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Phenotypic comparison of clinical and plant-beneficial strains of *Pantoea agglomerans*

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Summary. Certain strains of *Pantoea* are used as biocontrol agents for the suppression of plant diseases. However, their commercial registration is hampered in some countries because of biosafety concerns. This study compares clinical and plant-beneficial strains of *P. agglomerans* and related species using a phenotypic analysis approach in which plant-beneficial effects, adverse effects in nematode models, and toxicity were evaluated. Plant-beneficial effects were determined as the inhibition of apple fruit infection by *Penicillium expansum* and apple flower infection by *Erwinia amylovora*. Clinical strains had no general inhibitory activity against infection by the fungal or bacterial plant pathogens, as only one clinical strain inhibited *P. expansum* and three inhibited *E. amylovora*. By contrast, all biocontrol strains showed activity against at least one of the phytopathogens, and three strains were active against both. The adverse effects in animals were evaluated in the plant-parasitic nematode *Meloidogyne javanica* and the bacterial-feeding nematode *Caenorhabditis elegans*. Both models indicated adverse effects of the two clinical strains but not of any of the plant-beneficial strains. Toxicity was evaluated by means of hemolytic activity in blood, and genotoxicity with the Ames test. None of the strains, whether clinical or plant-beneficial, showed any evidence of toxicity. [**Int Microbiol** 2014; 17(2):81-90]

Keywords: *Pantoea agglomerans* · *Erwinia amylovora* · *Meloidogyne javanica* · *Penicillium expansum* · *Caenorhabditis elegans* · biocontrol · biosafety · toxicity · hemolytic activity · Ames test

Introduction

Pantoea agglomerans (formerly known as *Enterobacter agglomerans*, *Erwinia herbicola*, or *Erwinia milletiae*) is a Gram-negative enterobacterium that has been subjected to numerous taxonomic rearrangements, grouping strains of diverse ecological origin [3,9,10,18,20,22,33,34]. *P. agglomerans* is a ubiquitous epiphytic bacterium found on a wide-range of plant species. It is also frequently isolated from animal, aquatic, and soil environments [1,24,25,35]. Certain strains of *P. agglomerans* and *P. vagans* (formerly included in *P. agglomerans*) isolated from plant environments are among the most beneficial biological control agents for the suppression of plant diseases caused by phytopathogenic bacteria and fungi [7,27,28,30,41,53,56,57]. Several such strains have been developed as active ingredients of microbial biopesticides registered as plant protection products against fire blight caused by *Erwinia amylovora* [38].

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Clinical reports have implicated *P. agglomerans* as an opportunistic human pathogen, but these have typically been descriptive, indicate polymicrobial isolations, and lack verification of pathogenicity or demonstrate a role for the bacterium in disease [4,14,32,55]. Inaccurate identification of many clinical strains [48] has further exaggerated the association of *P. agglomerans* with human infections. Although sometimes reported as a plant pathogen [14,23,47], only strains belonging to the pathovars *gypsophilae* and *betae* have been shown to be truly phytopathogenic, as defined by Koch's postulates [13,37]. Plasmids with virulence factors (e.g., type III secretion system genes) that are responsible for phytopathogenicity [37] are carried by both strains but not by other plant and clinical strains [48].

Regulatory decisions for the registration of biopesticides as alternatives to chemical/antibiotic plant protection products rely upon available data [39]. Currently, *P. agglomerans* is classified in Europe as a biosafety level 2 species, precluding its consideration for beneficial applications (EU Directive 2000/54/EC). However, only a few studies have compared beneficial and clinical strains, in contrast to the many studies of other bacterial species, e.g., *Serratia marcescens* [62], *Burkholderia cepacia,* [5,42,59], and *Pseudomonas aeruginosa* [16].

Recent reports of comparative molecular and biochemical analyses found no clear distinction between clinical and plant *P. agglomerans sensu stricto* strains. Clinical and biocontrol strains clustered together according to standard microbiological, metabolic, or biochemical characteristics [48], pattern polymorphisms of total or partial genomic DNA (fAFLPs, ITS, and ERIC/REP-DNA), single-locus sequence analyses [45,48], or DNA-DNA hybridization [8]. Clinical and biocontrol strains also showed no difference in their ability to colonize soybean roots or embryonated chicken eggs [58]. Registration in the USA and Canada of plant strains C9-1 (Blight Ban C9-1, NuFarm) and E325 (BloomTime, Verdesian Life Sciences) and in New Zealand of P10c (BlossomBless, Gro-Chem New Zealand Ltd.) demonstrated a lack of animal pathogenicity, allergenicity, and toxicity; however these data are proprietary and do not extend to strain comparisons.

The aim of our study was to complement genotypic analyses with phenotypic comparisons between *Pantoea* clinical strains and plant-beneficial strains. The strains were compared based on hemolysis, genotoxicity and nematode infectivity. Differential adaptation to plant habitats was evaluated using biocontrol models against fungal and bacterial phytopathogens.

