Identification of Loci of *Pseudomonas syringae* pv. *actinidiae* Involved in Lipolytic Activity and Their Role in Colonization of Kiwifruit Leaves

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ABSTRACT

Bacterial canker disease caused by *Pseudomonas syringae* pv. *actinidiae*, an emerging pathogen of kiwifruit plants, has recently brought about major economic losses worldwide. Genetic studies on virulence functions of *P. syringae* pv. *actinidiae* have not yet been reported and there is little experimental data regarding bacterial genes involved in pathogenesis. In this study, we performed a genetic screen in order to identify transposon mutants altered in the lipolytic activity because it is known that mechanisms of regulation, production, and secretion of enzymes often play crucial roles in virulence of plant pathogens. We aimed to identify the set of secretion and global regulatory loci that control lipolytic activity and also play important roles in in planta fitness. Our screen for altered lipolytic activity phenotype identified a total of 58 Tn5 transposon mutants. Mapping all these Tn5

mutants revealed that the transposons were inserted in genes that play roles in cell division, chemotaxis, metabolism, movement, recombination, regulation, signal transduction, and transport as well as a few unknown functions. Several of these identified *P. syringae* pv. *actinidiae* Tn5 mutants, notably the functions affected in phosphomannomutase AlgC, lipid A biosynthesis acyltransferase, glutamate–cysteine ligase, and the type IV pilus protein Pill, were also found affected in in planta survival and/or growth in kiwifruit plants. The results of the genetic screen and identification of novel loci involved in in planta fitness of *P. syringae* pv. *actinidiae* are presented and discussed.

Additional keywords: bacterial canker of kiwifruit, emerging pathogen, virulence loci.

Gram-negative *Pseudomonas* plant-pathogenic bacteria are known to cause disease in a broad range of economically important host plants with a variety of disease symptoms. The *Pseudomonas syringae* group contain model pathogens that are distinguished into at least nine genomospecies and 60 pathovars (Gardan et al. 1999; Marcelletti and Scortichini 2014). Examples of three agriculturally important pathogens of this group are *P. syringae* pv. *tomato* DC3000 (Preston 2000), *P. syringae* pv. *phaseolicola* 1448A (Joardar et al. 2005), and *P. syringae* pv. *syringae* B728a (Hirano and Upper 2000). These have been widely studied and used as model systems to study the virulence functions in plant pathogen interactions. The majority of plant pathogenic pseudomonads studied are pathogenic to herbaceous plants while pathogens from woody plants have been understudied. The increasing economic value of woody plants and the growing knowledge of pathogens and hosts are now helping to address these diseases and their pathogens in woody plants.

In the past 2 decades, emerging and re-emerging plant pathogens have caused new threats to the production of several economically important crops. The kiwifruit pathogen *P. syringae* pv. *actinidiae* is an emerging woody plant pathogen that has recently received considerable attention due to its increasing incidence and global spread (Scortichini et al. 2012). *P. syringae* pv. *actinidiae* causes

canker or leaf spot on *Actinidia* plants (Serizawa et al. 1989) and was first described in Japan in 1984 (Takikawa et al. 1989) and later was isolated in South Korea (Koh et al. 1994) and Italy (Scortichini 1994). In 2008, a serious re-emergence of *P. syringae* pv. *actinidiae* was found on *Actinidia chinensis* (kiwigold) plants in central Italy causing huge economic losses (Ferrante and Scortichini 2010). This outbreak was caused by a different population of *P. syringae* pv. *actinidiae* from the original one that caused fewer problems in 1992 (Marcelletti et al. 2011; Vanneste et al. 2010). *P. syringae* pv. *actinidiae* has also recently caused important outbreaks in several countries including China (Yang et al. 2015), Portugal (Balestra et al. 2010), France (Vanneste et al. 2011), Chile (Anonymous 2011), New Zealand ((Everett et al. 2011), Spain (Abelleira et al. 2011), Switzerland (EPPO 2011), and Turkey (Bastas and Karakaya 2012).

In plant–*Pseudomonas* pathogen interactions, several virulence factors contributing to bacterial pathogenicity have been studied in great detail. These include the type III secretion system (T3SS) (Hauser 2009) bacterial toxins (Lindeberg et al. 2008) and exopolysaccharides (Yu et al. 1999; Laue et al. 2006). Moreover, global regulatory mechanisms like quorum sensing, two component systems, and alternative sigma factors have also been found to play an important role in virulence of *Pseudomonas* pathogens. *P. syringae* pv. *actinidiae* studies are so far focused mainly on its identification and pathogen differentiation (Koh and Nou 2002; Koh et al. 2014; Lee et al. 2016), its origin, evolution and epidemic spread (Butler et al. 2013; Chapman et al. 2012; Ciarroni et al. 2015; Cunty et al. 2015; Mazzaglia et al. 2012; Scortichini et al. 2012; Vanneste et al. 2013), genome sequence analyses (Fujikawa and Sawada 2016; Marcelletti et al. 2011; McCann et al. 2013; Templeton et al. 2015), proteomic analyses of different host

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and three supplementary tables are published online.

tissues (Petriccione et al. 2013, 2014), and its management either of using resistant host lines or use of bacteriophages (Di Lallo et al. 2014; Yu et al. 2016) and chemical compounds (Cameron et al. 2014; Ghods et al. 2015).

