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Friend or foe: genetic and functional characterization of plant endophytic *Pseudomonas aeruginosa*

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Summary

Endophytic *Pseudomonas aeruginosa* strain BP35 was originally isolated from black pepper grown in the rain forest in Kerala, India. Strain PaBP35 was shown to provide significant protection to black pepper against infections by *Phytophthora capsici* and *Radopholus similis*. For registration and implementation in disease management programmes, several traits of PaBP35 were investigated including its endophytic behaviour, biocontrol activity, phylogeny and toxicity to mammals. The results showed that PaBP35 efficiently colonized black pepper shoots and displayed a typical spatiotemporal pattern in its endophytic movement with concomitant suppression of *Phytophthora* rot. Confocal laser scanning microscopy revealed high populations of PaBP35::*gfp2* inside tomato plantlets, supporting its endophytic behaviour in other plant species. Polyphasic approaches to genotype PaBP35, including BOX-PCR, *rec*N sequence analysis, multilocus sequence typing and comparative genome hybridization analysis, revealed its uniqueness among *P. aeruginosa* strains representing clinical habitats. However, like other *P. aeruginosa* strains, PaBP35 exhibited resistance to antibiotics, grew at 25–41°C and produced rhamnolipids and phenazines. PaBP35 displayed strong type II secretion effectors-mediated cytotoxicity on mammalian A549 cells. Coupled with pathogenicity in a murine airway infection model, we conclude that this plant endophytic strain is as virulent as clinical *P. aeruginosa* strains. Safety issues related to the selection of plant endophytic bacteria for crop protection are discussed.

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Introduction

Pseudomonas species are ubiquitous in plant-associated environments and play an important role in the natural protection of plants against pathogens (Weller *et al.*, 2002; Haas and Défago, 2005; Mendes *et al.*, 2011). Several species, notably *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas chlororaphis*, are well known for their ability to control a range of plant pathogenic fungi and oomycetes (Haas and Défago, 2005; Loper *et al.*, 2012; Raaijmakers and Mazzola, 2012). Multiple strains of *Pseudomonas aeruginosa*, especially those from tropical countries, have been reported to inhibit a wide range of plant pathogens and are being prospected as biocontrol agents in several crops (Buysens *et al.*, 1996; de Meyer and Höfte, 1997; Parveen *et al.*, 1998; Anjaiah *et al.*, 2003; Bano and Musarrat, 2003; Kishore *et al.*, 2005; Kumar *et al.*, 2005; Prakob *et al.*, 2007; Siddiqui and Akhtar, 2007; Azadeh and Meon, 2009; Minaxi and Saxena, 2010). *Pseudomonas aeruginosa* strains suppress plant pathogens through secretion of antimicrobial compounds (Mavrodi *et al.*, 2006; Abdel-Mawgoud *et al.*, 2010) and also induced systemic resistance is an important mechanism involved in pathogen control (Audenaert *et al.*, 2002). Given their biocontrol efficacy, several *P. aeruginosa* strains are considered as potential constituents of integrated pest management programmes in tropical countries. However, *P. aeruginosa* is also known as an opportunistic pathogen colonizing diverse groups of other organisms, including humans, mice, insects, nematodes, fungi and amoebae (Chitkara and Feierabend, 1981; Lyczak *et al.*, 2000; Mahajan-Miklos *et al.*, 2000; Cao *et al.*, 2001; Pukatzki *et al.*, 2002; Rabin and Hauser, 2003; Walker *et al.*, 2004). Therefore, in-depth genetic and phenotypic characterization of plant-associated *P. aeruginosa* strains is essential for their safe implementation as biocontrol agents in agriculture and horticulture.

To date, however, detailed information on *P. aeruginosa* strains from soil and plant-associated habitats *vis-à-vis*

their relation to strains from other environments and clinical habitats is limited. In a recent study, Wu and colleagues (2011) compared the complete genome of *P. aeruginosa* M18, a strain isolated from sweet melon rhizosphere, with the previously sequenced nosocomial strain LESB58; they found that strain M18 was more susceptible to several antimicrobial agents and easier to be erased in a mouse acute lung infection model than strain LESB58. In a similar study, Steindler and colleagues (2009) reported that the rice rhizosphere strain *P. aeruginosa* PUPa3 was pathogenic in two non-mammalian infection models, *Caenorhabditis elegans* and *Galeria mellonella*; they further reported that the virulence-related *Las*I/R and *Rhl*I/R quorum sensing machinery contributed to plant colonization by strain PUPa3.

In the present study, we assessed a wide range of genetic and phenotypic traits of plant endophytic strain *P. aeruginosa* BP35 (PaBP35), including its antibiotic resistance spectrum and virulence in mammalian infection models. Strain PaBP35 was originally isolated from internal tissues of black pepper vines collected from Peruvannamuzhi, one of the major pepper growing regions in a tropical rainforest of Western Ghats in Kerala, India (Aravind *et al.*, 2009). Biolog-based phenotypic profiling of strain PaBP35 as well as 16S rDNA sequence showed high similarities between PaBP35 and *P. aeruginosa* type strains (Aravind *et al.*, 2009). The strain identity and its genetic relatedness to *P. aeruginosa* strains originating from clinical habitats were determined by a panel of genotyping methods such as *rec*N sequence analyses (Zeigler, 2005), multilocus sequence typing (MLST) (Curran *et al.*, 2004) and comparative genome hybridization (CGH) using the *P. aeruginosa* AT chip (Wiehlmann *et al.*, 2007). We also investigated the endophytic behaviour of strain PaBP35 in black pepper and tomato plants, its biocontrol activity against *Phytophthora* rot as well as its toxicity and pathogenicity in mammalian models.

Results

Endophytic colonization of black pepper by PaBP35 and control of Phytophthora capsici

In cut shoots of black pepper, strain PaBP35 displayed a spatiotemporal pattern in endophytic colonization (Fig. 1A). Results of the colony counts and PCR amplification with PaBP35-specific primers indicated a progressive increase in population densities in surface-disinfected stem tissues (Fig 1B). The endophytic population size was found to stabilize in the cut shoots after 12 min of bacterization and positively correlated with the duration of bacterization ($R^2 = 0.80$). With increasing inoculum density of strain PaBP35, the size of the lesions caused by *P. capsici* on black pepper shoots decreased significantly (Fig. 2A). At an inoculum density of $10^{8–10}$ cells ml^{-1} and an endogenous population size of $10^{4–6}$ colony-forming units (cfu) per gram of tissue, the level of disease control was similar to that observed for metalaxyl, the chemical compound commonly used to control *P. capsici* and other oomycete pathogens (Fig. 2B). Next to the bioassays conducted with stem cuttings, we also investigated endophytic colonization of rooted black pepper plantlets. The results showed that with an increasing duration of bacterization, there was a concomitant increase in the endogenous population size of PaBP35 (Fig. S1A). In general, the population densities were higher in roots (5.7 $\log_{10}$ cfu g^{-1}) than in stem (5.2 $\log_{10}$ cfu g^{-1}) and leaves (4.5 $\log_{10}$ cfu g^{-1}). The bacterial population in roots stabilized after 7 days whereas an increase in population size was observed in the stem (Fig. S1A). The presence of PaBP35 in the plant tissues was again confirmed by PCR (Fig. S1B).

Localization of PaBP35 in stem and leaves of tomato seedlings

Strain PaBP35 was tagged with a *gfp* reporter construct (Koch *et al.* 2001) to visualize its endophytic behaviour in tomato seedlings. Strain PaBP35::*gfp2* could be visualized on the root surface and in the interiors of roots, stem and leaf of tomato seedlings at 14 days of plant growth on a soil inoculated with PaBP35::*gfp2* at an initial density of 10^8 cfu g^{-1} soil (Fig. 3A). PaBP35::*gfp2* established population densities inside the tomato stem and leaf of approximately 5.0 and 3.5 $\log_{10}$ cfu g^{-1} respectively. Strain PaBP35::*gfp2* colonized the interior tissues of the tomato seedlings much better (up to three orders of magnitude) than the clinical strain *P. aeruginosa* PAO1 (Fig. 3B).