Materials and methods

***Pantoea agglomerans* strains and growth conditions.** Twelve *P. agglomerans* strains and *P. vagans* C9-1 were used, including clinical isolates and plant epiphytes previously described to have beneficial activity as biocontrol agents against plant diseases (Tables 1 and 2). The clinical isolates included the type strain ATCC 27155 (syn. LMG 1286) as well as other strains from research or culture collections. Plant-beneficial bacteria were those typical of most biocontrol strains that are either commercial or have been the subject of considerable research. All strains were previously identified as *P. agglomerans* based upon the results of biochemical tests. According to 16S rRNA gene sequences, the strains belonged to *P. agglomerans* (including *P. vagans* strain C9-1) [48], except for EM13cb and EM17cb, which are closely related to *P. agglomerans* but are not members of this species. Based on Phoenix analysis, they are *P. agglomerans* but along with 16S rRNA sequencing both *gyrB* analysis and MALDI-TOF mass spectrometry

Table 1. Relevant characteristics of strains of plant-beneficial *Pantoea agglomerans* and *P. vagans* used in the present work

Strain	Plant host/material	Country of origin	Target disease	Toxicology CFU/kg[a]	Identification as *P. agglomerans* (*Phoenix, 16S rRNA, gyrB, MALDI-TOF MS*)[b]
CPA-2	apple fruit surface	Spain	postharvest rot	4.3×10^{11}	nd + + +
EPS125	pear fruit surface	Spain	postharvest rot	$>10^{10}$	nd + + +
C9-1	apple stem	USA	fire blight	5×10^{11}	+ + – –
P10c	apple flower	New Zealand	fire blight	nr	nd + + +
Eh252	*Malus pumila*	USA	fire blight	nr	+ + – –
Eh318	apple stem	USA	fire blight	nr	nd + + +
Eh1087	apple flower	New Zealand	fire blight	nr	nd + + +

[a]Toxicology, acute oral toxicity on mammals; nr, not reported; strain CPA-2 in ref. [41]; strain EPS125 in ref. [7]; strain C9-1 in US-EPA Code 006470 [www.epa.gov].
[b]According to ref. [48,49]. +, positive result; –, negative result; nd, not done.

Table 2. Relevant characteristics of strains of *Pantoea agglomerans* of clinical origin used in the present work

Strain	Country of origin	Material of origin	Nature of the infection	Identification as *P. agglomerans* (*Phoenix, 16S rRNA, gyrB, MALDI-TOF MS*)[b]
ATCC 27155 (LMG 1286)	Zimbabwe	knee	laceration	+ + + +
CIP A181	France	blood	bacteremia	+ + + +
VA21971	Switzerland	wound	infected wound	+ + + +
EM13cb[a]	Spain	blood	bacteremia	+ – – –
EM17cb[a]	Spain	blood	bacteremia	+ – – –
EM22cb	Spain	blood	bacteremia	+ + + +

[a]Based on Phoenix these strains are *P. agglomerans* but 16S rRNA, *gyrB* and MALDI-TOF mass spectrometry do not support that identification; instead they belong to *Pantoea conspicua* for EM13cb and *Pantoea anthophila* for EM17cb.
[b]According to ref. [22,48,49]. +, positive result; –, negative result.

place them close to but nonetheless distinct from *P. agglomerans*. Rather, based on the binary data, EM13cb is *Pantoea conspicua* and EM17cb *Pantoea anthophila*. Sequence analysis of housekeeping genes including *gyrB* confirmed the identity of *P. agglomerans* strains and assigned C9-1 to *P. vagans*. Strains for this study were chosen to be representative of different environments of isolation (human/plant, tissue, country) and of the variability in relation to biochemical properties (Phoenix, MALDI-TOF mass spectrometry) [49] and *gyrB* sequences [48]. Strains were recovered from cultures preserved at –80 ºC and cultured overnight on Luria-Bertani (LB) agar at 25 ºC. Colonies were scraped from the agar surface and suspended in sterile distilled water. The cell culture was adjusted to a cell density corresponding to 1×10^8 CFU/ml. Appropriate concentrations were prepared by dilution with sterile distilled H_2O.

Biocontrol of blue mould, a postharvest decay caused by *Penicillium expansum*. *Penicillium expansum* EPS46 [21] was used for the blue mould infection inhibition assay and maintained on potato dextrose agar (PDA) at 4 ºC. Conidia were collected from 7-day PDA cultures incubated at 25 ºC in darkness. The colonies were scraped with a moist cotton swab and resuspended in distilled H_2O containing 0.5 % Tween 80. Spore concentration was adjusted to 10^4 spores/ml using a hemocytometer, and a fresh suspension was used for each trial. Apples (*Malus* × *domestica* L. 'Golden Delicious') were surface-disinfected for 1 min by immersion in dilute sodium hypochlorite (1 % active chlorine), washed twice in sterile H_2O, and air-dried prior to use. Fruit were wounded with a flame-sterilized 3-mm diameter cork-borer to a uniform depth of 5-mm at three equidistant points around the middle and then placed on polystyrene mats in plastic incubation boxes. Each wound was first inoculated with a 50 µl of a bacterial suspension of a *Pantoea* strain (1×10^8 CFU/ml) and incubated for 24 h at 20 °C in sealed boxes with high humidity (*ca.* 100 % RH). Each wound was then inoculated with 20 µl of a *P. expansum* (1×10^4 conidia/ml) suspension and incubated for 5 days. Non-treated controls consisted of fruit inoculated with water or with the pathogen alone. Treatments consisted of three replicates with three pieces of fruit each (three wounds) and arranged in a completely randomized design with two independent trials. Disease incidence for each replicate was determined as the percentage of wounds infected 5 days after inoculation.

