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Mechanisms of antagonism of *Pseudomonas fluorescens* EPS62e against *Erwinia amylovora*, the causal agent of fire blight

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Summary. *Pseudomonas fluorescens* EPS62e was selected during a screening procedure for its high efficacy in controlling infections by *Erwinia amylovora*, the causal agent of fire blight disease, on different plant materials. In field trials carried out in pear trees during bloom, EPS62e colonized flowers until the carrying capacity, providing a moderate efficacy of fire-blight control. The putative mechanisms of EPS62e antagonism against *E. amylovora* were studied. EPS62e did not produce antimicrobial compounds described in *P. fluorescens* species and only developed antagonism in King's B medium, where it produced siderophores. Interaction experiments in culture plate wells including a membrane filter, which physically separated the cultures, confirmed that inhibition of *E. amylovora* requires cell-to-cell contact. The spectrum of nutrient assimilation indicated that EPS62e used significantly more or different carbon sources than the pathogen. The maximum growth rate and affinity for nutrients in immature fruit extract were higher in EPS62e than in *E. amylovora*, but the cell yield was similar. The fitness of EPS62e and *E. amylovora* was studied upon inoculation in immature pear fruit wounds and hypanthia of intact flowers under controlled-environment conditions. When inoculated separately, EPS62e grew faster in flowers, whereas *E. amylovora* grew faster in fruit wounds because of its rapid spread to adjacent tissues. However, in preventive inoculations of EPS62e, subsequent growth of EPS101 was significantly inhibited. It is concluded that cell-to-cell interference as well as differences in growth potential and the spectrum and efficiency of nutrient use are mechanisms of antagonism of EPS62e against *E. amylovora*. [**Int Microbiol** 2007; 10(2):123-132]

Key words: *Erwinia amylovora* · *Pseudomonas fluorescens* · fire blight disease · biological antagonism

Introduction

Fire blight of rosaceous plants is an economically important disease caused by *Erwinia amylovora* that affects mainly apple and pear production and several woody ornamental plants. The disease can be partially controlled through the use of appropriate culture measures and treatment with antibiotics, copper derivatives, or other chemical compounds [35, 46]. However, the use of antibiotics is not authorized in several countries and may result in the selection of resistant strains of the pathogen, thus limiting disease control [24,26].

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Biological control of fire blight offers an alternative or complementary approach to the use of chemical control [31,32,34,46]. Several strains have been reported to be effective antagonists of *E. amylovora* with respect to *Pseudomonas fluorescens* [21], *Pantoea agglomerans* (syn. *Erwinia herbicola*) [2,48], and *Bacillus subtilis* [7]. *P. fluorescens* A506, *P. agglomerans* E325 and *Bacillus subtilis* QST713 are already registered or in the process of registration as biological products for fire-blight control in the USA. However, due to restrictions imposed by new regulations, only *B. subtilis* QST713, formulated as Serenade, is registered in Europe for fire-blight control.

Understanding the mechanism of action of a biological control agent may allow the optimum conditions for implementing biocontrol in a given pathosystem to be determined [25,30]. However, assessment of the mechanisms of antagonism is a complex and difficult task, starting with prospec-

tive studies to reveal the implications of a given process (e.g., antibiosis, nutrient competition, host colonization). In some cases, molecular methods based on defective mutants will provide a more robust confirmation of the putative mechanism [17]. Several mechanisms have been proposed to explain the inhibition of *E. amylovora* and control of fire blight, depending on the strain of antagonist, but most studies have focused on antibiosis [43,46]. The implication of pantocin antibiotics produced by a strain of *P. agglomerans* (syn. *E. herbicola*) against *E. amylovora* was shown in vivo [51]. Information on *P. fluorescens* is restricted to strain A506, in which the mechanism proposed was initially based on competitive exclusion [49] but antibiosis was later identified as an additional mechanism [44]. In the case of strains of *B. subtilis*, antibiosis has not been demonstrated [1,7], but its implication is expected due to the ability of this genera to produce antibiotics and exoenzymes [4,22]. Therefore, it seems that the most efficient biological control agents of fire blight reported thus far rely on antibiosis.

Apart from antibiosis, other putative mechanisms have been shown following analysis of the spectrum of nutrient use and nutrient competition [17,50], interaction studies using membrane filters in plant extracts [5,16], and growth vs. nutrient concentration response analysis [3,13,20]. However, these experimental approaches, all of which have been tested in post-harvest pathogen–biocontrol-agent systems, have not been applied to study the mechanism of fire-blight biocontrol. We are interested in biological control agents of fire blight that do not produce antibiotics, because of restrictions in Europe for the registration of such agents producing secondary metabolites [36]. Therefore, non-antibiotic-producing strains of bacteria isolated from fruit tree environments were screened for antagonism to *E. amylovora*. Accordingly, strain EPS62e of *P. fluorescens* was isolated using an ex vivo selective-enrichment procedure and further selected among several candidate strains based on its wide range of activity [6,37].

The purpose of the present work was to determine the putative mechanisms of *E. amylovora* inhibition by *P. fluorescens* EPS62e. Antibiosis, cell-to-cell interaction, nutrient competition, and competitive exclusion by colonization of entry sites on the plant host were thus evaluated.

