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* (*Psa*), an emerging pathogen of kiwifruit plants, has recently brought about major economic losses worldwide. Genetic studies on virulence functions of *Psa* 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 as 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 Tn*5* transposon mutants. Mapping all these Tn*5* 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 *Psa* Tn*5* mutants, notably the functions affected in phosphomannomutase AlgC, lipid A biosynthesis acyltransferase, glutamate--cysteine ligase and the type IV pilus protein PilI, 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 *Psa* are presented and discussed.

Keywords: *Pseudomonas syringae* pv. *actinidiae,* bacterial canker of kiwifruit, emerging pathogen, virulence loci

INTRODUCTION

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 *P. syringae* group contain model pathogens which are distinguished into at least 9 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 two 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* (*Psa*) is an emerging woody plant pathogen that has recently received considerable attention due to its increasing incidence and global spread (Scortichini et al., 2012). *Psa* 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 *Psa* was found on *Actinidiae chinensis* (kiwigold) plants in central Italy causing huge economic losses (Ferrante and Scortichini 2010). This outbreak was caused by a different population of *Psa* from the original one that caused fewer problems in 1992 (Marcelletti et al. 2011; Vanneste et al. 2010). *Psa* 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 (Service 2011) and Turkey (Bastas and Karakaya 2011).

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 (QS), two component systems and alternative sigma factors have also been found to play an important role in virulence of *Pseudomonas* pathogens. *Psa* studies are so far focused mainly on its identification and pathogen differentiation (Koh et al. 2014; Koh and Nou 2002; 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 tissues (Petriccione et al. 2013; Petriccione et al. 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 *Psa* virulence have been performed (Cellini et al. 2014), we decided to perform an exhaustive screen of a Tn*5* genomic mutant library of *Psa* 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. *Psa* 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 *Psa* 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. *Psa* strain 10,22 is clonal to the Psa CRAFRU strains which have been isolated in the province of Latina (central Italy) from spot lesions of *A. chinensis* (Ferrante and Scortichini, 2010). *Psa* strains were grown at 25°C in Luria Bertani (LB), Nutrient sucrose agar (NSA; composition for one litre: peptone 20g, sucrose 5g, K2HPO4 0.5gm, Mgso4.7H2O 0.25g, agar 12g) 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 *Psa* strains and Km, 50 µg/ml; Gm, 15 µg/ml; Tc, 15 µg/ml and Ampicillin (Ap), 100 µg/ml for *E. coli* strains. *Psa* 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 labelling 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 (Birnboim 1983); total DNA from *Psa* 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 *Psa* were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figurski and Helinski 1979).

Generation of a *Psa* genomic Tn*5* mutant library. Tn5 mutagenesis was performed by using triparental matings between donor *E. coli* (pSUP2021) containing the transposon Tn*5* (Km resistance), a helper *E. coli* strain (pRK2013) and recipient *Psa* strain. Briefly, *Psa* (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^{o}C. Cells of *Psa*, donor *E. coli* and helper *E. coli* were pelleted, washed twice in plain LB and re-suspended in 20 ml of sterile LB media. The absorbance of all three strains was measured at OD_{600} and cells were mixed in the following ratio: recipient *Psa*, $2x10^8$ colony forming units (CFU/ml); helper *E. coli*, $4x10^9$ CFU/ml; donor *E. coli*, $4x10^9$ CFU/ml. The mixture of cells was pelleted, re-suspended 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 re-suspended 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 2-3 days to allow the growth of exconjugants (Tn*5* mutants). The *Psa* genomic Tn*5* 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 *Psa* Tn*5* mutants for lipolytic activity. Lipolytic activity for *Psa* 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 60-80 Herts using a Bandeline Sonoplus

153 HD270 homogenizer, Berlin, D) until the solution became homogenous white. This
154 homogenous tributyrin mix was added to pre-warmed 400 ml of LB Agar media containing
155 Km antibiotic, mixed well and poured onto petri plates. *Psa* Tn*5* mutant colonies randomly
156 picked from plates were spotted one by one on LB Agar-tributyrin plates using a toothpick.
157 Plates were further incubated for 3-5 days at room temperature in order to see the halo
158 generated due to lipolytic activity. The halo size of *Psa* Tn*5* mutants was compared to wild
159 type halo size by visual inspection and the *Psa* Tn*5* mutants that showed any alteration in the
160 level of lipolytic activity were selected for a further round of screening. After three
161 confirmatory rounds of screening *Psa* Tn*5* mutants with altered lipolytic activity phenotype
162 were selected, grown in liquid LB broth with Nf + Km and their glycerol stocks were stored
163 at -80^{o}C. All mutants were grown in liquid in LB medium and did not show any significant
164 difference with respect to the wild type.
165 **Localization of Tn*5* insertion.** In order to clone DNA of *Psa* adjacent to the transposon
166 insertions, an arbitrary PCR was performed using a pair of primers listed in Supplementary
167 Table S2 as previously described (O'Toole and Kolter 1998) with some modifications.
168 Arbitrary PCR products were purified and directly sequenced using Tn*5* specific Tn*5*-
169 Intprimer. Genomic DNA was also isolated from selected *Psa* Tn*5* mutants and double
170 digested either with BamHI + EcoRI, BamHI + SacI or BamHI + ClaI. These double digested
171 products were ligated in pBluescript (double digested with the similar set of enzymes),
172 transformed into DH5α *E. coli* cells and selected on LB agar plates with Ap + Km.
173 Sequences obtained were subjected to homology searches using NCBI Blast with the draft
174 genome sequence of *Psa* (str. M302091; taxid:629266). The exact position and orientation of
175 the Tn*5* insertions were mapped in *Psa* draft genome. In order to test if mutants have single
176 Tn*5* insertion, the Southern analysis was performed on mutants Lap38, Lap41, Lap45 and

Lap 276 using radio labeled Tn*5* DNA fragment as a probe. It was confirmed that all these mutants contained a single Tn*5* insertion in their chromosome (Supplementary Figure S1).