Phenotypic and functional traits of PaBP35

Strain PaBP35 exhibited resistance to multiple antibiotics, grew at 25–41°C and produced several known secondary metabolites and enzymes (Table 1). PaBP35 exhibited a characteristic floral-like swarming pattern with substantial accumulation of biomass within 12–16 h of incubation at 37°C on soft KMB agar (0.6%); this phenotype was absent at 25°C (Fig. S2A). In dual culture assays on 1/5th PDA, PaBP35 significantly inhibited mycelial growth of *P. capsici* (Fig. S2B). RP-HPLC analysis of cell-free culture supernatant of PaBP35 revealed a single major peak at retention time 19.8 min (Fig. S2C). The spectral characteristics of this peak were consistent with these of phenazine antibiotics (Fig. S2D). Genes involved in the biosynthetic pathway of phenazine were detected in PaBP35 with phenazine-specific primers (Mavrodi *et al.*, 2010) that yielded an amplicon of 427 bp (Fig. S2E). When assayed for activity, the MeOH extract containing

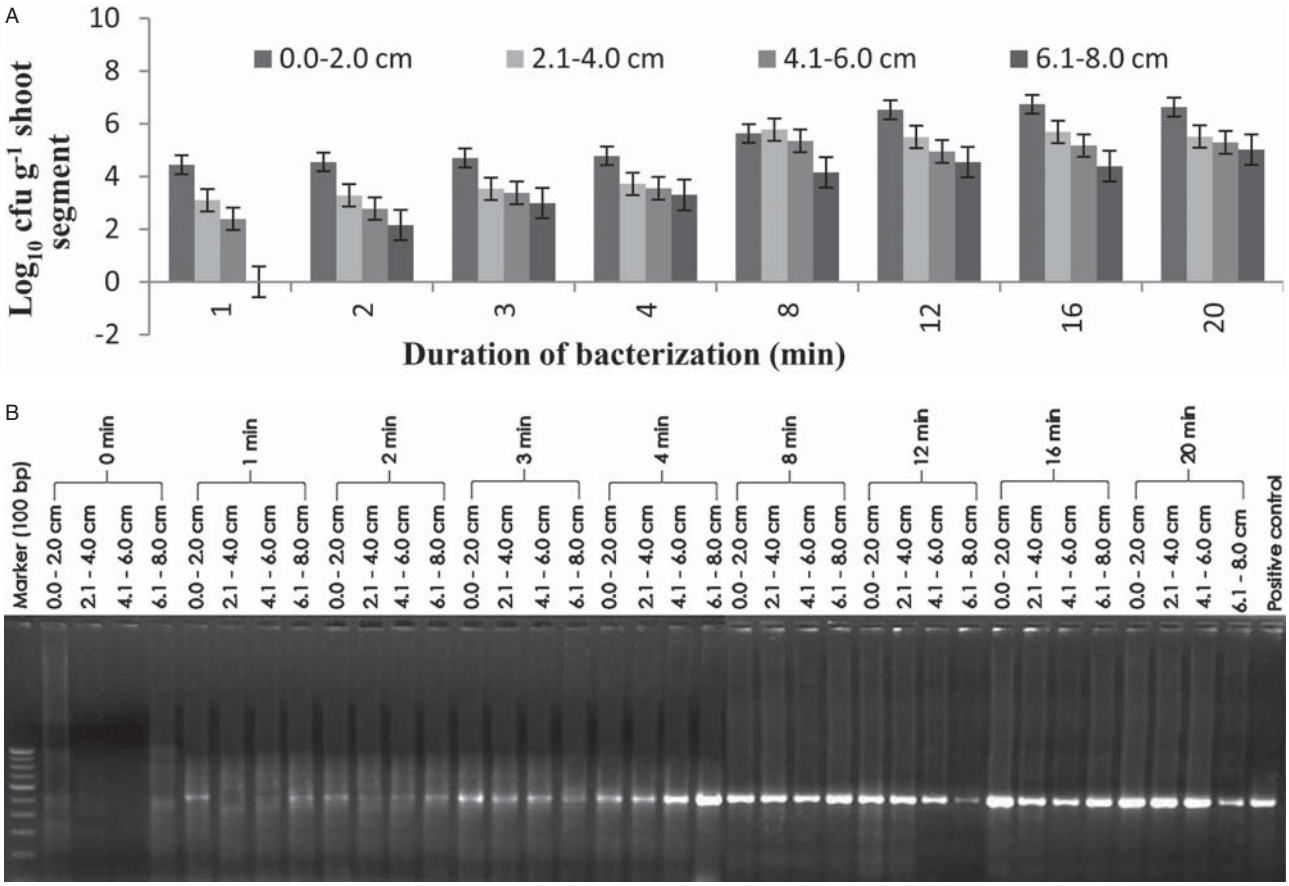

Fig. 1. Spatiotemporal pattern in endophytic colonization of *P. aeruginosa* strain PaBP35 in black pepper shoots. Only the lower cut end (0.0–2.0 cm) was treated with a suspension of strain PaBP35 (10^{9-10} cells ml^{-1}). The treated shoots were partitioned in 2 cm segments after varying duration (up to 20 min) of bacterial treatment and subjected to bacterial isolation and enumeration as well as to DNA extraction followed by a PaBP35-specific PCR detection.

A. Population density of PaBP35 recovered from partitioned segments of cut shoots of black pepper plants during 0–20 min of bacterization. Population densities of PaBP35 were determined by dilution plating of PBS extract of stem tissues onto nutrient agar medium amended with kanamycin (60 μg ml^{-1}) and 2,3,5 triphenyl tetrazolium chloride (50 μg ml^{-1}). Mean values of five replicates are given; error bars represent the standard error of the mean.

B. PCR-based detection of strain PaBP35 in partitioned segments of cut shoots of black pepper plants during 0–20 min of bacterization. The presence of PaBP35 was determined by PCR amplification (560 bp PaBP35-specific fragment) of DNA extracted from the treated black pepper shoot segments. DNA from PaBP35 pure cultures served as positive control.

the phenazine derivative inhibited mycelial growth of *P. capsici* to a similar level as was observed with PaBP35 cells (Fig. S2F). Strain PaBP35 did not harbour genes for 2,4-DAPG, pyrrolnitrin or pyoluteorin (data not shown).

When spot-inoculated on SW medium, PaBP35 formed a halo around its colony that is typical for rhamnolipids and other surfactants (Fig. S3A). The surfactant activity of cell suspensions and culture supernatant of PaBP35 was confirmed in drop collapse assays and in tensiometric analysis: the surface tension of PaBP35 extracts was 41 mN m^{-1} as compared with 72 mN m^{-1} of the growth medium alone. The surfactant extract showed typical zoosporicidal activity against *Phytophthora capsici*, *P. palmivora* and *P. megakarya*, for which the zoospores ceased their motility in 30–60 s and burst within 2 min of exposure (Fig. S3B). At the molecular level, we detected rhamnolipid genes *rhl*A (1100 bp), *rhl*C (1200 bp) (Fig. S3C), *rhl*I (377 bp) and *rhl*R (266 bp) (Fig. S3D) in strain PaBP35.

Genotyping of PaBP35

PaBP35 was genotyped by polyphasic approaches including BOX-PCR, *rec*N sequence analysis, MLST and CGH analysis. The polymorphic amplicon profile of the BOX-PCR was converted into binary data and subjected to phylogenetic analysis by FreeTree (Pavlícek *et al.*, 1999). The similarity index, obtained based on Dice coefficient, between PaBP35 and PAO1 was 0.69 and 0.13 between PaBP35 and *P. fluorescens* strain SS101. Phylogenetic analysis of the *rec*N gene of PaBP35 (1180 bp, GenBank JN159935), with the minimum evolution method

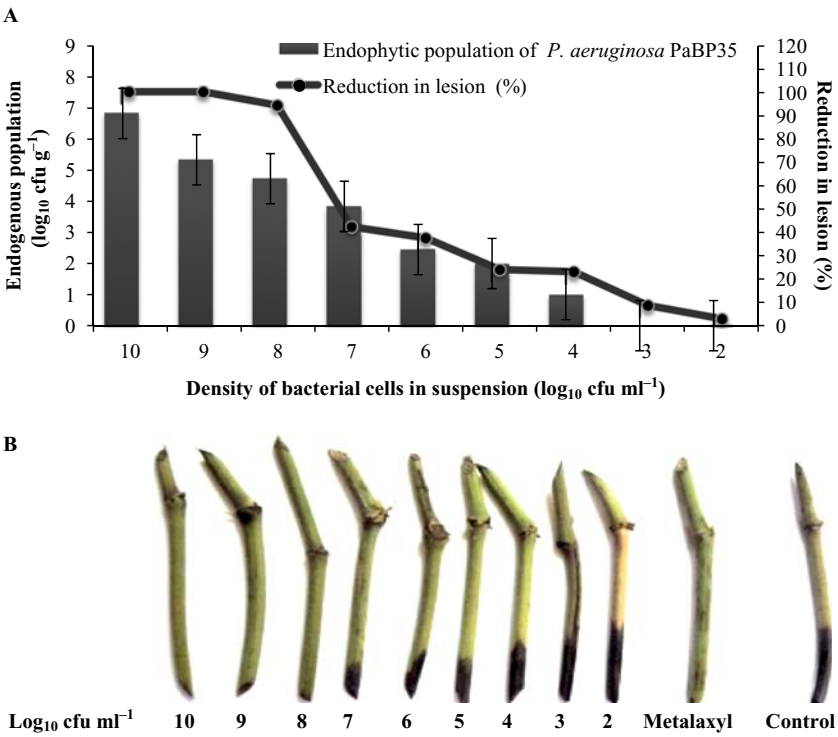

Fig 2. Density-dependent endophytic colonization by *P. aeruginosa* strain PaBP35 in black pepper shoots and concomitant suppression of lesion expansion caused by *Phytophthora capsici*.
A. Population density of PaBP35 recovered from partitioned segments of cut shoots of black pepper plants treated with different densities of bacterial cells. Population densities of PaBP35 were determined by dilution plating of PBS extract of stem tissues onto nutrient agar medium amended with kanamycin (60 μg ml^{-1}) and 2,3,5 triphenyl tetrazolium chloride (50 μg ml^{-1}). Mean values of five replicates are given; error bars represent the standard error of the mean. The line diagram indicates the reduction in the size of the lesions caused by *Phytophthora capsici*.
B. Visual presentation of *P. capsici* lesions on black pepper cuttings treated with different densities of *P. aeruginosa* strain PaBP35. Metalaxyl-Mz and untreated cut shoots served as positive and negative control.

using maximum composite likelihood model (Mega 5.01), showed that strain PaBP35 clustered close to PaM18, a strain isolated from the rhizosphere of melon (Lu *et al.*, 2009). All other clinical *P. aeruginosa* strains clustered separately (Fig. S4).