As very few genetic and molecular studies of *P. syringae* pv. *actinidiae* virulence have been performed (Cellini et al. 2014), we decided to perform an exhaustive screen of a Tn5 genomic mutant library of *P. syringae* pv. *actinidiae* for alteration of an easily discernable phenotype shown to be virulence-associated in other pathogenic bacteria. In phytobacteriology, mechanisms of regulation, production, and secretion of enzymes often play crucial roles in virulence of plant pathogens (Buttner and Bonas 2010; Chang et al. 2014; Poueymiro and Genin 2009). With this aim, we screened several thousands of transposon mutants for the secreted lipase/esterase activity (lipolytic activity) phenotype. *P. syringae* pv. *actinidiae* mutants altered for lipolytic activity and the genes carrying insertions in these mutants were identified. Role of these genes in determining lipolytic activity phenotype was verified by generating independent mutants in identified genes and complementation analysis. The role of these genes were then assessed for their contribution to survival and/or growth of *P. syringae* pv. *actinidiae* in plant infection models; the results are presented and discussed.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1 and Supplementary Table S1, respectively. *P. syringae* pv. *actinidiae* strain 10.22 is clonal to the *P. syringae* pv. *actinidiae* CRAFRU strains, which have been isolated in the province of Latina (central Italy) from spot lesions of *A. chinensis* (Ferrante and Scortichini 2010). *P. syringae* pv. *actinidiae* strains were grown at 25°C in Luria Bertani (LB), nutrient sucrose agar (NSA; composition for 1 liter: peptone 20 g, sucrose 5 g, K₂HPO₄ 0.5 gm, MgSO₄·7H₂O 0.25 g, and agar 12 g) or King's B medium (King et al. 1954). The concentrations of antibiotics used in this study were as follows: nitrofurantoin (Nf), 150 µg/ml; kanamycin (Km), 50 µg/ml; gentamycin (Gm), 30 µg/ml; and tetracycline (Tc), 30 µg/ml for *P. syringae* pv. *actinidiae* strains and Km, 50 µg/ml; Gm, 15 µg/ml; Tc, 15 µg/ml; and ampicillin (Ap), 100 µg/ml for *E. coli* strains. *P. syringae* pv. *actinidiae* is naturally resistant to nitrofurantoin.

Recombinant DNA techniques. Routine DNA manipulation steps such as digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligations with T4 ligase, Southern analysis, radioactive labeling by random priming, and transformation of *E. coli* were done using standard procedures as described previously (Sambrook et al. 1989). Colony hybridizations were performed using Hybond-N+ membrane (Amersham Biosciences); plasmids were purified using the EuroGold plasmid columns (Euro Clone) or with the alkaline lysis method (Birboim 1983); total DNA from *P. syringae* pv. *actinidiae* strains were isolated by Sarkosyl/Pronase lysis as described previously (Better et al. 1983). PCR amplifications were performed using Go-Taq DNA polymerase or pfu DNA polymerase (Promega). The oligonucleotide primers used in this study are listed in Supplementary Table S2. Automated sequencing was performed by Macrogen sequence service (Europe). Triparental matings between *E. coli* and *P. syringae* pv. *actinidiae* were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figurski and Helinski 1979).

Generation of a *P. syringae* pv. *actinidiae* genomic Tn5 mutant library. Tn5 mutagenesis was performed by using triparental matings between donor *E. coli* (pSUP2021) containing the transposon Tn5 (Km resistance), a helper *E. coli* strain (pRK2013), and recipient *P. syringae* pv. *actinidiae* strain. Briefly, *P. syringae* pv. *actinidiae* (strain 10.22) was grown overnight in 100 ml of LB media supplemented with Nf at room temperature. Donor *E. coli* and helper *E. coli* strains were also grown overnight in 20 ml of LB media supplemented with appropriate antibiotics at 37°C. Cells of *P. syringae* pv. *actinidiae*, donor *E. coli*, and helper *E. coli* were pelleted, washed twice in plain LB, and resuspended in 20 ml of sterile LB media. The absorbance of all three strains was measured at OD₆₀₀ and cells were mixed in the following ratio: recipient *P. syringae* pv. *actinidiae*, 2 × 10⁸ colony forming units (CFU/ml); helper *E. coli*, 4 × 10⁹ CFU/ml; and donor *E. coli*, 4 × 10⁹ CFU/ml. The mixture of cells was pelleted, resuspended in a small volume of LB media and spotted onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) that was overlaid on LB agar. Overnight incubated cells grown at room temperature were scraped from the membrane and resuspended in 1 ml of sterile LB media. The cell suspension (50 µl each) was plated on LB agar plates containing Nf and Km. The plates were incubated at room temperature for 2 to 3 days to allow the growth of exconjugants

TABLE 1. Bacterial strains used in this study

Strains	Relevant characteristics ^a	Reference/source
<i>Escherichia coli</i>		
DH5α	Cloning strain, Nal ^r	(Grant et al. 1990)
C118 λpir	Δ(are-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1 lysogenized with λpir phage	(Herrero et al. 1990)
pRK2013	Helper strain for triparental conjugation, Km ^r	(Figurski and Helinski 1979)
<i>Pseudomonas syringae</i> pv. <i>actinidiae</i> (Psa)		
Psa 10.22	Wild type; Italian isolate; Nf ^r	Lab collection
Psa Lap mutants	Psa::Tn5 mutants with alteration in lipase secretion, Nf ^r , Km ^r	This work
Psa Lap38-pKNOCK	Lap38::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap41-pKNOCK	Lap41::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap42-pKNOCK	Lap42::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap45-pKNOCK	Lap45::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap169-pKNOCK	Lap169::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap200-pKNOCK	Lap200::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap208-pKNOCK	Lap208::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap276-pKNOCK	Lap276::pKNOCK; Nf ^r , Km ^r ; derivative of wild type	This work
Psa Lap38+pCosLap38	Psa Lap38 carrying cosmid clone for Lap38; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap38	This work
Psa Lap41+pCosLap41	Psa Lap41 carrying cosmid clone for Lap41; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap41	This work
Psa Lap42+pCosLap42	Psa Lap42 carrying cosmid clone for Lap42; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap42	This work
Psa Lap45+pCosLap45	Psa Lap45 carrying cosmid clone for Lap45; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap45	This work
Psa Lap169+pCosLap169	Psa Lap169 carrying cosmid clone for Lap169; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap169	This work
Psa Lap200+pCosLap200	Psa Lap200 carrying cosmid clone for Lap200; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap200	This work
Psa Lap208+pCosLap208	Psa Lap208 carrying cosmid clone for Lap208; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap208	This work
Psa Lap276+pCosLap276	Psa Lap276 carrying cosmid clone for Lap276; Nf ^r , Km ^r , Tc ^r ; derivative of Psa Lap276	This work

^a Nal^r, Km^r, Tc^r, Gm^r, and Nf^r indicate resistance to nalidixic acid, kanamycin, tetracycline, gentamycin, and nitrofurantoin, respectively.