Biocontrol of fire blight caused by *Erwinia amylovora*. Biocontrol activity against *E. amylovora* EPS101 was evaluated using apple flowers, following the methods of Cabrefiga and Montesinos [11]. Newly opened pear flowers were collected from an experimental orchard at the Mas Badia Agricultural Experiment Station (Girona, Spain). Individual flowers were placed with the cut peduncle submerged in 1 ml of a 10 % sucrose solution in a single 1.5-mL Eppendorf plastic tube [45]. Tubes containing flowers were supported in tube racks placed in incubation boxes. Flower hypanthia were treated with 20 µl of a *Pantoea* suspension (1×10^8 CFU/ml) and incubated overnight at 20 ºC in sealed boxes with high relative humidity (*ca.* 100 % RH). The flowers were then inoculated with 10 µl of a 1×10^7 CFU/ml suspension of *E. amylovora* deposited on the hypanthia and incubated for 5 days as described above. Non-treated controls consisted of water or pathogen-alone treatments. Treatments consisted of three replicates with eight flowers, and were arranged in a completely randomized design with two trials. Disease severity was evaluated 5 days after inoculation with a severity scale from 0 to 3, in which 0 indicated no symptoms; 1, partial hypanthia necrosis; 2, total hypanthia necrosis; and 3, necrosis progression through the peduncle. Data analysis included calculation of the mean disease severity for each replicate and maximum severity observed within an experiment, as described previously [6].

Adverse effects on nematodes. Toxicity of the *Pantoea* strains to the plant-parasitic nematode *Meloidogyne javanica* and pathogenicity to the bacterial-feeding nematode *Caenorhabditis elegans* were studied.

A population of *M. javanica* was maintained on the root system of tomato plants (*Lycopersicon esculentum* L. ['Rio Grande'] in the greenhouse by periodic transfer to new plant material every 2-3 months. Prior to each trial, *M. javanica* eggs were collected from root galls following Cobb's method [50]. The volume of suspension collected was measured and the egg concentration was determined using a counting chamber at 40× magnification (Olympic Equine Products, Issaquah, WA, USA). *M. javanica* egg suspensions were disinfected by adding sodium hypochlorite (3 % active chlorine) to the suspension and gently agitating for 20 min. Eggs were collected on a sterile 500-mesh sieve and washed with sterile distilled H_2O to remove residual sodium hypochlorite. Eggs were aerated at 20 ºC for 48 h to induce hatching; stage J2 juveniles were collected and the concentration adjusted. Bacterial cell suspensions and cell-free culture supernatants were prepared from 48-h *P. agglomerans* liquid LB cultures centrifuged at 10,000 ×*g* for 15 min. Cell pellets were resuspended in glucose minimal medium (GMM) containing (per liter) 5 g of glucose, 1 g NH_4Cl, 3 g KH_2PO_4, 2.4 g Na_2HPO_4, 0.5 g NaCl, and 0.2 g $MgSO_4$ at pH 7. The concentration was adjusted to 1×10^8 CFU/ml. Culture supernatants (bacterial culture extracts) were separated from the pel-

lets and filtered through a 0.22-µm pore size filter membrane. Toxicity was assayed by placing 100 µl of the *M. javanica* J2 suspension (2000 juveniles/ml) in 30-ml sterile tubes, and adding either 100 µl of bacterial suspension in 1.8 ml of sterile GMM or 1.9 ml of cell-free culture supernatant. Negative control treatments consisted of J2 *M. javanica* incubated with only GMM. A positive control treatment was included, consisting of the chemical nematicide Vydate P (100 mg/l, Du Pont Ibérica, Barcelona, Spain). The tubes were sealed with Parafilm and incubated for 24 h at 25 ºC in darkness. Toxicity was determined as the concentration of viable J2 nematodes by observation at 40× magnification. Dead individuals were determined based on immobility and the presence of straight rigid bodies. Treatments consisted of three replicate tubes arranged in a completely randomized design. Two independent trials were conducted.