For the MLST analysis, different housekeeping genes of PaBP35 were sequenced (*acs*A – JN180235; *aro*E – JN159932; *gua*A – JN159933; *mut*L – JN180236; *nuo*D – JN180237; *pps*A – JN159934; *trp*E – JN180238). Allele numbers for each of the loci were obtained from http://pubmlst.org/paeruginosa. The allelic profile of PaBP35 is *acs*A-22, *aro*E-5, *gua*A-83, *mut*L-2, *nuo*D-4, *pps*A-13 and *trp*E-7, which indicated that this strain is a novel sequence type for *P. aeruginosa*. The eBURST algorithm (Feil *et al.*, 2004), available at the eBURST (version 3) website http://eburst.mlst.net, was used to display the *P. aeruginosa* population of 1210 isolates (http://pubmlst.org/paeruginosa/) in June 2012 (Fig. 4). The following settings for the creation of the eBURST-based snapshot were used: number of loci per isolate, 7; minimum number of identical loci for group definition, 0; minimal single-locus variant count for subgroup definition, 3; and number of

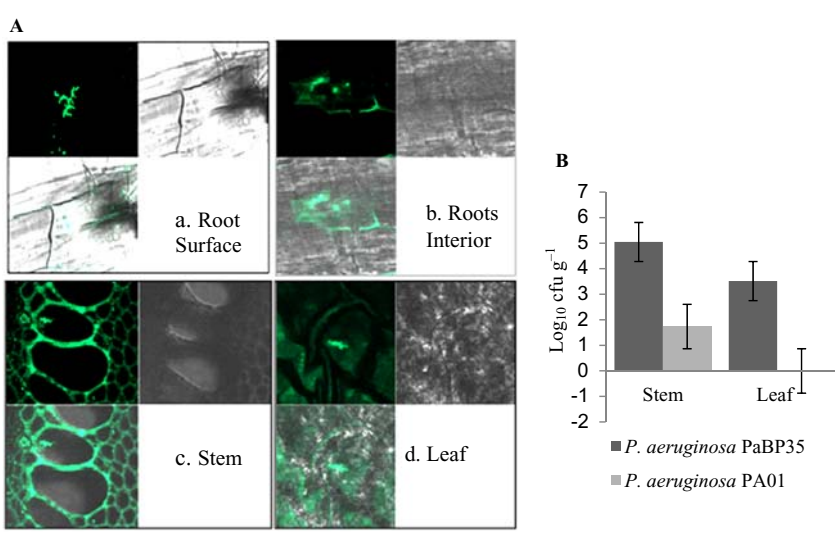

Fig. 3. A. Confocal laser scanning microscopy images (100 × magnification) of *P. aeruginosa* PaBP35::*gfp2* cells on the root surface, in the root interior, stem interior and leaf interior of 14 day-old tomato seedlings.
B. Endogenous population (log_{10} cfu g^{-1}) of PaBP35::*gfp2* or *P. aeruginosa* PAO1 in surface-sterilized stem and leaf of tomato seedlings grown on soil inoculated with these strains. PAO1 could not be detected in leaf tissues. Mean values of five replications are given; error bars represent the standard error (SEM 0.92).

Table 1. Phenotypic characteristics of *P. aeruginosa* strain PaBP35.

Taxonomic/functional traits	Results
General features	
Plant origin	Stem tissues of black pepper
Geographical origin	Rainforest of Western Ghats in Kerala, India
Growth temperature range	25–41°C (best at 37°C)
Biolog^{R} analysis (95 biochemical tests)	Closest match to *P. aeruginosa* (100% similarity)
16S rDNA analysis	Closest match to *P. aeruginosa* (99% similarity)
Swarming motility on soft KB medium	+ (typical 'floral type pattern' of swarming)
Antibiotic resistance	Kanamycin (up to 1000 μg ml^{-1}), tetracycline (up to 50 μg ml^{-1}), gentamycin (up to 60 μg ml^{-1}), amoxycillin (30 μg ml^{-1}), ampicillin (25 μg ml^{-1}), bacitracin (10 units), cephalexin (30 μg ml^{-1}), cephaloridine (30 μg ml^{-1}) Cephalothin (30 μg ml^{-1}), lincomycin (15 μg ml^{-1}) Novobiocin (30 μg ml^{-1}), penicillin G (10 units) Spectinomycin (100 μg ml^{-1}), trimethoprim (30 μg ml^{-1})
Antibiotic sensitivity	No growth occurred at cephotaxime (30 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), ciprofloxacin (30 μg ml^{-1}), colistin (25 μg ml^{-1}), doxycycline hydrochloride (30 μg ml^{-1}), nalidixic acid (30 μg ml^{-1}), neomycin (30 μg ml^{-1}), oxacillin (5 μg ml^{-1}), piperacillin (100 μg ml^{-1}), polymyxin B (100 units), rifampicin (30 μg ml^{-1}), streptomycin (25 μg ml^{-1})
Functional traits	
Biological activity	Inhibition of hyphal growth of *Phytophthora capsici*; suppression of lesion expansion of *P. capsici* on stem cutting of black pepper; protects rooted plants of black pepper against *P. capsici*-induced rot; zoosporicidal activity against *P. capcisi*, *P. megasperma* and *P. palmivora*
Secondary metabolites and enzymes	
HCN	+
Phenazines	+
Rhamnolipids	+ (best at 37°C, no or very scanty at 25°C)
Lipase, protease	+
Chitinase, cellulase, pectinase	–
Plant growth promotion-related features	
Phosphate solubilization, ammonia production	+
Indole acetic acid (IAA)	–

+, positive for production; –, negative for production.

resamplings for bootstrap analysis, 1000. The approach specifically examines the relationships within clonal complexes and showed that PaBP35 was a single-locus variant of HA-D14 (ST575) (Fig. 4) – a strain reported from a clinical habitat in the Netherlands (http://pubmlst.org/paeruginosa). For the analysis of the single nucleotide polymorphisms (SNPs) in the core genome of PaBP35, the typing scheme of Wiehlmann and colleagues (2007) was used to compare PaBP35 with strains from multiple origins and specifically from clinical settings. The 16-binary data set from multilocus SNP genotype representing the core genome was used to analyse the strain relatedness using the eBURST algorithm. The burst diagram revealed that the strain PaBP35 is a singleton in the *P. aeruginosa* population (Fig. S5). Strains from most of the environmental sources were clustered with clinical strains of *P. aeruginosa*.

Toxicity analysis

The cytotoxic activity of strain PaBP35 for A549 human airway epithelial cells was compared with that of three other *P. aeruginosa* strains, two environmental (EC21 from sea water and 0822 from tomato plants) and one clinical isolate (OC2E) from a CF patient. Taking the release of lactate dehydrogenase (LDH) as a surrogate parameter for mammalian cell damage, the selected time points of 0, 2, 4 and 6 h roughly reflect the action of constitutively produced virulence effectors ($t = 0$ h), of type III secretion effectors ($t = 2$ h), of type II secretion effectors ($t = 4$ h) and of other secondary metabolites such as siderophores or quinolones ($t = 6$ h) (Lee *et al.*, 2005). Strain PaBP35 showed no constitutive or type III secretion-mediated cytotoxicity, but it exerted moderate to strong cytotoxicity on A549 cells at the time points 4 and 6 h respectively (Fig. 5).