(Tn5 mutants). The *P. syringae* pv. *actinidiae* genomic Tn5 insertion mutants were then patched onto LB agar plates with Nf and Km and grown in liquid media with the similar set of antibiotics.

Screening of *P. syringae* pv. *actinidiae* Tn5 mutants for lipolytic activity. Lipolytic activity for *P. syringae* pv. *actinidiae* strains was performed as mentioned previously with some modifications (Smeltzer et al. 1992). Briefly, for plate assays 1 ml of tributyrin (Sigma) solution was added to a 10 ml of LB broth and sonicated using a sonicator (with four pulses of 60 to 80 Hertz using a Bandeline Sonoplus HD270 homogenizer, Berlin) until the solution became homogenous white. This homogenous tributyrin mix was added to prewarmed 400 ml of LB agar media containing Km antibiotic, mixed well and poured onto petri plates. *P. syringae* pv. *actinidiae* Tn5 mutant colonies randomly picked from plates were spotted one by one on LB agar-tributyrin plates using a toothpick. Plates were further incubated for 3 to 5 days at room temperature in order to see the halo generated due to lipolytic activity. The halo size of *P. syringae* pv. *actinidiae* Tn5 mutants was compared with wild-type halo size by visual inspection and the *P. syringae* pv. *actinidiae* Tn5 mutants that showed any alteration in the level of lipolytic activity were selected for a further round of screening. After three confirmatory rounds of screening *P. syringae* pv. *actinidiae* Tn5 mutants with altered lipolytic activity phenotype were selected and grown in liquid LB broth with Nf + Km, and their glycerol stocks were stored at -80°C . All mutants were grown in liquid in LB medium and did not show any significant difference with respect to the wild type.

Localization of Tn5 insertion. In order to clone DNA of *P. syringae* pv. *actinidiae* adjacent to the transposon insertions, an arbitrary PCR was performed using a pair of primers as previously described in O'Toole and Kolter (1998) with some modifications. Arbitrary PCR products were purified and directly sequenced using Tn5-specific Tn5-Intprimer. Genomic DNA was also isolated from selected *P. syringae* pv. *actinidiae* Tn5 mutants and double digested either with *Bam*HI + *Eco*RI, *Bam*HI + *Sac*I, or *Bam*HI + *Cla*I. These double-digested products were ligated in pBluescript (double digested with the similar set of enzymes), transformed into DH5 α *E. coli* cells, and selected on LB agar plates with Ap + Km. Sequences obtained were subjected to homology searches using NCBI Blast with the draft genome sequence of *P. syringae* pv. *actinidiae* (str. M302091; taxid:629266). The exact position and orientation of the Tn5 insertions were mapped in *P. syringae* pv. *actinidiae* draft genome. In order to test if mutants have single Tn5 insertion, the Southern analysis was performed on mutants Lap38, Lap41, Lap45, and Lap 276 using radio labeled Tn5 DNA fragment as a probe. It was confirmed that all these mutants contained a single Tn5 insertion in their chromosome (Supplementary Fig. S1).

Regeneration of knock-out mutants and complementation. The selected Tn5 mutants were regenerated via single homologous recombination using the pKNOCK-Km suicide delivery system. Briefly, internal fragments of the loci were PCR amplified and cloned into a pGEMT-easy vector. *Eco*RI-digested internal fragments were ligated to *Eco*RI-digested pKNOCK-Km and transformed into *E. coli* C118 λ pir cells yielding pKNOCK plasmids having internal fragments from selected Tn5 loci. These pKNOCK plasmids were then used as a suicide delivery system in a triparental mating as described earlier. The eight mutants (lipolytic altered phenotype [Lap]: Lap38, Lap41, Lap42, Lap45, Lap169, Lap200, Lap208, and Lap276) generated using this strategy were selected on NSA plates supplemented with Nf + Km. *P. syringae* pv. *actinidiae* mutant strains were verified by PCR analysis and sequencing.

In order to complement the selected *P. syringae* pv. *actinidiae* Tn5 mutants, a cosmid library of *P. syringae* pv. *actinidiae* 10.22 strain was constructed by using the cosmid pLAFR3 (Staskawicz et al. 1987) as a vector. Insert DNA was prepared by partial *Eco*RI digestion of the genomic DNA and then ligated into the corresponding site in pLAFR3. The ligated DNA was then packaged into λ phage heads using Gigapack III Gold packaging extract (Stratagene) and the phage particles were transduced to *E. coli* HB101 as recommended by the

supplier. In order to identify the cosmid containing the genes of interest, the cosmid library was screened using the same PCR product as a radiolabeled probe in colony hybridization products that were used to generate pKNOCK mutants. The required cosmid clones were obtained and were further introduced into respective mutants by triparental conjugation (mutant strain + helper + cosmid containing the expected loci) for complementation analysis. Exconjugants were selected on NSA plates supplemented with Nf, Km, and Tc.

