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Multifactorial suppression of *Erwinia amylovora* by the apple flower endophyte *Pantoea agglomerans* AFF2001

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ABSTRACT

Fire blight, caused by *Erwinia amylovora*, represents a significant threat to apple and pear production worldwide. The negative environmental impact of copper and the emergence of antibiotic resistance have led to the development of more eco-friendly management strategies for *E. amylovora*. It is now well established that apple flowers harbour bacterial taxa capable of limiting the ability of *E. amylovora* to colonise floral tissues. Because apple flowers are the primary infection sites of *E. amylovora*, their endophytic microbiota are reservoirs of potential biological control agents. In this study, endophytic bacteria were isolated from healthy apple flowers (*Malus domestica* cv. Golden Delicious) collected in Trentino and screened for their ability to control *E. amylovora*. Based on 16S rRNA gene sequencing, the bacterial isolates mainly belonged to the families Enterobacteriaceae, Pseudomonadaceae and Microbacteriaceae. Among the tested bacterial strains, *Pantoea agglomerans* AFF2001 exhibited the highest efficacy in suppressing *E. amylovora* on newly opened apple flowers and immature pear slices. *In vitro* and *in planta* assays were combined to investigate interactions between *P. agglomerans* AFF2001 and *E. amylovora*. The selected bacterial strain showed a significant contact-dependent inhibition of *E. amylovora* growth. It significantly acidified a stigma-mimicking medium, thereby reducing the viability and motility of *E. amylovora* cells. Furthermore, active competition for iron was associated with increased siderophore production by *P. agglomerans* AFF2001 in the presence of *E. amylovora*. The analysis of the *P. agglomerans* AFF2001 genome identified biosynthetic gene clusters responsible for producing siderophores and toxic secondary metabolites, as well as genes encoding a Type VI secretion system that may be involved in contact-dependent antagonism. Random transposon mutagenesis with pUT-miniTn5-Km revealed that genes involved in regulatory pathways, motility, and amino acid biosynthesis contribute to the biocontrol activity of *P. agglomerans* AFF2001. These results demonstrate that suppression of *E. amylovora* by *P. agglomerans* AFF2001 involves multiple interacting mechanisms, including nutrient and iron competition, environmental acidification, interference with the plant pathogen motility, and contact-dependent interactions. The findings emphasise apple flower endophytes as a valuable reservoir of biological control agents and identify *P. agglomerans* AFF2001 as a promising candidate for the development of sustainable fire blight management strategies.

1. Introduction

Fire blight is caused by *Erwinia amylovora*, a Gram-negative bacterium listed by the European and Mediterranean Plant Protection Organization (EPPO) as an A2 quarantine pest [1]. Because apple (*Malus domestica* L.) and pear (*Pyrus communis* L.) are its primary hosts, fire blight poses a serious threat to apple and pear production in many regions worldwide. To date, antibiotics and copper-based compounds have been the most effective tools for limiting the spread and severity of the disease. However, their use raises serious concerns due to environmental pollution and the emergence of antibiotic-resistant and copper-tolerant populations of *E. amylovora* [2,3]. Consequently, there is an increasing need for sustainable, environmentally friendly alternatives to fire blight management.

Microbial Biological Control Agents (BCAs), mainly bacteria and fungi that can reduce the pathogenicity and virulence of plant pathogens, represent a promising alternative to conventional chemical treatments [4]. Several (micro)organisms isolated from the apple

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phyllosphere have been tested against *E. amylovora* and are currently used as active ingredients in commercial biopesticides, including Bloomtime, BlightBan C9-1 and Blossom Protect [5,6]. Nevertheless, field trials conducted over multiple seasons have shown that these products do not consistently provide complete suppression of fire blight under all environmental conditions [6–8]. Therefore, identifying new and more effective BCAs remains a priority.

During the fire blight disease cycle, flowers constitute the primary sites of infection [9]. *E. amylovora* cells initially grow epiphytically on stigmatic surfaces and subsequently reach the plant vascular system through the hypanthium [10]. As reported for several other plant–pathogen interactions, the host-associated microbiota can significantly influence disease development [11,12]. In this context, metagenomic analyses have shown that different apple cultivars harbour a highly conserved floral bacterial community, predominantly composed of members of the families Pseudomonadaceae and Enterobacteriaceae [11,12]. Recently, Cui and colleagues demonstrated that the combined application of *Pantoea* and *Pseudomonas* strains isolated from apple stigmas significantly reduced the incidence of fire blight in flowers. However, streptomycin treatment remained the most effective control strategy [11]. Although the potential of endophytic bacteria isolated from apple flowers to control *E. amylovora* is well established, little is known about the mechanisms underlying their antagonistic activity.

In the present study, endophytic bacterial isolates were isolated from healthy apple flowers collected in Trentino and screened for antagonistic activity against *E. amylovora*. Bacterial strains exhibiting the highest efficacy in suppressing disease development on detached apple flowers and pear slices were selected as potential BCA candidates. A series of *in vitro* assays was subsequently performed to investigate the mechanisms underlying the observed antagonism. To more closely replicate the nutritional and environmental conditions encountered in floral tissues, a Partial Stigma-Based Medium (PSBM) was developed based on the chemical composition of apple stigmatic exudates [13]. The genome of the most effective BCA candidate, *P. agglomerans* AFF2001, was sequenced to identify genetic features that may be involved in antagonism against *E. amylovora*. Subsequently, untargeted mutagenesis was performed to generate knockout mutants, which were characterised *in vitro* and *in vivo* to elucidate potential molecular mechanisms underlying the antagonistic activity observed in *P. agglomerans* AFF2001.

2. Materials and methods

2.1. Isolation of endophytic bacteria from apple flowers

Apple (cv. Golden Delicious) flowers were collected from apple orchards in Trentino at the balloon stage in 2021. After weighing, the flowers were surface-sterilised by immersion in 70% (v/v) ethanol for 1 min with gentle agitation. Ethanol was discarded and replaced with 2% (v/v) sodium hypochlorite (NaClO), then the mixture was gently shaken for 90 s. The NaClO solution was then removed, and flowers were rinsed with 70% ethanol for 30 s to neutralise residual hypochlorite. Subsequently, flowers were washed five times with sterile distilled water (SDW).

Surface-sterilised flowers were transferred to sterile Petri dishes using sterile forceps and allowed to dry for 30 min under a laminar flow hood. Once dried, flowers were placed in sterile jars kept on ice, and a saline solution (0.85% NaCl, w/v) supplemented with Tween 80 (0.1%, v/v) was added to each jar based on the previously determined flower weight. Samples were homogenised using a mixer mill (Mixer Mill MM200, Retsch) for 20 s at 25 Hz. The homogenate was transferred to 2 mL tubes with cut tips, briefly vortexed, and centrifuged to separate plant debris from the suspension.

Serial dilutions were prepared, and 100 µL of each dilution was plated onto Reasoner's 2A agar (R2A; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with cycloheximide (50 µg mL^{-1}) to inhibit fungal growth. R2A, a low-nutrient medium, was chosen because it enables enumeration of groups of bacteria that are often underrepresented on nutrient-rich media [14]. Dishes were incubated at 27°C until bacterial colonies became visible.

2.2. Maintenance of bacterial strains and preparation of cell suspensions

All bacterial strains and growth media used in this study are listed in Tables S1 and S2, respectively. Bacterial strains were stored long-term in 40% (v/v) glycerol at −80°C and routinely cultured on R2A agar and/or Nutrient Agar (NA; Oxoid Ltd., Basingstoke, Hampshire, UK) at 27°C for 48 h.

Erwinia amylovora Ea21 (afterwards Ea21), isolated from a diseased apple tree (cv. Golden Delicious) in Trentino (Italy) [15], was used to generate its spontaneous rifampicin-resistant mutant, *E. amylovora* $Ea21Rf^R$ (afterwards $Ea21Rf^R$). To do that, Ea21 was cultured on NA supplemented with increasing concentrations of rifampicin up to 100 µg mL^{-1}. Differences between Ea21 and $Ea21Rf^R$ in growth kinetics were evaluated in PSBM supplemented with thiamine (afterwards PSBMT) [13], and virulence was also assessed on apple flowers and pear slices as reported below. Their ability to induce the hypersensitive response (HR) in tobacco (*Nicotiana tabacum*) leaves was assessed following Klement et al. [16]. As no significant differences in growth, virulence, or HR induction were observed between the two strains, $Ea21Rf^R$ was used in experiments requiring selective enumeration.

Bacterial cell suspensions of Ea21, $Ea21Rf^R$, and apple flower endophytic bacteria were prepared by adding 3 mL of sterile saline solution (0.85% NaCl, w/v) to agar dishes, gently scraping the bacterial biomass with a sterile spatula, and collecting the suspension into sterile 15 mL tubes. For assays involving detached apple flowers, cells were resuspended in SDW rather than saline to avoid potential phytotoxic effects of NaCl. Cell suspensions of Ea21, $Ea21Rf^R$ and apple flower endophytic bacteria were adjusted to an optical density at 600 nm (OD_{600}) of 0.1, corresponding to approximately 1×10^8 colony forming units (CFU) mL^{-1}, unless otherwise specified.

2.3. Identification of bacterial isolates and comparison of metabolic profiles

Bacterial isolates were initially selected based on colony morphology and subsequently identified by 16S rRNA gene sequencing. The 16S rRNA gene was amplified using GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA) with the universal primer pair 27f and 1492r, as described by Lane [17]. PCR products were verified by agarose gel electrophoresis prior to sequencing using BigDye Terminator v3.1 chemistry. The 16S rRNA gene sequences obtained in this study were submitted to the NCBI GenBank database [18] and assigned accession numbers PX981877-PX981889. These sequences were analysed using BLASTN against the GenBank database to identify closely related taxa. The closest relatives were identified based on the highest sequence identity and query coverage. A threshold of sequence identity ≥98.5% was used for species-level assignment, while sequences showing lower similarity were assigned at the genus level. When multiple matches were highly similar, taxonomic assignment was based on the best combination of sequence identity and query coverage. Phylogenetic analyses were performed using MEGA 12 software [19]. Sequence alignment was conducted using MUSCLE [20] implemented in MEGA. Phylogenetic trees were inferred using the maximum likelihood method [21], and branch confidence was assessed by bootstrap analysis with 1000 replicates [22]. Reference 16S rRNA gene sequences used for phylogenetic analyses were retrieved from GenBank (accession numbers: AJ233423, AJ583501, CP031695, EU557337, KR233790, KT614051, KY606575, MG269632, MH329946, MK246112, NR_026395, OK235614, OM670225). The 16S rRNA gene sequence of *Escherichia coli* ATCC 11775 (CP033092) was used as the outgroup.

