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# Selection of a biocontrol agent based on a potential mechanism of action: degradation of nicotinic acid, a growth factor essential for *Erwinia amylovora*

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Summary. This work describes a medium-based screening method for selecting microbial biocontrol agents against *Erwinia amylovora* based on the degradation of a specific growth factor. *Erwinia amylovora*, the causal agent of the devastating fire blight disease, requires nicotinic acid or nicotinamide as an essential growth factor. Potential biocontrol agents are either selected for antimicrobial production in plate or directly on immature pears or apple blossoms. In this work, we have attempted to streamline the selection of a new potential biocontrol agent with a lower risk of non-target effects by isolation based on the ability to degrade nicotinic acid in vitro, using therefore few plant materials. A total of 735 bacteria and 1237 yeast were isolated from apple blossoms and pre-screened for nicotinic acid-degradation. *Pseudomonas rhizosphaerae* strain JAN was able to degrade both nicotinic acid and nicotinamide. Mutants deficient in this ability were constructed. JAN, but not the mutants, controlled *E. amylovora* on pear slices. On detached apple blossoms, JAN colonized apple hypanthia and strongly suppressed *E. amylovora* growth. Under greenhouse conditions, JAN was more effective in controlling blossom blight than *P. fluorescens* A506, a commercial biocontrol agent of fire blight unable to degrade nicotinic acid and nicotinamide. [**Int Microbiol** 2010; 13(4):195-206]

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## Introduction

The gram-negative bacterium *Erwinia amylovora* is the causal agent of fire blight, a disease affecting several species of the *Rosaceae* family. This disease is found in most of the temperate regions of the world and can cause significant losses in important crops, such as apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.) [32]. Fire blight can affect all aerial parts of the plant, but blossoms are the most important site of infection for epidemic blossom blight. On blossoms, *E. amylovora* reaches the hypanthium—the cup-shaped structure, typical of Rosaceae and other few plant families, on which the sepals, petals and stamens are born—after an epiphytic phase on the stigma. Infection via nectarthodes—the opening at the base of the flower from which nectar exudes—in the hypanthium occurs when a large bacterial population and suitable environmental conditions (high relative humidity and/or rain) are present [30].

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Expensive exclusion measures (quarantine) are commonly used to limit the spread of the disease, but when they are not successful and the pathogen becomes established, control can only rely on the application of antibiotics, prohibited in several countries, or on the use of copper and resistance inducers that control the disease only partially [32]. The use of microbial antagonists against *E. amylovora* is an alternative or complementary measure to the application of antibiotics [24]. Few species, mainly *Pseudomonas fluorescens* [4,34], *Pantoea agglomerans* (synonym *Erwinia herbicola*) [9,31,37] and *Bacillus subtilis* [2], are active against *E. amylovora*, and only very few isolates have been developed for field application [2,6,35].

To date, the selection of biological control agents against fire blight has been based either on the massive screening for efficacy of numerous isolates on immature pear fruit and/or apple stigma [4,25] or on screening for inhibition on artificial media [1,23,36]. These screening procedures, however, have the disadvantage of either being extremely time-consuming and subjected to the availability of blossoms and fruits or selecting for biocontrol agents able to produce antimicrobial compounds and therefore potentially having a higher risk of non-target effects.

*Erwinia amylovora* requires nicotinic acid (NiAc), nicotinamide ($NiNH_2$) and/or 6-hydroxynicotinic acid (6-HNiAc) as an essential growth factor when cultured on minimal medium in the laboratory [21,29]. NiAc and $NiNH_2$, but not 6-HNiAc, are present on the hypanthia of apple and pear blossoms [21]. The need for NiAc is not common in the *Erwinia* genus, and it has been proposed as a biochemical test for the identification of *E. amylovora* [7]. The selection of microorganisms that do not produce inhibitory compounds and can compete with *E. amylovora* for NiAc and $NiNH_2$ could therefore represent an innovative method to select new biocontrol agents for the control of the disease with low risk of non-target effects.