C57Bl/6J mice were inoculated intranasally with 2.0×10^6 cfu of *P. aeruginosa*. This dose of PaBP35 induced a reversible acute infection in the mice as indicated by a drop of body temperature, troubled behaviour and impaired lung function (Fig. 6). Disease symptoms peaked at 6 h after inoculation. At this time point, 1% to 50% of the bacterial inoculum was retrieved from the individual murine lungs (Fig. 7A). Mice recovered spontaneously, appeared unaffected at 24 h (Fig. 6B) and had cleared 98% to 99.9% of the initial dose of PaBP35. In line with previous experiments (Wölbeling *et al.*, 2010), lung function required about 72 h to return to values prior to

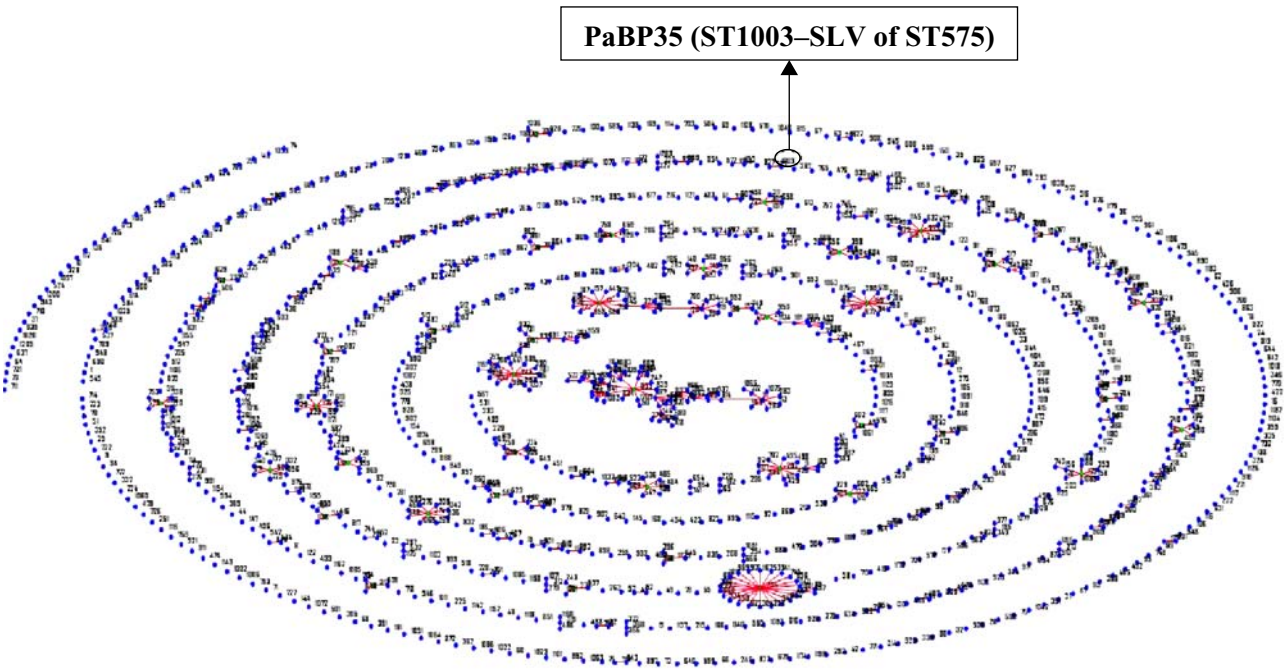

Fig. 4. Snapshot of 1210 *P. aeruginosa* isolates on the basis of MLST allelic profiles using the eBURST algorithm. Numbers and blue dots represent sequence types. Pink lines connect single-locus variants, which are STs that differ in sequence for only one of the seven housekeeping genes. The snapshot shows all clonal complexes (connected STs), singletons (unconnected STs) and patterns of evolutionary descent. The placement of singletons as well as the length of the nodes is arbitrary. Green shaded dots indicate probable ancestors of clonal complexes. Strain PaBP35 (ST1003) is a single-locus variant of ST575; it does not have any double-locus variants (DLVs) and has 15 other triple-locus variants (TLVs) in the population.

infection. The same intranasal dose of the seawater isolate EC21 as well as of the clinical CF isolate OC2E caused irreversible infectious disease (Fig. 6B) that was more severe than the infection with the PaBP35 strain. *Pseudomonas aeruginosa* BP35 invaded lung cells and was growing intracellularly (Fig. 7B).

Discussion

Several strains of *P. aeruginosa*, especially those from tropical countries, were shown to inhibit a wide range of plant pathogens and hence are being promoted as potential biocontrol and growth-promoting agents in several agricultural crops (Viji *et al.*, 2003; Prakob *et al.*, 2007; Siddiqui and Akhtar, 2007; Azadeh and Meon, 2009; Lu *et al.*, 2009; Minaxi and Saxena, 2010). The probable reason for this spate of reports on *P. aeruginosa* as biocontrol strain could be attributed to a bias in the selection criteria such as *in vitro* antagonism. Several plant-associated *P. aeruginosa* strains, i.e. 7NSK2 (Buysens *et al.*, 1996), PNA1 (Anjaiah *et al.*, 2003), NJ-15 (Bano and Musarrat, 2003), PUPa3 (Kumar *et al.*, 2005) and M18 (Hu *et al.*, 2005), have been found to show excellent biocontrol property against various phytopathogens, and can potentially be utilized for biopesticide development.

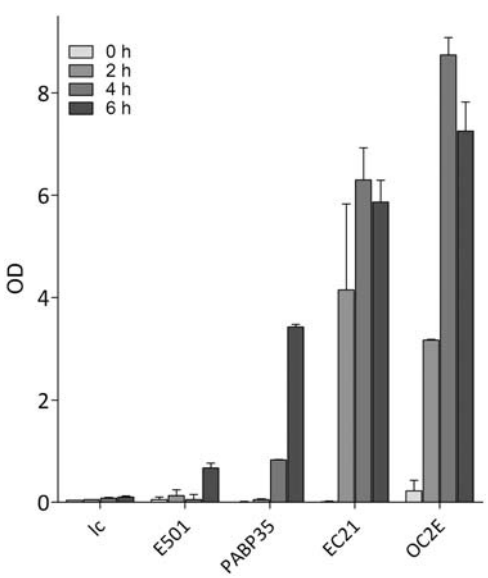

Fig. 5. Cytotoxicity of *P. aeruginosa* strains E501, PaBP35, EC21 and OC2E on A549 cells. Cytotoxicity was assessed by the release of LDH at the time points 0, 2, 4 and 6 h after incubation. lc, mock control of non-infected A549 cells processed in parallel. The ordinate indicates the optical density of undiluted supernatants. Experiments were performed twice in triplicate. Bars show mean values with SEM.