Material and methods

Bacterial strains and culture conditions. *Pseudomonas fluorescens* EPS62e was isolated from a healthy pear of cultivar Conference in a commercial orchard near Girona (Spain). This strain was selected from a collection of 600 isolates of *P. agglomerans* and *P. fluorescens* for its high efficacy in the inhibition of *E. amylovora* infections in immature fruits. A spontaneous mutant of EPS62e resistant to 50 µg nalidixic acid/ml but which retained the phenotypical and genotypical characteristics and performance of the wild-type parental strain was selected. *E. amylovora* EPS101, a highly aggressive wild-type strain [9], was isolated from an infected shoot of a Conference pear in Lleida (Spain). A spontaneous mutant of *E. amylovora* EPS101 resistant to 100 µg rifampicin/ml was selected and confirmed to be as aggressive as the wild-type parental strain. In some experiments, *E. amylovora* Ea273 (kindly provided by S. V. Beer), isolated from *Malus sylvestris*, was used. Bacterial suspensions of the antagonist and pathogenic strains were obtained from ultra-freeze-preserved cultures (–80ºC) grown overnight at 25ºC in Luria Bertani (LB) agar. Colonies were scraped from the agar surface and suspended in sterile distilled water. The cell concentration was adjusted to 10^8 colony-forming units (CFU) per ml and diluted in sterile distilled water until the proper concentration was obtained.

Source of plant material. Immature fruits and flowers of pear were obtained from commercial orchards. The pear cultivars used were Doyenne du Comice, Blanquilla, Conference, and Passe Crassane. Fruits were collected in early June at the 6-week stage following fruit set and kept in the dark at 0–4°C. The fruits were used prior to one month of storage to avoid significant physiological changes that could have affected the assay results. Before inoculation, the fruits were surface-disinfected by immersion for 1 min in a diluted solution of sodium hypochlorite (1% active chlorine) and washed twice in distilled water; excess water was removed under air flow in a sterile cabinet. Each fruit was wounded four times on opposite sides with a cork-borer (approximately 2 mm diameter and 5 mm depth). Fruits were placed in polystyrene-let to remove in boxes. Individual pear flowers were obtained from detached pear branches, age two years, taken from orchards during the winter and kept at 0–4ºC until use.

Detached pear branches, bearing 7–15 dormant flower buds, were forced to bloom in an environmental chamber following the procedure described by Montesinos and Vilardell [28]. The open blossoms were detached from branches and the individual flowers were maintained with the cut peduncle submerged in 1 ml of a 10% sucrose solution in a single plastic Eppendorf vial of 1.5 ml. Vials containing flowers were placed in plastic tube racks for treatment with the biocontrol agent and inoculation with the pathogen [38].

Self-rooted pear plants were obtained by micropropagation (Agromillora Catalana, S.A., Barcelona, Spain). Two- to 3-year-old plants were grown in 20-cm-diameter plastic pots and left outside the greenhouse during the winter in order to chill. During the early spring, the plants were pruned such that three or four shoots per plant remained and then forced to bud in the greenhouse. Fertilizer (200 ppm N-P-K solution; 20-10-20) was applied once a week. The plants were used when the shoots were 3 or 4 cm long and had 5 or 6 young leaves. Standard insecticide and miticide sprays were applied. Before the plants were treated with the biocontrol agent and pathogen inoculation, the three youngest expanded leaves of each shoot were wounded by a double incision (~1 mm) perpendicular to the midrib, approximately in the middle of the leaf.

Efficacy assays. Controlled environment assays were carried out in immature pear fruits (cv. Passe Crassane, Blanquilla, Conference and Doyenne du Comice), pear flowers (cv. Conference and Doyenne du Comice), and whole pear plants (cv. Conference). For the *P. fluorescens* EPS62e treatments, 10 µl of the antagonist suspension was deposited at 10^8 CFU/ml in each of the wounds produced in the immature fruits and young leaves or on the surface of the hypanthium in flowers. Treated plant material was covered with plastic bags and after 24 h of incubation at 21ºC, exposed to high relative humidity and 16 h of fluorescent light. Ten µl of a pathogen suspension containing 10^7 CFU/ml was then deposited at the same site as the antagonist. The treated and inoculated plant material was covered again with plastic bags and incubated at 21ºC, high relative humidity, and 16 h of fluorescent light for 10 days. The experimental design consisted of three repetitions of nine immature fruits, eight flowers, and three plants per treatment. Non-treated controls inoculated with water or with the pathogen were included.

Incidence per wound was evaluated for each repetition 7 days after pathogen inoculation.

In field studies, EPS62e was applied in the field during bloom to determine the degree of colonization of pear flowers and the extent of protection against *E. amylovora* infection. Two trials were carried out, one in a Conference cultivar and another in a Doyenne du Comice cultivar, in an experimental orchard located in Mas Badia Agricultural Experiment Station (Girona). Trees were sprayed three times with a suspension of EPS62e (10^8 CFU/ml) until the runoff point during the bloom period (20, 75, and 100% full bloom). A non-treated control using water was included. The experimental design consisted of three replicates per treatment with five trees per replicate. Disease expression was assessed in the laboratory under biological security conditions. For those experiments, six branches per replicate with approximately three cluster flowers per branch were collected after 12 days of field incubation. Detached branches were artificially inoculated with *E. amylovora* by deposition of 10 µl of pathogen suspension adjusted to 10^7 CFU/ml in the central flower of each flower cluster. Once inoculated, branches were maintained in a sucrose nutrient solution at 25ºC and covered with a plastic bag. Incidence per cluster flowers was evaluated per each repetition 7 days after pathogen inoculation.

Production of antimicrobial compounds. The capacity of EPS62e to produce the antibiotics described for the species *P. fluorescens* was determined using a method based on the PCR detection of biosynthetic genes. Specific gene sequences corresponding to the antibiotic biosynthetic genes for phenazine-1-carboxylic acid (PCA), 2,4-diacetylphlorogucinol (PHL), and pyrrolnitrin (PRN) were evaluated. PCA was determined using the primers PCA2a (5′ TTGCCAAGCCTCGCTCCAAC 3′) and PCA3b (5′ CCGCGTTGTTCCTCGTTCAT 3′), developed from two genes described inside the PCA biosynthesis cluster of *P. fluorescens* 2-79 (*phzC* and *phzD*) [40]. PHL was determined using the primers Phl2a (5′ GAGGACGTCGAAGACCACCA 3′) and Phl2b (5′ ACCGCAGCATCGTGTATGAG 3′), targeted to the *phlD* gene [40]. PRN was determined using the primers Prna (5′ TCAAGGACAAGCCGACCGAGT 3′) and Prnb (5′ GCAGCCCGAACAGCACGAAGT 3¢), developed for the *prnD* gene encoding for the aminopyrrolnitrin oxidase [8].