Regeneration of knock-out mutants and complementation. The selected Tn*5* mutants were regenerated via single homologous recombination using the pKNOCK-Km suicide delivery system. Briefly, internal fragments of the loci were PCR amplified using primers listed in Supplementary Table S2 and cloned into a pGEMT-easy vector. EcoRI digested internal fragments were ligated to EcoRI digested pKNOCK-Km and transformed into *E. coli* C118 λpir cells yielding pKNOCK plasmids having internal fragments from selected Tn*5* loci (Supplementary Table S1). 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 38, Lap 41, Lap 42, Lap 45, Lap 169, Lap 200, Lap 208 and Lap 276) generated using this strategy were selected on NSA plates supplemented with Nf + Km. *Psa* mutant strains were verified by PCR analysis and sequencing.

In order to complement the selected *Psa* Tn*5* mutants, a cosmid library of *Psa* 10,22 strain was constructed by using the cosmid pLAFR3 (Staskawicz et al. 1987) as a vector. Insert DNA was prepared by partial EcoRI 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 radiolabelled 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.** *Psa in planta* assay was performed as described previously (Patel et al. 2014). Briefly, one-year-old, potted plants of *A. deliciosa* cv. Hayward were maintained in a climatic room and watered regularly. For inoculation, *Psa* strains were grown for 48 h on NSA medium supplemented with antibiotics, at 23-25°C. Bacterial cultures were pelleted, washed with sterile saline (0.85% NaCl in distilled water) and adjusted to $1–2\times10^6$ 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 of diameter were sampled from the inoculation site immediately after infiltration (at 0 h) then ground in 1 ml of sterile saline, and serial ten-fold dilutions were plated onto NSA supplemented with antibiotics. Colonies were counted two days after incubation at 23-25°C. CFU/0.5 cm^2 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 3rd and 7th day post inoculation. Confirmation of colony identity was achieved by following well established phenotypic and molecular procedures including duplex-PCR (Ferrante and Scortichini 2009; Ferrante and Scortichini 2010; Galelli et al. 2011).

RESULTS

Psa genes involved in lipolytic activityare functionally diverse

It was of interest to determine plate visible phenotypes of *Psa* which could provide a convenient screen to identify mutants of potential virulence loci. *Psa* strains were found to exhibit secreted lipase/esterase activity on plate media (Supplementary Figure S2). It was therefore decided to screen for mutants altered in lipolytic activity with the aim of identifying secretion and global regulatory mutants which are important for *Psa* virulence. A genome wide Tn*5* mutagenesis and screening experiment for detecting alteration in lipolytic activity were therefore set up in *Psa*. A Tn*5* mutant library of *Psa* was generated as described in Materials and Methods section and 30,000 mutants were tested for alteration in lipolytic activity on LB Agar-tributyrin plates (Supplementary Figure S2). In the first round of screening, a total of 254 mutants were obtained with lipolytic altered phenotype (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 *Psa*. In fact, the genome sequence of several *Psa* strains including *Psa* 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 3 different groups from 1 to 3, in increasing order (Table 2, Figure 1). Out of a total of 56 mutants in which the position of the Tn5 insertion could be localized (see

247 below), 27 Lap mutants were classified into group 1, 17 Lap mutants were group 2 and 12
248 Lap mutants were group 3.

249 The position of Tn*5* insertion sites was determined using an arbitrary PCR method as
250 described in the Materials and Methods section; this was successful for 56/65 Lap mutants
251 (Table 2). For the remaining 9 Lap mutants a molecular cloning approach was used without
252 success as described in the Materials and Methods. The reason for this is not known and it is
253 most likely the methodology used had limitations that are currently unknown. The 56 Lap
254 mutants were localized to 38 different ORFs (several Lap mutants were isolated more than
255 once) belonging to 8 different functional categories: cell division, chemotaxis, hypothetical
256 proteins, metabolism, motility, recombination, regulation and signal transduction and
257 transport (Table 2; Figure 2 and Supplementary Figure S3). Mutations in genes encoding for
258 Lipid A biosynthesis, MotA/TolQ/ExbB and TolB mediated transport, chemotaxis and porin
259 OprF caused a drastic increase (group 3) in lipolytic activity whereas mutation in genes
260 encoding cell division proteins FtsX and FtsE, putative hydrolases, redox homeostasis
261 functions like GshA, DsbA and DsbB, Phosphomannomutase AlgC, Putative lipoprotein,
262 ATP-dependent protease La, Glucan biosynthesis protein D, LacI family transcriptional
263 regulator and glycine/betaine/L-proline ABC transporter, caused a moderate increase (group
264 2) in lipolytic activity. Mutations in the remaining genes including genes coding for
265 metabolic and regulatory functions caused a slight and consistent increase (group 1) in
266 lipolytic activity (Table 2).

