* genomes.

**Presence of pectolytic enzymes and catabolism of plant-derived aromatic compounds**

The three *Psa* strains contain pectin lyase and polygalacturonase genes that display complete identity to the orthologues of *Pto* T1. These enzymes are also present in the soft-rot bacterium *P. marginalis* [60]. Similar to other *P. syringae* pathovars that infect woody hosts, such as *P. s. pv. aesculi* and *P. savastanoi* *pv. savastanoi* [39,40], *Psa* also displays genes involved in the catabolism of plant-derived aromatic compounds using both the catechol branch of the β-choroecdipate and the protocatechuate 3,4-dioxigenase pathways. In fact, all the *Psa* genomes encode putative proteins involved in the degradation of anthranilate to catechol (i.e. anthranilate dioxigenase reductase and anthranilate phosphor-ibosyranferase) as well as proteins involved in the catabolism of catechol (catechol 1,2-dioxigenase, muconolactone delta-isomerase, muconate cycloisomerase N-terminal and dienelactone hydrolase). Moreover, the *Psa* strains have putative *pcaG* and *pcaH* genes encoding the two subunits (α and β) of protocatechuate 3,4-dioxigenase, an enzyme involved in the degradation of protocatechuate, which is present in soil-inhabiting bacteria.

**Differential multiplication trend of *Psa* strains in *Actinidia* spp. leaves and pathogenicity test to tomato**

Field evidence from the recent epidemics of bacterial canker in Italy indicates that the current *Psa* population (I2-*Psa*) is aggressive to both *A. chinensis* and *A. deliciosa*. In the latter species, I2-*Psa* incited more severe symptoms compared with the *Psa* population of the past outbreaks in Italy (I-*Psa*). As only *A. deliciosa* was cultivated, inoculations of *A. delicosa* and *A. chinensis* leaves revealed different multiplication trends between J-*Psa*, I-*Psa* and I2-*Psa* (Figure 8). In fact, I2-*Psa* performed better than J-*Psa* and I-*Psa* in *A. chinensis* leaves. I2-*Psa* performed well also on *A. delicosa*, although not as well as I-*Psa* and J-*Psa*, as three weeks after the inoculation still showed high cell levels (i.e. between 10^9 and 10^10 cfu/ml). Conversely J-*Psa* and I-*Psa*, that reached a cell concentration of about 10^9 cfu/ml 3–4 days after the inoculation with either 10^8 or 10^9 cfu/ml when inoculated in *A. delicosa* leaves, only grew poorly in *A. chinensis* leaves where the cell concentration of the strains decreased significantly 21 days after the inoculation. The control leaves did not show any sign of infection. These results indicate that the *Psa* population currently causing severe damage in Italy and New Zealand is capable to infect both *Actinidia* species, whereas the *Psa* population causing outbreaks of bacterial canker to cv. Hayward in Japan and Italy about 20–25 years ago displays more affinity for *A. delicosa*. The three *Psa* strains incited a typical hypersensitivity reaction on tomato leaves, whereas *Pto*DC3000 caused typical symptoms of bacterial speck.

**Discussion**

Through Illumina sequencing technology, we have performed a genome-wide survey of genetic variation in *Psa* strains, the causative agent of bacterial canker of *Actinidia* spp. worldwide. This study has determined putative variable genomic regions and sets of genes related to the pathogenicity and virulence that could differentially modulate the aggressiveness of pathogen populations towards *Actinidia* species as well as genes involved in the environmental fitness and adaptation of the bacterium *in planta*. We confirmed that the re-emerging wave of bacterial canker to *A. delicosa* and *A. chinensis* is being raised by a population of *Psa* (i.e., I2-*Psa*) distinct from the population that has led to past outbreaks in Japan and Italy (i.e. J-*Psa* and I-*Psa*) [10,12]. Moreover, we stress that the current epidemics of bacterial canker are being caused by a *Psa* population that, most probably, did not originate from that found in Italy about 20 years ago. The origin of this new epidemic wave has rised a large debate worldwide, and it has not been easy to provide convincing answers using conventional approaches. Conversely, the large amount of sequence data obtained in this work has provided unmatched solidity to the reconstructed genealogy of the *Psa* strains examined.