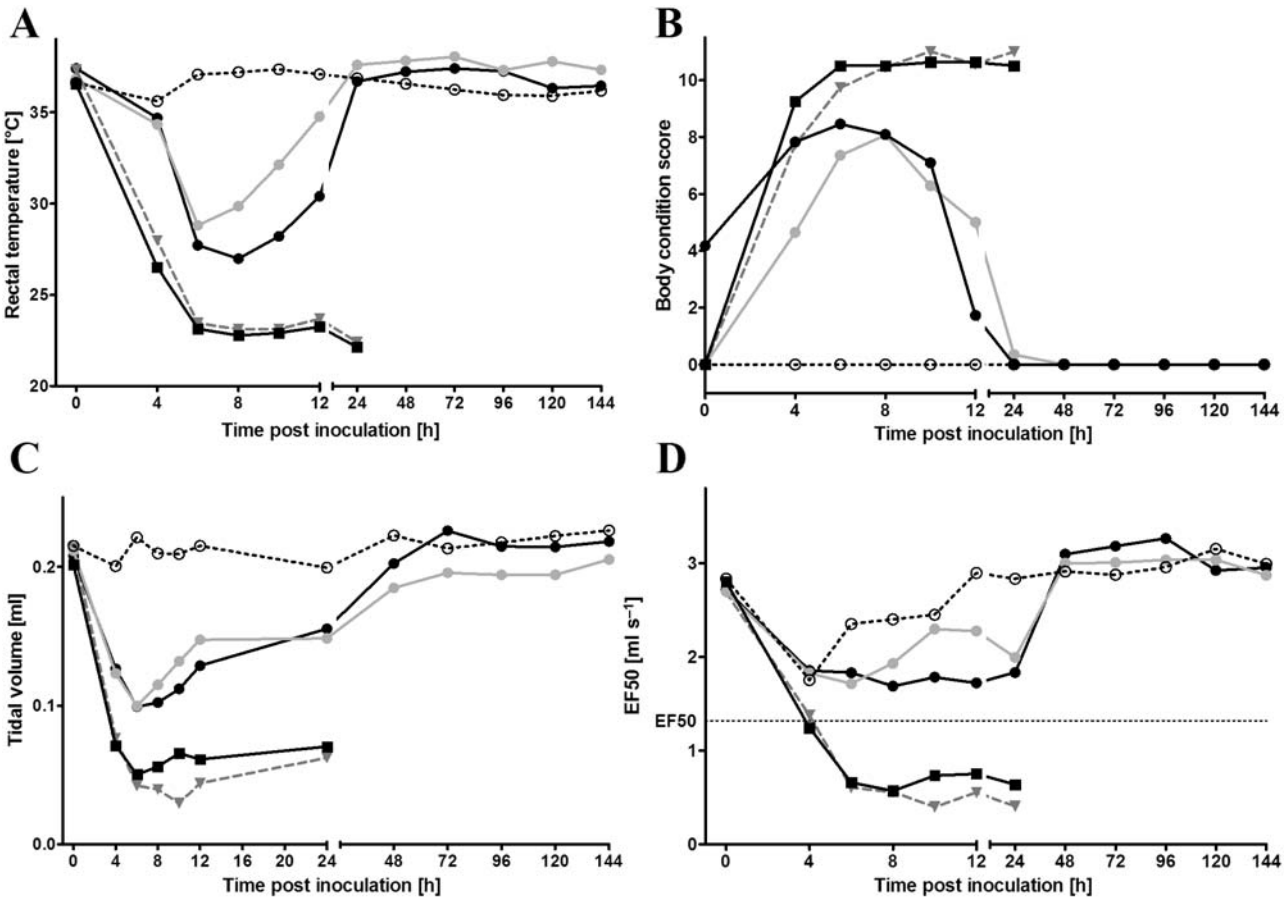

Fig. 6. Course of an acute airway infection with *P. aeruginosa* in C57BL/6J mice. Mice were infected intranasally with 2.0×10^6 cfu per millilitre of physiological saline of *P. aeruginosa* strains E501 (light grey circles), PaBP35 (black circles), EC21 (dark grey triangles with dashed line) and OC2E (black squares). Mock animals (open circles with broken line) received physiological saline only. Graphs show the course of the rectal temperature (A), the body condition score (B), the lung function parameters tidal volume (C) and the expiratory flow EF50 (D). Data points represent median values of 20 mice.

Although biocontrol, in general, has long been considered as an environmentally sound and safe alternative to synthetic pesticides, the advancements in importance of opportunistic human pathogens has led to public concern in recent years about the widespread use of certain microbes in agriculture (Handelsman, 2002). The emergence of immune-compromising infectious diseases and the increase in organ transplants have made opportunistic pathogens a perceptible threat to human health (Handelsman, 2002; Berg *et al.*, 2005). If not addressed, the concerns may be a hindrance to public acceptance, registration, adoption and practice of biocontrol agents in agriculture especially in developing nations where the production, marketing and field applications of biocontrol agents prevails.

Here, we characterized a naturally occurring plant endophytic *P. aeruginosa* strain for several traits, including its toxicity to mammalian infection models. Strain PaBP35 was originally isolated from visually healthy aerial black pepper shoots collected from a remote tropical rainforest in India as an effective antagonist of the oomycetes pathogen *P. capsici*, which causes foot rot of black pepper (Aravind *et al.*, 2009). Strain PaBP35 displayed a characteristic pattern of swarming motility with remarkable 'floral-shaped islands' of bacterial biomass within 12–16 h when incubated at 37°C – a character not seen at 25°C. Such a temperature dependency for swarming motility was also demonstrated in certain strains of *Serratia marcescens* (Lai *et al.*, 2005). The mechanism underlying the temperature-dependent swarming phenotype for strain PaBP35 is yet unclear but may be linked to temperature-dependent production of rhamnolipids, the biosurfactants that are crucial for swarming motility (Caiazza *et al.*, 2005).

Strain PaBP35 showed characteristic endophytic behaviour within the plant as the bacterium could be tracked in the interiors of tomato and black pepper plantlets 7 days post rhizosphere application. When PaBP35

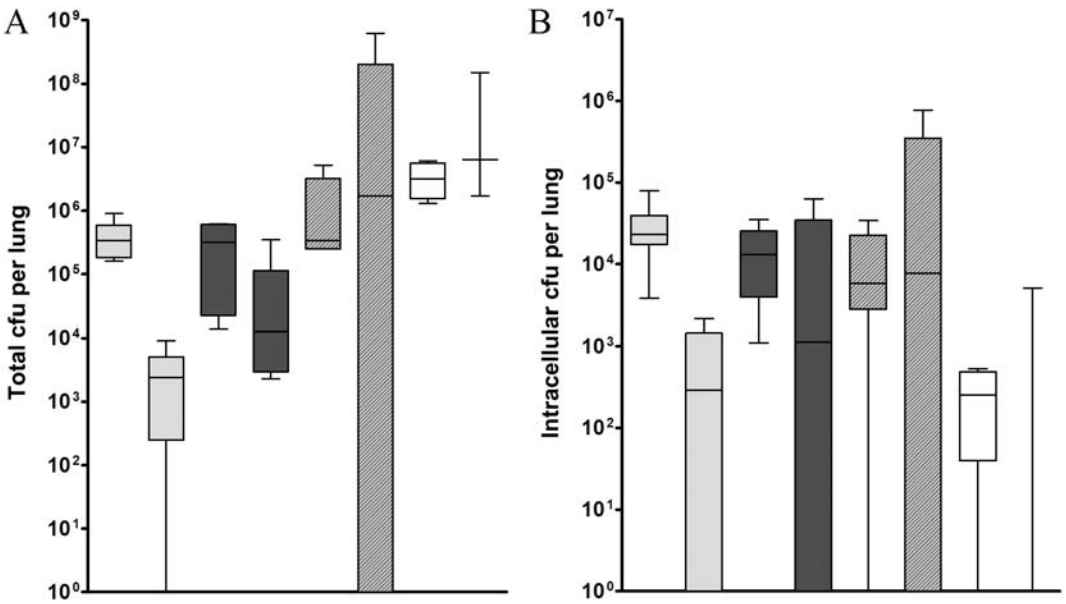

Fig. 7. Boxplots of bacterial cfu in murine lungs 6 h (left boxes of each strain) and 24 h (right boxes of each strain) after infection. (A) Total cfu, (B) intracellular cfu of strains E501 (light grey), PaBP35 (black), EC21 (dark grey, shaded) and OC2E (white). Bottom and top of the boxes displaying the 25th and 75th percentile, the median is presented as line within the box and the ends of the whiskers represent the minimum and maximum of the group.

was inoculated in soil, it could reach the various tomato seedling interiors in a systemic way as the bacterium could be found in the root, stem and leaf endosphere within 7 days and progressively increased in population density. The population build-up was 4 to 5 $\log_{10}$ cfu in stem tissue and 3 to 5 $\log_{10}$ cfu in leaf tissue at 21 and 28 days post bacterization. For black pepper, its endophytic behaviour was shown for cut shoots and the intact rooted plantlets. Possibly, the bacterium moved from the open cut end of the black pepper shoots to the distant parts of the plant through transpirational pull and attained an endogenous population size of 10^{5-6} cfu g^{-1} of tissue within 30 min after bacterization. *Pseudomonas aeruginosa* is endowed with twitching motility at 0.3 mm per minute by their type IV pilli (Darzins and Russell, 1997). Whether twitching motility is involved in the endophytic movement and colonization of PaBP35 is yet unknown. Significantly high population of PaBP35 tracked in endosphere as compared with clinical strain of *P. aeruginosa* PAO1 further confirms this specific affinity of the plant endophytic PaBP35 for the plant tissue. The intrinsic ability of *P. aeruginosa* BP35 to colonize the plant tissue could be attributed to its plant origin. The strain was originally isolated as an endophyte from black pepper collected from Kerala which is an endemic area for *Phytophthora*-induced rot not only in black pepper but also for diverse crops such as coconut, areca nut, taro, rubber, cardamom and cocoa. Upon this endophytic colonization the bacterium offered systemic protection against *P. capsici* in black pepper (Aravind *et al.*, 2009). The endophytic colonization of black pepper cuttings and plantlets by strain PaBP35 was found to have profound effects on the *Phytophthora* rot of black pepper as evident from the significant reduction in lesion size. Collectively, these data indicated that the minimum endogenous population of about 5–6 $\log_{10}$ units per gram of tissue was needed to protect the plantlets against *P. capsici*. In order to achieve this density, the cut shoots need to be dipped at least for 16–20 min in bacterial suspension (10^{9-10} cfu ml^{-1}).

Strain PaBP35 displayed a strong mycelial inhibitory activity against *P. capsici in vitro* and protected not only the cut shoots but also the rooted plantlets of black pepper against *Phytophthora* infection (Aravind *et al.*, 2012). The inhibitory activity of PaBP35 on this oomycete pathogen may be attributed to the secretion of phenazine antibiotics as well as rhamnolipids. The role of phenazines for plant pathogen suppression has been reported (Anjaiah *et al.*, 1998; Blankenfeldt *et al.*, 2004; Mavrodi *et al.*, 2006; Gibson *et al.*, 2009; Huang *et al.*, 2009). Besides mycelial inhibition, PaBP35 displayed zoosporicidal activity against *P. capsici*, *P. megakarya* and *P. palmivora* which is most likely attributed to secretion of the rhamnolipids. *Pseudomonas aeruginosa* is a well-known producer of rhamnolipids at industrial scale (Soberón-Chávez *et al.*, 2005). To determine the role of these metabolites in pathogen control, bioassays will be

needed with mutants of PaBP35 deficient in the production of each of these compounds.