267 **Several of Lap mutants are affected in growth or survival *in planta***

268 It was of interest to assess the possible role of identified *Psa* Tn*5* mutants in survival
269 and/or growth in kiwifruit leaves. All the *Psa* Lap mutants were inoculated on *A. deliciosa*
270 kiwifruit leaves and bacterial colonization was determined at 0 h and after the 3rd and 7th day
271 post inoculation by bacterial count (CFU/0.5cm^2). At 0 h, there was no significant difference

272 in the level of bacterial inoculum (0.5-1E+04 CFU/0.5cm^2) for all the strains tested. The
273 analysis of three rounds of experiments revealed that several *Psa* mutants were significantly
274 impaired in *in planta* survival and/or growth compared to wild type *Psa* at 3rd and 7th day post
275 inoculation (Table 2). We assigned three different *in planta* survival and/or growth score
276 categories to the mutants; (i) a 'wild type' group which display *in planta* survival and/or
277 growth phenotype similar to the wild-type or within a 10 fold difference (CFU/ 0.5cm^2 score
278 1E+07and above either at 3 or 7 DAI), (ii) a fitness *in planta* deficient group displaying a 10-
279 100 fold difference (CFU/0.5cm^2 score less than 1E+07 and higher than 1E+06 either at 3 or 7
280 DAI) and (iii) a severely fitness *in planta* deficient group displaying more than 100 fold
281 difference compared to wild-type (CFU/0.5cm^2 score 1E+06 or less either at 3 or 7 DAI). Out
282 of the total 56 mutants in which the disrupted gene could be localized, 27, 20 and 9 Lap
283 mutants were placed into categories i, ii and iii respectively. In general, some metabolic
284 functions were found to be associated with reduced growth or survival *in planta*. The mutants
285 affected in genes encoding for transporters and cell division belonged to the middle category
286 whereas the mutants in the two-component regulatory genes displayed in most cases a
287 phenotype similar to the wild type. A few mutants displayed a more significant difference in
288 survival and/or growth in the kiwifruit leaves including loci involved in motility and
289 metabolism (Table 2).

290 **Validation of the results by regeneration of the mutants and their complementation**

291 In order to validate the results, genes affected in some of the transposon mutants were
292 regenerated by homologous recombination. The *in planta* fitness and lipolytic activity
293 phenotypes of these mutants and the complemented strains were then verified. Mutations
294 were generated in genes identified in the eight out of nine Tn*5* mutants drastically affected in
295 fitness *in planta* as described in the Materials and Methods section. Importantly, all the eight
296 regenerated knock-out mutants were found to display similar phenotypic behavior as the Tn*5*

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 (Figure 3, Supplementary Figure S3 and Table 3). Four of these mutants originally isolated from the Tn*5* genomic mutant library were also tested for the presence of a single Tn*5* insertion in the chromosome; as depicted in Supplementary Figure S1 the four mutants had one Tn*5* 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 3). 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 multicopy allele effects of these genes which 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 *Psa* 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 *Psa* to colonize the kiwifruit plant. The approach used was selection via a genetic screen of 30,000 Tn*5* mutants for alteration in lipolytic activity phenotype; this resulted in the identification of 56 Tn*5* 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 than 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 *Psa* 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), *Xanthomonas. 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 *Psa*. How these genes modulate the lipolytic activity or *Psa in planta* growth/survival needs to be understood.

Lipase (triacylglycerol acylhydrolase, EC. 3.1.1.3) is an important class of enzymes that hydrolyzes the carboxyl ester bonds in mono-, di- and tri-glycerides 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 *Psa* 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 to other Lap mutants. These genes are known to be directly or indirectly involved in bacterial membrane integrity (Nikaido 2003).

Differently 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 dienelactone hydrolase appear to affect lipolytic activity in *Psa* 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

356 enzyme formation in *P. aeruginosa*; the *dsbA* mutant was defective for lipase production
357 whereas mutation in *dsbC* increased the secretion of lipase by two fold (Urban et al. 2001). A
358 new esterase characterized in a strict *Vibrio* sp. marine bacterium (Park et al. 2007) showed
359 primary structure similarity to a putative dienelactone hydrolase of *Psa* suggesting that this
360 protein might have esterase/lipase activity. It is possible that due to a mutation in the gene
361 coding for this lipase *Psa* compensates by over production of other lipase/esterase(s).

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

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

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

388 Functions involved in chemotaxis and twitching motility were also affected in fitness
389 *in planta*. Methyl-accepting chemotaxis proteins (MCPs) are important for bacterial
390 movement, biofilm formation, virulence and competitive fitness in several bacteria
391 (Ferrandez et al. 2002; Garvis et al. 2009; McLaughlin et al. 2012; Yao and Allen 2006);
392 On the other hand, the type IV pili is required for host colonization and adherence, biofilm
393 formation, antimicrobial tolerance and pathogenicity functions (Roine et al. 1998;
394 Romantschuk and Bamford 1986; Suoniemi et al. 1995; Taguchi and Ichinose 2011) and
395 thus the PilI mutant of *Psa* might be affected for *in planta* survival and/or growth due to one
396 or more of these reasons. The phosphomannomutase AlgC in *P. aeruginosa* is involved in the
397 synthesis of alginate (a type of exopolysaccharide; EPS) and LPS (Ye et al. 1994). EPS
398 provides a selective advantage to bacteria in multiple ways, including water absorption,
399 minerals and nutrients accumulation, protection from hydrophobic and toxic macromolecules
400 (Denny 1995), biofilm formation and virulence in several phytopathogenic bacteria, including
401 *P. syringae* (Fett and Dunn 1989; Yu et al. 1999), *Ralstonia* (*Pseudomonas*) *solanacearum*
402 (Kao et al. 1992; Saile et al. 1997), *E. stewartii* (Dolph et al. 1988) and *X. campestris*

(Katzen et al. 1998). Therefore mutation in *algC* homolog of *Psa* 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 (Godlewska et al. 2009). In soft rot causing pathogen *Erwinia chrysanthemi*, TolB, TolQ and other Tol function mutants were found affected for growth under stress conditions, cell morphology, motility and virulence (Dubuisson et al. 2005). Therefore the transport and/or membrane integrity functions of the Tol system are important for *Psa* 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 *Psa* pathogenesis process.