We refined the taxonomic position of *Psa* within genomospecies *S. sspns* Gardan et al. [7] using MLST analysis and housekeeping genes, and we found that *Psa* is phylogenetically closer to *P. s. pv. theae* than to *P. axellana*. Our analysis, performed with all nine genomospecies (except genomospecies 7) currently circumscribes the majority of the *P. syringae* pathovars and related phytopathogenic pseudomonads and also confirms that the genomospecies 3, including *Pto*, is the most closely-related cluster. The very high genetic similarity between *Psa* and *P. s. pv. theae*, a phytopathogen so far reported solely in Japan, reinforces the assumption that *Psa* might be of Asian origin. In fact, there are several reports on the occurrence of this pathogen isolated from both *A. chinensis* and *A. delicosa* in China [15,16,20,21], South Korea [11,13,22] and from *A. delicosa* and wild *A. arguta* and *A. kolomikta* plants in Japan [10,23,24]. The possibility of an Asian origin has been already argued [23].

However, some of the genetic features found in the *Psa* strains of the past and recent epidemic in Italy and New Zealand indicate that different evolutionary routes have been followed by the ancestor(s) of the two different *Psa* populations which are represented by J-*Psa*/I-*Psa* and by I2-*Psa*. Our genome-wide analysis indicated that the two strains J-*Psa* and I-*Psa*, which were isolated in different years from the same kiwigreen cultivar, in geographically distant areas that were affected by outbreaks of bacterial canker of different severity, are extremely similar. This evidence indicates the major role of climatic conditions on the epidemic of bacterial canker to *A. delicosa*. Furthermore, our comparative study of *Psa* strains shows that despite the apparent prevalence worldwide of a clonal population, a reservoir of diversity of the pathogen has been maintained, which has allowed for a new population, represented by I2-*Psa*, to emerge about 25 years later from an independent evolutionary line with different genetic characteristics and enhanced epidemic potential. Bacterial canker caused by *Psa* to both *A. chinensis* and *A. delicosa* has also been recently reported in New Zealand. The molecular typing performed using the MLST analysis of seven housekeeping genes
and the detection of 12 effector protein genes allowed to ascertain that the \textit{Psa} strains of the current epidemics in Italy, here represented by \textit{I2-Psa}, are identical to those highly virulent strains found in New Zealand, which also lack of the phaseolotoxin gene cluster [27]. Notably, in that country, another \textit{Psa} population, genetically different and less virulent (i.e. apparently causing only leaf spots) than \textit{I2-Psa} but capable to infect both kiwigreen and kiwigold has also been identified. Such a population also differs from the two other genetically different \textit{Psa} populations of the past outbreaks of bacterial canker on \textit{A. deliciosa} in Asia (i.e. Japan and South Korea) and has been retained as endemically in New Zealand [27]. Whether the highly virulent \textit{Psa} population currently causing severe economic losses to \textit{A. chinensis} and \textit{A. deliciosa} in Italy and New Zealand originated in the area of the pathogen or from the less virulent endemic \textit{Psa} population recently isolated in New Zealand still remain to be verified. However, during the last 30 years, four genetically distinct \textit{Psa} populations have infected, to different extents, different \textit{Actinidia} species on different continents which is a remarkable case of multiple convergent evolution of phytopathogenic pseudomonad populations of the same pathovar to one single plant genus.

We cannot establish with certainty the origin of this re-emerging wave of bacterial canker in Italy although a likely scenario could be hypothesised. The past outbreaks only caused leaf spot and twig die-back but never the death of thousands of plants. The current \textit{I2-Psa} population could have been introduced from abroad through latently infected propagative material or infected pollen. Once established in central Italy, it further reached the other Italian regions by means of latently infected propagative material.