PCA and Phl production was also assessed using a modified high-performance liquid chromatography (HPLC) technique [18]. Compounds were extracted over liquid cultures of a strain grown in LB broth amended with 1% glucose during 48 h at 25ºC.

Siderophore production was determined in vitro by means of the Chrome Azurol S (CAS) agar-plate method, as described by Schwyn and Neilands [41]. EPS62e was grown previously in King's B (KB) medium in order to stimulate siderophore production and was inoculated in CAS agar plates by transferring the colonies to the surface of the agar plates with toothpicks. Plates were incubated at 22ºC for 2 days. An orange-colored halo around the colony was considered positive for siderophore production. Strain BL915 of *P. fluorescens* was included as a positive control.

In vitro antagonism against strains of *E. amylovora* and other phytopathogenic bacteria. The spectrum of inhibition of EPS62e on agar media was determined against 16 strains of *E. amylovora* from different host plants and geographical origins (EPS100, EPS101, Ea273, CFBP1430, PMV6076, UPN529, UPN611, USV1000, OMP-BO1185, IVIA1614.2, Ea115.22, EAZ4, NCPPB1819, NCPPB2080 and NCPPB3159) and six phytopathogenic bacteria, including *Xanthomonas fragariae* (CFBP3549-95), *Ralstonia solanacearum* (CECT125), *Agrobacterium tumefaciens* (CECT472), *P. syringae* pv. *tomato* (DC3000), *P. syringae* pv. *phaseolicola* (CFBP3635-95), and *P. syringae* pv. *syringae* (EPS94). Antagonism on agar media was tested with agar overlays prepared by mixing 4.5 ml of melted agar and 0.5 ml of bacterial suspension at 10^8 CFU/ml. Antagonist colonies were transferred to the surface of the overlay agar plates with toothpicks and the plates were incubated at 25ºC. Antagonistic activity against *E. amylovora* strains was assessed on LB, KB, KB amended with iron (50 µM $FeCl_3$), glucose-asparagine medium (GA), and GA amended with iron. Three repetitions for each strain were performed. Antagonism was detected as the presence of a halo of inhibition of the indicator microorganism around the colony after 48 h of incubation at 21ºC [27].

The effect of spent media from cultures of EPS62e was also tested. The antagonist was grown on GA broth for 48 h at 25ºC. The culture was then filtrated through a 0.45-µm membrane filter. Half of the filtrated spent medium was amended with concentrated fresh GA and the remaining half was unmodified. Thereafter, *E. amylovora* was inoculated on fresh, spent, and spent-amended GA broth and growth was monitored after 48 h.

Interactions through membrane filters. Competition for nutrients, antibiosis, and direct cell interaction between *P. fluorescens* EPS62e and *E. amylovora* EPS101 were also studied using a modification of the method developed by Janisiewicz et al. [16]. Each well of a 24-well tissue-culture plate (Costar-Corning, Corning, NY, USA) contained a Millicell culture-plate cylindrical insert with a hydrophilic membrane of 0.45 µm pore size (Millipore Corp., Bedford, MA, USA) as the inner compartment. Immature pear fruit extract prepared from immature Passe Crassane fruits was assayed. For the preparation of extract, fruits were surface-disinfected and homogenized using a Waring blender. The slurry material thus obtained was centrifuged at 4,000 rpm for 5 min and diluted to 10% in sterile distilled water. Diluted extract was filter-sterilized through a 0.45-µm pore filter (Millicell-CM, Millipore, Bedford, MA, USA). Interaction experiments were carried out to test the effect of initial populations of EPS62e (0.5×10^6, 5×10^7 and 5×10^8 CFU/ml), direct contact between cells (presence or absence of membrane), and pear-extract concentration (1 or 10%) on *E. amylovora* inhibition. In the assay in which strains were in direct contact with the test substances, 0.6 ml of pear extract was placed in the outside well, and 0.2 ml of the corresponding concentration of EPS62e plus 0.2 ml of EPS101 at 10^7 CFU/ml were inoculated inside the cylinder insert. In the assay in which strains were separated from the test substances by the membrane filter, 0.4 ml of pear extract and 0.2 ml of the corresponding concentration of EPS62e were placed in the outside well and 0.2 ml of a suspension of EPS101 plus 0.2 ml of pear extract were placed inside the cylinder insert. A non-treated control inoculated with water instead of EPS62 was included. Each assay was replicated three times. Plates were incubated at 25ºC for 48 h after which 100 µl were taken from the inside cylinder insert and from the outside well and serially diluted ten-fold in sterile distilled water. Aliquots of appropriate dilutions were seeded on LB agar plates amended with 100 µg rifampicin/ml for assessment of strain EPS101 or with 50 µg nalidixic acid/ml for assessment of EPS62e. Colony counts of *E. amylovora* EPS101 and *P. fluorescens* EPS62e were assessed after 24 h of incubation at 25ºC. ANOVA was used to test the effect on growth of *E. amylovora* EPS101 with respect to the ratio of biocontrol agent to pathogen and the effects of physical separation between pathogen cells and biocontrol agent, and of nutrient concentration. Statistical analyses were done using SAS (version 8.2, SAS Institute, NC, USA).

Spectrum of nutrient use and niche-overlap index. Nutritional profiles of carbon source utilization by *P. fluorescens* EPS62e and *E. amylovora* EPS101 were determined using Biolog GN microplates (Biolog, Hayward, CA, USA) according to the manufacturer's instructions. Microplates corresponding to *E. amylovora* EPS101 were incubated for 24 h at 25ºC. EPS62e microplates were incubated for 6 h at 25ºC. Each well was scored as positive or negative according to the optical density at 405 nm. Wells with an optical density higher than 0.25 were considered positive. The niche overlapping index (*NOI*) was calculated as the number of carbon sources utilized by both bacteria respect to the total number of carbon sources utilized by either EPS62e or EPS101 [15].