In planta survival and/or growth assay. *P. syringae* pv. *actinidiae* in planta assay was performed as described previously (Patel et al. 2014). Briefly, 1-year-old potted plants of *A. deliciosa* 'Hayward' were maintained in a climatic room and watered regularly. For inoculation, *P. syringae* pv. *actinidiae* strains were grown for 48 h on NSA medium supplemented with antibiotics at 23 to 25°C. Bacterial cultures were pelleted, washed with sterile saline (0.85% NaCl in distilled water), and adjusted to 1 to 2×10^6 CFU/ml in sterile saline. Leaf areas of approximately 1 cm in diameter were inoculated using a needleless sterile syringe with the bacterial suspension. For each strain, 10 leaves were inoculated in four sites and control plants were treated in a similar manner using sterile saline. In order to determine the initial in planta bacterial load, leaf disks of about 0.5 cm diameter were sampled from the inoculation site immediately after infiltration (at 0 h) and then ground in 1 ml of sterile saline, and serial 10-fold dilutions were plated onto NSA supplemented with antibiotics. Colonies were counted 2 days after incubation at 23 to 25°C. CFU/0.5 cm² were then determined for wild type and randomly selected mutant strains as log values and tabulated in order to know the range of initial bacterial load onto the leaves. The fitness in planta over time, the in planta survival and/or growth score of wild type, and all the mutants and their respective complements were then obtained for 3 and 7 days postinoculation (DPI). Confirmation of colony identity was achieved by following well established phenotypic and molecular procedures including duplex-PCR (Ferrante and Scortichini 2009, 2010; Gallelli et al. 2011).

RESULTS

***P. syringae* pv. *actinidiae* genes involved in lipolytic activity are functionally diverse.** It was of interest to determine plate visible phenotypes of *P. syringae* pv. *actinidiae* that could provide a convenient screen to identify mutants of potential virulence loci. *P. syringae* pv. *actinidiae* strains were found to exhibit secreted lipase/esterase activity on plate media (Supplementary Fig. S2). It was therefore decided to screen for mutants altered in lipolytic activity with the aim of identifying secretion and global regulatory mutants that are important for *P. syringae* pv. *actinidiae* virulence. A genome-wide Tn5 mutagenesis and screening experiment for detecting alteration in lipolytic activity was therefore set up in *P. syringae* pv. *actinidiae*. A Tn5 mutant library of *P. syringae* pv. *actinidiae* was generated as described in the Materials and Methods section and 30,000 mutants were tested for alteration in lipolytic activity on LB agar-tributyrin plates. In the first round of screening, a total of 254 mutants were obtained with Lap. Some of the Lap mutants were also affected in growth on LB agar-tributyrin plates, suggesting the alteration of the size of the halo might be due to a growth defect; these Lap mutants were not selected for further analysis. All mutants were then further tested for reproducibility of these phenotypes and finally a total of 65 Lap mutants, which were all showing more lipolytic activity than the wild-type strain, were chosen for further study. Surprisingly, in the screen for altered lipolytic activity, no Lap mutants were completely abolished in lipolytic activity (no halo), indicating that probably more than one lipase/esterase enzyme is produced by *P. syringae* pv. *actinidiae*. In fact, the genome sequence of several *P. syringae* pv. *actinidiae* strains including *P. syringae* pv. *actinidiae* ICMP1884 and strain ICMP19096 were found to possess at least three proteins with a lipase domain annotated protein. Based on the zone of clearance (halo) size in LB agar-tributyrin plates, Lap mutants were placed into three different groups from 1 to 3, in increasing order (Table 2;

TABLE 2. The details of Tn5 mutants and their in planta survival and/or growth score