We also found, by assessing the \textit{Psa} growth in the leaves, that \textit{A. deliciosa} cv. Hayward was more susceptible than \textit{A. chinensis} cv. Hort16A when inoculated with the \textit{Psa} strains causing past outbreaks of bacterial canker in Japan and Italy. By contrast, \textit{I2-Psa} multiplication trend was higher in \textit{A. chinensis}. However, a relevant inoculum was also found in \textit{A. deliciosa} three weeks after the inoculation, thus confirming that the re-emerging wave of bacterial canker has been caused by a \textit{Psa} population that has a high fitness for both \textit{Actinidia} species. In the Latium region (central Italy), \textit{A. chinensis} probably largely contributed to the very rapid

Figure 8. Multiplication trends of \textit{Psa} strains in \textit{Actinidia} species. Multiplication in \textit{A. deliciosa} cv Hayward (a) and in \textit{A. chinensis} cv Hort16A (b) leaves. Bacteria were inoculated at 1–2×10^3 and 1–2×10^6 cfu/ml. Data represent the mean log of bacterial cell number and standard deviation (SD) as obtained from eight inoculation sites per each sample. doi:10.1371/journal.pone.0027297.g008
expansion of such population in the area of kiwifruit cultivation, which also acted as reservoir of infection for A. deliciosa.

The question then arises as to how this newly virulent Psa population originated. The importance of stress factors in promoting bacterial evolution has been recently pointed out. In fact, under stress condition in the host (i.e. nutrient deficiency outside the host, attack by antimicrobial compounds inside the host, and low temperatures), the bacterial competency to uptake DNA is activated and the pathogen can acquire exogenous genetic material that could help it to escape from the stress [61]. In addition, a possible loss of mobile genetic elements carrying avirulence genes can lead to an enhanced virulence [62]. I2-Psa does not have the 50 kb plasmid and the phaseolotoxin gene cluster present in J-Psa and I-Psa but has gained a 160 kb plasmid and a putative prophage that does not occur in the other population. It remains to be verified if any of these stress factors could have promoted the rise of such new Psa population. We did not investigated in details the structure of plasmins but, similarly to other P. syringae pathovars, the three Psa strains have the repA gene that is retained essential for the replication of the pPT25A-like plasmid family [63].

One of the more striking pieces of evidence of the difference between the two populations is the presence of the phasedolatoxin gene cluster, argK-tox, in J-Psa and I-Psa and its absence in I2-Psa and in all the other strains of the current epidemic assessed so far in Italy and New Zealand [19,27]. Phasedolatoxin is considered a major virulence factor for both Psa and Pph [21,64]. The argK-tox gene cluster is located on the chromosome and was supposedly acquired through lateral gene transfer from bacteria distantly related to P. syringae or from non pathogenic or avirulent P. syringae strains [54,55]. Additionally, one Psa strain, which was isolated from Vicia sativa, displays such a gene cluster [65], although it showed a nucleotide identity of only 85,3% with that displayed by Psa and Pph [66]. The presence of the argK-tox cluster in one variable region of the J-Psa and I-Psa genomes confirms the acquisition of such genetic trait by lateral gene transfer. Also within Pph have been found many pathogenic strains lacking the phasedolatoxin cluster but very aggressive, similarly to I2-Psa, towards their host plant [67]. Tamura et al. [29], using an argK-tox mutant of KW11 J-Psa found that the bacterium induced the same type of symptoms in the plant similar to the wild-type, except for the chlorotic halo surrounding the leaf spots. However, the toxin did not promote bacterial growth in planta. It has been postulated that phasedolatoxin, by inhibiting ornitine-carbamoyltransferase, can reduce or inhibit the growth of other microorganisms [64]. The high identity of the argK-tox clusters found in strains of Psa and Pph suggests a recent acquisition of the cluster by the two pathovars [66]. The worldwide occurrence of many highly virulent strains lacking the argK-tox gene cluster in both Psa and Pph allows us to speculate that the ancestral genomes of these two phytopathogens did not include the phasedolatoxin gene cluster.