To compare metabolic capabilities, Biolog Phenotype MicroArray™

plates (Biolog Inc., Hayward, CA, USA) were used to assess utilisation of carbon (PM1, PM2A) and nitrogen (PM3B) sources. Plates were inoculated according to the manufacturer's instructions and incubated at 27°C for 24 h. Metabolic activity was monitored using an OmniLog system, and data were analysed using OmniLog PM software v1.7. Results were visualised as heatmaps, with colour intensity reflecting metabolic activity measured in OmniLog Units (OU).

2.4. Evaluation of bacterial strains for protection of detached apple flowers against E. amylovora

The detached apple flower assay was performed according to Pusey [23], with minor modifications. Newly opened apple (cv. Golden Delicious) flowers were collected and placed in 5 mL tubes, with the peduncles submerged in 4.5 mL of sterile sucrose solution (10%, w/v). Flower stigmas were inoculated with 2 μL of bacterial suspension (10 μL per flower) and allowed to dry under laminar airflow.

Flowers were then incubated in sealed plastic containers at 25°C and 80% relative humidity. After 24 h, 10 μL of an Ea21 suspension was applied to the stigmas. Control treatments included flowers treated with SDW alone and flowers inoculated with Ea21 only. Disease severity was evaluated after four days using a 0-4 scale, where 0 = green stigmata; 1 = necrotic area is limited to the tips of stigmata; 2 = half of the stigmata surface is necrotic; 3 = most of the stigmata surface is necrotic; 4 = the entire stigmata surface is necrotic. Plant protection efficacy (%) was calculated using the following formula: $\text{Plant protection efficacy (\%)} = \frac{\text{Mean(Control)} - \text{Treated}}{\text{Mean(Control)}} \times 100$, where 'Mean (Control)' represents the average disease severity recorded in the pathogen-only control, and 'Mean(Treated)' represents the average disease severity recorded when Ea21 was applied on apple flowers, preventively treated with apple endophytic bacteria. Each treatment comprised 10 replicates (apple flowers), and the experiment was repeated twice.

2.5. Evaluation of bacterial strains for protection of immature pear slices against E. amylovora

Immature pears cv. Williams were surface sterilised by immersion in 2% (v/v) sodium hypochlorite for 5 min with gentle agitation (60 rpm). The solution was discarded and replaced with 0.5% (v/v) sodium hypochlorite for 1 min, followed by three washes with SDW.

Sterile Petri dishes (90 mm) were lined with three layers of sterile filter paper moistened with 3 mL of SDW. Pears were cut into uniform slices using a sterile chopper, and each slice was immersed in a bacterial cell suspension at 1×10^8 CFU mL^{-1} until the entire surface was evenly wetted. Slices were placed in Petri dishes, dried under laminar airflow and incubated at 25°C and 80% relative humidity.

After 24 h, 10 μL of an Ea21 suspension at 1×10^8 colony-forming units CFU mL^{-1} was deposited onto each slice and allowed to dry. Slices treated with saline solution alone and inoculated only with Ea21 were used as negative and positive controls, respectively. Disease severity was assessed after five days using a 0-6 scale, as previously described [24], where 0 = symptomless; 1 = low exudate production with browning affecting approximately one-quarter of the slice; 2 = exudate production with browning affecting approximately half of the slice; 3 = exudate production with light browning of the slice; 4 = exudate production with dark browning of the slice; 5 = enhanced exudate production with intense dark browning of the slice; and 6 = completely scorched tissue. Plant protection efficacy (%) was calculated using the following formula: $\text{Plant protection efficacy (\%)} = \frac{\text{Mean(Control)} - \text{Treated}}{\text{Mean(Control)}} \times 100$, where 'Mean(Control)' represents the average disease severity recorded in the pathogen-only control, and 'Mean(Treated)' represents the average disease severity recorded when Ea21 was applied on pear slices, preventively treated with apple endophytic bacteria. Each treatment comprised three biological replicates (Petri dishes) with three technical replicates (three pear slices per dish), and the experiment was repeated twice.

2.6. Assessment of hypersensitive response suppression in tobacco plants

The HR assay was performed with slight modifications to the protocol described by Klement et al. [16]. Bacterial strains were resuspended in sterile saline solution and mixed at a 1:1 ratio with Ea21Rf^R to obtain final concentrations of approximately 1×10^8 CFU mL^{-1} for each bacterium. One millilitre of the bacterial suspension was infiltrated into the intercellular spaces of tobacco leaves using a needleless syringe. Three infiltration sites were used per leaf, and one plant was assigned to each treatment to minimise cross-contamination. Bacterial strains isolated in this study were also infiltrated individually to assess their ability to elicit HR. Sterile saline solution and Ea21Rf^R alone served as the negative and positive controls, respectively. After infiltration, the total infiltrated area was marked with a black permanent marker. Then, plants were maintained in a greenhouse under controlled conditions (25 ± 1°C, 70 ± 10% relative humidity, 16 h photoperiod).

HR symptoms were evaluated 48 h post-infiltration by quantifying necrotic leaf areas using ImageJ software [25]. The extent of HR was calculated as the ratio of necrotic leaf area to total infiltrated leaf area. Plant protection efficacy was then calculated based on HR values as the percentage reduction of HR in the treated sample compared to the control, according to the following formula: $\text{Plant protection efficacy (\%)} = \frac{\text{Mean(Control)} - \text{Treated}}{\text{Mean(Control)}} \times 100$, where 'Mean (Control)' represents the average HR value in the pathogen-only control, and 'Mean(Treated)' represents the average HR value recorded when Ea21Rf^R was infiltrated with apple flower endophytic bacteria. Each treatment included three replicates (infiltration areas per leaf), and the assay was performed twice.

To assess the effect of the apple flower endophytic bacteria on Ea21Rf^R population size, the total infiltrated leaf sectors were excised, homogenised in 3 mL of sterile saline solution, serially diluted, and plated onto NA supplemented with rifampicin (100 μg mL^{-1}) and cycloheximide (100 μg mL^{-1}).

2.7. Evaluation of contact-dependent and contact-independent antibacterial activity

Contact-dependent inhibition was evaluated using Ea21Rf^R to enable selective recovery. Cell suspensions of Ea21RfR and potential biocontrol bacterial strains were each prepared at two concentrations (1×10^7 and 1×10^8 CFU mL^{-1}). Five microliters of Ea21Rf^R and the selected bacterial strain were co-spot-inoculated onto PSBMT agar dishes (Supplementary Table 2). The following combinations were tested: (i) Ea21Rf^R (1×10^8 CFU mL^{-1}) with potential biocontrol strain (1×10^8 CFU mL^{-1}) and (ii) Ea21Rf^R (1×10^8 CFU mL^{-1}) with potential biocontrol strain (1×10^7 CFU mL^{-1}). Individual inoculations of each bacterial strain were included as controls.

After 48 h of incubation at 27°C, the inoculation spots were excised with a sterile cork borer (5 mm in diameter), resuspended in 1 mL of sterile saline, and vortexed. Serial dilutions were plated onto NA supplemented with rifampicin (100 μg mL^{-1}) to quantify Ea21Rf^R viable cells, and onto NA without antibiotics to quantify the biocontrol strains. Each treatment included three replicates (dishes), and the experiment was repeated twice.

Contact-independent antibacterial activity was evaluated following the protocol of Puopolo et al. [26]. Briefly, 20 μL of each bacterial suspension (1×10^8 CFU mL^{-1}) was spot-inoculated onto PSBMT or R2A agar dishes and incubated at 27°C for 48 h. Bacterial cells were then killed by exposure to chloroform vapours for 1 h. Dishes were aerated under a laminar airflow for 30 min and exposed to UV light for 20 min to eliminate residual contamination. Control dishes without bacterial inoculation were included.

A suspension of Ea21 in phosphate-buffered saline amended with agar (PBSA; 0.4 g L^{-1}) was prepared at 1×10^8 CFU mL^{-1} and overlaid onto the treated dishes (3 mL per dish). Dishes were incubated at 27°C, and inhibition zones surrounding the original bacterial spots were evaluated after 48 h. Each treatment included three replicates (dishes), and the assay was repeated twice.

2.8. Assessment of siderophore production and interspecific interactions

Siderophore production was assessed using a chrome azurol S (CAS) agar assay adapted from Schwyn and Neilands [27] and Vasseur-Coronado et al. [28]. PSBMT agar was poured into Petri dishes (90 mm) and allowed to solidify. Half of the agar surface was removed aseptically and replaced with CAS agar.

Cell suspensions of Ea21 and selected apple flower endophytic bacteria were prepared, and 5 µL of each suspension was spot-inoculated onto the PSBMT side of the dish, 1 cm apart from each other and from the CAS agar interface. Individual inoculations of each bacterial strain were included as controls. Dishes were incubated at 27°C for five days.

Siderophore production was quantified by measuring the area of CAS agar discolouration using ImageJ software. For co-inoculated dishes, measurements were taken on the side of the halo opposite the interacting bacterial strain to avoid interference. Each treatment included three biological replicates (dishes), and the experiment was conducted twice.

2.9. Growth curves in partial stigma-based medium

Selected bacterial strains and Ea21 were centrifuged at 5000 rpm for 10 min, washed twice with sterile saline solution, and resuspended in liquid PSBMT to an OD_{600} of 0.1. One millilitre of each bacterial suspension was transferred into wells of a sterile 48-well microplate.