Caenorhabditis elegans SS104 was maintained on nematode growth medium (NGM) prepared as follows: 3 g of NaCl, 17 g of agar, and 2.5 g of peptone were added to 975 ml of distilled H_2O. The solution was autoclaved and then cooled to 55 °C. One ml of 1 M $CaCl_2$, 1 ml of 5 mg cholesterol/ml (prepared in ethanol), 1 ml of 1 M $MgSO_4$, and 25 ml of 1 M KPO buffer (108.3 g KH_2PO_4, 25.6 g K_2HPO_4, H_2O to 1 liter, pH 6). *Escherichia coli* OP50 was used as the food source. Strain SS104 is a temperature-sensitive mutant unable to reproduce when incubated at 25 ºC, which ensured a constant number of nematodes during the assay. The nematode age distribution was synchronized before the experiment by a bleaching procedure as previously described [43]. Briefly, the surface of agar plates containing eggs was washed with 5 ml sterile M9 buffer, centrifuged at 1500 ×*g* for 2 min, and the pellet suspended in 4.5 ml of bleaching solution (0.5 ml water, 2.5 ml sodium hydroxide 1 M, and 4 ml sodium hypochlorite ~4 %). The tube was gently mixed intermittently for approximately 5 min to kill all nematode forms except eggs. The reaction was stopped by centrifugation for 1 min at 1500 ×*g*. The pellet was washed three times with M9, resuspended, and then incubated for 14 h at 25 ºC with gentle agitation. Synchronized nematodes (L1 larval stage) were placed on NGM agar plates with *E. coli* OP50 and incubated at 25 ºC for 2 day to obtain stage L4 adult nematodes, that were recovered from the agar plates as described above. Bacterial cell suspensions of the *Pantoea* strains, *E. coli* OP50, and *Salmonella enterica* CECT 4595 (ATCC 14028), used as positive controls in this test, were plated on NGM and grown for 24 h at the appropriate temperature. For the survival assay, collected worms were transferred to fresh lawn plates (150 per plate) of the bacteria (treatments). Each assay was carried out in triplicate. For the assay with cell-free culture supernatants of the bacterial strains, cultures were grown for 48 h in LB broth at 28 ºC, except *S. enterica* CECT 4594, which was incubated at 37 ºC. The cultures were centrifuged at 10,000 ×*g* for 10 min and the supernatants were filter sterilized through a 0.22-µm pore size filter membrane. Four hundred µl of the effluent was dispensed into 24-well plates containing 50 µl of an *E. coli* OP50 suspension in M9 buffer and 50 µl of synchronized L4 nematodes (approximately 100 individuals). Worm mortality was scored over 7 days for bacterial cell suspension and 5 days for cell-free culture supernatants by observation at 40× magnification. Dead individuals were determined based on immobility and the presence of straight rigid bodies. The assay was carried out in triplicate. Sodium azide at 750 µM was used as a positive control.

Hemolytic activity. Hemolytic activity was evaluated in solid and liquid media. *Streptococcus pyogenes* ATCC 19615 and melittin were used as positive controls. In solid medium, hemolytic activity was scored by the presence of a clear halo around bacterial colonies plated in triplicate at the adequate dilutions on blood agar base (Oxoid Limited, Basingstoke, UK) with either 5 % (v/v) sheep or horse erythrocytes (Oxoid Limited) after incubation at 28 ºC for 48 h. In liquid medium, both cells suspended in Ringer's and cell-free culture supernatants obtained after centrifugation at 10,000 × *g* for 10 min of 48-h cultures in LB broth were used. Hemolysis was evaluated by determining hemoglobin release from erythrocyte suspensions of sheep or horse blood (5 % v/v, Oxoid) [2]. Blood was centrifuged at 6000 ×*g* for 5 min, washed three times with Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.2), and diluted ten-fold. Red blood cell aliquots (65 µl) were mixed with 65 µl of bacterial cell suspension (5×10^9 CFU/ml) or cell-free culture supernatants in triplicate in a 96-well reaction plate and incubated with continuous shaking for 1 h at 37 ºC. After the incubation, the tubes were centrifuged at 3500 ×*g* for 10 min. Supernatant aliquots (80 µl) were transferred to 100-well microplates (Oy Growth Curves Ab, Helsinki, Finland) and diluted with 80 µl of deionized H_2O (Milli-Q, Millipore, Billerica, MA, USA). Hemolysis was measured as the absorbance at 540 nm using a Bioscreen C plate reader (Oy Growth Curves Ab). Complete hemolysis was determined in Tris buffer amended with melittin (100 µM) (Sigma-Aldrich, Madrid, Spain) as a positive control.

Genotoxicity. Prior to the Ames test, the culture supernatants were assayed to ensure that they were not cytotoxic to the *Salmonella* strains (survival above 50 %) [32,31]. The bacterial reverse mutation test (Ames test) was performed as described in the Test Guideline 471 (OECD) [19] using two bacterial strains of *Salmonella typhimurium* as reference strains (TA98, to detect frameshift mutations and TA100, for base-pair substitutions), without metabolic activation. Cell-free *Pantoea* culture supernatants were prepared as described above for the hemolytic activity test. Overnight cultures of two strains of *Salmonella* were prepared in LB broth. Genotoxicity was assayed by mixing 0.1 ml of *Pantoea* cell-free supernatants with 3 ml of overlay agar, 0.2 ml of histidine-biotin solution (0.5 mM), and 0.2 ml of *S. enterica* suspension at a concentration of 5×10^8 CFU/ml and plated in minimal medium. Sodium azide at 1.25 and 2.5 µM was used as the mutagenic agent for strain TA100 and 2-nitrofluorene at 2.5 and 5.0 µM as the mutagenic agent for strain TA98. The assay was performed in triplicate. Genotoxicity was scored as positive when the ratio of induced to natural revertants was ≥2.

Statistical analysis. The significance of the effect of treatments was determined using a one-way analysis of variance. Means were separated using the Waller-Duncan test at $P < 0.05$. The analysis was performed with the GLM procedure of the PC-SAS (SAS Institute, Cary, NC, USA).

Results and Discussion

Recent genotypic and biochemical analyses have provided limited discrimination of *P. agglomerans* biocontrol and clinical strains [8,15,48,58], whereas here we used phenotypic comparisons to obtain a greater degree of differentiation. Thus, plant-beneficial strains could be clearly distinguished from clinical strains on the basis of antagonistic activity against plant pathogens. We found no evidence for the toxicity of any of the biocontrol strains, and some evidence for the pathogenicity of the two clinical strains using nematode models. These findings support the previously reported lack of genetic virulence determinants in clinical and biocontrol strains [48].