Other differences between the two Psa populations were found by the assessment of the effector protein genes. The three strains display an identical core repertoire of 33 hop and 6 avr effector genes. However, the Psa strains also possess some unique effector protein genes. hopX1, hop4R, hopBD2 and hopPmalB are unique to J-Psa and I-Psa, whereas hopA1, hopA1-2, hopH1, and hopC2-like are unique to I2-Psa. Such a different effector repertoire found in the three Psa strains might account for the differential aggressiveness shown by the pathogen to Actinidia species. However we still do not know the roles played by these effector genes in terms of pathogenicity and host range for the pathovar Actinidia. In a recent extensive study regarding the evolution of pathogenicity within the P. syringae complex, Baltrus et al. [68] sequenced also a Psa strain, namely MAFF302091, isolated from A. deliciosa in Japan, in 1984 in the Kanegawa district. Interestingly, this strain differs from J-Psa, isolated in Shizuoka district, for the presence/absence of some effector genes. This would indicate that each single strains might possess a distinct effector repertoire. In a comparative study on the occurrence of effector genes in many P. syringae strains, also Sarkar et al. [69] analysed Psa MAFF302091. Similar to J-Psa, also Psa MAFF302091 also does not contain hopAI and hopAI-2. Noteworthy, these two effectors are rarely found contemporarily present in the 91 P. syringae strains tested and only three Psa strains showed such effectors in their repertoire [69]. However, our pathogenicity test showed that the three Psa strains did not cause infection to tomato. Vanneste et al. [70] claimed that hopAI was also present in I2-Psa but, according to the genome sequencing, their results appear as an artifact.

Phages, prophages and their morons (i.e. DNA elements inserted between a pair of genes in one phage genome) are known to shape the pathogenicity and virulence of bacterial pathogens and their presence within the bacterial genome can largely contribute to the genetic and phenotypic diversity of bacteria and to the emergence of pathogenic variants [71]. In fact, by carrying various elements contributing to virulence, prophages can also contribute to the individuality of bacteria strains as found in Salmonella, Lactobacillus and Burkholderia [71,72,73]. In Xylella fastidiosa, the causative agent of infectious diseases of many cultivated crops, prophage-associated chromosomal rearrangements and deletions have been found to be largely responsible for strain-specific differences [74]. Interestingly, in a variable region of I2-Psa, we found the presence of many putative proteins related to the assembly and acquisition of prophage PSSPHO6, which are also present in Pph 1448A. It remains to be ascertained if there is a link between the relevant virulence of I2-Psa to Actinidia spp. and the presence of this prophage which was acquired by horizontal gene transfer and if it could have contributed to the further adaptation of Psa to Actinidia spp.

It is interesting to observe that all three Psa strains sequenced here were isolated from leaf spot symptoms but display a set of genes involved in the degradation of lignin derivatives and other phenolics. In fact, similarly to other P. syringae pathovars associated with woody hosts such as P. syringae pv. aeuculi and P. savastanoi pv. savastanoi and to P. putida, a soil-inhabiting species [39,40], the three Psa strains have genes putatively related to the degradation of the anthranilate to the catechol branch of the β-ketoisopropate pathway and to the protocatechuate degradation via the proto-catechuate 4,5-dioxigenase pathway. These pathways allow for the utilisation of unsubstituted lignin-related compounds and other plant derived phenolic compounds such as mandelate and phenol [75]. This could explain one of the most striking symptoms induced by Psa on Actinidia spp., the extensive degradation of the woody tissues of the main trunk and leaders mainly occurring during winter. In Italy, I2-Psa incites canker of larger dimensions on A. chinensis cultivars compared to A. deliciosa and sometimes also causes the complete destruction of all external woody tissues, as shown in Figure 1c. It is also worth noting that I2-Psa survived more than 45 days in infected A. chinensis twigs that were pruned and subsequently brought into the lab, without receiving any amendment (i.e. water) [Marchetti and Scortichini, unpublished data].