Plates were incubated at 27°C in a Synergy 2 microplate reader (BioTek® Instruments) with continuous orbital shaking. Bacterial growth was monitored by measuring OD_{600} at hourly intervals over 24 h. At the end of the incubation period, cultures were serially diluted and plated onto NA or R2A agar to determine final bacterial cell concentrations. Each assay included three replicates (wells) per bacterial strain and was repeated twice.

2.10. Effect of bacterial culture filtrates on E. amylovora growth, motility and biofilm formation

Selected bacterial strains and Ea21 were cultured in 12 mL of liquid PSBMT in sterile 15 mL tubes and incubated at 27°C with orbital shaking (180 rpm). PSBMT without bacterial inoculation served as the untreated control. After 24 h, cultures were centrifuged at 5000 rpm for 10 min, and supernatants were sterilised by filtration through 0.2 µm membranes.

The pH of each culture filtrate was measured; half of the volume was adjusted to pH 7, while the remaining portion was left at its original pH (talis qualis, TQ). All filtrates were filter-sterilised again and aliquoted into sterile 5 mL tubes. Each filtrate was inoculated with 100 µL of an overnight Ea21 culture and incubated at 27°C with shaking (180 rpm) for 24 h. Cultures were serially diluted, plated on NA and incubated at 27°C for 48 h before colony enumeration. Each treatment included three replicates (tubes), and the experiment was repeated three times.

To assess the impact of culture filtrates on Ea21 motility, selected bacterial strains were grown in 300 mL PSBMT contained in 500 mL flasks under the same conditions described above. Culture filtrates were processed as described previously. Sterile bottles containing distilled water and agar were prepared to obtain final agar concentrations of 0.25% for swimming assays [29] and 0.5% for swarming assays [30]. Sixty millilitres of each filtrate were mixed with molten agar and poured into 60 mm Petri dishes, which were allowed to solidify overnight.

Ea21 cells grown on NA for 48 h were inoculated at the centre of each dish using a sterile 1 µL loop. Dishes were incubated at 27°C, and motility areas were quantified using ImageJ software after 24 h (swimming) or 48 h (swarming). Each treatment included ten replicates (dishes) and was repeated three times.

Biofilm formation was evaluated as described by Peng et al. [31], with minor modifications. Selected apple flower endophytic bacteria were cultured in 5 mL PSBMT for 24 h at 27°C with shaking (180 rpm). Ea21 was grown in 10 mL Lysogeny Broth (LB) for 18 h under the same conditions. Cultures were centrifuged, and supernatants were processed as described above.

Ea21 cells were washed twice with sterile saline solution and resuspended in LB to an OD_{600} of 2. Wells of sterile 96-well polystyrene microplates (Costar 3595; Corning Inc., Corning, NY, USA) were treated with acetone for 20 s, air-dried and filled with 100 µL of the Ea21 suspension. An additional 100 µL of PSBMT, bacterial culture filtrate (TQ or pH-adjusted), or SDW water was added to each well, resulting in a final Ea21 OD_{600} of 1. The control treatment consisted of PSBMT mixed 1:1 with LB medium.

Plates were incubated statically at 27°C for 48 h. Biofilms were stained with 10% crystal violet, washed with distilled water and air-dried. Crystal violet was solubilised using a methanol-acetic acid solution (40% and 10%, respectively), and absorbance was measured at 594 nm. Each biofilm measurement was normalised to the growth of Ea21 measured at OD_{600}. The modulation (%) of Ea21 biofilm production by culture filtrates of biocontrol bacteria was calculated using the formula: $\text{Modulation } (\%) = \frac{\text{Mean(Control)} - \text{Treated}}{\text{Mean(Control)}} \times 100$, where Mean(Control) is the average biofilm production in the untreated control. Each treatment included seven replicates (wells), and the experiment was repeated twice.

2.11. Genome sequencing and assembly of Pantoea agglomerans AFF2001

Genome sequencing of *P. agglomerans* AFF2001 was performed to identify biosynthetic gene clusters potentially involved in antagonistic activity and to provide a reference genome for the subsequent molecular characterisation of transposon insertion mutants generated in this study, as reported below. Genomic DNA of *P. agglomerans* AFF2001 was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. Whole-genome sequencing was performed using Oxford Nanopore technology at the Edmund Mach Foundation (San Michele all'Adige, Italy), employing a MinION flow cell (version 9.4). Raw reads were assembled using the Flye assembler implemented in the EPI2ME bacterial genomes pipeline (v2.9.3-b1797) [32] and polished with Medaka (v1.11.3) [33]. Genome annotation was performed using the RAST server [34], and biosynthetic gene clusters were predicted with antiSMASH 7.0 [35].

2.12. Construction and screening of P. agglomerans AFF2001 knockout mutants

A spontaneous rifampicin-resistant mutant of *P. agglomerans* AFF2001 was generated by sequentially culturing the wild-type strain in Nutrient Broth (NB, Oxoid Ltd., Basingstoke, Hampshire, UK) containing gradually increasing concentrations of rifampicin. Differences in growth kinetics between the wild-type and rifampicin-resistant mutant were evaluated as described for Ea21Rf^R. The rifampicin-resistant mutant was then used as the recipient in triparental mating for transposon mutagenesis using a mini-Tn5 system [36]. Triparental mating was performed as described by Williams and Stavrinides [37], with *E. coli* DH5α pUT-miniTn5-Km as the donor strain, *E. coli* HB101 pRK2013 as the helper strain [38], and the rifampicin-resistant *P. agglomerans* AFF2001 as the recipient.

Overnight cultures were grown in LB supplemented with appropriate antibiotics, then centrifuged, washed with saline, and mixed at a 20:20:1

donor:helper:recipient ratio. Aliquots (100 μL) were spot-inoculated onto LB agar dishes and incubated at 30°C for 24 h. Mating mixtures were resuspended and plated onto NA containing kanamycin (25 μg mL^{-1}) and rifampicin (100 μg mL^{-1}) to select for transconjugants.

To screen for mutants with impaired antagonism, Ea21 was transformed with the pBBRMCS5-GFP plasmid [39] through electroporation [40], yielding Ea21GFP. Growth kinetics of Ea21GFP were compared to the wild-type strain as described for the rifampicin-resistant mutant. Knockout mutant strains were co-spot-inoculated with Ea21GFP on PSBMT dishes and incubated at 27°C for 48 h. Spots exhibiting fluorescence or altered morphology compared to the wild-type control were selected for further analysis. The 16S rRNA gene sequencing was performed as described above to confirm that the mutants were derivatives of *P. agglomerans* AFF2001.

2.13. Molecular characterisation of P. agglomerans AFF2001 mutants

To identify transposon insertion sites, genomic DNA from selected mutants was extracted and digested with the restriction enzyme *Hin*cII. Digested DNA fragments were ligated and used as templates for inverse PCR with primers npt+772 and npt−41 [41,42]. Amplified products were sequenced and mapped to the *P. agglomerans* AFF2001 draft genome using BLAST, and gene annotations were retrieved from the RAST server.

Growth rates of *P. agglomerans* AFF2001 mutants were assessed in NB and PSBMT to exclude growth-related biases. Then, they were characterised for their ability to control Ea21 on detached apple flowers and pear slices as reported above. Additionally, cell motility, siderophore production, changes in growth medium pH, and the effects of their bacterial culture filtrates on Ea21 traits were assessed as described above. Moreover, the ability of *P. agglomerans* AFF2001 mutants to form biofilms was evaluated as described by Nagórska et al. [43] and Yaryura et al. [44].

2.14. Statistical analysis

Data from all experiments were analysed using PAST4 (PAleontological Statistics, version 4.07) [45]. Statistical differences between treatments were determined using one-way analysis of variance (ANOVA). Data were analysed for normality using the Shapiro–Wilk test ($p > 0.05$) and for homogeneity of variances using Levene's test. Tukey's HSD and Student's t-test were used as post hoc tests. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Endophytic bacteria isolated from apple flowers mainly belong to the Enterobacteriaceae, Microbacteriaceae and Pseudomonadaceae families, and exhibit metabolic profiles partially overlapping with E. amylovora

Following an initial selection based on colony morphology, 13 endophytic bacteria isolated from healthy apple flowers were identified by 16S rRNA gene sequencing. Phylogenetic analysis revealed that the majority of the endophytic bacteria belonged to the families Enterobacteriaceae, Microbacteriaceae and Pseudomonadaceae (Fig. 1, Table S1). Among the Enterobacteriaceae, *Erwinia billingiae* AFF4005 clustered with *E. amylovora*, a similarity reflected in colony morphology. *P. extremaustralis* AFC1001 displayed a transparent colony morphology on R2A agar, whereas the remaining *Pseudomonas* strains formed white or yellow colonies. *Acinetobacter* sp. AFF2002 was the sole