Growth vs. nutrient concentration response analysis. The maximum cell yield (Y_{max}), maximum growth rate (μ_{max}), and half-saturation constant for immature pear extract (K_s) were determined for *E. amylo*vora strains Ea273 and EPS101, and for *P. fluorescens* EPS62e. Experiments were done using immature pear extract, obtained as previously described.

Pear extract was used at different concentrations (0.62, 0.31, 0.155, 0.124, 0.078, 0.039, 0.019, 0.009, 0.006, and 0.005 g soluble solutes/l). Growth curves at each nutrient concentration were determined using the Bioscreen system (Labsystems, Helsinki, Finland). A 20-µl suspension of the bacteria at 10^8 CFU/ml was transferred to each well of a 100 well-microtiter plate containing 180 µl per well of the corresponding medium concentration. Each treatment was replicated three times. Measurements were taken at 600 nm. Samples were shaken at medium intensity for 10 s prior to OD readings, obtained at an incubation temperature of 25ºC. Growth was measured every 30 min during 72 h. A calibration curve was previously done for each bacterium to relate the optical density at 600 nm to viable-cell concentration. Growth rates (μ) for each strain in the corresponding nutrient concentration were estimated by linear regression from linearized growth curves, assuming an exponential growth function during the exponential phase. The maximum growth rate (μ_{max}) and the half-saturation constant (K_s) were estimated by linear regression using double-reciprocal plot transformation of the growth rate (μ) and the initial nutrient concentration (S), according to the hyperbolic saturation function. Maximum cell yield for each strain was determined considering the growth attained at the end of the incubation period for the highest nutrient concentration. ANOVA was performed to test significant differences in Y_{max}, K_s, and μ_{max} between the *E. amylovora* strains and EPS62e. Means were separated by Tukey's test ($P \leq 0.05$). Statistical analyses were done using SAS (version 8.2, SAS Institute, NC, USA).

Interactions between antagonist, pathogen, and host-plant material. The ability of EPS62e to colonize and inhibit growth of *E. amylovora* EPS101 in immature fruit and flowers of pear was investigated. Experiments were done on Passe Crassane immature fruits and Doyenne du Comice flowers. Fruits and flowers were obtained and prepared as previously described for the efficacy assays. Fruit wounds and hypanthia of flowers were treated with 10 µl of EPS62e at 10^8 CFU/ml 12 h before inoculation with 10 µl of *E. amylovora* EPS101 at 10^7 CFU/ml. Two controls were included, one inoculated only with EPS62e at 10^8 CFU/ml and another inoculated only with EPS101 at 10^7 CFU/ml. Three replicates of three flowers or fruits per replicate were used for each treatment and time. Population levels of the antagonist and pathogen were monitored by the withdrawal of samples at different times during 72 h. Samples of flowers and fruits were homogenized in a sterile plastic bag with 20 ml of buffered peptone water (1 g peptone/l, 0.05 M Na_2HPO_4, 0.02 M KH_2PO_4, pH 7.0) using a stomacher (Masticator, IUL Instruments, UK). Extracts were serially diluted and 0.1-ml aliquots of appropriate dilutions were spread on LB agar plates amended either with 50 µg nalidixic acid/ml, for analysis of EPS62e, or with 100 µg rifampicin/ml for assessment of EPS101. Plates were incubated at 25ºC and colonies counted after 24 h. Population levels were expressed as CFU/wound or CFU/flower. Growth rates of *E. amylovora* and EPS62e were calculated using the exponential model as the slope of the log CFU vs. time relationship during the exponential phase.

Results

Efficacy assays. EPS62e was tested with different plant material (immature fruits, flowers, whole plants, and different cultivars) in several assays. The results confirmed its efficacy in significantly decreasing the incidence of infections caused by *E. amylovora* (Table 1). Figure 1 shows the effect of treatment of immature fruit wounds with EPS62e on infection intensity by *E. amylovora*. In both experiments performed in the field, EPS62e colonized flowers and remained at population levels in the range of 6.5–7.7 log CFU/flower during the blossom period. Upon inoculation with *E. amylovora* in the laboratory and expression of symptoms, the incidence of infected flowers was very high in the non-treated control (~89.6–89.9%) but was reduced to 61.9% (Conference) and 54.0% (Doyenne du Comice) in EPS62e-treated trees (Table 1). Therefore, in both trials the treatment of EPS62e was significantly effective and efficacy was moderate, with incidences of 31–40%.

Table 1. Disease incidence in immature fruits, flowers and plants of several pear cultivars inoculated with *Erwinia amylovora* EPS101 and treated with *Pseudomonas fluorescens* EPS62e

			Disease incidence (%)		
Cultivar	Trial No.	Plant Material	Non-treated control	Treated	P < F[a]
Passe Crassane	1	Immature fruits	97.9	16.7	<0.001
Blanquilla	1	Immature fruits	93.8	70.7	0.027
Conference	1	Immature fruits	96.9	66.6	0.042
	2	Detached flowers	95.0	30.4	<0.001
	3	Whole plants	94.3	2.0	<0.001
	4	Field assay	89.6	61.9	0.008
Doyenne du Comice	1	Immature fruits	90.6	37.5	0.010
	2	Detached flowers	95.8	16.7	<0.001
	3	Field assay	89.9	54.0	0.019

[a]Significance obtained in a one-way analysis of variance (P < 0.05).