Reports on the genetic relationship between biocontrol strains of *P. aeruginosa* and strains of clinical or disease habitat are scarce. In an elegant study, Alonso and colleagues (1999) reported that clinical and non-clinical *P. aeruginosa* strains might be functionally equivalent in several traits relevant for their virulence or environmental properties. We used a polyphasic genetic approach in order to shed new light on the issue of relatedness of environmental and clinical strains of opportunistic human pathogens. Strain PaBP35 was subjected to a series of genetic analyses using highly discriminatory genomic tools. These analyses confirmed its close relationship with *P. aeruginosa* M18, a strain isolated from sweet melon rhizosphere in 1996 (Hu *et al.*, 2005). Clinical *P. aeruginosa* PAO1, PA7, PA14 and LESB58 clustered separately in two other clusters. Nearly congruent observations were made when MLST-based analysis was adopted for our strain which clearly formed a new sequence type (ST1003) in a large collection of 1210 strains of *P. aeruginosa* predominantly from the human disease habitat. PaBP35 clustered with ST575 as single-locus variant in MLST and a singleton in CGH analysis of a large population of *P. aeruginosa*. The versatile functional and adaptive behaviour of *P. aeruginosa* is attributed to its high proportion of regulatory genes (Kiewitz and Tuemmler, 2000; Stover *et al.*, 2000). The various *P. aeruginosa* strains originating from different environment niches differ in accessory genome regions, genome expression profiles, virulence activities and antibiotic resistance spectrum (Wu *et al.*, 2011). Two independent MLST studies on large collections indicated that *P. aeruginosa* has a non-clonal epidemic population structure (Kiewitz and Tuemmler, 2000; Pirnay *et al.*, 2002; Curran *et al.*, 2004). Microarray-based CGH analysis of 53 *P. aeruginosa*-specific genes in the core and accessory genome as well as phylogenetic analyses of housekeeping genes suggests that strain PaBP35 is a singleton.

However, PaBP35 displayed strong cytotoxicity on mammalian A549 cells indicating its proficiency in type II secretion effectors. The CGH data on PaPB35 suggested that the strain lacks *Exo*U but possess *Exo*S – one of the two mutually exclusive cytotoxins. The apparent late lethality of PaPB35 could be attributed to presence of T2SS and *Exo*S which is 100-fold less toxic than *Exo*U (Lee *et al.*, 2005; Jyot *et al.*, 2011). Consistent with the detection of rhamnolipids and phenazines described above, strain PaBP35 appeared to be proficient in the production of type II secretion effectors and secondary metabolites. *Pseudomonas aeruginosa* is known to shield itself from innate immunity-related polymorphonuclear neutrophilic leukocytes by upregulating synthesis of a number of QS-controlled virulence determinants including rhamnolipids, all of which are able to cripple and eliminate cells of the host defence (Alhede *et al.*, 2009). Coupled with pathogenicity in a murine airway infection model, we conclude that this plant endophytic strain is as virulent as clinical *P. aeruginosa* type strains.

Conclusions

This study addresses genotyping and functional analysis of environmental *P. aeruginosa* isolated from *Piper nigrum* collected from a remote forest ecosystem in Southern Indian state of Kerala. Although the polyphasic genetic analysis revealed its uniqueness among the global collection of *P. aeruginosa* strains, PaBP35 like other *P. aeruginosa* strains exhibited resistance to multiple antibiotics, grew at 25–41°C and produced rhamnolipids, HCN and phenazines. Furthermore, PaBP35 displayed strong cytotoxicity on mammalian A549 and was also able to cause clinical infection in an acute murine airway infection model. The results of our work underscore the importance of multiple criteria including appropriate toxicological testing for selection of suitable strains for biocontrol purpose in agriculture. Based on the results obtained in this study, strain PaBP35 cannot be further pursued as a biocontrol agent of *Phytophthora* rot of black pepper and other plant diseases. It is in the interest of public safety as well as the continuance of sustainable crop protection technologies to develop an unambiguous strain selection policy with due importance to biosafety.

Experimental procedure

Bacterial strain and culture conditions

Bacterial strain PaBP35 used in this study was isolated from stem tissue of black pepper cultivar *Panniyur* I. Species identity of PaBP35 was established as *P. aeruginosa* by: (i) Biolog-based identification scheme using BiologGN plates (Biolog, USA) by adopting manufacture's instructions and (ii) sequence comparison of 16S rDNA with GenBank entries by closest match (Aravind *et al.*, 2009). The strain was deposited in the Microbial Type Culture Collection (MTCC) in India with Accession Number MTCC5410. The bacterium was routinely cultured on King's Medium B at 37°C. For swarming motility the bacterium was placed in the middle of soft KB agar (0.6% w/v) and incubated at 25°C and 37°C by adopting procedures described previously (de Bruijn and Raaijmakers, 2009). The plates were examined every 12 h up to 48 h. For toxicity studies *P. aeruginosa* strain PaBP35 [hexadecimal code (Wiehlmann *et al.*, 2007): 843A] isolated from stem tissue of black pepper was compared with strain 100 (hexadecimal code: EC21) isolated from the deep sea and strain E501 (hexadecimal code: 0822) isolated from the surface of a tomato. The strain was characterized for various phenotypic features such as intrinsic antibiotic resistance against range of antibiotics, growth at 37–41°C, swarming motility, produc-

tion of secondary metabolites, hydrolytic enzymes and growth promotion-related features by adopting standard methodologies.

Endophytic colonization of PaBP35 in black pepper transplants

Spatiotemporal endophytic colonization of PaBP35 in cut shoots. Endophytic colonization of PaBP35 in black pepper *vis-à-vis* suppression of *P. capsici* rot was determined on cut shoots as described by Dinu and colleagues (2007). Cut shoots (*cv*: IISR Subhakara) of about 8.0 cm length with at least one nodal primordia were washed thoroughly with water, surface disinfected with 1% sodium hypochlorite solution for 10 min, washed five times with sterile distilled water, blot dried on a sterile blotting paper and used.

Effect of varying duration of PaBP35 treatment on its endogenous population size vis-à-vis Phytophthora *rot.* The lower end of cut shoots was bacterized in mid log phase culture of PaBP35 (6×10^8 cells ml^{-1}) for varying durations, viz. 1, 2, 3, 4, 8, 12, 16 and 20 min, challenged with mycelial disk of *P. capsici*, wrapped with moist absorbent cotton and incubated at 25 ± 1°C with 100% RH for 4 days. The lesion length (mm) was measured and subjected to statistical analysis. Endogenous population size of PaBP35 in shoot was assessed 0, 6, 12, 18, 24 and 36 h post bacterization for 30 min by serial dilution plating and confirmed using PCR assay. Bacterized cut shoots was surface disinfected as described above, partitioned into segments of 2.0 cm and were ground in phosphate-buffered saline (PBS, g l^{-1}: NaCl 8.0, KCl 0.2, Na_2HPO_4 1.44, KH_2PO_4 0.24; distilled water 1 l, pH 7.4). The supernatant collected after centrifugation (1000 *g* for 2 min) was serially diluted up to 10^{-4} and 1 ml of the supernatant was plated out on nutrient agar amended with kanamycin (60 μg ml^{-1}) and 2,3,5 triphenyl tetrazolium chloride (50 μg ml^{-1}). Colonies of PaBP35 were counted and population size was expressed as $\log_{10}$ cfu g^{-1} of tissue. For PCR assay, shoot genomic DNA was subjected to PCR using primer combination (ITS1F: 5′-AAG TCG TAA CAA GGT AG-3′ and ITS2R: 5′-GAC CAT ATA TAA CCC CAA-3′) which was found to amplify 560 bp in our strain PaBP35. Briefly, PCR was set up in 10.0 μl of total volume with 1 × of Taq buffer, 3.0 mM $MgCl_2$, 400 μM dNTPs, 5% DMSO, 20 μg of BSA, 20 pmol each of ITS1F and ITS2R, 0.5 units DNA polymerase, 100 ng of template DNA. The reaction mixture was subjected to initial denaturation for about 3 min at 94°C followed by 35 cycles of 92°C for 1 min, annealed at 28°C for 1 min and extended at 72°C for 2 min with the final extension of 72°C for 10 min. The PCR product was visualized in 2% agarose gel stained with ethidium bromide.

Effect of varying density of PaBP35 on endogenous PaBP35 population vis-à-vis Phytophthora *rot.* Effect of varying cell densities of PaBP35 (10^{-1}, 10^{-2} . . . 10^{-9}, 10^{-10}) on *Phytophthora* rot was determined in cut shoot assay as mentioned above. Cut shoots were bacterized for 30 min, challenged with *P. capsici*, incubated for 4 days and the lesion length measured. Endophytic population size of PaBP35 was assessed by serial dilution plating and confirmed using PCR assay as described above.