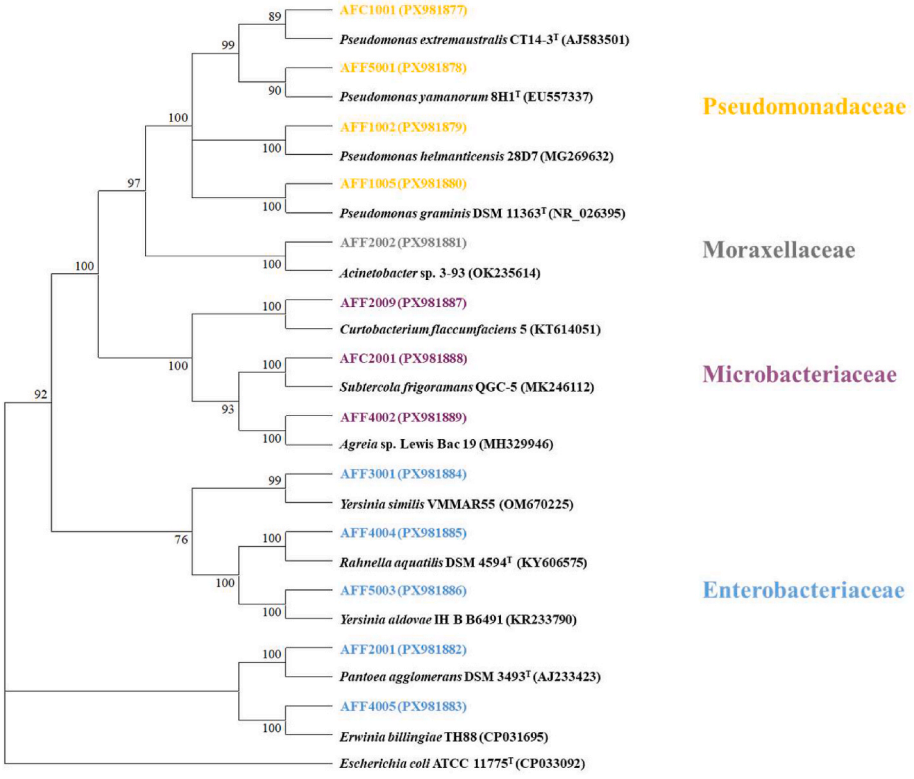

Fig. 1. Phylogenetic tree of the endophytic bacteria isolated from apple flowers. Bacterial strains were identified as members of the Enterobacteriaceae (blue), Microbacteriaceae (violet), Moraxellaceae (grey), and Pseudomonadaceae (yellow) families. The phylogenetic analysis was conducted using 16S rRNA gene sequences from bacterial strains isolated in this study (PX981877-PX981889) and from other bacterial strains in GenBank (AJ233423, AJ583501, CP031695, EU557337, KR233790, KT614051, KY606575, MG269632, MH329946, MK246112, NR_026395, OK235614, OM670225). Bootstrap values (1000 replicates) [18] higher than 70 are shown at the branch points. The 16S rRNA gene sequence of the type strain *E. coli* ATCC 11775^T was used as the outgroup. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

representative of the Moraxellaceae family (Fig. 1). Gram-negative bacteria predominated among the isolates, with only three Gram-positive strains belonging to the Microbacteriaceae family (Fig. 1).

To assess potential competition for floral nutrients, the metabolic profiles of each endophytic bacterium were compared with that of Ea21 using Biolog Phenotype MicroArray plates. Members of the Enterobacteriaceae, including *E. billingiae* AFF4005, *Pantoea agglomerans* AFF2001, *Rahnella aquatilis* AFF4004, *Yersinia rochesterensis* AFF5003, and *Y. similis* AFF3001, exhibited carbon utilisation profiles closely resembling that of Ea21, particularly showing a strong preference for glucose and its derivatives (PM1 and PM2A; Figs. S1 and S2). These included β-methyl-D-glucoside, fructose, galactose, mannitol, N-acetyl-D-glucosamine, ribose, sorbitol and D-trehalose. High metabolic activity was also detected for gentiobiose, pectin and sucrose.

Among the tested bacterial strains, *P. agglomerans* AFF2001 showed the greatest overlap with Ea21 in carbon source utilisation, particularly for N-acetyl-D-glucosamine, fructose, succinic acid, D-galactose, D-trehalose, and pectin, as indicated by growth curve analyses and heatmap data (Figs. S2 and S3). In contrast, *Acinetobacter* sp. AFF2002 and *Subtercola frigoramans* AFC2001 exhibited limited utilisation of carbon sources present in the PM1 and PM2A plates.

Nitrogen source utilisation (PM3B) varied among the bacterial strains (Fig. S3). Ea21 preferentially assimilated specific amino acids, including asparagine, aspartic acid, cysteine, glutamic acid, glutamine, proline and tryptophan. These substrates were also utilised by several Enterobacteriaceae (*P. agglomerans* AFF2001, *R. aquatilis* AFF4004, *Y. rochesterensis* AFF5003), Pseudomonadaceae strains (*P. extremaustralis* AFC1001, *P. helmanticensis* AFF1002, *P. yamanorum* AFF5001) and *Acinetobacter* sp. AFF2002. Overall, most endophytic bacterial strains assimilated a broad range of amino acids, whereas *Acinetobacter* sp. AFF2002, *Curtobacterium flaccumfaciens* AFF2009, and *S. frigoramans* AFC2001 showed limited nitrogen utilisation.

3.2. *Acinetobacter sp. AFF2002, Curtobacterium flaccumfaciens AFF2009, Pantoea agglomerans AFF2001, and Pseudomonas extremaustralis AFC1001 effectively suppressed E. amylovora on apple flowers and pear slices*

The endophytic bacterial strains were initially screened for their ability to suppress fire blight development on newly opened apple flowers. Untreated flowers inoculated only with Ea21 developed necrotic areas on the stigmas. The preventive application of *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001 and *P. extremaustralis* AFC1001 significantly reduced fire blight symptom severity, with control efficacies of 44.44 ± 5.73%, 51.11 ± 5.54%, 62.22 ± 5.02%, and 26.66 ± 3.39%, respectively, compared with the untreated control (Fig. 2A, Fig. S41). In most cases, necrosis was limited to the stigmatic tips, particularly in flowers treated with *P. agglomerans* AFF2001, although no statistically significant differences were observed among the four effective bacterial strains. Flowers treated with the remaining bacterial strains showed no significant reduction in disease symptoms (data not shown) and were therefore excluded from further analyses.

These four bacterial strains were subsequently evaluated for their ability to reduce the virulence of Ea21 on immature pear slices. Untreated immature pear slices inoculated only with Ea21 showed extensive tissue browning and abundant bacterial ooze. In contrast, the preventive application of *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 significantly reduced symptom development, including ooze production and tissue browning, with control efficacies of 86.77 ± 3.40%, 76.86 ± 6.13%, and 100.00 ± 0.00%, respectively (Fig. 2B, Fig. S5). Notably, pear slices treated with *P. agglomerans* AFF2001 showed complete protection, with no visible symptoms. When ooze was present, it was limited in extent and accompanied by minimal browning. In contrast, treatment with *P. extremaustralis* AFC1001 induced extensive browning of the pear

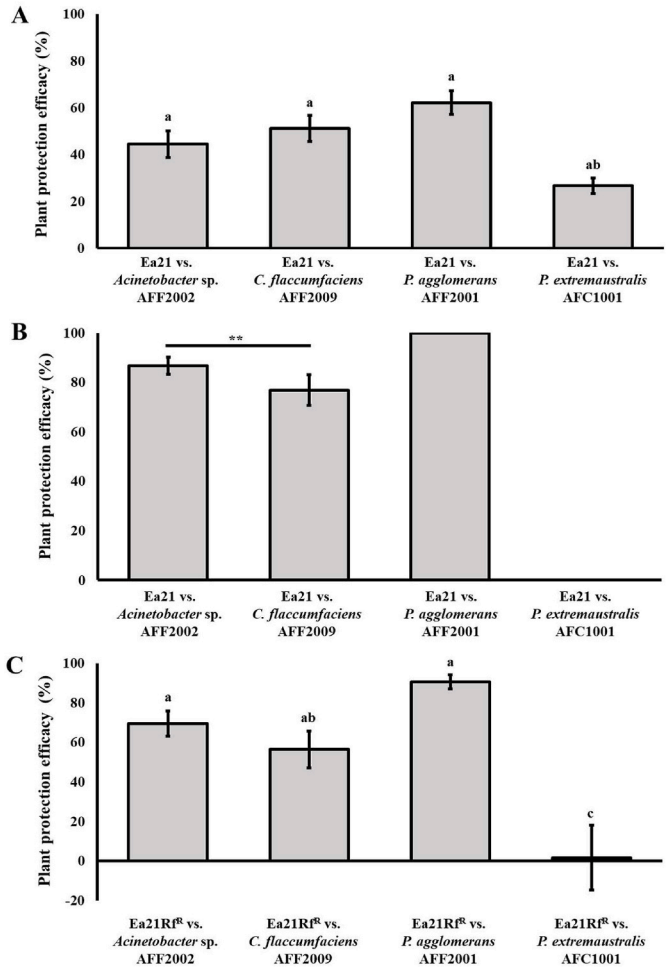

Fig. 2. Biocontrol efficacy of apple flower endophytic bacteria against *E. amylovora* Ea21 in different plant systems. *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001, and *P. extremaustralis* AFC1001 were evaluated on detached apple flowers (A), immature pear slices (B), and for suppression of the hypersensitive response in tobacco leaves (C). Plant protection efficacy is expressed as the percentage reduction compared with the pathogen-only control. Data are the mean ± standard error of one representative experiment from a series of independent experiments showing similar results. Different letters indicate significant differences according to Tukey's HSD test ($\alpha = 0.05$). Asterisks indicate significant differences according to Student's t-test ($p < 0.01$).

tissue, including the peel. This response made it difficult to determine the effect of this bacterial strain on Ea21 virulence, which could not be reliably assessed.

3.3. *Pantoea agglomerans AFF2001 reduced hypersensitive response development and E. amylovora population size in tobacco leaves*

The HR assay on tobacco was conducted to evaluate whether the selected bacterial strains affected the pathogenicity of Ea21. Leaf areas infiltrated with Ea21 alone exhibited extensive necrosis accompanied by an HR, resulting in tissue collapse (Fig. S6). Among the tested bacterial strains, *P. extremaustralis* AFC1001 failed to suppress HR development, as necrotic areas closely matched those observed in leaves infiltrated with Ea21 alone. Although tissue collapse appeared slightly delayed, HR was not prevented (Fig. 2C, Fig. S6).

In contrast, co-infiltration with *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 reduced HR severity (Fig. 2C, Fig. S6), with control efficacies of 69.50 ± 6.29%, 56.41 ± 9.29%, and 90.61 ± 3.55%, respectively, with only mild

chlorosis and limited bacterial ooze observed. Treatment with *P. agglomerans* AFF2001 was the most effective at limiting disease symptoms.