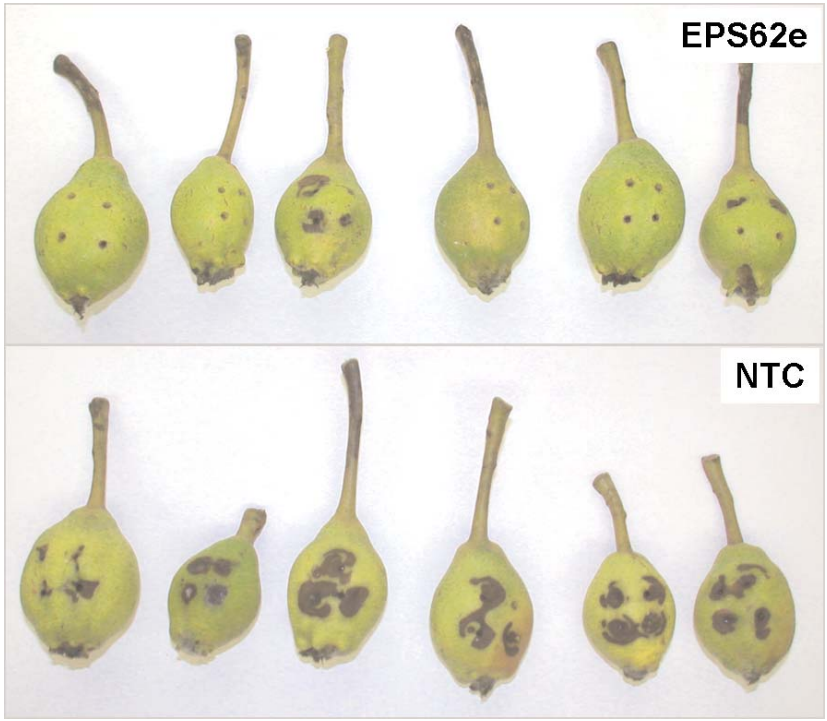

Fig 1. Effect of the treatment of wounds of immature pears with *P.seudomonas luorescens* (EPS62e) or non-treated (NTC) on the intensity of infections after inoculation with *Erwinia amylovora.*

Production of antimicrobial compounds and range of antagonism in vitro. Strain EPS62e does not have the biosynthetic genes to produce the common antimicrobial compounds described in *P. fluorescens* species, e.g., 2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid, and pyrrolnitrin. These results agree with the lack of true antibiosis against *E. amylovora*. EPS62e only developed antagonism on KB medium against seven out of the 16 strains of *E. amylovora* tested, including EPS101, CFBP1430, PMV6076, UPN529, OMP-BO1185, Ea115.2, and NCPPB1819, but this activity was lost when iron was amended to the medium. In addition, for plant pathogens other than *E. amylovora*, antagonism was essentially limited to the genus *Xanthomonas* as well as to *Ralstonia solanacearum* and *P. syringae*, although in all cases inhibition on KB also disappeared upon iron amendment. These results suggested that inhibition was mediated by siderophore production, which was confirmed by the presence of an orange halus in CAS agar around the EPS62e colonies. Spent medium inhibited growth of *E. amylovora*, but was not inhibitory after restoration of the carbon sources, indicating that antibiosis due to the production of inhibitory substances was not present.

Interaction through membrane filters. Growth of *E. amylovora* in pear extract was inhibited following the addition of EPS62e cells (Table 2). A significant effect of inhibition of *E. amylovora* growth was observed for the initial population of *P. fluorescens* EPS62e ($P < 0.0001$) and cell-to-cell biocontrol agent and pathogen contact ($P < 0.0001$). The effect was dependent also on the concentration of pear extract ($P < 0.0001$).

When the experiment was done at 0.62 g soluble solutes/l pear extract, and biocontrol agent and pathogen were cultured separated by a membrane filter, growth of *E. amylovora* EPS101 was not affected at initial EPS62e populations of 10^7 CFU/ml and 10^8 CFU/ml (ratio EPS62e/EPS101 1:1 and 10:1), but was slightly reduced when the initial population was very high, at 10^9 CFU/ml (ratio 100:1). In contrast, when the pathogen and biocontrol agent were incubated together, growth of *E. amylovora* was reduced in all cases compared to the non-treated control (without EPS62e). In addition, the level of inhibition of *E. amylovora* growth increased with an increase of the initial population of EPS62e.

When the experiment was carried out in diluted pear extract (0.062 g soluble solutes/l) and pathogen and biocontrol agent were separated by the filter membrane, the reduction of growth of *E. amylovora* by EPS62e was not significant at the initial population of 10^7 CFU/ml compared to the non-treated control, but was significant at 10^8 and 10^9 CFU/ml. When EPS62e and *E. amylovora* were cultured together, growth of *E. amylovora* was inhibited at all EPS62e concentrations. In contrast, growth of EPS62e was unaffected by *E. amylovora* and achieved concentrations after 48 h of around 10^8 CFU/ml in all cases.

Table 2. Effect of the initial concentration of *Pseudomonas fluorescens* EPS62e on population levels of *Erwininia amylovora* upon incubation for 48 h in pear extract, dependent upon soluble solutes concentration and separation by a permeable membrane

Treatment			Growth of EPS101 (log CFU/ml)	
Well	Membrane cylinder	Ratio EPS62e:EPS101	Pear extract (0.062 g/l)	Pear extract (0.62 g/l)
–	EPS101	–	8.08 a[a]	8.56 a
EPS62e	EPS101	1:1	8.19 a	8.60 a
EPS62e	EPS101	10:1	7.85 b	8.54 a
EPS62e	EPS101	100:1	6.39 e	7.87 b
–	EPS62E+EPS101	1:1	7.23 c	7.43 c
–	EPS62E+EPS101	10:1	6.90 d	7.23 c
–	EPS62E+EPS101	100:1	5.47 f	6.18 d

[a]Means in the same column followed by different letters are significantly different ($P \leq 0.05$) according to the Tukey's test.