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FIGURE LEGENDS

Figure 1: Lipolytic activity score.

Lipolytic activity is visible as clearance zone (halo) on the LB Agar-tributyrin plate. The zone of clearance of wild-type *Psa* was used as a standard or no change in the level of lipolytic activity. Compare to the wild-type level, the increasing size of the zone of clearance on LB Agar-tributyrin plate, lipolytic activity score was categorized from 1 to 3. Score 3 indicates a maximum level of increase in the zone of clearance (lipolytic activity).

Figure 2. Functional categorization of *P. syringae* pv. *actinidiae* lipolytic activity altered phenotype (Lap) mutants and associated ORFs. A: Distribution of number of Tn*5* mutants in different functional categories based on analysis of all 56 Lap mutants. Sign of semicolon (;) separates number of Tn*5* mutants and their percentage (%) distribution. B: Distribution of number of mutated genes (ORFs) in different functional categories based on analysis of all the 38 ORFs in lipolytic activity altered phenotype screening. Sign of semicolon (;) separates number of ORFs and their percentage (%) distribution.

Figure 3. Lipolytic activity phenotype of phosphomannomutase AlgC mutant and complemented strain on LB Agar-tributyrin plate. Lipolytic activity is visible as clearance zone (halo) from WT: wild type, A: Tn*5*-phosphomannomutase AlgC mutant, B: pKnock-phosphomannomutase AlgC mutant and C: Complemented strain of phosphomannomutase AlgC mutant.

Supplementary Figure S1: Southern blotting of both wild type and Tn*5* mutants with radioactive Tn*5* fragment as probe.

Genomic DNA of *Psa* wild-type (WT) and four randomly selected Tn*5* mutants; Lap38 (A), Lap41 (B), Lap45 (C) and Lap276 (D) were isolated and digested using KpnI (NEB). These samples were subjected to Southern blot analysis using radioactive labeled Tn*5* fragment as a probe. No band in WT (negative control) and a single band in the lanes A, B, C and D indicate one copy of Tn*5* insertion in these mutants. M stand for DNA ladder.

Supplementary Figure S2: Lipolytic activity and screening of Tn*5* mutants.

A. Lipolytic activity of wild-type *Psa* (strain 10,22) on LB Agar-tributyrin plate.

B. Screening of *Psa* Tn*5* mutants for altered lipolytic activity phenotype.

LB Agar-tributyrin plates (1 ml of tributyrin in 400 ml of LB Agar) were used to assess the lipolytic activity of WT and Tn*5* mutants of *Psa*. The *Psa* Tn*5* mutants were spotted one by one on LB Agar-tributyrin plates using a toothpick and incubated for 3-5 days at room temperature. The halo observed, indicate lipolytic activity. In the screening, the halo size of *Psa* Tn*5* mutants was compared to wild-type halo size by visual inspection and the *Psa* Tn*5* mutants that showed any alteration in the level of lipolytic activity were selected.

Supplementary Figure S3: Number of Lap mutants localized to each ORFs in lipolytic activity altered phenotype screening.

The frequency of mutants obtained in each of the identified ORF was plotted based on localization of the Tn*5* insertion in the 56 Lap mutants.

769 Table 1. Bacterial strains used in this study

Strains	Relevant characteristics[a]	Reference/Source
Escherichia coli		
$DH_5\alpha$	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 tri-parental conjugation, Km^r	(Figurski and Helinski 1979)
***Pseudomonas syringae* pv. *actinidiae* (*Psa*)**		
Psa 10,22	Wild type; Italian isolate; Nf^r	Lab collection
Psa Lap mutants	*Psa*::Tn*5* 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	Lap 41::pKNOCK; Nf^r, Km^r; derivative of wild type	This work
Psa Lap42- pKNOCK	Lap 42::pKNOCK; Nf^r, Km^r; derivative of wild type	This work
Psa Lap45- pKNOCK	Lap 45::pKNOCK; Nf^r, Km^r; derivative of wild type	This work
Psa Lap169- pKNOCK	Lap 169::pKNOCK; Nf^r, Km^r; derivative of wild type	This work
Psa Lap200- pKNOCK	Lap 200::pKNOCK; Nf^r, Km^r; derivative of wild type	This work
Psa Lap208- pKNOCK	Lap 208::pKNOCK; Nf^r, Km^r; derivative of wild type	This work
Psa Lap276- pKNOCK	Lap 276::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.