Quantification of Ea21Rf^R populations recovered from leaf tissue revealed that only *P. agglomerans* AFF2001 significantly reduced pathogen abundance to 5.28 ± 0.06 $\log_{10}$ CFU mL^{-1}, with approximately a three-order-of-magnitude decrease compared with the positive control (8.05 ± 0.08 $\log_{10}$ CFU mL^{-1}) (Fig. S7). Other bacterial treatments did not result in a significant reduction of Ea21Rf^R populations.

3.4. *P. agglomerans AFF2001 exhibits strong contact-dependent antibacterial activity against E. amylovora*

Based on the results obtained in the plant assays, *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 were selected for further investigation of their modes of action. Their ability to inhibit the growth of Ea21 was evaluated using contact-dependent and contact-independent assays.

In the contact-dependent assay, Ea21Rf^R was co-spot-inoculated with each bacterial strain at different cell concentrations on PSBMT agar to assess contact-dependent interactions (Fig. 3A). Recovery of Ea21Rf^R cells from the untreated control resulted in a bacterial cell density of 11.59 ± 0.20 $\log_{10}$ CFU mL^{-1} (Fig. S8).

All three bacterial strains reduced the recovery of Ea21Rf^R viable cells, even when the antagonist was applied at a concentration lower than that of the pathogen. Notably, no viable Ea21Rf^R cells were recovered in the presence of *P. agglomerans* AFF2001, indicating complete inhibition of *E. amylovora* growth and suggesting a strong contact-dependent antagonistic activity. This effect was independent of the initial concentration of the biocontrol bacterial strain (10^7 or 10^8 CFU mL^{-1}) (Fig. 3A). In contrast, *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009 exhibited only partial inhibition of Ea21Rf^R growth, with residual viable cells consistently recovered. Their inhibitory effect was substantially weaker than that observed for *P. agglomerans* AFF2001 (Fig. 3A).

In the contact-independent assay with chloroform-treated bacterial cultures, none of the tested strains produced detectable inhibition zones against Ea21 (data not shown), indicating that, under the conditions tested, they did not release any stable, diffusible antibacterial compounds.

3.5. *Siderophore production by P. agglomerans AFF2001 is enhanced in the presence of E. amylovora*

To evaluate potential competition for iron, siderophore production by the selected bacterial strains was assessed in PSBMT using a CAS agar assay. No CAS agar discolouration was observed for *Acinetobacter* sp. AFF2002 or *C. flaccumfaciens* AFF2009, either when grown alone or in the presence of Ea21, indicating the absence of detectable siderophore production under the tested conditions (Fig. S9). Notably, Ea21 displayed reduced siderophore production (−26.75 ± 8.29% and −23.29 ± 7.70%, respectively) when grown in the presence of *Acinetobacter* sp. AFF2002 or *C. flaccumfaciens* AFF2009, indicating a possible lack of competition for iron (Fig. 3B).

In contrast, morphological changes in Ea21 microcolonies were observed in co-inoculation assays with *P. agglomerans* AFF2001. Colonies appeared less dense and more transparent on the side facing the *P. agglomerans* AFF2001 spot compared with control colonies grown alone. Moreover, Ea21 showed increased siderophore production by 36.8 ± 6% when grown in proximity to *P. agglomerans* AFF2001, indicating a potential competitive interaction for iron (Fig. 3B).

Interestingly, *P. agglomerans* AFF2001 produced a clear CAS discolouration halo, which increased significantly by 26.54 ± 4.95% when Ea21 was co-inoculated at a distance of 1 cm (Fig. S9). This observation suggests that the presence of *E. amylovora* stimulates siderophore production by *P. agglomerans* AFF2001.

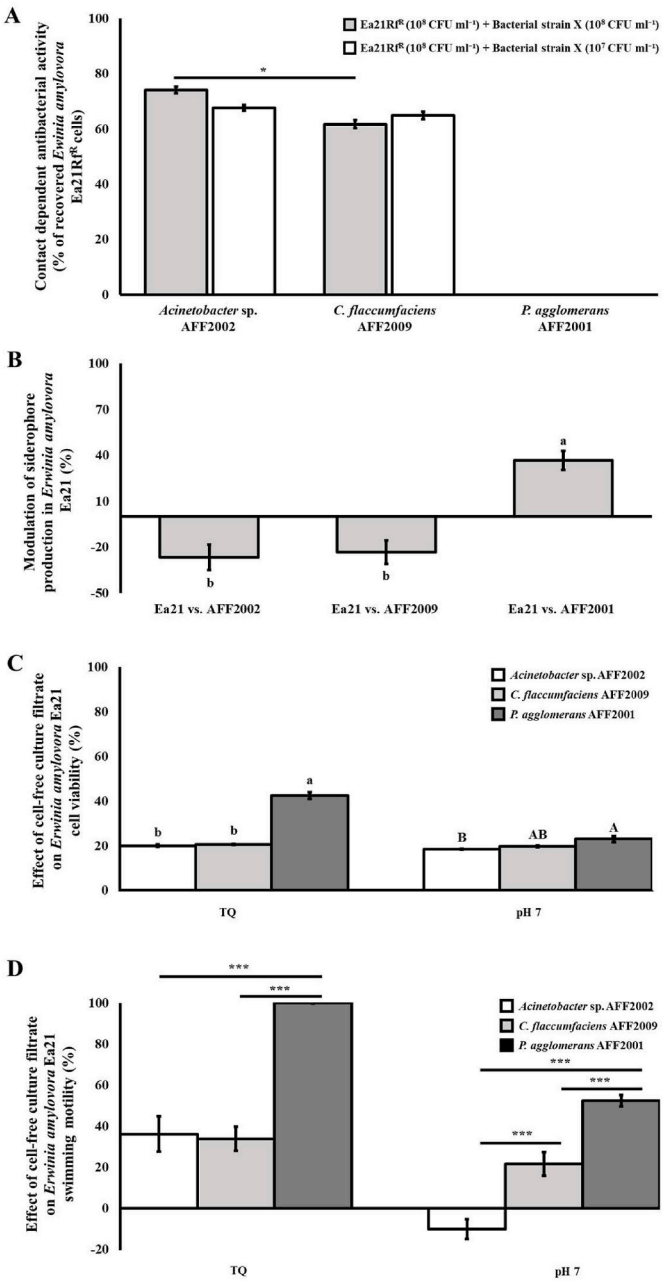

Fig. 3. Effects of potential biocontrol bacteria on growth, viability, siderophore production, and motility of *E. amylovora* Ea21. *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001, and *P. extremaustralis* AFC1001 were evaluated for contact-dependent growth inhibition (A), modulation of siderophore production by the selected bacterial strains (B), cell viability in bacterial culture filtrates (C), and swimming motility (D). Values represent the percentage change relative to the untreated control. Data are the mean ± standard error of one representative experiment from a series of independent experiments showing similar results. Different letters indicate significant differences according to Tukey's HSD test (α = 0.05). Asterisks indicate significant differences according to Student's t-test ($p < 0.01$).

3.6. *Bacterial culture filtrates affect E. amylovora growth and motility*

To further elucidate potential mechanisms underlying antagonistic activity, the effects of cell-free culture filtrates from the selected bacterial strains on Ea21 growth and motility were investigated. Cultivation in PSBMT led to marked acidification of the medium by *P. agglomerans* AFF2001, with the pH decreasing from 7.00 ± 0.01 to 3.51 ± 0.03 after 24 h (Table 1). In contrast, culture filtrates of *Acinetobacter* sp. AFF2002

Table 1
Assessment of the pH of *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001 culture filtrates in Partial Stigma-Based Medium (PSBMT).

Bacterial strain	pH value
Acinetobacter sp. AFF2002	6.33 ± 0.05a[a]
Curtobacterium flaccumfaciens AFF2009	6.45 ± 0.03 a
Pantoea agglomerans AFF2001	3.51 ± 0.03 b

[a] Values are expressed as the mean ± standard error of one representative experiment from a series of independent experiments showing similar results. Values followed by the same letters within the same column are not significantly different according to Tukey's HSD test (α = 0.05).

and *C. flaccumfaciens* AFF2009 showed only moderate acidification, with pH values of 6.33 ± 0.05 and 6.45 ± 0.03, respectively (Table 1).

In PSBMT, Ea21 reached a population density of 10.98 ± 0.03 $\log_{10}$ CFU mL^{-1}. When Ea21 was grown in culture filtrates of the potential BCAs without pH adjustment (talis qualis, TQ), there was a reduction in Ea21 cell viability compared with the control medium (Fig. 3C). Specifically, the culture filtrates of *P. agglomerans* AFF2001 determined a reduction of 42.48 ± 1.45% Ea21 cell viability, whereas *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009 culture filtrates caused reductions of 19.94 ± 0.54% and 20.56 ± 0.24%, respectively (Fig. 3C).

This inhibitory effect was attenuated when the culture filtrates were adjusted to pH 7 (Fig. 3C). Under neutral pH conditions, growth inhibition was reduced to 18.49 ± 0.25%, 19.70 ± 0.38%, and 22.97 ± 1.45% for *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001, respectively. These results indicated that the medium acidification contributed substantially to growth suppression, particularly for *P. agglomerans* AFF2001. Nevertheless, even at neutral pH, culture filtrates of *P. agglomerans* AFF2001 retained a significant inhibitory effect on Ea21 growth, suggesting the involvement of additional antagonistic factors (Fig. 3C).

Motility assays revealed that Ea21 swimming and swarming were completely inhibited in the TQ culture filtrate of *P. agglomerans* AFF2001 (Fig. 3D, Figs. S10 and S11). Importantly, partial inhibition of motility persisted even when the pH of the filtrate was adjusted to 7, with reductions of approximately 52.36 ± 2.73% and 27.69 ± 3.70% in swimming and swarming motility, respectively.

Culture filtrates of *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009 also affected motility, although less consistently. In particular, the *Acinetobacter* sp. AFF2002 filtrate adjusted to pH 7 appeared to enhance swimming motility, whereas swarming motility was reduced in the TQ filtrate but not after pH adjustment (Fig. 3D, Figs. S10 and S11).