Spectrum of nutrient use and niche overlap. Of the 94 carbon sources studied, *E. amylovora* utilized 27 and *P. fluorescens* EPS62e 51. Twenty-one out of the 27 carbon sources used by *E. amylovora* were also used by EPS62e. The six carbon sources used by *E. amylovora* but not by EPS62e were β-methyl D-glucoside, gentiobiose, saccharose, glycyl-L-aspartic acid, glucose-1-phosphate and glucose-6-phosphate. Only 21 out of the 51 carbon sources used by EPS62e were also utilized by *E. amylovora*: D-fructose, bromosuccinic acid, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, inosine, uridine, glycerol, *N*-acetyl-D-glucosamine, D-galactose, L-serine, α–D-glucose, D-mannitol, D-sorbitol, D-trehalose, methyl pyruvate, L-proline, mono-methyl succinate, succinic acid and D-gluconic acid. The 30 carbon sources used by EPS62e and not used by *E. amylovora* were acetic acid, urocanic acid, cisaconitic acid, succinamic acid, citric acid, α-ketoglutaric acid, D-galactonic lactone, D-galacturonic acid, D-alanine, D,L-lactic acid, L-alanine, *m*-inositol, malonic acid, 2-aminoethanol, D-glucosaminic acid, propionic acid, L-asparagine, adonitol, D-glucoronic acid, chinic acid, D-saccharic acid, L-threonine, D,L-α-glycerol-phosphate, D,L-carnitine, β-hydroxybutyric acid, D-arabitol, D-mannose, and γ-hydroxybutyric acid. Therefore, EPS62e used more carbon sources than *E. amylovora*, including most of those used by *E. amylovora*, while the opposite was not true. The resulting niche overlapping index (*NOI*) calculated from 95 carbon sources was 0.78 for *E. amylovora* on EPS62e and 0.41 for EPS62e on *E. amylovora*.

Growth potential in relation to nutrient concentration. Relationships between cell yields and growth rates with respect to the initial concentration of nutrients in pear extract for the biocontrol agent EPS62e and for two strains of pathogen are shown in Fig. 2. EPS62e grew faster than *E. amylovora* at all substrate concentrations. However, differences were not observed in cell yield between pathogen and biocontrol agent ($P = 0.9090$). The maximum cell yield was in the range of 9.05–9.16 log CFU/ml at 0.6 g soluble solutes/l. In contrast, EPS62e had a μ_{max} significantly higher ($P < 0.0001$) and a K_s significantly lower ($P = 0.010$) than the two *E. amylovora* strains. EPS62e showed a μ_{max} of 0.352 h^{-1} and K_s of 0.152 g soluble solutes/l, while the *E. amylovora* strains had a μ_{max} of around 0.237 h^{-1} and a K_s *of* around 0.063 g soluble solutes/l. Therefore, EPS62e had a more efficient response to pear-extract nutrients than *E. amylovora*, with higher growth potential and nutrient affinity than *E. amylovora*.

***Ex vivo* interaction between antagonist and pathogen in host plant material.** *E. amylovora* EPS101 and *P. fluorescens* EPS62e were able to colonize and survive in wounds of immature pear fruits and intact pear flowers (Fig. 3). When inoculated separately, after 72 h, EPS62e attained stable population levels around 1.5×10^7 CFU/fruit wound or flower, whereas the values in *E. amylovora* were around 10^9 CFU/fruit wound and 10^8 CFU/flower. These results indicated that the cell yield of *E. amylovora* was higher than that of EPS62e in plant tissues. However, the growth rate of EPS62e in flowers was higher than that of *E. amylovora*, but this was not the case in fruit wounds, where the growth rate of *E. amylovora* was higher.

When EPS62e was inoculated before *E. amylovora*, either in immature pear fruits or flowers, the growth of *E. amylovora* was strongly inhibited. Population levels of *E. amylovora*

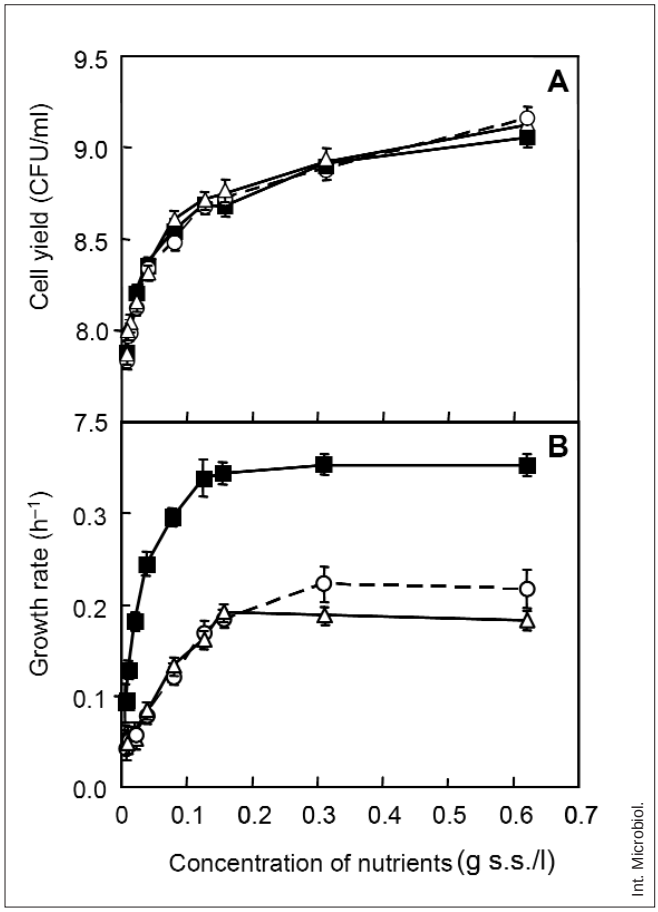

Fig 2. Effect of the concentration of immature pear fruit extract (g soluble sugars/l) on cell yield (**A**) and growth rate (**B**) of *Pseudomonas fluorescens* EPS62e (black squares) and strains EPS101 (open circles) and Ea273 (open triangles) of *Erwinia amylovora.*