All seven plant-beneficial strains significantly suppressed at least one of the two plant diseases in assays with the commercial strains (C9-1 and P10c), and Eh252, which showed significant biocontrol activity in both assays. Only two plant-beneficial strains (Eh318 and Eh1087), both originally selected

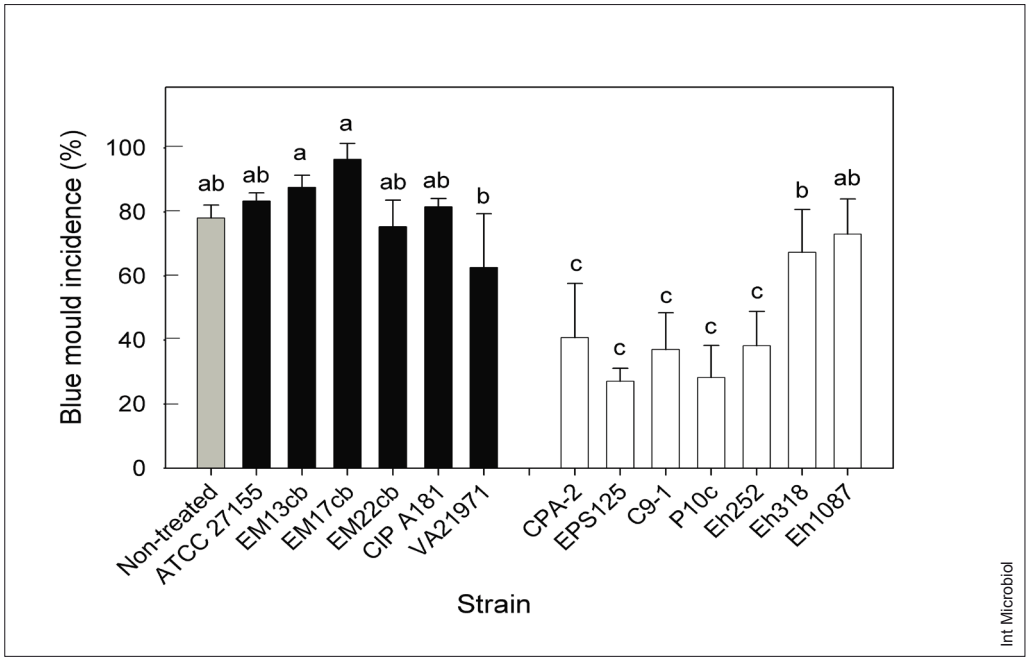

Fig. 1. Biological control of blue mould caused by *Penicillium expansum* on 'Golden Delicious' apples by clinical (black bars) and plant-beneficial (white bars) strains of *Pantoea* spp. Values are the mean of three replicates, each consisting of three fruits, each with three wounds. Error bars represent the 95 % confidence interval of the mean. Bars for blue mould rot incidence labeled with the same letter do not differ significantly ($P < 0.05$) according to the Waller-Duncan test.

as fire blight biocontrol agents, failed to suppress the fungal pathogen *P. expansum* (Fig. 1). Similarly, only the two strains originally selected as postharvest rot biocontrol agents (CPA-2 and EPS125) failed to suppress the bacterial pathogen *E. amylovora* in flowers (Fig. 2), while all of the fire blight biocontrol agents were effective. The inhibitory activity observed

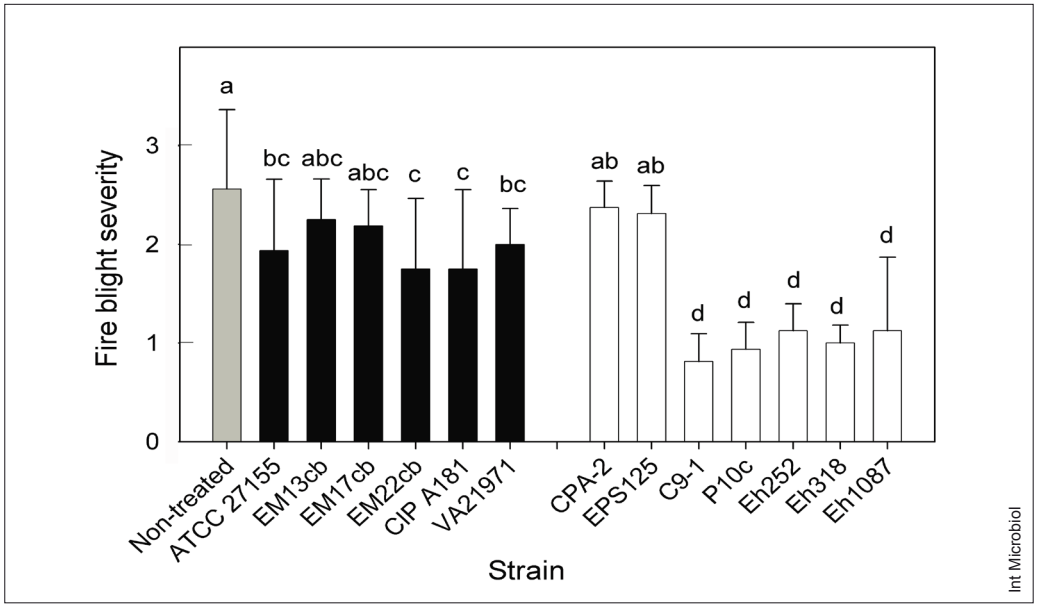

Fig. 2. Biological control of fire blight on pear flower by *Erwinia amylovora* by clinical (black bars) and plant-beneficial (white bars) strains of *Pantoea* spp. Values are the mean of three replicates consisting of eight flowers. Error bars represent the 95 % confidence interval of the mean. Bars for fire blight severity labeled with the same letter do not differ significantly ($P < 0.05$) according to the Waller-Duncan test.