Details of Tn5 mutants obtained in the screening of altered lipolytic activity										
Tn5 mutants ^a	Lipolytic activity score ^b	Tn5 insertion position ^c	Orientation of Tn5 insertion ^d	ORF/protein ID of Tn5 insertion ^e	ORF length ^f	BlastX information (ORF function) ^g	Functional Categorization ^h	In planta survival and/or growth score ⁱ		Virulence category ^j
								3 days	7 days	
Lap20	1	2429	+/-	EGH66838.1	2,754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction	2.43E+07	6.00E+07	i
Lap33	1	2517	+/+	EGH66838.1	2,754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction	1.20E+07	1.70E+07	i
Lap35	1	747	+/-	EGH67056.1	837	Hypothetical protein	Hypothetical	1.57E+07	9.77E+07	i
Lap36	1	404	+/+	EGH64339.1	897	site-specific tyrosine recombinase XerD	Recombination	1.83E+07	2.63E+07	i
Lap37	1	354	+/-	EGH64482.1	873	XerC	Recombination	3.57E+07	2.61E+07	i
Lap38	1	113	+/-	EGH67827.1	813	Septum site determining protein minD	Cell division	1.46E+06	1.68E+06	ii
Lap52	1	590	+/-	EGH66838.1	2,754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction	8.20E+07	9.70E+07	i
Lap54	1	167	+/-	EGH67427.1	1,113	Glycosyl transferase	Metabolism	4.33E+06	5.55E+06	ii
Lap55	1	544	+/-	EGH65930.1	1,788	Sensory box histidine kinase	Regulation and signal transduction	2.37E+07	2.40E+07	i
Lap62	1	905	+/-	EGH66807.1	2,493	Helicase domain containing protein	Metabolism	2.93E+07	2.97E+07	i
Lap71	1	1048	+/+	EGH66370.1	1,176	3-Ketoacyl-CoA thiolase	Metabolism	2.23E+06	2.55E+06	ii
Lap73	1	278	+/-	EGH68302.1	747	HAD- superfamily hydrolase	Metabolism	3.48E+06	3.80E+06	ii
Lap81	1	288	+/+	EGH65338.1	528	Disulfide bond formation protein DsbB	Metabolism	3.70E+07	3.73E+07	i
Lap82	1	502	+/-	EGH66828.1	1,347	Sensor protein PhoQ	Regulation and signal transduction	1.07E+07	1.10E+07	i
Lap83	1	713	+/-	EGH66147.1	1,938	Cobaltochelatase subunit CobN	Metabolism	2.47E+07	2.94E+07	i
Lap84	1	1200	+/+	EPM67430.1	1,683	Helicase, putative	Metabolism	2.10E+07	2.13E+07	i
Lap106	1	3	+/+	EGH67057.1	378	Hypothetical protein	Hypothetical	1.40E+07	1.43E+07	i
Lap108	1	401	+/-	EGH66177.1	642	Hypothetical protein	Hypothetical	1.14E+07	1.31E+07	i
Lap121	1	375	+/-	EGH66177.1	642	Hypothetical protein	Hypothetical	1.23E+07	1.55E+07	i
Lap131	1	428	+/+	EGH66177.1	642	Hypothetical protein	Hypothetical	1.03E+07	1.07E+07	i
Lap153	1	437	+/+	EGH66177.1	642	Hypothetical protein	Hypothetical	4.23E+07	2.00E+07	i
Lap161	1	181	+/+	EGH65029.1	801	Sec-independent protein translocase TatC	Transport	8.07E+06	8.39E+06	ii
Lap194	1	283	+/-	EGH68302.1	747	HAD family hydrolase	Metabolism	2.17E+06	2.49E+06	ii
Lap195	1	415	+/+	EGH64055.1	540	Type IV pilus protein pili	Motility	4.67E+04	1.96E+04	iii
Lap249	1	66	+/+	EGH65067.1	825	Glycosyl transferase, group 4 family protein	Metabolism	7.60E+06	7.92E+06	ii
Lap276	1	422	+/-	EGH66838.1	2,754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction	1.27E+07	1.30E+07	i
LapC2	1	723	+/-	EPM47746.1	1,164	phosphoglycerate kinase	Metabolism	2.37E+06	2.69E+06	ii
LapC3	1	288	+/+	EGH65338.1	528	Disulfide bond formation protein DsbB	Metabolism	4.50E+06	4.82E+06	ii
Lap3	2	600	+/-	EGH67743.1	888	Dienelactone hydrolase family protein	Metabolism	6.67E+07	8.73E+07	i
Lap18	2	832	+/+	EGH65110.1	1,119	Putative lipoprotein	Metabolism	3.47E+07	2.40E+07	i
Lap34	2	29	+/+	EGH65738.1	846	Glycine/betaine/L-proline ABC transporter,	Transport	4.73E+06	5.05E+06	ii
Lap39	2	672	+/-	EGH65700.1	672	Cell division ATP-binding protein FtsE	Cell division	1.43E+07	1.63E+07	i
Lap45	2	208	+/+	EGH65936.1	645	Thiol:disulfide interchange protein DsbA	Metabolism	5.23E+04	3.72E+05	iii
Lap53	2	903	+/+	EGH64518.1	1,398	Phosphomannomutase AlgC	Metabolism	3.02E+05	1.10E+05	iii
Lap60	2	750	+/-	EGH64518.1	1,398	Phosphomannomutase AlgC	Metabolism	5.17E+06	5.49E+06	ii
Lap74	2	2208	+/-	EGH63578.1	2,397	ATP-dependent protease La	Metabolism	1.90E+07	1.93E+07	i
Lap76	2	155	+/-	EGH64016.1	735	LPS biosynthesis protein	Metabolism	2.20E+07	2.23E+07	i
Lap78	2	269	+/-	EGH64681.1	1,620	Glucan biosynthesis protein D	Metabolism	2.73E+07	2.88E+07	i
Lap79	2	475	+/+	EGH67201.1	966	LacI family transcriptional regulator	Regulation and signal transduction	5.63E+06	7.23E+06	ii
Lap105	2	861	+/+	EGH65701.1	1,035	Cell division protein FtsX	Cell division	1.13E+07	1.19E+07	i
Lap107	2	856	+/+	EGH65701.1	1,035	Cell division protein FtsX	Cell division	9.33E+06	9.65E+06	i ^k
Lap168	2	1201	+/+	EGH64518.1	1,398	Phosphomannomutase AlgC	Metabolism	4.67E+05	7.87E+05	iii
Lap169	2	1115	+/+	EGH64518.1	1,398	Phosphomannomutase AlgC	Metabolism	9.00E+05	8.84E+05	iii
Lap208	2	1130	+/-	EGH65920.1	1,590	Glutamate-cysteine ligase	Metabolism	3.70E+03	4.17E+03	iii
Lap13	3	674	+/-	EGH65387.1	880	Lipid A biosynthesis lauroyl acyltransferase	Metabolism	1.40E+05	1.60E+05	iii
Lap14	3	166	+/-	EGH65087.1	696	MotA/TolQ/ExbB proton channel	Transport	1.40E+06	3.30E+06	ii
Lap15	3	165	+/-	EGH65087.1	696	MotA/TolQ/ExbB proton channel	Transport	1.49E+06	3.70E+06	ii
Lap16	3	558	+/+	EGH65090.1	1,302	Translocation protein TolB	Transport	1.37E+06	2.10E+06	ii
Lap17	3	515	+/-	EGH65087.1	696	MotA/TolQ/ExbB proton channel	Transport	1.39E+06	3.80E+06	ii
Lap41	3	840	+/+	EGH67601.1	1,035	Outer membrane porin OprF	Transport	3.37E+06	3.69E+06	ii
Lap42	3	621	+/+	EGH65387.1	880	Lipid A biosynthesis lauroyl acyltransferase	Metabolism	6.67E+04	3.97E+04	iii
Lap61	3	1092	+/+	EPM75923.1	1,548	Wbdc	Metabolism	1.83E+07	1.87E+07	i
Lap77	3	161	+/+	EGH67825.1	553	Lipid A biosynthesis lauroyl acyltransferase	Metabolism	5.33E+06	6.65E+06	ii
Lap176	3	867	+/+	EGH65490.1	1,305	Methyl-accepting chemotaxis protein	Chemotaxis	6.88E+06	7.92E+06	ii
Lap188	3	322	+/-	EGH67825.1	933	Lipid A biosynthesis lauroyl acyltransferase	Metabolism	7.50E+06	7.82E+06	ii
Lap200	3	868	+/+	EGH65490.1	1,305	Methyl-accepting chemotaxis protein	Chemotaxis	1.46E+05	4.66E+05	iii