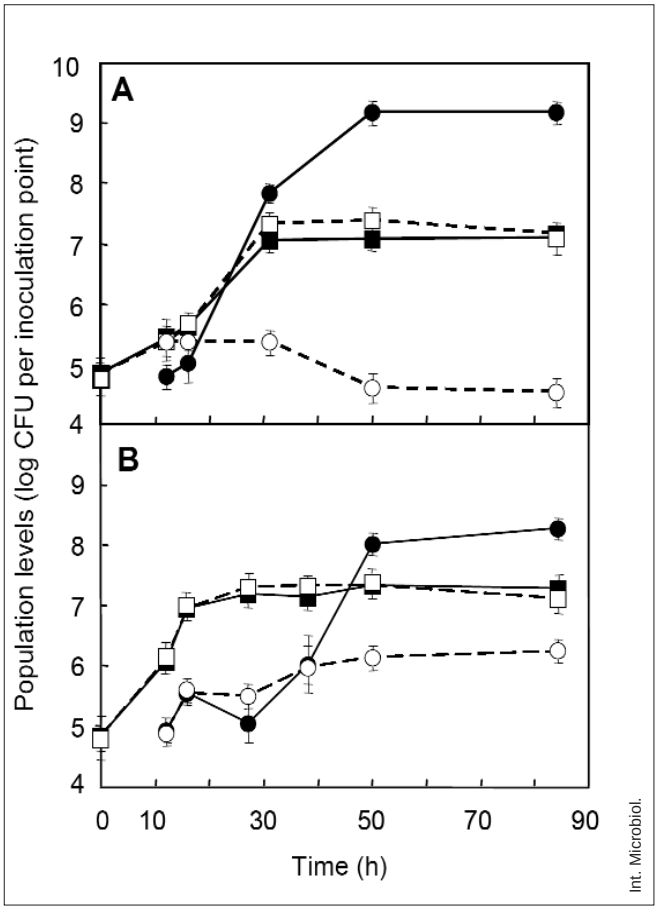

Fig. 3. Population levels of *Erwinia amylovora* (circles) and *Pseudomonas fluorescens* (squares) on immature fruits (**A**) and detached flowers (**B**) of pear upon inoculation of one strain alone (open symbols) or combined the two strains (closed symbols).

decreased from initial values of 2.3×10^5 to 8.3×10^4 CFU/wound in immature pear fruits and slightly increased from 3.6×10^4 to 1.7×10^6 CFU/flower in intact pear flowers. Therefore, the growth potential of *E. amylovora* decreased by 3.9 log CFU/wound in immature fruits pre-inoculated with EPS62e and by 2.1 log CFU/flower in pear flowers pre-inoculated with EPS62e.

Discussion

Several mechanisms have been suggested to operate in the biocontrol of fire blight by different strains of antagonists, including antibiosis [43,51], induced resistance in the host [19], and competition for space and limited resources [48,50] between the biocontrol agent and the pathogen. Strain EPS62e does not synthesize the antibiotics described in *P. fluorescens* (PCA, Phl and Prn) nor does it carry the corresponding biosynthetic genes. The lack of antibiosis was confirmed by the absence of inhibition of *E. amylovora* by spent medium from growing EPS62e, in dual-culture agar tests, and in the presence of immature pear fruit extract in interaction experiments using membrane-filter separation devices. Nevertheless, EPS62e inhibited some of the plant pathogens and *E. amylovora* strains on KB medium but lost its inhibitory activity upon iron amendment. These results are in agreement with those of other studies, in which antagonistic activity was lost by iron amendment due to siderophore production [23,29]. In the present work, production of siderophores by EPS62e was confirmed in Schwin-Neidlands medium. The role of siderophores, produced by many *Pseudomonas* species, in the control of some plant diseases has been described [47]. In some reports, siderophores have been shown to suppress several pathogen-induced diseases by conferring a competitive advantage of the biocontrol agent over the pathogen under conditions in which there is a limited supply of essential trace

Table 3. Carbon sources reported to be present in pear and other pome fruit tissues and surfaces in relation to their use by *Pseudomonas fluorescens* EPS62e and *Erwinia amylovora*

Compound	EPS62e	*E. amylovora*	Reported in[a]
Glucose	+	+	fr, fl, le, ne, ph, xy [10]
Fructose	+	+	fr, fl, le, ne, ph, xy [10]
Sucrose	-	+	fr, fl, le, ne, ph, xy [10]
Sorbitol	+	+	fr, le [12]
Asparagine	+	–	fr, ph, xy [14]
Aspartate	+	+	fr, ph, xy [14]
Glutamine	ND	ND	ph, xy [14]
Lactate	+	–	fr [44]
Citrate	+	–	fr [44]
Malate	ND	ND	fr [44]
Chinate	+	–	fr (pears) [44]

[a]fr, fruit; fl, flowers; le, leaf; ne; nectar; ph, phloem; xy, xylem. [Reference] ND: Not determined.

minerals, such as iron, in natural habitats [11].

In studying the interaction of EPS62e with *E. amylovora* in membrane-filter devices, we used immature pear fruit extract as a culture medium for two reasons. First, immature pear fruits are among the most susceptible plant materials to *E. amylovora* infection and have been used for biocontrol and pathogen aggressiveness studies [9,33]. Second, because the extract mimics the composition of pear plant tissues better than synthetic culture media. The interaction experiments showed that the inhibition of *E. amylovora* by EPS62e required cell-to-cell contact, because inhibition was suppressed upon separating the bacterial cultures by a membrane filter. However, *E. amylovora* population levels were significantly reduced when the initial population of EPS62e was very high (10^9 CFU/ml; ratio EPS62e/EPS101 100:1) or when the extract concentration was slightly diluted (1%). These results indicate that inhibition is mediated by nutrient competition since it only happened at very high EPS62e concentrations in the membrane-separation device. The fact that *E. amylovora* did not affect growth of EPS62e either under membrane separation or in mixed culture is also of interest. From the membrane-filter interaction experiments it can be concluded that cell-to-cell interaction is the main process implicated in the suppression of growth of *E. amylovora* by EPS62e, whereas antibiosis does not play a role. Cell-to-cell interaction has been reported as a mechanism in the biological control agent of post-harvest fruit diseases, *P. agglomerans* EPS125, in interaction experiments with *Monilia laxa* and *Rhizopus stolonifer* in nectarine peel leachate [5].