Spatiotemporal colonization of rooted plantlets. Endophytic colonization of PaBP35 in black pepper root, shoot and leaf of bacterized black pepper-rooted cuttings (5–7 leaf stage) was estimated by serial dilution plating as well as by PCR as described above. The roots of apparently healthy pepper cultivar were dipped in cell suspension ($10^{9–10}$ cells ml^{-1}) of PaBP35 for 30, 60 and 120 min, respectively, and planted in mixture of soil + sand + farmyard manure (2:1:1). Plant was partitioned into roots, stem and leaf at 0, 7th, 14th, 21st and 28th day post bacterization and assayed. One gram of surface disinfected root, stem and leaf were ground aseptically with 2.0 ml of PBS, serially diluted up to 10^{-3} and plated as described above. For PCR assays, supernatant from the undiluted extract was used as template after centrifugation at 1000 r.p.m. for 1 min.

Endophytic localization of PaBP35 in tomato

GFP tagging of PaBP35. For tracking and localization of bacterium, green fluorescent protein (GFP)-tagged PaBP35 was used. For tagging, stable GFP construct *pBKminiTn7gfp2Gm10* maintained in *E. coli* XL1 Blue (gentamycin 25 μg ml^{-1}), the helper plasmids *pUXBF13Amp100* maintained in *E. coli* XL1 Blue (ampicillin 100 μg ml^{-1}) and *pRK2013* maintained in HB101 (kanamycin 50 μg ml^{-1}) were used. Four parental mating was conducted on sterilized nitrocellulose membrane placed on LB at 37°C for 12 h. The transformants were selected on KMB amended with rifamycin (50 μg ml^{-1}) and gentamycin (75 μg ml^{-1}) and PCR confirmed (amplicon size 668 bp) using primers according to the protocols of Koch and colleagues (2001).

Assay for endophytic behaviour of PaBP35 in tomato. Rhizosphere of 4 week-old tomato cultivar Moneymaker was drenched with suspension (25 ml) of PaBP35::*gfp2* (OD_{600}: 1.0) or *P. aeruginosa* PAO1. The plants were harvested 2 and 3 weeks post inoculation, partitioned into roots, stem and leaves, washed in sterilize water and the thin sections fixed in paraformaldehyde (4%) for 12 h at 4°C. The tissues were scanned and imaged in confocal laser scanning microscope (Carl Zeiss, Germany). The endogenous population of PaBP35::*gfp2* or PAO1 in bacterized tomato plantlets was quantified by serial dilution plating. Plant was surface disinfected as described above, partitioned into stem and leaf and were ground in PBS. The supernatant collected after centrifugation (1000 *g* for 2 min) was serially diluted up to 10^{-4} and 1 ml of the supernatant was plated on PSA amended with rifamycin 50 μg ml^{-1} + gentamycin 100 μg ml^{-1} + kanamycin 50 μg ml^{-1} for selective isolation of *P. aeruginosa* BP35::*gfp2* or PSA amended with kanamycin 50 μg ml^{-1} for selective isolation of PAO1. Colonies of *P. aeruginosa* were counted after incubation at 37°C for 2 days and population size was expressed as $\log_{10}$ cfu g^{-1} of tissue.

Statistical analysis

Population densities estimated and expressed as cfu g^{-1} of tissue were normalized by logarithmic transformation prior data analysis (Loper *et al.*, 1984). Lesion inhibition estimated by percentage values was normalized by angular transforma-

tion. The data were analysed using MstatC and their mean separated using Duncan's multiple range test at 95% probability level.

Phenotypic and functional biocontrol traits

Strain PaBP35 was analysed for number of traits such as: (i) *in vitro* mycelial inhibition, (ii) zoosporicidal activity against *P. capsici*, (iii) suppression of *Phytophthora* rot on cut shoots of black pepper and (iv) endophytic colonization of black pepper as well as tomato transplants. The antagonistic activity of the PaBP35 towards hyphal growth of *P. capsici* strain LT3229 was tested on one-fifth strength potato dextrose agar medium (pH 7.0) (Oxoid, Hampshire, England) as described previously (de Souza *et al*., 2003a). Zoosporicidal activity of PaBP35 was tested on one-fifth strength potato dextrose agar medium (pH 7.0) (Oxoid, Hampshire, UK) as described previously (de Souza *et al*., 2003b).

Analysis culture supernatants of strain PaBP35. Cell free culture filtrates were analysed and activity tested against *P. capsici* strain LT3229 as prescribed by de Souza and colleagues (2003a). For detection of phenazines 2, 4-diacetyl phloroglucinol (DAPG), pyrrolnitrin and pyoluteorin, RP-HPLC linked to a photodiodearray spectrophotometer was adopted (de Souza *et al*., 2003b). One hundred microlitres of extract was used for RP-HPLC analysis in C18 reverse phase column [40 min gradient (0.5 ml min^{-1}) from 0% to 100% CAN + 0.1% TFA] and spectral analysis was carried out from 200 to 450 nm. MeOH extract was assayed on PDA for activity against *P. capsici* LT3229. Growth inhibition was recorded after 72 h incubation at 25°C. Surfactant production by *P. aeruginosa* BP35 was detected on SW medium (Siegmund and Wagner, 1991) as well as by drop collapse assay and quantified by tensiometric analysis of the surfactant extracted as described by de Souza and colleagues (2003b). Surface tension measurements were carried out with a K6 tensiometer (Krüss GmbH, Hamburg, Germany). Measurements were performed at 25°C, and sterile distilled water was used to calibrate the tensiometer. LC-MS analysis was carried out to establish its identity. Zoosporicidal activities of a 3 µl aliquot of the surfactant were mixed on a glass slide with an equal volume of zoospore suspensions of *P. palmivora*, *P. megakarya* and *P. capsici*. The behaviour of the zoospores was observed under a light microscope (Dialux20 EB; Ernst Leitz GmbH, Wetzlar, Germany) at × 100 magnification for up to 5 min.

PCR detection of genes involved in antibiotic biosynthetic/regulatory pathway. PCR-based detection of genes involved in the biosynthesis of pyrrolnitrin, DAPG, phenazines and pyoluteorin in PaBP35 was performed as described by de Souza and Raaijmakers (2003) and Mavrodi and colleagues (2010) with slight modifications. Rhamnolipid production in *P. aeruginosa* has been reported to require both the *rhl* system and *rhl*A (encoding a rhamnosyltransferase) and *rhl*C [encoding the rhamnosyltransferase involved in di-rhamnolipid (L-rhamnose-L-rhamnose-β-hydroxydecanoyl-β-hydroxydecanoate)]. *rhl*A gene (amplicon size 1100 bp) (Pamp and Tolker-Nielsen, 2007) and *rhl*C gene (amplicon size 1200 bp) (Rahim *et al*., 2001) were amplified using GoTaq PCR system according to manufacture's instruction. Cycling reactions (96°C 1 min 35 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 1 min with the final extension of 72°C for 10 min) were carried out in MJ Research PTC-200 thermocycler. Detection of N-acyl homoserine lactone systems (RhlI/R) was performed by adopting the procedures of Steindler and colleagues (2009).

Genotyping of P. aeruginosa *BP35*

BOX-PCR. Total genomic DNA isolated from PaBP35 using DNeasy kit (Qiagen) was used in the genotyping schemes. PaBP35 was compared with clinical *P. aeruginosa* strain PAO1 for BOX amplicons (Koeuth *et al*., 1995; Rademaker *et al*., 1998). Image analysis was performed with Quantity-One (Bio-Rad) software and the presence (1) or absence (0) of strain-specific polymorphic amplicons was converted into binary data. Phylogenetic and molecular evolutionary analyses were conducted with FreeTree and similarity index was obtained based on Dice coefficient (Dice, 1945; Pavlícek *et al*., 1999).

rec*N sequence analysis.* PCR amplification of a 1200 bp fragment of the *rec*N gene was performed using high-fidelity Phusion PCR according to the manufacturer's instruction. The *rec*N gene fragment was amplified with primers RecN1F (5′GAA CTG CTG ATC GAC ATC CA-3′) and RecN1R (5′AGC AAC TGT CCG ACC ACT TC-3′) designed from *rec*N sequences (PA4763) of completely sequenced genome of *P. aeruginosa* PAO1 (http://www.pseudomonas.com) using Primer 3 plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated *in silico* using oligo calculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html). The PCR product was purified using a commercial kit (GE-illustra GFX PCR DNA and Gel Band Purification kit) according to manufacturer's instruction and sequenced by Macrogen (Amsterdam, the Netherlands). The obtained sequences were trimmed, edited and contig assembled in Vector NTI Suite 8 (Invitrogen) and compared with database sequences. A rooted phylogenetic tree was constructed using the sequences reported here with diverse *Pseudomonas rec*N sequences previously deposited in GenBank in Mega 5 (Tamura *et al*., 2011). Nodal robustness of the inferred tree was assessed by 1000-bootstrap replicates (Felsenstein, 1985).