770 **Table 2: The details of Tn*5* mutants and their *in planta* survival and/or growth score**

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Details of Tn*5* mutants obtained in the screening of altered lipolytic activity							
Tn*5* mutants	**Lipolytic activity score[a]**	**Tn*5* insertion position[b]**	**Orientation of Tn*5* insertion[c]**	**ORF/ Protein ID of Tn*5* insertion[d]**	**ORF length[e]**	**Blast-X information (ORF function)[f]**	**Functional Categorizat**
Lap20	1	2429	+/-	EGH66838.1	2754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction
Lap33	1	2517	+/+	EGH66838.1	2754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction
Lap35	1	747	+/-	EGH67056.1	837	Hypothetical protein	Hypothetical
Lap36	1	404	+/+	EGH64339.1	897	site-specific tyrosine recombinase XerD	Recombination
Lap37	1	354	+/-	EGH64482.1	873	XerC	Recombination
Lap38	1	113	+/-	EGH67827.1	813	Septum site determining protein minD	Cell division
Lap52	1	590	+/-	EGH66838.1	2754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction
Lap54	1	167	+/-	EGH67427.1	1113	Glycosyl transferase	Metabolism
Lap55	1	544	+/-	EGH65930.1	1788	Sensory box histidine kinase	Regulation and signal transduction
Lap62	1	905	+/-	EGH66807.1	2493	Helicase domain containing protein	Metabolism
Lap71	1	1048	+/+	EGH66370.1	1176	3-ketoacyl-CoA thiolase	Metabolism
Lap73	1	278	+/-	EGH68302.1	747	HAD- superfamily hydrolase	Metabolism
Lap81	1	288	+/+	EGH65338.1	528	Disulfide bond formation protein DsbB	Metabolism
Lap82	1	502	+/-	EGH66828.1	1347	Sensor protein PhoQ	Regulation and signal transduction
Lap83	1	713	+/-	EGH66147.1	1938	Cobaltochelatase subunit CobN	Metabolism
Lap84	1	1200	+/+	EPM67430.1	1683	Helicase, putative	Metabolism
Lap106	1	3	+/+	EGH67057.1	378	Hypothetical protein	Hypothetical
Lap108	1	401	+/-	EGH66177.1	642	Hypothetical protein	Hypothetical
Lap121	1	375	+/-	EGH66177.1	642	Hypothetical protein	Hypothetical
Lap131	1	428	+/+	EGH66177.1	642	Hypothetical protein	Hypothetical
Lap153	1	437	+/+	EGH66177.1	642	Hypothetical protein	Hypothetical
Lap161	1	181	+/+	EGH65029.1	801	Sec-independent protein translocase TatC	Transport
Lap194	1	283	+/-	EGH68302.1	747	HAD family hydrolase	Metabolism
Lap195	1	415	+/+	EGH64055.1	540	Type IV pilus protein pill	Motility
Lap249	1	66	+/+	EGH65067.1	825	Glycosyl transferase, group 4 family protein	Metabolism
Lap276	1	422	+/-	EGH66838.1	2754	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction
LapC2	1	723	+/-	EPM47746.1	1164	phosphoglycerate kinase	Metabolism
LapC3	1	288	+/+	EGH65338.1	528	Disulfide bond formation protein DsbB	Metabolism
Lap3	2	600	+/-	EGH67743.1	888	Dienelactone hydrolase family protein	Metabolism

Lap18	2	832	+/+	EGH65110.1	1119	Putative lipoprotein	Metabolism
Lap34	2	29	+/+	EGH65738.1	846	glycine/betaine/L-proline ABC transporter,	Transport
Lap39	2	672	+/-	EGH65700.1	672	Cell division ATP-binding protein FtsE	Cell division
Lap45	2	208	+/+	EGH65936.1	645	Thiol:disulfide interchange protein DsbA	Metabolism
Lap53	2	903	+/+	EGH64518.1	1398	Phosphomannomutase AlgC	Metabolism
Lap60	2	750	+/-	EGH64518.1	1398	Phosphomannomutase AlgC	Metabolism
Lap74	2	2208	+/-	EGH63578.1	2397	ATP-dependent protease La	Metabolism
Lap76	2	155	+/-	EGH64016.1	735	LPS biosynthesis protein	Metabolism
Lap78	2	269	+/-	EGH64681.1	1620	Glucan biosynthesis protein D	Metabolism
Lap79	2	475	+/+	EGH67201.1	966	LacI family transcriptional regulator	Regulation and signal transduction
Lap105	2	861	+/+	EGH65701.1	1035	Cell division protein FtsX	Cell division
Lap107	2	856	+/+	EGH65701.1	1035	Cell division protein FtsX	Cell division
Lap168	2	1201	+/+	EGH64518.1	1398	Phosphomannomutase AlgC	Metabolism
Lap169	2	1115	+/+	EGH64518.1	1398	Phosphomannomutase AlgC	Metabolism
Lap208	2	1130	+/-	EGH65920.1	1590	Glutamate--cysteine ligase	Metabolism
Lap13	3	674	+/-	EGH65387.1	880	Lipid A biosynthesis lauroyl acyltransferase	Metabolism
Lap14	3	166	+/-	EGH65087.1	696	MotA/TolQ/ExbB proton channel	Transport
Lap15	3	165	+/-	EGH65087.1	696	MotA/TolQ/ExbB proton channel	Transport
Lap16	3	558	+/+	EGH65090.1	1302	Translocation protein TolB	Transport
Lap17	3	515	+/-	EGH65087.1	696	MotA/TolQ/ExbB proton channel	Transport
Lap41	3	840	+/+	EGH67601.1	1035	Outer membrane porin OprF	Transport
Lap42	3	621	+/+	EGH65387.1	880	Lipid A biosynthesis lauroyl acyltransferase	Metabolism
Lap61	3	1092	+/+	EPM75923.1	1548	Wbdd	Metabolism
Lap77	3	161	+/+	EGH67825.1	553	Lipid A biosynthesis lauroyl acyltransferase	Metabolism
Lap176	3	867	+/+	EGH65490.1	1305	Methyl-accepting chemotaxis protein	Chemotaxis
Lap188	3	322	+/-	EGH67825.1	933	Lipid A biosynthesis lauroyl acyltransferase	Metabolism
Lap200	3	868	+/+	EGH65490.1	1305	Methyl-accepting chemotaxis protein	Chemotaxis