Competition for certain available nutrients is another mechanism that may be involved in the biocontrol of *E. amylovora* by EPS62e. EPS62e exhibits a more versatile spectrum of nutrient sources, since it used 51 out of 95 carbon sources compared to the 27 used by *E. amylovora*. Nutritional similarity between *E. amylovora* and EPS62e was quantified using *NOI*, defined as the ability to utilize carbon sources not utilized by a competing strain [50]. EPS101 presented a high *NOI* (0.78), which indicated that most of carbon sources used by EPS101 were also used by EPS62e. In contrast, EPS62e showed a low *NOI* (0.41), indicating that EPS101 was unable to use most of the carbon sources used by EPS62. Table 3 shows the carbon sources that have been reported as more abundant in pear and pome fruits [12,14,45] in relation to the ability of EPS62e and *E. amylovora* to utilize them. Nine of these carbon sources were tested in this study, and eight of these sources were used by EPS62e whereas only five were used by *E. amylovora*. Globally, the most abundant carbon sources in nectar and pear tissues, such as glucose and fructose, were used by both the antagonist and the pathogen. Sucrose, which is found in all organs, was only used by *E. amylovora*. Therefore, in terms of the effects of nutrient use and availability on plant host tissues, EPS62e has the potential to outcompete *E. amylovora*. This finding is in agreement with a report that suppression of bacterial speck of tomato (*P. syringae* pv. *tomato*) was related to nutritional similarity between nonpathogenic and pathogenic bacteria, suggesting that pre-emptive utilization of carbon sources was involved in biological control of the disease [17].

In the present work, we developed a new approach to analyze the growth potential of a biological control agent with respect to the pathogen, based on microbial growth kinetics at different nutrient concentrations. This approach is commonly used in competition studies in several fields of microbial ecology and technical microbiology [3,20]. We estimated apparent values of maximum growth rate (μ_{max}) and affinity for medium nutrients (K_s) and cell yield (Y) of the pathogen and biocontrol agent from batch cultures obtained at different initial nutrient concentrations. The growth potential in terms of maximum growth rate (μ_{max}) and affinity for nutrients in the medium (K_s) differed between EPS62e and *E. amylovora*, but cell yields (Y) were similar. EPS62e showed a higher μ_{max} and lower K_s (higher affinity) for immature pear fruit extract than *E. amylovora*. From these results, it can be argued that EPS62e outcompetes *E. amylovora* by the depletion of nutrients. Thus, competition on plant tissues likely involves sugars used by both bacteria, such as glucose and fructose, which are the major components of plant tissues and surfaces (fruit, flower, leaf, nectar, phloem and xylem sap) [10]. This hypothesis is supported by the observation that nutrient availability on the leaf surface limits the population levels of many epiphytic bacteria and that the carrying capacity for *P. fluorescens* A506 of several plants is directly relat-

ed to the amount of sugars present on the leaf surface [27]. However, conclusions cannot be extrapolated directly to the situation in fruit or leaf wounds or the hypanthia of flowers.

The capacity to colonize and survive in different plant organs and the ability to grow in the same ecological niche as the pathogen are critical aspects of disease control, since these properties are essential to competition with the pathogen for sites and nutrients, as demonstrated for *P. fluorescens* and *P. agglomerans* [21,39,42]. EPS62e and EPS101 colonized and quickly multiplied until the carrying capacity of the wounds made in immature pear fruits and of the hypanthia in intact pear flowers was reached. In flowers, EPS62e was better able to initiate colonization than *E. amylovora*, in agreement with the former's higher μ_{max}, range of carbon.source assimilation, and lower NOI and K_s. In contrast, the ability of *E. amylovora* to initialize infection was poor, although it was well-able to colonize wounds and hypanthia and to infect and spread to adjacent tissues. These properties allowed it to surpass the carrying capacity of the wounds and hypanthia, reaching values of 10^8–10^9 CFU/flower or fruit wound. Growth rates found on flowers agreed with those in immature fruit extract, but not with those of fruit wounds. This difference was probably due to the fact that *E. amylovora* can infect and spread from the wounds of immature fruits. This ability may play an important role when *E. amylovora* is the first colonizer and was related to the loss of efficacy of EPS62e in post-inoculation treatments (data not shown). However, when the biocontrol agent was pre-inoculated, the growth of EPS101 was significantly reduced in wounds and hypanthia whereas the growth of EPS62e was unaffected. Therefore, pre-emptive colonization of plant material by EPS62e reduces potential colonization by *E. amylovora*.

Favorable field conditions for colonization of pear trees by EPS62e and the ability to out-compete *E. amylovora* are expected especially during high fire-blight risk periods, which occur during bloom and after hail or thunderstorms produce wounds and surface lesions on plant organs (e.g., immature fruits, leaves). This was confirmed in trials performed in the present work, in which EPS62e colonized flowers until the carrying capacity was reached. This approach prevented infections by *E. amylovora* with a moderate efficacy. These results agree with those of Pujol et al. [37] and Bonaterra et al. [6], who tested strain EPS62e in field assays of traceability and colonization.

In conclusion, the putative inhibitory mechanisms of *E. amylovora* by EPS62e rely on its superior fitness in colonizing wounds and flowers and on its direct cell-to-cell antagonistic interactions, but do not involve antibiosis.

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