Since biofilm production is considered a virulence factor of *E. amylovora*, we investigated the effect of bacterial culture filtrates on biofilm production in *E. amylovora* Ea21. No significant effect on Ea21 biofilm formation was observed in all the tested culture filtrates (Table S3).

Values are expressed as the mean ± standard error of one representative experiment from a series of independent experiments showing similar results.

3.7. Genome assembly and genomic features of P. agglomerans AFF2001

The draft genome of *P. agglomerans* AFF2001 (JBUXNA000000000) comprised 5,054,504 bp assembled into five contigs, with a G + C content of 54.8%, comparable to that reported for the type strain FDAARGOS 1447 (ASM1904838v1) (Fig. 4). Genome annotation predicted 4932 protein-coding sequences and 98 RNA genes (Table S4).

The largest contig (3,994,567 bp) was identified as the chromosome. BLAST analyses revealed that three additional contigs showed high sequence similarity to known plasmids from *P. agglomerans* strains, namely pBH6cv2_D, pASB05p2, and pASB05p1 (Table 2). Genome

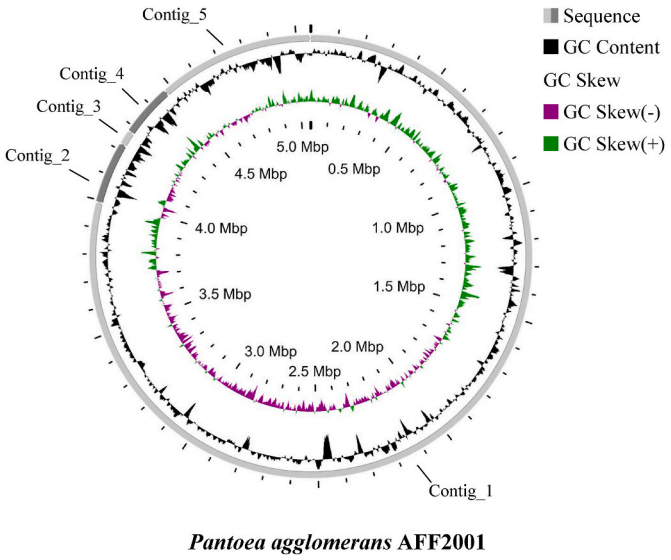

Fig. 4. Genomic map representation of the *P. agglomerans* AFF2001 draft genome. Rings show (from the inside): (1) GC skew; (2) GC percent; (3) nucleotide sequence. Genomic maps were constructed using Proksee [46].

Table 2
Contigs of *P. agglomerans* AFF2001 with associated features and secondary metabolite predictions.

Contig ID	Length (bp)	Notes	antiSMASH analysis
Contig_1	3,994,567	Predicted chromosome	frederiksenibactin (similarity 84%), aryl polyenes (similarity 94%)
Contig_2	230,479	No homologies	-
Contig_3	54,201	DNA regions homologous to plasmid pBH6cv2_D (identity 97.55%, coverage 57%)	-
Contig_4	196,450	Homologous to plasmid pASB05p2 (identity 99.42%, coverage 100%)	griseoluteic acid (similarity 66%)
Contig_5	578,807	DNA regions homologous to plasmid pASB05p1 (identity 99.09%, coverage 90%)	desferrioxamine E (similarity 100%), carotenoid (similarity 100%)

mining using antiSMASH identified biosynthetic gene clusters associated with the production of aryl polyenes and the siderophore frederiksenibactin on the chromosomal contig. In addition, Contig_5 harboured gene clusters responsible for the biosynthesis of desferrioxamine E and carotenoids, whereas a putative griseoluteic acid biosynthetic gene cluster showing 66% similarity to reference clusters was identified on Contig_4 (Table 2).

Genes encoding components of the Type VI Secretion System (T6SS) were identified on the chromosome (Table S5). These included structural proteins, regulatory elements, and accessory factors that form a complete T6SS machinery. Moreover, two putative T6SS effector genes homologous to those reported in *P. agglomerans* FDAARGOS 1447 were identified, suggesting a potential role for T6SS-mediated interbacterial interactions (Table S5).

3.8. P. agglomerans AFF2001 mutants show reduced biocontrol activity on apple flowers and pear slices

To elucidate the molecular basis of the biocontrol activity of *P. agglomerans* AFF2001, random transposon mutagenesis was performed. More than 2000 mutants were initially screened by co-spot-

inoculation with *E. amylovora* Ea21GFP on PSBMT agar. Mutants that failed to inhibit Ea21GFP growth, as indicated by fluorescent co-spots, or that displayed altered colony morphology were selected for further analysis.

Three mutants, designated *P. agglomerans* AFF2001-AP2, AFF2001-AP13, and AFF2001-AP24, consistently exhibited altered phenotypes. Mutant AFF2001-AP2 formed co-spots surrounded by a fluorescent ring, while mutant AFF2001-AP24 produced transparent colonies. Mutants AFF2001-AP2 and AFF2001-AP13 displayed a lumpy colony morphology without pigmentation changes. The 16S rRNA gene sequencing confirmed that all three mutants were derived from *P. agglomerans* AFF2001.

Before functional characterisation, the growth kinetics of the mutants were assessed in NB and PSBMT to exclude growth-related artefacts. Although minor differences in growth curves were observed, all mutants reached final cell densities comparable to the wild-type strain, with an average of 9.18 ± 0.11 $\log_{10}$ CFU mL^{-1} in both PSBMT and NB (Fig. 5, Fig. S12).

In plant assays, all mutants except AFF2001-AP2 exhibited significantly reduced plant protection efficacy on detached apple flowers compared with the wild-type, with values of 46.02 ± 8.68% for AFF2001-AP13, 22.40 ± 10.46% for AFF2001-AP24, and 54.45 ± 4.30% for *P. agglomerans* AFF2001 wild-type (Fig. 6A). Mutant AFF2001-AP24 showed the most pronounced loss of activity, with extensive stigmatic necrosis observed (Fig. S13).

In immature pear slice assays, only slices treated with mutant AFF2001-AP24 developed Ea21 exudates and visible browning (Fig. S14), indicating a marked reduction in protective efficacy (63.28 ± 8.81%) compared with the other treatments (Fig. 6B). Enumeration of Ea21 cells confirmed that pathogen populations recovered from AFF2001-AP24 -treated pear slices (9.56 ± 0.22 $\log_{10}$ CFU g^{-1} of pear slice) were comparable to those of the untreated control (9.59 ± 0.05 $\log_{10}$ CFU g^{-1} of pear slice) (Fig. 6C).

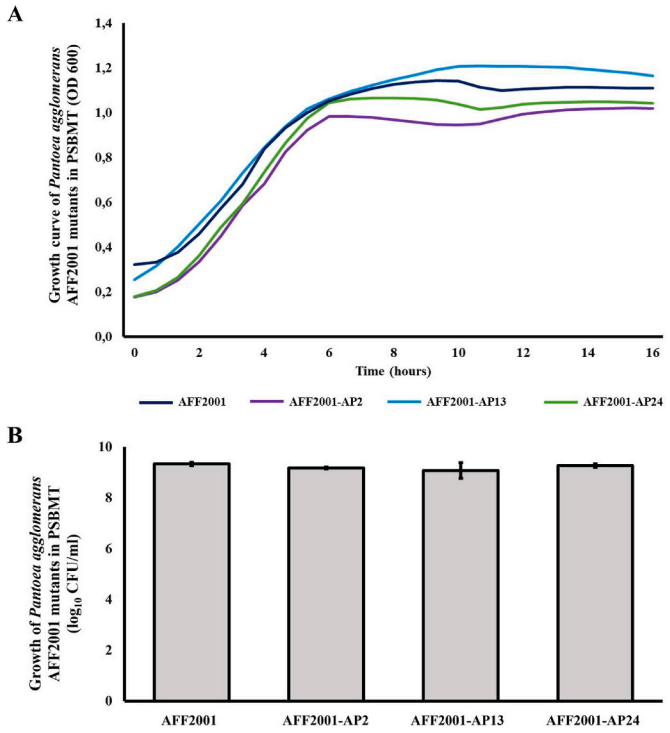

Fig. 5. Growth curves of *P. agglomerans* AFF2001 knockout mutants in Partial Stigma-Based Medium (PSBMT). *P. agglomerans* AFF2001, *P. agglomerans* AFF2001-AP2, *P. agglomerans* AFF2001-AP13, and *P. agglomerans* AFF2001-AP24 were resuspended in PSBMT (OD_{600} = 0.1) and incubated in 48-well microplates at 27°C with shaking. Growth was monitored by measuring OD_{600} hourly for 24 h, and final cell concentrations were determined by plating serial dilutions on Nutrient Agar. (A) Growth curve. (B) Bacterial cell counts. Columns represent the mean bacterial cell number ± standard error of three replicates of one representative experiment from a series of independent experiments showing similar results. Columns bearing the same letters are not significantly different according to Tukey's HSD test (α = 0.05).