The fact that Psa strains can infect both herbaceous (i.e. leaves and young twigs) and woody tissues of the same host plant could
mean that differential set of genes are activated when the pathogen is multiplying and infecting different organs of the plant. It would be interesting to further investigate the P. syringae complex to determine whether the capability of infecting herbaceous tissues appeared before or after that of infecting woody tissues.

All Psa strains display also sets of genes that are virulence factors or are important for the survival of the bacterium in planta or for competing with other micro-organisms. In fact, Psa has genes involved in the inhibition of nitric oxide metabolism, namely nitric oxide dioxygenase and anaerobic nitric oxide reduction [76]. Nitric oxide plays a fundamental role in plant disease resistance by acting as a signal-inducing plant gene to synthesise defense-related compounds [77]. The inhibition of nitric oxide synthesis consequently promotes the bacterial growth in planta.

Psa strains contain copA and copB genes that play a key role in copper resistance [56]. Copper ions are essential for bacterial species but can incite toxic cellular effects if levels of free ions are not controlled. It has been observed that strains of P. syringae with no known history of exposure to copper selection accumulate copper and are resistant. In these bacteria, copper accumulation may have a beneficial role other than in resisting high levels of copper [56]. Interestingly, Nakajima et al. [57] found that at the beginning of bacterial canker outbreaks in Japan (i.e. 1984–1987) all the Psa strains displayed solely copA and copB. However, after repeated spray treatments with copper-based bactericides, the Psa strains also showed additional genes responsible for the maximal resistance to copper, namely copR and copB.

Psa can counteract the lethal effect of antibiotics by means of multidrug efflux pumps of the multidrug resistance systems encoded by chromosomal genes. These features apparently confer a relevant fitness for the in planta growth of Psa. Notably, I2-Psa also displays a lantibiotic dehydratase protein that might putatively inactivates this class of antibiotics which is produced by Gram-positive bacteria and characterised by its high specific activity against multidrug-resistant bacteria [79].

The efficient uptake and utilisation of iron through siderophores is regarded as an important virulence factor for phytopathogenic pseudomonads, especially in iron-limited environments [79]. The Psa strains have a set of genes coding for the production of siderophores such as pyoverdine, haemin, enterobactin and versinibactin. These last two siderophores are primarily described in the Enterobacteriaceae, and some of them are characterized by a very high affinity for iron [80,81]. Similar to P. syringae pv. asaehi, Psa strains also contain hemagglutinin-like proteins. Although they have not been investigated in pseudomonads, in other phytopathogenic bacteria such as Erwinia chrysanthemi, Xylella fastidiosa and Xanthomonas oryzae pv. oryzae these protein have been shown to specifically act in adhesion between the bacterial cell and the plant host cell [82,83].

One laboratory claimed that Psa NCPPB3871 (I-Psa) which was directly received from the National Collection of Plant Pathogenic Bacteria, is not to be a genuine Psa strain [84]. We and other labs [66] did not find such discrepancy. A putative contamination in the former lab could have occurred.

The comparative genome-wide analysis performed with these three Psa strains representing two different populations of the pathovar, provides important insights into the evolution and adaptation of this pathogen to A. eucalypti spp. and highlights how a virulence factor like the phaseolotoxin can be lost without decreasing the relative virulence of the bacterium. We also demonstrate how the mobile arsenal of phytopathogenic bacteria (i.e. plasmids and prophages) can be lost and gained by populations of the same pathovar that, consequently, can modulate their virulence accordingly.

Materials and Methods

Bacterial strains

Psa NCPPB3739 is the type strain of the pathovar and was isolated in 1984, in Japan (Shizuoka district) from a leaf spot lesion of A. deliciosa cv. Hayward [10]. Psa NCPPB3871 was isolated in 1992 in the Roma province (central Italy) from a leaf spot lesion on A. deliciosa cv. Hayward [12]. Psa CRA-FRU 8.43 was isolated in the province of Latina (central Italy) from a leaf spot lesion of A. chinensis cv. Hort16A [14,19], and is the first isolate from the epidemic of bacterial canker currently causing severe damage to the cultivation of A. chinensis and A. deliciosa in Italy [25]. In this paper, the Psa strains are referred to as J-Psa (NCPPB3739), I-Psa (NCPPB3871) and I2-Psa (CRA-FRU 8.43). Figure 1 shows the symptomatic leaves of A. deliciosa and A. chinensis in Italy from where isolations were performed. For this study, the Psa strains were maintained on nutrient agar amended with 5% w/v sucrose (NSA) and incubated at 25±1°C.