^a Lap: lipolytic altered phenotype.^b Lipolytic activity score of Tn5 mutants on Luria Bertani agar-tributyryl plates (score scale from 1 to 3).^c position of Tn5 insertion in the open reading frame (ORF).^d Orientation of Tn5 insertion with respect to ORF.^e Protein ID of ORF in which Tn5 transposon was found inserted.^f Length of ORF (in bp) in which Tn5 transposon was found inserted.^g Annotated function of ORF in which Tn5 transposon was found inserted.^h Functional categorization assigned to the functions.ⁱ In planta survival and/or growth score (CFU/ml) at 3 and 7 days postinoculation (DPI). At 0 h, there was no significant difference in the level of bacterial inoculum (0.5E+04 to 1E+04 CFU/0.5 cm² leaf area) for all of the strains tested.^j In planta survival and/or growth category (i: a "wild type" group that displays in planta phenotype similar to the wild type or within a 10-fold difference to wild type [CFU/0.5 cm² leaf area score 1E+07 and above either at 3 or 7 DPI]; ii: a fitness in planta deficient group displaying a 10- to 100-fold difference [CFU/0.5 cm² score less than 1E+07 and higher than 1E+06 either at 3 or 7 DPI]; and iii: a severely fitness in planta deficient group displaying more than 100-fold difference compared with wild type [CFU/0.5 cm² leaf area score 1E+06 or less either at 3 or 7 DPI]).^k Score is close to category i.

Fig. 1). Out of a total of 56 mutants in which the position of the Tn5 insertion could be localized (see below), 27 Lap mutants were classified into group 1, 17 Lap mutants were group 2, and 12 Lap mutants were group 3.

The position of Tn5 insertion sites was determined using an arbitrary PCR method as described in the Materials and Methods section; this was successful for 56 of 65 Lap mutants (Table 2). For the remaining nine Lap mutants, a molecular cloning approach was used without success as described in the Materials and Methods. The reason for this is not known and it is most likely that the methodology used had limitations that are currently unknown. The 56 Lap mutants were localized to 38 different ORFs (several Lap mutants were isolated more than once) belonging to eight different functional categories: cell division, chemotaxis, hypothetical proteins, metabolism, motility, recombination, regulation, and signal transduction and transport (Table 2; Fig. 2; Supplementary Fig. S3). Mutations in genes encoding for lipid A biosynthesis, MotA/TolQ/ExbB and TolB mediated transport, chemotaxis and porin OprF caused a drastic increase (group 3) in lipolytic activity, whereas mutation in genes encoding cell division proteins FtsX and FtsE, putative hydrolases, redox homeostasis functions like GshA, DsbA, and DsbB, phosphomannomutase AlgC, putative lipoprotein, ATP-dependent protease La, Glucan biosynthesis protein D, LacI family transcriptional regulator, and glycine/betaine/L-proline ABC transporter caused a moderate increase (group 2) in lipolytic activity. Mutations in the remaining genes including genes coding for metabolic and regulatory functions caused a slight and consistent increase (group 1) in lipolytic activity (Table 2).

Several of Lap mutants are affected in growth or survival in planta. It was of interest to assess the possible role of identified *P. syringae* pv. *actinidiae* Tn5 mutants in survival and/or growth in kiwifruit leaves. All of the *P. syringae* pv. *actinidiae*

Fig. 1. Lipolytic activity score. Lipolytic activity is visible as clearance zone (halo) on the Luria Bertani (LB) agar-tributyryn plate. The zone of clearance of wild-type *Pseudomonas syringae* pv. *actinidiae* was used as a standard or no change in the level of lipolytic activity. Compared with the wild-type (WT) level, the increasing size of the zone of clearance on LB agar-tributyryn plate, lipolytic activity score was categorized from 1 to 3. A score of 3 indicates a maximum level of increase in the zone of clearance (lipolytic activity).

Fig. 2. Functional categorization of *Pseudomonas syringae* pv. *actinidiae* lipolytic activity altered phenotype mutants and associated open reading frames (ORFs). **A**, Distribution of number of Tn5 mutants in different functional categories based on analysis of all 56 lipolytic altered phenotype mutants. Semicolon (;) separates number of Tn5 mutants and their percent (%) distribution. **B**, Distribution of number of mutated genes (ORFs) in different functional categories based on analysis of all 38 ORFs in lipolytic activity altered phenotype screening. Semicolon (;) separates number of ORFs and their percent (%) distribution.

Lap mutants were inoculated on *A. deliciosa* kiwifruit leaves and bacterial colonization was determined at 0 h and after 3 and 7 DPI by bacterial count (CFU/0.5 cm²). At 0 h, there was no significant difference in the level of bacterial inoculum (0.5-1E+04 CFU/0.5 cm²) for all of the strains tested. The analysis of three rounds of experiments revealed that several *P. syringae* pv. *actinidiae* mutants were significantly impaired in in planta survival and/or growth compared with wild-type *P. syringae* pv. *actinidiae* at 3 and 7 DPI (Table 2). We assigned three different in planta survival and/or growth score categories to the mutants: (i) a “wild type” group that display in planta survival and/or growth phenotype similar to the wild type or within a 10-fold difference (CFU/0.5 cm² score 1E+07 and above either at 3 or 7 DPI), (ii) a fitness in planta deficient group displaying a 10- to 100-fold difference (CFU/0.5 cm² score less than 1E+07 and higher than 1E+06 either at 3 or 7 DPI), and (iii) in planta severely fitness deficient group displaying more than 100-fold difference compared with wild type (CFU/0.5 cm² score 1E+06 or less either at 3 or 7 DPI). Out of the 56 mutants total in which the disrupted gene could be localized, 27, 20, and 9 Lap mutants were placed into categories i, ii, and iii, respectively. In general, some metabolic functions were found to be associated with reduced growth or survival in planta. The mutants affected in genes encoding for transporters and cell division belonged to the middle category, whereas the mutants in the two-component regulatory genes displayed in most cases a phenotype similar to the wild type. A few mutants displayed a more significant difference in survival and/or growth in the kiwifruit leaves including loci involved in motility and metabolism (Table 2).