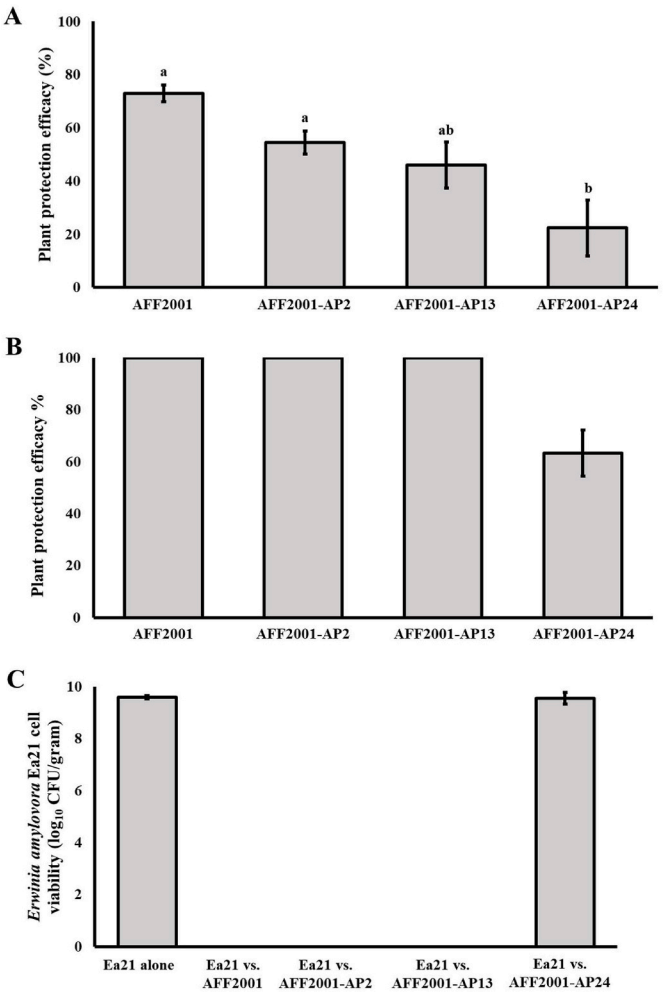

Fig. 6. Biocontrol efficacy of *P. agglomerans* AFF2001 mutants against *E. amylovora* Ea21. *P. agglomerans* AFF2001 and its mutant derivatives were evaluated for disease reduction on detached apple flowers (A), immature pear slices (B), and *E. amylovora* Ea21 populations recovered from pear tissue (C). Plant protection efficacy is expressed as the percentage reduction relative to the pathogen-only control (A–B). *E. amylovora* Ea21 cell viability is expressed as mean bacterial cell counts (C). Data are the mean ± standard error of one representative experiment from a series of independent experiments showing similar results. Different letters indicate significant differences according to Tukey's HSD test (α = 0.05).

3.9. Transposon insertion sites and phenotypic characterisation of *P. agglomerans AFF2001 mutants*

Identification of transposon insertion sites revealed that mutant AFF2001-AP2 carried an insertion in the *rcsA* gene, encoding a transcriptional regulator involved in capsular polysaccharide synthesis. Mutant AFF2001-AP13 harboured a disruption in the *pdeR* gene, encoding a c-di-GMP phosphodiesterase, whereas mutant AFF2001-AP24 was disrupted in the *leuA* gene, encoding 2-isopropylmalate synthase, a key enzyme in leucine biosynthesis (Table 3).

Phenotypic assays revealed that mutations in these genes affected several traits potentially associated with biocontrol activity. Mutant

Table 3
Mini-Tn5 insertion sites in *P. agglomerans* AFF2001.

Mutant	Genomic region	Gene	Protein	GenBank Accession (locus_tag)
P. agglomerans AFF2001-AP2	Contig_1: 2,141,131 – 2,141,766	*rcsA*	Colanic acid capsular biosynthesis activation accessory protein RcsA	ACZDUD_11120
P. agglomerans AFF2001-AP13	Contig_1: 1,860,136 – 1,862,121	*pdeR*	c-di-GMP phosphodiesterase	ACZDUD_09755
P. agglomerans AFF2001-AP24	Contig_1: 436,542 – 434,980	*leuA*	2-isopropylmalate synthase	ACZDUD_02885

AFF2001-AP2 displayed motility and siderophore production comparable to the wild-type (820.78 ± 43.11 mm^2 for swimming area, 20.45 ± 1.40 mm^2 for swarming area, and 112.26 ± 4.71 mm^2 for siderophore discolouration area) but exhibited reduced biofilm formation (0.04 ± 0.01 compared with 0.13 ± 0.01 for the wild-type) (Fig. S15). Mutant AFF2001-AP13 showed reduced swimming motility (290.49 ± 9.79 mm^2), enhanced swarming behaviour (43.93 ± 2.04 mm^2) and increased biofilm formation (0.22 ± 0.01), accompanied by reduced siderophore production (43.04 ± 4.33 mm^2) (Fig. S15). Mutant AFF2001-AP24 exhibited a complete loss of swimming and swarming motility, absence of biofilm formation and no detectable siderophore production (Fig. S15).

The pH values of the bacterial culture filtrates showed that all *P. agglomerans* AFF2001 mutants acidified the PSBMT medium. However, AFF2001-AP24 caused significantly less acidification than the wild-type (Table 4).

Culture filtrates from all mutants retained the ability to inhibit Ea21 growth, although the effect was weaker for AFF2001-AP24 (8.88 ± 1.99%) in comparison to the wild-type (38.63 ± 0.29%) (Fig. 7). After pH adjustment, no statistically significant differences were observed between the mutants and the wild-type (Fig. 7).

Overall, these results indicate that genes involved in motility regulation, biofilm formation, iron acquisition, and environmental acidification contribute to the biocontrol activity of *P. agglomerans* AFF2001.

4. Discussion

It is now widely recognised that the microbial communities associated with apple flowers can act as a natural barrier against the colonisation of *E. amylovora*, thereby limiting the development of fire blight. Building on this concept, the present study investigated the apple flower endophytic microbiota as a source of BCAs active against *E. amylovora*.

In this study, the majority of the endophytic bacteria isolated from healthy apple flowers (*M. domestica* cv. Golden Delicious) belonged to the families Enterobacteriaceae, Pseudomonadaceae, and Microbacteriaceae, consistent with previous studies reporting that Enterobacteriaceae and Pseudomonadaceae dominate the apple flower microbiome across different cultivars and environments [10–12]. This consistency suggests that the structure of the apple floral microbiota is relatively conserved and that members of these families may play functional roles in shaping the establishment of *E. amylovora*. Indeed, metabolic profiling using Biolog assays revealed a substantial overlap in carbon and nitrogen source utilisation between *E. amylovora* Ea21 and several endophytic bacterial strains, particularly members of the Enterobacteriaceae. Among these, *P. agglomerans* AFF2001 showed the highest similarity to Ea21, especially in the assimilation of glucose, fructose and related carbohydrates, as well as specific amino acids. These findings suggest that nutrient competition may be one mechanism by which *P. agglomerans* AFF2001 could limit the establishment of *E. amylovora* on floral tissues. This hypothesis is supported by previous work showing that competition for limiting nutrients is a key mode of action of several BCAs against fire blight [47–49]. Importantly, amino acids required by *E. amylovora*, such as glutamine, glutamic acid and aspartic acid, are present in apple stigma exudates at extremely low concentrations [50], which may intensify competition among microbial inhabitants.

Table 4
pH of *P. agglomerans* AFF2001 mutants culture filtrates in Partial Stigma-Based Medium. Values represent the mean ± standard error of three replicates from one representative experiment in a series of independent experiments showing similar results.

Bacterial strain	pH value
P. agglomerans AFF2001	3.51 ± 0.03
P. agglomerans AFF2001-AP2	3.44 ± 0.03
P. agglomerans AFF2001-AP13	3.54 ± 0.06
P. agglomerans AFF2001-AP24	5.19 ± 0.19 **[a]

[a] Asterisk indicates values that differ significantly according to the Student's t-test, $p < 0.01$.

The isolated endophytic bacteria were screened using a stepwise workflow that began with assays to determine their ability to control *E. amylovora in vivo*. This approach was chosen to avoid biases introduced by selecting candidates solely on the basis of *in vitro* antagonism, which does not always translate into effective disease suppression under biologically relevant conditions [51,52].

The evaluation of the endophytic bacteria on detached apple flowers led to the selection of four bacterial strains, namely *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001, and *P. extremaustralis* AFC1001, which were effective in reducing fire blight symptoms. Similar results have been reported in previous studies, in which bacterial strains belonging to *Pantoea*, *Pseudomonas* and *Curtobacterium* showed antagonistic activity against *E. amylovora* [6,11]. It is worth mentioning that *Curtobacterium flaccumfaciens* is a plant-associated bacterium that includes well-known phytopathogenic pathovars, such as *C. flaccumfaciens* pv. *flaccumfaciens* [53], the causal agent of bacterial wilt of bean, and *C. flaccumfaciens* pv. *betae* [54], the causal agent of vascular wilt and leaf spot in red beet. Although the potential to cause plant disease could limit its development as a commercial product [55,56], we decided to include it in the characterisation to determine its ability to control *E. amylovora* in other plant hosts and shed light on its modes of action.

Further assays revealed essential differences among these candidates. In immature pear slice assays and hypersensitive response tests on tobacco, *P. extremaustralis* AFC1001 induced tissue browning unrelated to typical fire blight symptoms and failed to suppress the hypersensitive response, suggesting undesirable side effects and limiting its suitability as a commercial product. In contrast, *P. agglomerans* AFF2001 consistently outperformed the other bacterial strains, achieving complete suppression of symptoms on pear slices and significantly reducing *E. amylovora* populations in tobacco leaf tissues.

Subsequent *in vitro* assays were conducted in PSBMT, which mimics the nutritional conditions of apple flowers [13]. Unlike many previous studies that relied on rich or non-specific media [10,51,57]. The use of PSBMT provided a clearer picture of how the selected endophytic bacteria could behave once they entered the apple flowers. All the results showed that *P. agglomerans* AFF2001 was the most effective in contrasting *E. amylovora*. Thus, we proceeded with genome sequencing and annotation to determine the molecular mechanisms underlying its biocontrol activities.

P. agglomerans AFF2001 exhibited the most vigorous contact-dependent antibacterial activity, completely inhibiting the growth of *E. amylovora* during co-culture. This behaviour resembles that of commercial fire blight BCAs such as *P. agglomerans* E325 and *P. vagans* C9-1, which are known to produce antibacterial compounds active against *E. amylovora* [13,58].