Library preparation and Illumina sequencing

Bacterial genomic DNA was extracted from 1 ml of overnight J-Psa, I-Psa and I2-Psa cultures grown in KB broth DNA using a Wizard DNA purification kit (Promega Italia, Padova, Italy) following the manufacturer’s instructions. DNA was measured and checked for quality using a NanoDrop (NanoDrop products, Wilmington, DE, USA). A total of 10 μg of DNA from each sample was fragmented by incubation for 70 min with 5 μl of dsDNA Fragmentase (New England Biolabs MA, USA). The reaction was stopped with EDTA and purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The eluate was end repaired using an End Repair kit (New England Biolabs, MA, USA) for 30 min at 20°C. The end-repaired DNA was A-tailed for 30 min at 37°C using a dA Tailing kit (New England Biolabs, MA, USA). After purification using the MinElute purification kit (QIAGEN), the DNA was ligated using Quick T4 DNA ligase (New England Biolabs) to 500 pmol of Illumina adaptors that had been previously annealed by heating at 98°C for 3 min and then slowly cooling to 16°C in a thermocycler. After further purification using the MinElute purification kit (QIAGEN), 1 μl of each reaction was quantified by labelling with biotin, spotted on nitrocellulose after a serial dilution, and detected using an anti-biotin-AP conjugate (Roche Diagnostics, Monza, Italy) following manufacturer’s instructions. Equal amounts of DNA from samples were pooled together and size fractionated by 2% MS-6 agarose (Conda, Madrid, Spain) gel electrophoresis in TAE buffer at 120 V for 60 min. Gel slices containing DNA in the 400 to 600 bp estimated range were cut and purified using QIAquick gel extraction kit (QIAGEN) and used for sample preparation according to the protocol for genomic DNA sequencing using the Illumina Genome Analyser IIx (Illumina, USA). The samples were run at the Istituto di Genomica Applicata (Udine, Italy).

The Illumina sequencing provided comprehensively nearly 10 millions 100 nt reads from the genomic DNA of the three Psa strains that passed the quality check. This amount of sequence represents approximately 27.7, 67.6 and 61.2 X coverage for J-Psa, I-Psa and I2-Psa, respectively, i.e. the expected genomic size of these strains, based on the previously sequenced genomes of the P. syringae pathovars is 6 Mb.

Whole-genome assembly and alignment of Illumina genomes

Paired reads of 100 nts were assembled into contigs using the de novo (i.e. without using a reference genome) assembly option of the CLC genomic workbench (CLC-bio, Aarhus, Denmark). Contigs
sequences were scanned for ORFs by GLIMMER, version 3.02. [85] which had been previously trained on the complete genome sequences of Pseudomonas syringae pv. tomato strain DC3000 (NC_004578.1, i.e. Pto DC3000), P. s. pv. phaseolicola strain 1448A (NC_005773.3, i.e. Pph 1448A), and P. s. pv. syringae strain B728a (NC_007005.1, i.e. Pys B728a). The putative proteins were annotated against the RefSeq database using a PERL script for recursive BLASTX searches. Additional genome sequence analyses and MAUVE [87]. Several strains submitted for plasmid detection were the object of this Miniprep System (Promega, Madison, WI, U.S.A.) protocol. The protein complement with respect to each one of the three assist the comparison of genome sequence draft and its putative Pto paper of Baltrus et al. [68]

Analysis of plasmid content

Plasmid isolation was performed using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, U.S.A.) protocol. The strains submitted for plasmid detection were the object of this study as well as Psa KW30 and Psa KW31, which were isolated in 1984 in Japan from leaf spot lesions of A. deliciosa cv. Hayward [10] and additional 11 Psa strains isolated in different regions of Italy during current epidemics [14,25,42]. Plasmids from Escherichia coli strain 39R061 were used as molecular weight marker [93].