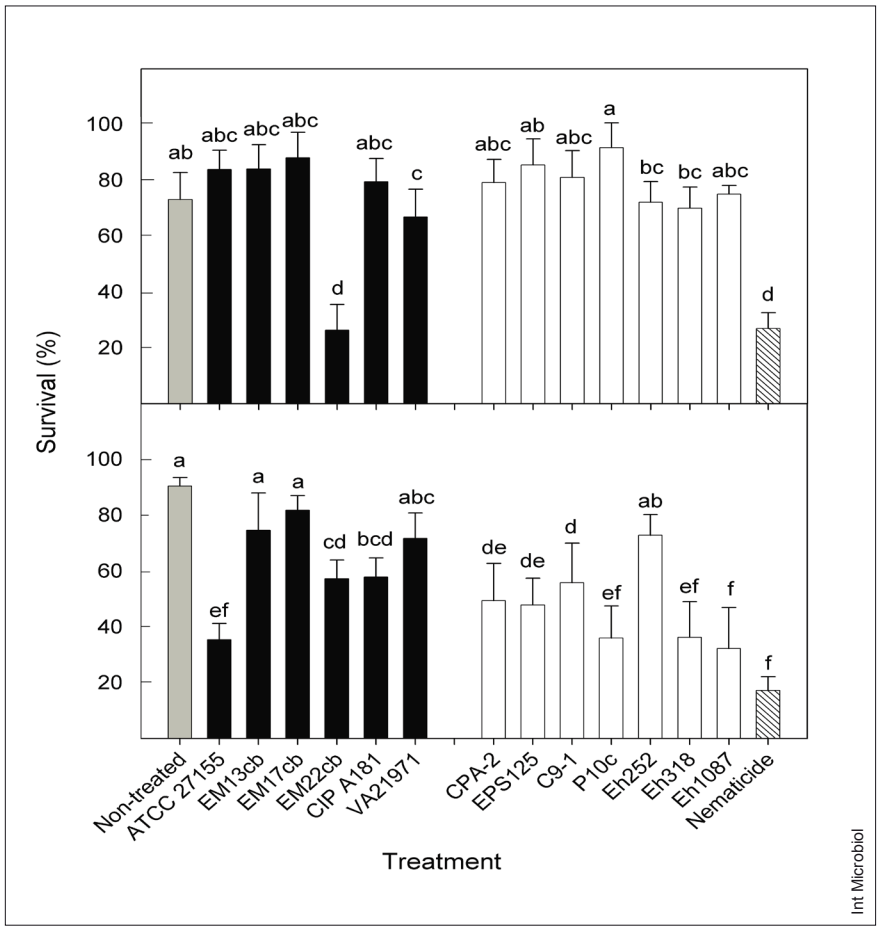

Fig. 3. Effect of cells (upper panel) and cell-free culture supernatants (lower panel) of clinical (black bars) and plant-beneficial (white bars) strains of *Pantoea* on the survival of the plant parasitic nematode *Meloidogyne javanica* (J2 stage juveniles) compared to the chemical nematicide Vydate P (dashed bars). Error bars represent the 95 % confidence interval of the mean. Bars for survival labeled with the same letter do not differ significantly ($P < 0.05$) according to the Waller-Duncan test.

for the biocontrol strains is not surprising since they were previously selected in large screenings of environmental isolates specifically on the basis of their superior biocontrol activity against target pathogens [38]. By contrast, the six clinical *Pantoea* strains were generally ineffective or had only a weak effect in either the postharvest assay or the flower assay. Four out of the six clinical strains inhibited *E. amylovora* infections in flowers, although generally to a lesser extent than the biocontrol strains (Fig. 2).

Our results provide no indication of toxicity or pathogenicity in either nematode models for plant-beneficial strains and only some evidence for the two clinical strains (ATCC 27155 and EM22cb). Cells of most *Pantoea* strains had no effect on the survival of the plant-parasitic nematode *M. javanica*, with only the clinical strain EM22cb killing J2 juveniles to a degree even close to that of the nematicide control (Fig. 3). The pathogenicity of this strain to the bacterial-feeding nematode *C. elegans* was similar to that of *Salmonella* ATCC 4594 (Fig. 4). Except for cells of *P. agglomerans* ATCC 27155, which were also effective in killing *C. elegans*, the remaining strains did not have significant effects on nematode survival in either of the models (Figs. 3 and 4). Cell-free culture supernatants from clinical or biocontrol strains also had no effect on *C. elegans* survival (data not shown) whereas those from most of the biocontrol strains had inhibitory effects on *M. javanica*. Note that only the cell-free culture supernatants of the clinical strain ATCC 27155, whose cells showed pathogenicity to *C. elegans*, also had adverse effects on *M. javanica*. Therefore, we conclude that the clinical strains EM-22cb and ATCC 27155 exhibited adverse effects in both of the nematode models studied.