Score is close to category i
Lap: Lipolytic activity altered phenotype,
a: Lipolytic activity score of Tn*5* mutants on LBA-tributyrin plates (score scale from 1 to 3)
b: position of Tn*5* insertion in the ORF
c: Orientation of Tn*5* insertion with respect to ORF
d: Protein ID of ORF in which Tn*5* transposon was found inserted
e: Length of ORF (in bp) in which Tn*5* transposon was found inserted
f: Annotated function of ORF in which Tn*5* transposon was found inserted
g: Functional categorization assigned to the functions
h: *In planta* survival and/or growth score (CFU/ml) at 3rd and 7th day after inoculation (DAI). At 0 h, there was no significant difference in the level of bacterial inoculum (0.5E+04 to 1E+04 CFU/0.5 cm^2 leaf area) for all the strains tested.

i: *In planta* survival and/or growth category (i: a 'wild type' group which display *in planta* phenotype similar to the wild-type or within a 10 fold difference to wild type [CFU/0.5 cm^2 leaf area score 1E+07 and above either at 3 or 7 days after inoculation (DAI)], ii: a fitness *in planta* deficient group displaying a 10-100 fold difference (CFU/ 0.5 cm^2 score less than 1E+07 and higher than 1E+06 either at 3 or 7 DAI) and iii: a severely fitness *in planta* deficient group displaying more than 100 fold difference compared to wild-type (CFU/ 0.5 cm^2 leaf area score 1E+06 or less either at 3 or 7 DAI).

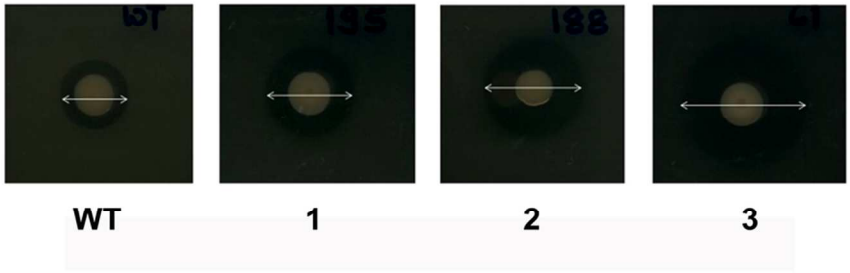

Figure 1. Lipolytic activity score

Figure 1: Lipolytic activity score.
Lipolytic activity is visible as clearance zone (halo) on the LB Agar-tributyrin plate. The zone of clearance of wild-type Psa was used as a standard or no change in the level of lipolytic activity. Compare to the wild-type level, the increasing size of the zone of clearance on LB Agar-tributyrin plate, lipolytic activity score was categorized from 1 to 3. Score 3 indicates a maximum level of increase in the zone of clearance (lipolytic activity).

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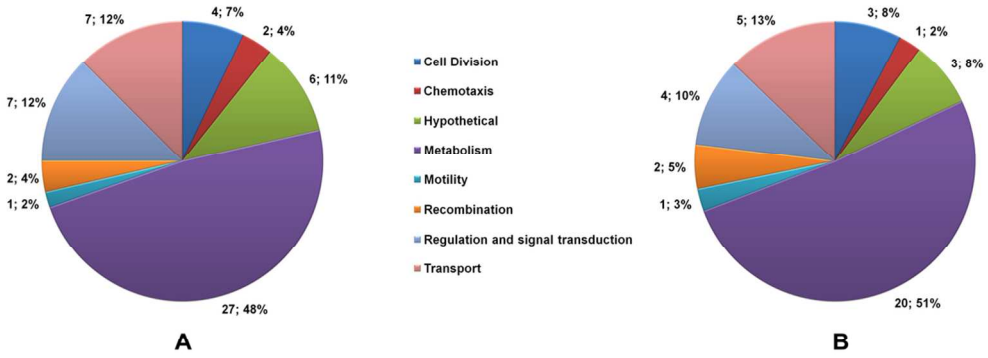

Figure 2. Functional categorization of *P. syringae* pv. *actinidiae* lap mutants and associated ORFs

Figure 2. Functional categorization of P. syringae pv. actinidiae lipolytic activity altered phenotype (Lap) mutants and associated ORFs. A: Distribution of number of Tn5 mutants in different functional categories based on analysis of all 56 Lap mutants. Sign of semicolon (;) separates number of Tn5 mutants and their percentage (%) distribution. B: Distribution of number of mutated genes (ORFs) in different functional categories based on analysis of all the 38 ORFs in lipolytic activity altered phenotype screening. Sign of semicolon (;) separates number of ORFs and their percentage (%) distribution.

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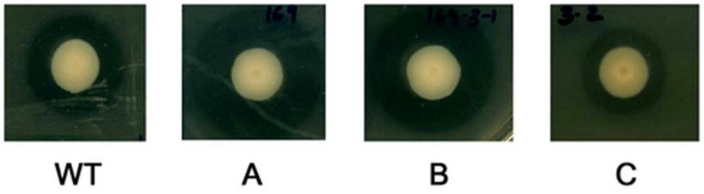

Figure 3. Lipolytic activity phenotype of phosphomannomutase AlgC mutant and complemented strain on LB Agar-tributyrin plate.

Figure 3. Lipolytic activity phenotype of phosphomannomutase AlgC mutant and complemented strain on LB 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.