Multilocus sequence typing. The MLST scheme was adopted from Curran and colleagues (2004) with slight modifications. PCR amplification of all seven housekeeping genes (*acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A and *trp*E) of *P. aeruginosa* BP35 was performed using high-fidelity Phusion PCR according to the manufacturer's instruction. The PCR products were purified with a commercial kit (GE-illustra GFX PCR DNA and Gel Band Purification kit) according to manufacturer's instruction. The purified amplicons were cloned into pGEM-T Easy vector and sequenced at Macrogen (Amsterdam, the Netherlands). Sequences were examined and edited in Vector NTI Suite 8 (Invitrogen). Allele number for each of the loci was obtained from http://pubmlst.org/paeruginosa by blasting the sequence with the database (Jolley *et al*., 2004). Based on the obtained allele numbers,

the allelic profile for strain PaBP35 was determined. To decipher the relatedness of strain PaBP35 to other *P. aeruginosa* strains, we adopted the eBURST algorithm. The following settings for the creation of the eBURST-based snapshot were used: number of loci per isolate, 7; minimum number of identical loci for group definition, 0; minimal single-locus variant count for subgroup definition, 3; and number of resamplings for bootstrap analysis, 1000. This approach specifically examines the relationships within clonal complexes.

Comparative genome hybridization analysis. The genotyping scheme suggested by Wiehlmann and colleagues (2007) was used to compare strain PaBP35 with other *P. aeruginosa* strains representing multiple sources including clinical origins. Briefly, labelled DNA was generated from the bacterial colony by cycles of multiplex primer extension reactions and then hybridized onto a microarray to yield an electronically portable 58-binary marker genotype that represents the core genome by SNPs and the accessory genome by markers of genomic islets and islands. All 58 targets for hybridization were amplified from *P. aeruginosa* colony DNA by cycles of multiplex primer extension reactions with Terminator DNA polymerase whereby the nascent strands are randomly labelled by incorporation of biotin-16-dUTP. The multiplex amplicons were hybridized under high stringency with the oligonucleotide microarray of target sequences that are inserted into the tip of a standard Eppendorf-like microtube. The hybridization signals were detected by colorimetric analysis with streptavidin–horseradish peroxidase conjugate and tetramethylbenzidine. The signals were automatically converted to the multilocus genotype by a Web-based server that compares the genotype with all other entries in the database. The 16-binary data set from multilocus SNP genotype representing the core genome was used to analyse the strain's relatedness to other *P. aeruginosa* strains using the eBURST algorithm as described above (Feil *et al.*, 2004).

Toxicity analysis of PaBP35

Cytotoxicity assay. A549 cells (human lung adenocarcinoma epithelial cell line) were routinely grown in DMEM medium supplemented with 10% fetal calf serum (FCS). Prior to infection, confluent A549 cells were washed and covered with DMEM medium. *Pseudomonas aeruginosa* strains PaBP35, EC21, E501 and OC2E were grown overnight in Luria broth (LB), subcultured into fresh LB and grown to mid-log. A549 cells were infected with mid-log *P. aeruginosa* at an initial multiplicity of infection (MOI) of 10. Culture supernatants were collected at the indicated times and centrifuged for 10 min at 3000 *g* to sediment bacteria and A549 cells. LDH in the supernatant was measured using a Roche LDH kit as per manufacturer's instructions.

Murine airway infection model

Mice. C57BL/6J mice were maintained at the Central Animal Facility of the Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. They were held in groups of three to five animals in microisolator cages (910 cm^2) with filter top lids and free access to sterilized standard laboratory chow (Diet No. 1324; Altromin, Lippe, Germany) and autoclaved, acidulated water at 21 $\pm$ 2°C, 55 $\pm$ 5% humidity and a 10:14 light–dark cycle. All mice were regularly monitored for infection by typical pathogens according to the FELASA recommendations. All procedures performed on mice were approved by the local district governments (AZ. 33.9-42502-04-08/1528) and carried out according to the FELASA guidelines for the veterinary care of laboratory animals.

Infection protocol. Twenty mice were infected intranasally with 2.0×10^6 cfu of a *P. aeruginosa* strain under light anaesthesia. To characterize the course of the bacterial infection, the body condition, weight, rectal temperature and lung function of the mice were evaluated as described previously (Munder *et al.*, 2005; 2011). In brief, the overall health of the animals was assessed by vocalization, piloerection, posture, locomotion, breathing, curiosity, nasal secretion, grooming and dehydration. Dysfunctions in single parameters were assessed by zero, one or two points. The overall fitness of the mice was determined by adding the points resulting in the following score of the mouse body condition: unaffected (0–1), slightly affected (2–4), moderately affected (5–7), severely affected (8–10) and moribund ($\geq$ 11). Non-invasive head-out spirometry was performed daily at the 5 days prior to inoculation and at time points 4, 6, 8, 10, 12, 18, 24, 48, 72, 96, 120 and 144 h post inoculation. Six hours and 24 h after challenge subgroups of mice were euthanized and their left lungs were taken for the determination of bacterial counts (cfu).

Spirometry. Non-invasive head-out spirometry with 14 parameters was performed as outlined elsewhere (Wölbeling *et al.*, 2010). In brief, mice were restrained in inserts where respiration caused air to flow through a pneumotachograph positioned above the thorax of the animals. The airflow was digitalized and analysed with the Notocord Hem 4.2.0.241 software package (Notocord Systems SAS, Croissy Sur Seine, France). Spirometric equipment was supplied by Hugo Sachs Elektronik-Harvard Apparatus (March Hugstetten, Germany). Here, the two robust and significant parameters tidal volume (time required for one breath) and EF50 (expiratory flow at 50% expiration) were shown exemplarily.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Ascending endophytic colonization of PaBP35 in rooted cuttings of *Piper nigrum*. Pre-planting bacterization (OD_{600}: 1.0) was carried out in roots for varying duration.

A. Each bar represents population size of PaBP35 in black pepper plant parts (1.0 g) excised from bacterized rooted cutting. LSD ($P = 0.05$): 0.113. Error bar indicates standard error.

B. Each lane represents PCR amplification of DNA (560 bp) extracted from black pepper plant parts, viz. roots, stem and leaf, excised from bacterized rooted cutting after various duration (0, 30, 60, 120 h) of bacterization. Each gel image represents 0, 7, 14, 21 and 28 days' samples.

*DPT, days post transplanting.

Fig. S2. A. Swarming motility of *P. aeruginosa* strain PaBP35 on KMB soft (0.6% w/v) agar. Cell suspension of strain PaBP35 were spot-inoculated (10 μl) in the centre of the soft agar plates and incubated at 25°C (Petri plate on left) and at 37°C (Petri plate on right) for 18 h.

B. Mycelial inhibition of *P. capsici* LT3229 by PaBP35 (Petri plate on right); control plate (Petri plate on left) did not show any inhibition.

C. RP-HPLC chromatogram at 367 nm of a cell free culture extract of PaBP35.

D. Spectral characteristics of the peak at RT 19.8 min as compared with the spectral characteristics of a pure phenazine standard (phenazine-1-carboxylic acid).

E. Gel picture with PCR products amplified from genomic DNA of *P. aeruginosa* strains PaBP35 and PAO1 with primers specific for the phenazine (*phz*) gene.

F. Inhibition of hyphal growth of *P. capsici* by phenazine-containing MeOH extracts of cell free culture filtrate of PaBP35; MeOH-spotted plate did not show any growth inhibition (Petri plate on left) whereas phenazine-spotted plate showed concentration-dependent inhibition of mycelium (Petri plate on right). Each paper disk was spotted with 0, 5, 10 and 20 μl of MeOH extract containing phenazine.

Fig. S3. A. Secretion of surfactants by *P. aeruginosa* strain PaBP35 on SW medium.

B. Zoosporicidal (*P. capsici*) activity of surfactants extracted from culture filtrates of PaBP35.

C. PCR amplification of two rhamnolipid biosynthesis genes, *rhl*A and *rhl*C, in PaBP35.

D. PCR amplification of two regulatory genes of rhamnolipid biosynthesis, *rhl*I and *rhl*R, in PaBP35. For PCR-based detection, *P. aeruginosa* strain PAO1 was used as a reference.

Fig. S4. Neighbour-joining phylogenetic tree of 1180 bp *rec*N sequences from multiple *Pseudomonas* species and strains from diverse habitats. The phylogenetic tree was generated

with the minimum evolution method using maximum composite likelihood model in Mega 5.01 program (Tamura *et al*., 2011). *Azotobacter vinelandi* served as an outgroup in the analysis. Numbers on the nodal support represents bootstrap values (1000 resamplings). ● *P. aeruginosa* from plant habitat; ▲ *P. aeruginosa* from clinical habitats.

Fig. S5. eBurst diagram of *P. aeruginosa* isolates from multiple habitats including strain PaBP35 (indicated as BP35). The strain relatedness was calculated based on core genome SNPs and presence of two multi-allele loci, *Fli*Ca or *Fli*Cb and *Exo*S or *Exo*U.