Multiplication of Psa in Actinidia spp. leaves and pathogenicity test on tomato

To compare the capability of infection in different Actinidia spp., we artificially inoculated both A. chinesis and A. deliciosa leaves with all three of the sequenced Psa strains. For inoculation, bacteria were grown for 48 h on TSA, at 25±1°C, and the plants were covered 24 h before the inoculation with a plastic bag. Leaf areas of approximately 1 cm in diameter on one-year-old potted A. deliciosa cv Hayward and A. chinesis cv Hort16A plants were inoculated using a needless sterile syringe with a bacterial suspension in sterile saline (0,65% NaCl in distilled water) at the concentrations of 1–2×10^7 and 1–2×10^6 cfu/mL. For each thesis, 10 leaves were inoculated in four sites. Control plants were treated using solely sterile saline. To determine bacterial growth in planta, leaf disks of about 0.5 cm of diameter were sampled from each species and inoculation site at regular intervals and ground in 1 mL of sterile saline, and serial ten-fold dilutions were spotted onto NSA medium. Colonies were counted two days after incubation at 25±1°C. According to a comparative study on host-specific virulence factors and effector genes [54], it has been observed that the contemporary presence of the effector genes hopA1, hopB, hopD and hopH in single strains is quite rare. Only three Psa strains displayed such a combination. To verify that Psa, in particular I2-Psa showing such effector genes, can multiply and also infect tomato plants, we performed a pathogenicity test by inoculating potted-seedlings of Lycopersicon esculentum (tomato) cv Lancdot. The inoculation was performed as described above and Pto DC3000 was used as positive control. Symptoms caused by inoculation of the three Psa strains were observed and compared with those caused by Pto DC3000.

Supporting Information

Figure S1 Evolutionary relationships of Psa strains to other phytopathogenic pseudomonads. Phylogenetic relationships were estimated from concatenated sequences from six housekeeping genes, acnB, fuk, glaA, pgI, rpoB and rpoD, for a total of 2,926 bp. PhyML [91] method was used to determine the best model of evolution for the ML analysis. Both the hierarchical likelihood ratio test (hLRT) and the standard Akaike Information Criterion (AIC) were used to evaluate the model scores. Bootstrap analysis was performed using the same software. The hypotheses about the genealogy of the Psa strains were tested using the likelihood-ratio test for monophyly which was developed by Huelsenbeck et al. [92]. Likelihoods were estimated using the Phangorn module [93] of the statistical package R (R development core team, 2007). The significance of the likelihood ratio was estimated by parametric bootstrap according to Huelsenbeck et al. [92] by simulation of 1,000 replicated datasets generated with Indel-Seq-Gen [94].

Figure S2 Presence and absence of phaseolotoxin in Psa strains. Diagrammatic representation of the phaseolotoxin gene cluster, avgK-lox, and the flanking regions. The phaseolotoxin cluster is present in J-Psa and 1-Psa (upper part) but not in I2-Psa (lower part). (DOC)

Table S1 Variable regions (VR) found in the draft genome of I2-Psa compared with J-Psa and 1-Psa draft genomes. (DOCX)

Table S2 Categorization according to TIGRFAMs of protein complement displayed by the draft genome of I2-Psa. (XLS)

Table S3 Similarity of the type III effector protein genes complement of J-Psa, 1-Psa and I2-Psa compared with the same complement of other sequences plant pathogenic pseudomonads. (XLS)
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Author Contributions

Conceived and designed the experiments: MS GF. Performed the experiments: SM GF PP MS. Analyzed the data: GF SM MP MS. Contributed reagents/materials/analysis tools: GF SM MP MS. Wrote the paper: MS GF.

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