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Supplementary Table S1. Plasmids used in this study

Plasmids	Relevant characteristics[a]	Reference/ Source[b]
pGEMT-easy	Cloning vector; Amp^r	Promega
pKNOCK-Km	Conjugative suicide vector; Km^r	(Alexeyev, 1999)
pLAFR3	Broad-host-range cloning vector, IncP1; Tc^r	(Staskawicz *et al.*, 1987)
Lap38-pGEMT	pGEMT-easy vector containing internal region from Lap38	This study
Lap41-pGEMT	pGEMT-easy vector containing internal region from Lap41	This study
Lap42-pGEMT	pGEMT-easy vector containing internal region from Lap42	This study
Lap45-pGEMT	pGEMT-easy vector containing internal region from Lap45	This study
Lap169-pGEMT	pGEMT-easy vector containing internal region from Lap169	This study
Lap200-pGEMT	pGEMT-easy vector containing internal region from Lap200	This study
Lap208-pGEMT	pGEMT-easy vector containing internal region from Lap208	This study
Lap276-pGEMT	pGEMT-easy vector containing internal region from Lap 276	This study
Lap38-pKNOCK	pKNOCK-Km vector containing internal region from Lap38	This study
Lap41-pKNOCK	pKNOCK-Km vector containing internal region from Lap41	This study
Lap42-pKNOCK	pKNOCK-Km vector containing internal region from Lap42	This study
Lap45-pKNOCK	pKNOCK-Km vector containing internal region from Lap45	This study
Lap169-pKNOCK	pKNOCK-Km vector containing internal region from Lap169	This study
Lap200-pKNOCK	pKNOCK-Km vector containing internal region from Lap200	This study
Lap208-pKNOCK	pKNOCK-Km vector containing internal region from Lap208	This study
Lap276-pKNOCK	pKNOCK-Km vector containing internal region from Lap276	This study
Lap38-pCos	Cosmid clone for Lap38	This study
Lap41-pCos	Cosmid clone for Lap41	This study
Lap42-pCos	Cosmid clone for Lap42	This study
Lap45-pCos	Cosmid clone for Lap45	This study
Lap169-pCos	Cosmid clone for Lap169	This study
Lap200-pCos	Cosmid clone for Lap200	This study
Lap208-pCos	Cosmid clone for Lap208	This study
Lap276-pCos	Cosmid clone for Lap276	This study

a; Nal^r, Nf^r Km^r, Tc^r and Amp^r indicates for nalidixic acid, nitrofurantoin, kanamycin, tetracycline and ampicillin respectively.

b; Reference:

Alexeyev, M. F. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. BioTechniques. 26 (5): 824-826, 828.

Staskawicz, B., Dahlbeck, D., Keen, N. & Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169: 5789-5794.

Supplementary Table S2. List of primers used in this study

Primers	Sequence (5' to 3')	Source
Tn5-Ext	GAACGTTACCATGTTAGGAGGTC	Lab collection
Tn5-Int	CGGGAAAGGTTCCGTTCAGGACGC	Lab collection
Arb-1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	Lab collection
Arb-2	GGCCACGCGTCGACTAGTAC	Lab collection
SP6	ATTTAGGTGACACTATAG	Lab collection
T7	TAATACGACTCACTATAGGG	Lab collection
pKNOCK-New F	CTTAACCGCTGACATGGGAA	Lab collection
pKNOCK-New R	TTTATTCGGACACGCGTCCT	Lab collection
Lap38-Int-Fw	AGCCAACCTGCAACAGGCTTT	This study
Lap38-Int-Rv	CGTCTTCAACGCCGAGCATCT	This study
Lap41-Int-Fw	TCTTGATGTCGCCCATGCTGTTAG	This study
Lap41-Int-Rv	GTCCATACGTTTCTGCCGGTTTCT	This study
Lap42-Int-Fw	TTCGGTACGAACTTGCTGGTCA	This study
Lap42-Int-Rv	TGGACATCGGCAAGACACTCA	This study
Lap45-Int-Fw	GTTGGAACGCCGGTTACTTCGTAT	This study
Lap45-Int-Rv	ATCGAAGTCGTTGAGCTGTTCTGG	This study
Lap169-Int-Fw	CTTGATCAGCGAGTGACCGGTTTT	This study
Lap169-Int-Rv	ACGAGCGCATCAAGACCAACA	This study
Lap200-Int-Fw	GTCCAGCACGTCAGGAAACTCTT	This study
Lap200-Int-Rv	CGGCTTCATCCACAGGACAAACA	This study
Lap208-Int-Fw	CAATACAGCACGAAGGCGTCGAT	This study
Lap208-Int-Rv	CGACTACCAGTCGCACTCCTACAT	This study
Lap276-Int-Fw	GCACAGTACCATCACCTTGCAGTT	This study
Lap276-Int-Rv	CATCGAGAAATCTGCCGACAACCT	This study

10 **Supplementary Table S3: Lipolytic activity and *in planta* survival and/or growth score of the mutant and complementation strains**