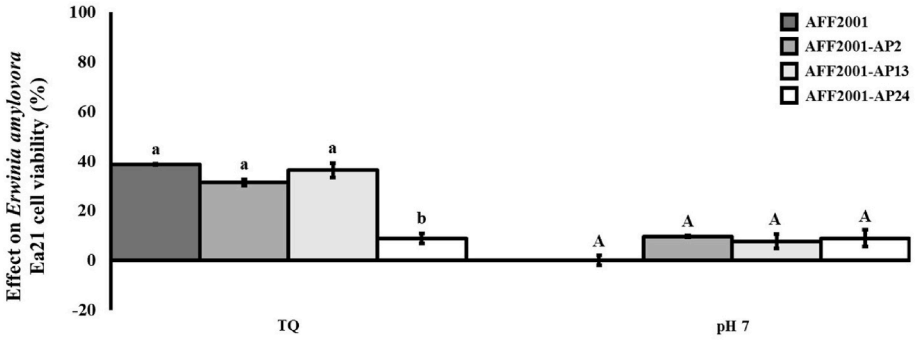

Fig. 7. Viability of *E. amylovora* Ea21 cells in *P. agglomerans* AFF2001 mutants' culture filtrates. *P. agglomerans* AFF2001 mutants were grown in liquid Partial Stigma-Based Medium (PSBMT) at 27°C with shaking, and culture supernatants were sterilised by filtration. Half of each filtrate was adjusted to pH 7, the rest was left unchanged (TQ). Filtrates were inoculated with *E. amylovora* Ea21, incubated for 24 h, then serially diluted and plated on NA for colony counts. Columns represent the mean effect ± standard error of three replicates ± standard error of one representative experiment from a series of independent experiments showing similar results. Columns bearing the same letters are not significantly different according to Tukey's HSD test ($\alpha = 0.05$).

Genome mining of AFF2001 revealed a putative D-alanylgriseoluteic acid biosynthetic gene cluster and a complete Type VI Secretion System (T6SS). Both features are consistent with contact-dependent antagonism, as D-alanylgriseoluteic acid is a phenazine-derived antimicrobial previously reported to inhibit *E. amylovora* colonisation of floral stigmas [59], while T6SSs are well-known mediators of interbacterial competition through direct effector delivery into neighbouring cells [60].

Although no diffusible antimicrobial activity was detected in chloroform assays, antibiotic production or T6SS-mediated killing may be induced specifically in the presence of competing bacteria, as reported for other systems [61–65]. Experimental validation of these hypotheses will require targeted mutagenesis of the relevant biosynthetic and secretion genes.

Iron competition also emerged as a potential mechanism contributing to the biocontrol activity of *P. agglomerans* AFF2001. This bacterial strain produced siderophores in PSBMT, and siderophore production increased in the presence of *E. amylovora*, suggesting active competition for iron. Similarly to *E. amylovora*, *P. agglomerans* AFF2001 has genes responsible for the biosynthesis of desferrioxamine E, a siderophore known to play a role in *E. amylovora* pathogenicity [66–69]. The observed reciprocal increase in siderophore production supports the idea that iron limitation may influence early stages of flower colonisation and *E. amylovora* fitness.

Another key finding of this study was the strong acidification of PSBMT by *P. agglomerans* AFF2001, which reduced the pH to values inhibitory to *E. amylovora* growth and motility. Acidification of floral tissues by *P. agglomerans* E325 has previously been reported both *in vitro* and *in vivo* [13,70], suggesting that pH modulation may be a conserved trait among effective *Pantoea* spp. BCAs. Although Ea21 exhibited limited growth under acidic conditions, motility was severely impaired, which is particularly relevant, given that *E. amylovora* motility is essential for migration from stigmas to nectaries during infection [71]. The persistence of inhibitory effects even after pH neutralisation of the *P. agglomerans* AFF2001 culture filtrate indicates that this BCA could release additional metabolites or signalling molecules with antagonistic effects against *E. amylovora*.

The generation and characterisation of *P. agglomerans* AFF2001 knockout mutants further supported the multifactorial nature of its biocontrol activity. Disruption of genes involved in capsular regulation (*rcsA*), cyclic di-GMP turnover (*pdeR*), and amino acid biosynthesis (*leuA*) resulted in environmental acidification, altered motility, biofilm formation, siderophore production, and reduced plant-protection efficacy.

A mutation in *rcsA*, encoding the auxiliary regulator RcsA, affected biofilm formation and siderophore production. RcsA is part of the Rcs phosphorelay system in *E. coli*, where it forms a heterodimer with RcsB to activate exopolysaccharides (EPSs) and biofilm-associated genes and repress flagellar synthesis [72,73]. Our results are consistent with this regulatory model. The partial reduction of biofilm formation suggests that EPS production is still maintained via RcsB-dependent regulation, since *rcsB* was not disrupted. Similarly, repression of flagellar synthesis may persist due to active RcsB-mediated control.

The *pdeR* mutant, encoding a c-di-GMP phosphodiesterase, showed increased swarming motility and biofilm formation, with reduced swimming motility and siderophore production. c-di-GMP is a central second messenger controlling the switch between motile and sessile lifestyles, generally promoting biofilm formation while repressing motility [74,75]. A mutation in a gene encoding a phosphodiesterase would likely result in intracellular accumulation of c-di-GMP, as it would no longer be efficiently degraded, leading to increased biofilm formation and reduced motility. Our observations are consistent with this model. However, the enhanced swarming motility suggests deviations from the canonical c-di-GMP regulatory model.

The last mutant, A24, was mutated in the *leuA* gene, encoding a 2-isopropylmalate synthase. This mutant exhibited a pleiotropic phenotype, with no detectable biofilm or siderophore production, reduced environmental pH, and markedly decreased plant protection efficacy. A24 is an auxotrophic mutant, as disruption of *leuA* abolishes leucine biosynthesis. Since the Biolog assay revealed that *P. agglomerans* AFF2001 can utilise leucine, albeit at low efficiency, loss of leucine biosynthesis may become critical under conditions of limited amino acid availability, as encountered in floral environments such as stigmas, where only a restricted set of amino acids is present [50]. However, the link between leucine metabolism and biocontrol activity remains unclear, although the severe phenotypic defects observed in the *leuA* mutant suggest that amino acid biosynthesis may be critical for the antagonistic performance of *P. agglomerans* AFF2001.

These results highlight the importance of regulatory networks that control surface colonisation, metabolic flexibility, and interbacterial interactions. To our knowledge, this is the first study directly linking *rcsA*, *pdeR*, and *leuA* to the biocontrol performance of *P. agglomerans* against *E. amylovora*.

Taken together, the data indicate that *P. agglomerans* AFF2001 suppresses *E. amylovora* through a combination of mechanisms, including nutrient and iron competition, environmental acidification, interference with motility and possibly contact-dependent killing. No single mechanism alone appears sufficient to explain the observed biocontrol efficacy, underscoring the complexity of microbial interactions on flower surfaces.

Despite its promising performance, the potential use of *P. agglomerans* AFF2001 as a BCA warrants careful biosafety evaluation. *Pantoea agglomerans* is classified as a biosafety level 2 organism under EU legislation [76], and some bacterial strains have been associated with plant or opportunistic human infections [77–80]. Therefore,

further genomic and phenotypic analyses, including pathogenicity tests on non-target plants and biosafety screening, are required before considering field application.

In conclusion, this study demonstrates that the apple flower endophytic microbiota represents a valuable reservoir of potential BCAs and highlights *P. agglomerans* AFF2001 as a particularly promising candidate. Beyond its potential use as a single BCA, *P. agglomerans* AFF2001 could be incorporated into a synthetic microbial community (SynCom) with other compatible bacterial strains, such as *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009, to enhance disease suppression through functional complementarity and microbial synergy [81–86]. Therefore, in the case of *C. flaccumfaciens* AFF2009, thorough genomic characterisation and pathogenicity assessments on non-target hosts are required to rule out potential phytopathogenicity and ensure biosafety prior to its inclusion in SynCom formulations or field applications. Future work will focus on validating the performance of these strains, individually and in SynCom formulations, under field conditions, and on assessing their long-term stability, efficacy, and biosafety.

5. Conclusions

This study aimed to advance the understanding of fire blight ecology to identify sustainable alternatives to conventional control strategies that rely on antibiotics and copper-based compounds. Although several BCAs have been developed to manage *E. amylovora*, their efficacy remains insufficient to consistently suppress disease under field conditions. Consequently, further exploration of the apple flower microbiota is required to identify novel and complementary BCAs.

Among the endophytic bacterial strains isolated from healthy apple flowers, *P. agglomerans* AFF2001 emerged as the most effective antagonist of *E. amylovora* Ea21. This endophytic bacterium significantly reduced fire blight symptom development on detached apple flowers and fully suppressed disease symptoms on immature pear slices. In addition, *P. agglomerans* AFF2001 impaired the virulence and pathogenicity of *E. amylovora* Ea21, as demonstrated by reduced ooze production on pear tissues and attenuation of the hypersensitive response in tobacco leaves.

Multiple mechanisms appear to contribute to the biocontrol activity of *P. agglomerans* AFF2001. These include competition for nutrients and iron, environmental acidification, inhibition of pathogen motility and contact-dependent antagonism. The multifactorial nature of these interactions suggests that no single mechanism is solely responsible for disease suppression; rather, their combined effects limit pathogen establishment and proliferation in floral tissues. Genomic analyses further support this conclusion by revealing biosynthetic gene clusters and secretion systems that may be involved in interbacterial interactions.

Overall, the findings of this study highlight *P. agglomerans* AFF2001 as a promising candidate for the development of novel biocontrol strategies against fire blight. Provided that biosafety and phytopathogenicity assessments confirm its suitability, this bacterial strain could be further developed either as a standalone biocontrol agent or as a component of a synthetic microbial community designed to enhance disease suppression through microbial complementarity and synergy.

CRediT authorship contribution statement

Anna Pedroncelli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Laura Franceschi:** Investigation, Methodology. **Malek Marian:** Investigation, Methodology. **Gerardo Puopolo:** Conceptualization, Data curation, Formal analysis, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2026.103306.

Data availability

The draft genome sequence of *Pantoea agglomerans* AFF2001 has been deposited in NCBI under BioProject accession number (PRJNA1422049).

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