Nematode assays are widely accepted as simple models to study bacterial virulence mechanisms [12,36,61] and to identify toxic compounds [40]. Although *C. elegans* is most often used, *Meloidogyne* is also sensitive to infection and killing by a wide-range of bacteria, including other Enterobacteriaceae such as *Enterobacter cloacae* [17,51]. Production of the antibacterial compounds that contribute to the biocontrol activity of some *P. agglomerans* strains [28,29,44] could be a factor in the adverse effects of the cell-free culture supernatants on

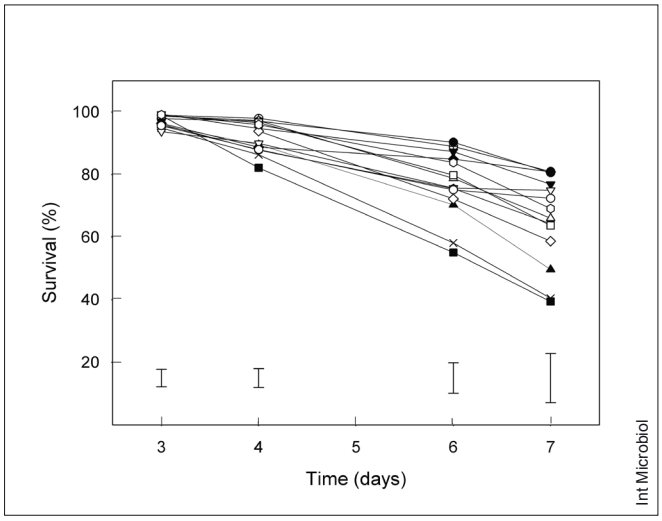

Fig. 4. Survival of *Caenorhabditis elegans* SS104 (adult stage) temperature-sterile mutants in the presence of cells of clinical strains of *Pantoea*: ATCC 27155 (up closed triangle), EM13cb (down closed triangle), EM17cb (closed circle), EM22cb (closed square), CIP A181 (closed diamond), VA21971 (closed hexagon), plant-beneficial strains CPA-2 (up open triangle), EPS125 (down open triangle), C9-1 (open circle), P10c (open square), Eh252 (open diamond), and Eh1087 (open hexagon) compared to the *E. coli* OP50 feeding strain (circle with ×) and to *Salmonella* 4594 (×), pathogenic to mammals. Error bars indicated the minimum significant difference according to the Waller-Duncan test.

plant-parasitic nematode killing, observed in our study.

Strains Eh252, Eh318, and *P. vagans* C9-1 produce the antibiotic pantocin A, and strain Eh318 also produce the antibiotic pantocin B [51,52,53,57,60]. The pantocin A biosynthetic genes *paaABC* are present in those plant-beneficial strains, but absent in the clinical strain EM22cb [48]. Another antimicrobial peptide, herbicolin I, is produced by the plant-beneficial strain C9-1 [27,29,51,52]. Phenazine is reportedly

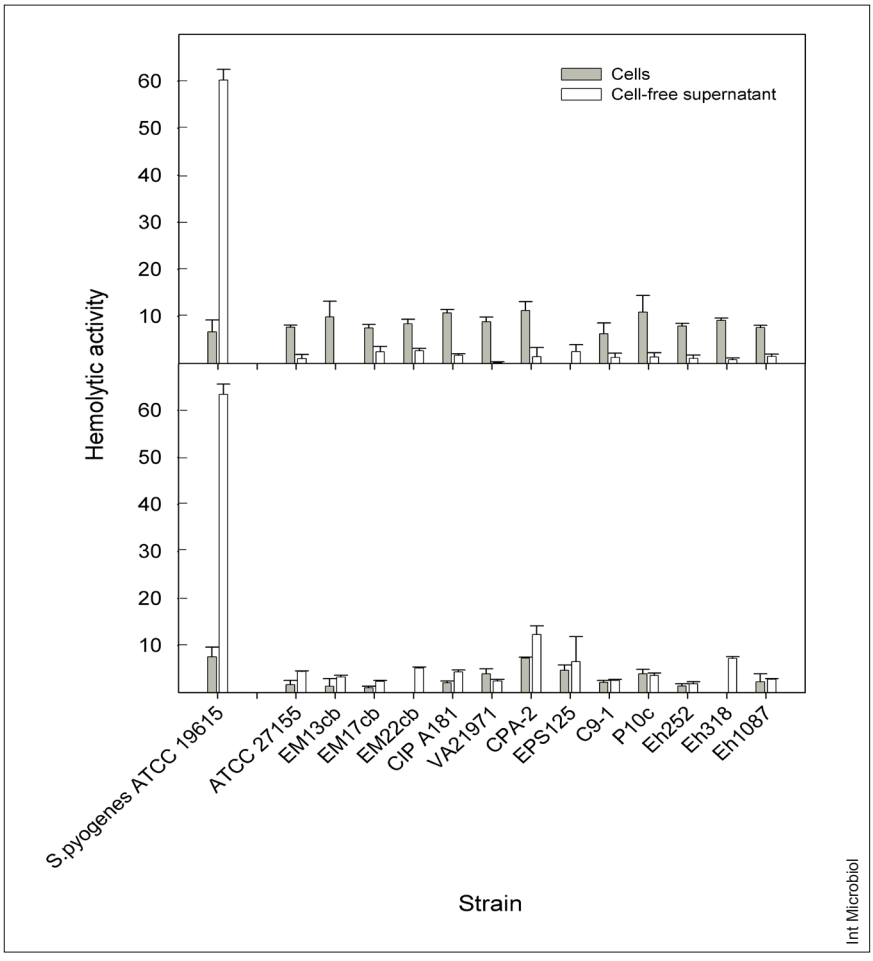

Fig. 5. Hemolytic activity of clinical (left bars) and plant-beneficial (right bars) strains of *Pantoea* compared to a reference hemolytic *Streptococcus pyogenes* strain. The assay was performed using cells and cell-free culture supernatants of *Pantoea* strains together with sheep blood (upper panel) and horse blood (lower panel). Error bars of hemolysis represent the 95 % confidence interval of the mean.