Strain[a]	Function[b]	Functional categorization[c]	*In planta* survival and/or growth score[d]		Summary of complementation for *in planta* survival and/or growth [e]	Lipolytic score[f]	Summary of complementation for lipolytic activity phenotype[g]
			3 DAI	7DAI			
WT			6.47E+07	1.36E+08		WT	
Lap38	Septum site determining protein minD	Cell division	4.33E+05	6.50E+05		1	
Lap38-pKNOCK			5.63E+05	8.45E+05		1	
Lap38-pCos			1.67E+03	2.50E+03	NC	WT	C
Lap41	Outer membrane porin OprF	Transport	1.40E+04	1.26E+05		3	
Lap41-pKNOCK			2.07E+05	5.17E+05		3	
Lap41-pCos			7.00E+02	1.75E+03	NC	3	NC
Lap42	Lipid A biosynthesis acyltransferase	Metabolism	3.00E+03	5.10E+03		3	
Lap42-pKNOCK			1.47E+03	3.67E+03		3	
Lap42-pCos			9.67E+03	2.42E+04	PC	WT	C
Lap45	Thiol:disulfide interchange protein DsbA	Metabolism	1.13E+06	1.93E+06		2	
Lap45-pKNOCK			2.07E+04	1.86E+05		2	
Lap45-pCos			3.53E+04	5.30E+04	NC	WT	C
Lap169	Phosphomannomutase AlgC	Metabolism	6.00E+02	9.00E+02		2	
Lap169-pKNOCK			9.67E+03	2.42E+04		2	
Lap169-pCos			1.27E+05	3.17E+05	PC	WT	C
Lap200	Methyl-accepting chemotaxis protein	Chemotaxis	3.57E+06	3.21E+07		1	
Lap200-pKNOCK			3.27E+05	4.90E+05		1	
Lap200-pCos			1.10E+05	2.75E+05	NC	1	NC
Lap208	Glutamate--cysteine ligase	Metabolism	9.00E+04	3.97E+05		2	
Lap208-pKNOCK			6.00E+03	5.40E+04		2	
Lap208-pCos			9.10E+05	1.55E+06	PC	WT	C
Lap276	Sensor histidine kinase/response regulator GacS	Regulation and signal transduction	2.53E+06	6.33E+06		1	

Lap276-pKNOCK			5.67E+05	2.50E+06		1	
Lap276-pCos			1.20E+05	3.01E+05	NC	1	NC

a: Strains; WT: wild type, Lap: lipolytic activity altered phenotype, strains with pKNOCK: pNKOCK generated mutants, strains with pCos: complemented strains using cosmid clone

b: Annotated function of ORF in which Tn*5* transposon was found inserted

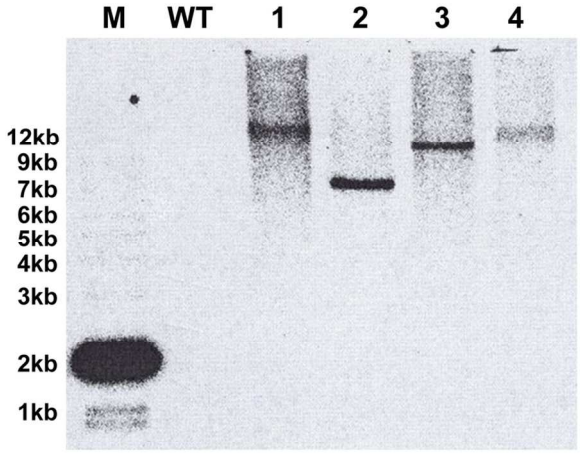
c: Functional categorization assigned to the functions

d: *In planta* survival and/or growth score (CFU/0.5 cm^2 leaf area) at 3^{rd} and 7^{th} day after inoculation (DAI). At 0 h, there was no significant difference in the level of bacterial inoculum (0.5E+04 to 1E+04 CFU/0.5cm^2 leaf area) for all the strains tested.

e: Summary of complementation for *in planta* survival and/or growth score; C: Complemented, NC: Not complemented, PC: Partially complemented

f: Lipolytic activity score (wt: similar to wild type and score scale from 1 to 3)

g: Summary of complementation for lipolytic activity score; C: Complemented, NC: Not complemented,



Supplementary Figure S1. Southern blotting of both wild type and mutants with radioactive labeledTn*5* fragment as a probe.

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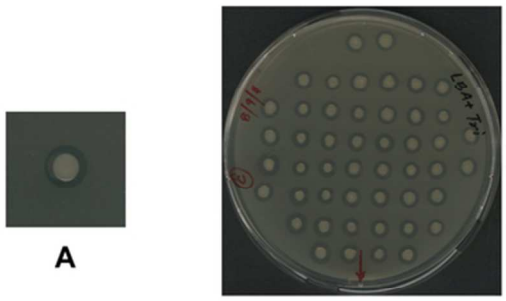

A B

Supplementary Figure S2. Lipolytic activity and screening of Tn*5* mutants

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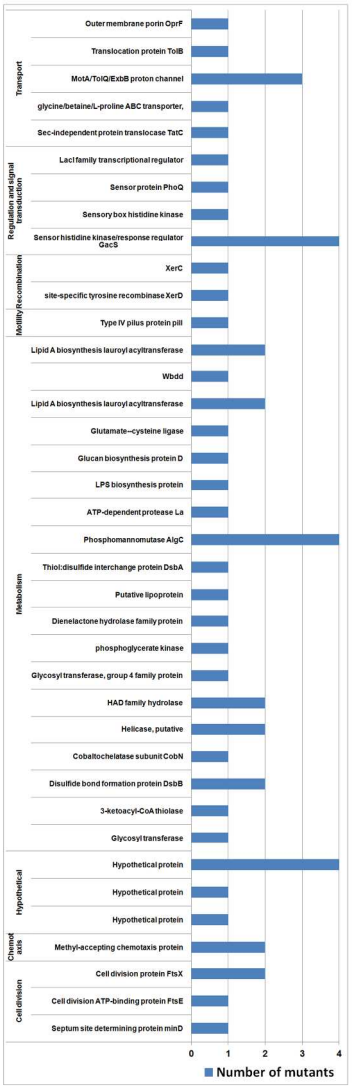

Supplementary Figure S3. Number of Tn*5* mutants localized to each ORFs in lipolytic activity altered phenotype screening.

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