Validation of the results by regeneration of the mutants and their complementation. In order to validate the results, genes affected in some of the transposon mutants were regenerated by homologous recombination. The in planta fitness and lipolytic activity phenotypes of these mutants and the complemented strains were then verified. Mutations were generated in genes identified in the eight out of nine Tn5 mutants drastically affected in fitness in planta as described in the Materials and Methods section. Importantly, all the eight regenerated knock-out mutants were found to display similar phenotypic behavior as the Tn5 mutants isolated in the screen. These results validated the screen indicating that the genetic locus in which the Tn5 was inserted was responsible for the observed phenotype (Fig. 3). Four of these mutants originally isolated from the Tn5 genomic mutant library were also tested for the presence of a single Tn5 insertion in the chromosome; the four mutants had one Tn5 in their genome. Of the eight mutants complemented with a cosmid clone, five displayed complementation for plate phenotype while three also showed partial restoration of in planta phenotype (Supplementary Table S3). The lack of

complementation in some of these mutants could be due to the cosmid clone not containing all the genetic material necessary for the complementation. Another possibility could be due to multi-copy allele effects of these genes that may cause instability or fitness cost. In planta complementation could have also failed due to an instability of the plasmid since there is no in planta selection.

DISCUSSION

Despite the importance of *P. syringae* pv. *actinidiae* as an emerging phytopathogen of kiwifruit plants worldwide, to date no major genetic studies have been performed to understand its virulence functions. The aim of this study was to begin to study the genetic and molecular mechanisms employed by *P. syringae* pv. *actinidiae* to colonize the kiwifruit plant. The approach used was selection via a genetic screen of 30,000 Tn5 mutants for alteration in lipolytic activity phenotype; this resulted in the identification of 56 Tn5 mutants. Lipase secretion in *Pseudomonas* group of bacteria is known to be mediated by either type I or type II secretion systems (Rosenau and Jaeger 2000). We expected to find mutations in genes associated with these secretion systems with lipolytic minus phenotype in the screen. Although we found a few mutants with reduced lipolytic activity compared with the wild-type strain, these were also affected in growth. It was therefore postulated that the reduced lipolytic activity could have been due to a defect in growth and therefore we did not select them in this study. In the screen, none of them were completely abolished for lipolytic activity. This suggested that either we did not find a mutation in protein secretion system genes or *P. syringae* pv. *actinidiae* has different protein secretion systems for different lipases and mutation in any one of them did not result in lipolytic minus phenotype.

Lipolytic enzyme LipA is an important virulence factor in several phytopathogens including *Xanthomonas oryzae* pv. *oryzae* (Aparna et al. 2009; Jha et al. 2007), *X. campestris* pv. *vesicatoria* (Tamir-Ariel et al. 2012), *Burkholderia glumae* (Devescovi et al. 2007) as well as the contribution of lipolytic enzymes to virulence of fungal pathogens like *Alternaria brassicicola* (Berto et al. 1999), *Blumeria graminis* (Feng et al. 2009), and *Fusarium* spp. (Bravo-Ruiz et al. 2013; Voigt et al. 2005). Production of secreted enzymes usually employs global loci involved in virulence. In line with this, our screen identified 38 genes coding for metabolic, transport, and regulatory functions involved in modulation of lipolytic activity; many of these genes affected in planta survival or growth of *P. syringae* pv. *actinidiae*. How these genes modulate the lipolytic activity or *P. syringae* pv. *actinidiae* in planta growth/survival needs to be understood.

Lipase (triacylglycerol acylhydrolase, EC. 3.1.1.3) is an important class of enzymes that hydrolyze the carboxyl ester bonds in mono, di-, and triglycerides to liberate fatty acids and alcohols; they have been studied mostly for biotechnological applications. Very few genetic studies have been reported for production and regulation of lipase/esterase. One of these has shown the involvement of quorum sensing in *Burkholderia glumae* (Devescovi et al. 2007). In this study, several genes involved in regulation of lipolytic activity phenotype of *P. syringae* pv. *actinidiae* were identified and their possible role in

lipase biosynthesis/production/secretion is discussed here. Lap mutants with the mutation in genes coding for transport associated functions such as TolB and TolQ and lipopolysaccharide associated functions (LPS biosynthesis protein, lipid A biosynthesis lauroyl acyltransferase, AlgC, WbdD) showed markedly increased levels of lipolytic activity in our screen compared with other Lap mutants. These genes are known to be directly or indirectly involved in bacterial membrane integrity (Nikaido 2003).

Different from the transport and LPS biosynthesis genes, Lap mutants in genes coding for cell division associated FtsE and FtsX, DsbA, and DsbB, and a putative diene lactone hydrolase appear to affect lipolytic activity in *P. syringae* pv. *actinidiae* to a lesser extent. In previous studies, *ftsX*, *ftsE*, and *ftsX* of *Aeromonas* spp. have been implicated in chitinase production and transport functions (Ahmadian et al. 2007; de Leeuw et al. 1999). DsbA and DsbB are disulfide bond introducing proteins that are known to promote the oxidative folding of secreted proteins (Inaba 2009). DsbA and DsbC have been shown to affect extracellular enzyme formation in *P. aeruginosa*; the *dsbA* mutant was defective for lipase production whereas mutation in *dsbC* increased the secretion of lipase by twofold (Urban et al. 2001). A new esterase characterized in a strict *Vibrio* sp. marine bacterium (Park et al. 2007) showed primary structure similarity to a putative diene lactone hydrolase of *P. syringae* pv. *actinidiae* suggesting that this protein might have esterase/lipase activity. It is possible that due to a mutation in the gene coding for this lipase, *P. syringae* pv. *actinidiae* compensates by over production of other lipase/esterase(s).