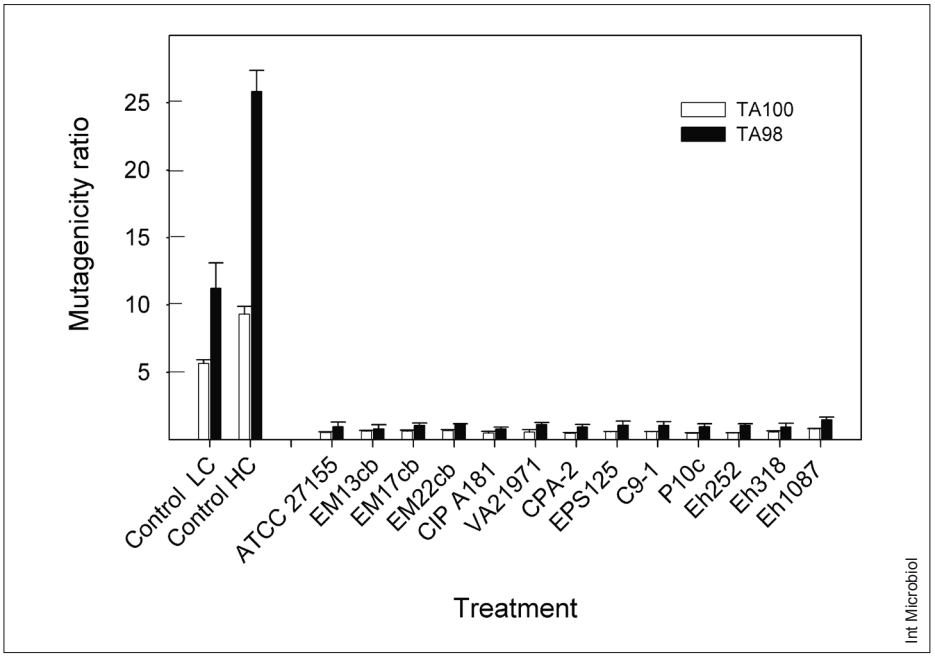

Fig. 6. Genotoxicity of cell-free culture supernatants of *Pantoea* strains (Ames test) compared to mutagenic agents. Mutant strains of *Salmonella* TA100 and TA98 were used as indicators. Sodium azide at 1.25 (LC) and 2.5 (HC) μM was used as the mutagenic agent to strain TA100 and 2-nitrofluorene at 2.5 (LC) and 5.0 (HC) μM as the mutagenic agent to strain TA98.

produced by the plant-beneficial strain Eh1087 [30], which also had adverse effects on *M. javanica* survival. The type III secretion system (T3SS) gene *hrcN*, an important factor in bacterial virulence against eukaryotes [26,46], is present in clinical strain VA2197, but absent in clinical strain EM22cb, the most lethal strain in the nematode assays [48]. It should be noted, however, that the T3SS present in *P. agglomerans* is phylogenetically more similar to the one present in biocontrol strains of *Pseudomonas* spp. than to the Inv/Spa system present in animal pathogens.

The data obtained in the present work with both nematode models are in agreement with the reported lethal oral dose of the biocontrol strains *P. vagans* C9-1 and *P. agglomerans* E325, CPA-2, and EPS125, which exceeded 10^8–10^{10} CFU/kg animal body weight in Sprague-Dawley CD rats [7,41, EPA Federal Registers 71:54928-54933 and 71:24590-24596]. Accordingly, the USA Environmental Protection Agency (EPA) has registered two commercial biopesticides containing strains C9-1 and E325 on the basis of several toxicological tests, labeling these strains as toxicity category IV (i.e., "practically non-toxic").

None of the plant-beneficial or clinical *Pantoea* strains had significant hemolytic activity in red blood cell assays conducted in liquid or agar culture, compared to the *S. pyogenes* ATCC 19915 positive control. In blood agar, a clear halo was observed and the percentage of hemolysis in the liquid assays for the supernatants was around 60–65 % of the melittin hemolysis for *S. pyogenes* using sheep and horse blood (Fig. 5). None of the cell-free culture supernatants, from either clinical or plant-beneficial *Pantoea* strains, showed genotoxicity to strains TA98 and TA100 in the Ames test (Fig. 6).

In conclusion, clinical *Pantoea* strains are indistinguishable from plant-beneficial strains on the basis of hemolytic or genotoxicity tests. However, the plant models permit differentiation of plant-beneficial from clinical strains. While the nematode models provided some proof of the adverse effects of the two clinical strains (ATCC 27155, EM22cb), negative effects were not observed with the plant-beneficial strains *P. agglomerans* and *P. vagans* C9-1. Finally, there was no evidence suggesting the toxicity of any of the plant-beneficial or clinical strains tested.

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