Lastly, *P. syringae* pv. *actinidiae* Lap mutants affected in regulatory functions including two component regulators such as GacS and PhoQ showed the least increase in lipolytic activity. The global two-component system GacA/GacS responds to a yet unknown signal (could be endogenously produced) and regulates many phenotypes including quorum sensing systems in pseudomonads (Aparna et al. 2009; Reimann et al. 1997). Elevated accumulation of the PhoP response regulator on the other hand, has been reported in a *lipC* mutant of *P. aeruginosa* (Rosenau et al. 2010). This suggests that PhoP could be a negative regulator of lipolytic activity as a mutation in PhoP results in increased lipolytic activity in *P. syringae* pv. *actinidiae*. It cannot be excluded that phosphate levels regulate lipolytic activity in *P. syringae* pv. *actinidiae*.

The Lap mutants that are affected in fitness in planta were found mutated for functions mainly in loci encoding for glutamate-cysteine ligase (group iii Lap mutant), methyl-accepting chemotaxis protein (group ii and iii Lap mutant), lipid A biosynthesis lauroyl acyltransferase (group iii Lap mutant), phosphomannomutase AlgC (group ii and iii Lap mutant), thiol:disulfide interchange protein DsbA (group ii Lap mutants), type IV pilus protein PilI (group iii Lap mutant), Tol operon (group ii Lap mutant), HAD family hydrolase (group i and ii Lap mutants), and outer membrane porin OprF (group ii Lap mutant). The possible roles in fitness in planta of these loci is discussed below.

Glutamate-cysteine ligase catalyzes the first and rate limiting step in the biosynthesis of glutathione (Meister and Anderson 1983). Glutathione acts as the central redox buffer system and plays a major role in cellular homeostasis against various oxidative stresses (Meister and Anderson 1983). Therefore, the glutamate-cysteine ligase Lap mutant in *P. syringae* pv. *actinidiae* could be affected in survival/colonization under adverse/stress conditions in planta. Another function involved in redox homeostasis is *dsbA* which has been shown to be important for expression of the type III secretion system, intracellular survival and twitching motility (Ha et al. 2003). Thus, the virulence deficiency of *P. syringae* pv. *actinidiae* *dsbA* mutant could also possibly be due to its effect on type III effector proteins.

Functions involved in chemotaxis and twitching motility were also affected in fitness in planta. Methyl-accepting chemotaxis proteins are important for bacterial movement, biofilm formation, virulence, and competitive fitness in several bacteria (Ferrandez

Fig. 3. Lipolytic activity phenotype of phosphomannomutase AlgC mutant and complemented strain on Luria Bertani agar-tributyrin plate. Lipolytic activity is visible as clearance zone (halo) from WT, wild type; A, Tn5-phosphomannomutase AlgC mutant; B, pKnock-phosphomannomutase AlgC mutant; and C, complemented strain of phosphomannomutase AlgC mutant.

et al. 2002; Garvis et al. 2009; McLaughlin et al. 2012; Yao and Allen 2006). On the other hand, the type IV pili is required for host colonization and adherence, biofilm formation, antimicrobial tolerance, and pathogenicity functions (Roine et al. 1998; Romantschuk and Bamford 1986; Suoniemi et al. 1995; Taguchi and Ichinose 2011) and thus the Pili mutant of *P. syringae* pv. *actinidiae* might be affected in in planta survival and/or growth due to one or more of these reasons. The phosphomannomutase AlgC in *P. aeruginosa* is involved in the synthesis of alginate (a type of exopolysaccharide [EPS]) and LPS (Ye et al. 1994). EPS provides a selective advantage to bacteria in multiple ways, including water absorption, minerals and nutrients accumulation, protection from hydrophobic and toxic macromolecules (Denny 1995), biofilm formation, and virulence in several phytopathogenic bacteria, including *P. syringae* (Fett and Dunn 1989; Yu et al. 1999), *Ralstonia* (*Pseudomonas*) *solanacearum* (Kao et al. 1992; Saile et al. 1997), *Erwinia stewartii* (Dolph et al. 1988), and *X. campestris* (Katzen et al. 1998). Therefore, mutation in *algC* homolog of *P. syringae* pv. *actinidiae* might affect virulence due to loss of any of these functions.

Among the transport functions, outer membrane porin OprF is a multifunctional porin involved in nonspecific diffusion of ionic molecules and small polar nutrients, including the polysaccharides (Nestorovich et al. 2006). OprF might affect virulence due to its role in transport or adhesion to host (Azghani et al. 2002) and biofilm formation (Yoon et al. 2002). TolQ and TolB functions are part of the Tol-Pal (peptidoglycan-associated lipoprotein) system that form a membrane-spanning multiprotein complex. The Tol-Pal complexes have been associated with several functions including role in transport, membrane integrity, and virulence (Godlewski et al. 2009). In soft rot causing pathogen *Erwinia chrysanthemi*, TolB, TolQ, and other Tol function mutants were found to be affected in growth under stress conditions, in cell morphology, in motility, and in virulence (Dubuisson et al. 2005). Therefore, the transport and/or membrane integrity functions of the Tol system are important for *P. syringae* pv. *actinidiae* fitness in planta.

In summary, in this study several loci employed in the biosynthesis/modulation of lipolytic activity have been unraveled, several of which are involved in the colonization of kiwifruit. Our genetic approach taking advantage of a secreted visible phenotype will be useful to identify virulence/colonization functions of other bacteria. Further studies on these genes are required to begin to understand the molecular mechanisms underlying *P. syringae* pv. *actinidiae* pathogenesis process.

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