The aim of our study was to develop a medium-based screening approach in which the ability of microorganisms to degrade NiAc (essential growth factor) would be the main criterion for the selection of a biocontrol agent candidate against *E. amylovora*. This approach consisted of an initial mass isolation of microorganisms from apple blossoms (microorganisms presumably well suited to the blossom environment), the selective screening on minimal medium of the NiAc-degraders, and the further selection of those most suitable for mass cultivation (quickly growing on the medium). For the selected microorganism, $NiNH_2$ degradation and the in vitro inhibition of *E. amylovora* through NiAc and $NiNH_2$ degradation was assessed. Production of antimicrobial compounds active against *E. amylovora* was evaluated. Biocontrol efficacy was evaluated on immature pear fruit slices, on detached apple blossoms and on apple trees maintained under controlled greenhouse conditions.

## Materials and methods

**Isolation of epiphytic microorganisms from apple blossoms.** Microorganisms were collected from three orchards, two located in Switzerland (St. Gallen, 43 samples; and Wädenswil, 100 samples) and one in northern Italy (Trento, 24 samples). Each sample was obtained by submerging the stigmas and styles of five randomly collected blossoms in 1.5 ml sterile saline solution (0.9% NaCl) in Eppendorf tubes and rotating them to dislodge the microorganisms. Serial decimal dilutions were prepared and plated on diluted (10%) tryptic soy agar (TSA; Difco, Detroit, MI, USA) and on potato dextrose agar (PDA; Difco) supplemented with kanamycin sulphate (50 µg/ml; Fluka, Buchs, Switzerland), to isolate bacteria and yeasts, respectively. After three days of incubation at 24°C, samples of individual colonies (at least one for each different colony morphology observed in each sample) were selected and transferred to 96-well microtiter plates, where they were cultured on diluted (10%) tryptic soy (TS, Difco) or potato dextrose broth (PDB; Difco). After two days of incubation at 24°C, sterile glycerol (AppliChem, Darmstadt, Germany) was added (30% of the final volume) to the wells and the plates were transferred to a freezer (–80°C) for storage.

**Selective screening of quick-growing, NiAc-degrading microorganisms and evaluation of their activity against *E. amylovora*.** We used a modified version of the method described by Wang et al. [33] to select microorganisms capable of degrading NiAc. A DF-NiAc salt minimal medium (pH = 6.0) was prepared, in which NiAc was added as the only N-source to DF salt minimal medium [5] to a final concentration of 2 mM. Isolates were transferred from the 96-well microtiter plates to DF-NiAc agar in Petri dishes in a 6 × 8 pattern suitable for replica plating, and incubated at 18°C for three days. The 12 best-growing isolates (those whose colonies had the largest diameters) were selected and tested for their ability to degrade $NiNH_2$ on DF salt minimal medium and to control *E. amylovora* in a pear-slice assay. We used a modified version of the immature pear-slice assay described by Kearns and Hale [10]. Immature pears (cv. Conference) with diameters of 2–3 cm were harvested in Valais, Switzerland, during the first week of June. The pears were surface-disinfected (10 min immersion in 1% sodium hypochlorite, Fluka), washed three times in sterile distilled water, dried, and stored at 3ºC in sealed plastic bags. Before use, pear fruits were surface sterilized with 70% ethanol and left to dry in a sterile hood. The 12 best-growing isolates were grown on TSA for 48 h at 18°C, gently scraped from the surface of the media, washed twice in 0.1 M potassium phosphate buffer, and then resuspended in 1.5 ml of 0.1 M potassium phosphate buffer. Bacterial suspensions at concentration $10^6$ colony forming units (CFU)/ml were prepared. The pears were cut into 3 mm-thick slices and dipped in the bacterial suspensions for 10 s. Potassium phosphate buffer (0.1 M) was used in the untreated control. Five pear slices were placed in each sealed Petri dish on wet, sterile filter paper. After 24 h of incubation at 18°C with a 16-h photoperiod, the slices were inoculated with 10 µl of a $10^6$ CFU/ml *E. amylovora* suspension and the Petri dishes were incubated at 18°C. Disease incidence, expressed as percentage of infected slices, was periodically assessed based on the appearance of bacterial exudate droplets on the slice surface. The efficacy of control was calculated using the following equation:

$$\text{Efficacy (\%)} = [(A–B)/A] \times 100$$

where $A$ is the incidence of disease on the pear slice samples pre-treated with 0.1 M potassium phosphate buffer and $B$ is the incidence of disease on the pear slices pre-treated with the biocontrol agent. Biocontrol efficacies were

evaluated 12 days after *E. amylovora* inoculation. Three independent experiments were carried out and each experiment included two replicates of five slices per each treatment.

Based on the results of this evaluation process, JAN was selected as the most effective microorganism against *E. amylovora* and subjected to further investigations.

**Identification of the NiAc-degrading strain JAN.** Total genomic DNA was extracted from an overnight suspension of JAN in 10% TS using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. A portion of the 16S rRNA gene was amplified with R1n and 1488r primers [11]. The PCR reaction mixture (20 µl) consisted of 1 × PCR buffer (Amersham Biosciences, Piscataway, NJ, USA) containing 100 µM of each dNTP (Amersham Biosciences), 0.20 µM of each primer, 0.07 U/µl Taq polymerase (New England Biolabs, Beverly, MA, USA) and approximately 200 pg of the extracted genomic DNA. After an initial denaturation step of 5 min at 94°C, there were 35 cycles of 40 s at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. The amplification was performed in a PTC-100 thermal cycler (MJ Research, Waltham, MA, USA). The PCR amplicon was purified from the PCR mix using the Wizard SV Gel and PCR Clean-up System purification kit (Promega), suspended in 50 µl bidistilled water, and visually quantified on agarose gel (Sigma-Aldrich, Steinheim, Germany). Sequencing was performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using the ABI PRISM BigDye Terminator 3.1 ready reaction cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions.

The homology of the amplicon was evaluated using the BLASTN software provided by NCBI [http://www.ncbi.nlm.nih.gov/]. 16S rRNA gene sequences of JAN and of representative species of the genus *Pseudomonas* [obtained from the Ribosomal Database Project II, http://rdp.cme.msu.edu] were aligned with ClustalW implemented in the BioEdit software [http://www.mbio.ncsu.edu/BioEdit/BioEdit.html] and a phylogenetic tree was constructed using the unrooted neighbor-joining method implemented in the MEGA 4 software package [http://www.megasoftware.net].

**Production of antimicrobial compounds against *E. amylovora* and in vitro inhibition of *E. amylovora* growth linked to NiAc degradation.** The pathogen strain used in this and the following experiments was *E. amylovora* CFBP 1430 [20]. Assays based on pear diffusion juice were used. The pear diffusion juice was obtained using immature pear (cv. Conference) slices cut (3 mm-thick) from surface sterilized pears under aseptic conditions. To avoid problems of oxidation, the slices were immediately dipped into a Petri dish filled with cold (3°C) sterile Millipore water (Millipore, Bedford, MA, USA) and peeled. Twenty-five slices (corresponding to about 30 g) were immersed in 300-ml Erlenmeyer flasks containing 100 ml cold sterile Millipore water, and incubated for 24 h at 3°C (without shaking). The resulting solution (pear diffusion juice) was sterile filtrated with a 0.22 µm polycarbonate filter (Millipore). The pear diffusion juice (50 ml) was inoculated with 500 µl of a suspension of JAN ($10^8$ CFU/ml) in 0.1 M potassium phosphate buffer and incubated with agitation (150 rpm) at 18°C until stationary phase was reached (about 36 h after inoculation). Cell-free supernatant was obtained by centrifugation at 4000 rpm for 5 min using an ALC multispeed refrigerated centrifuge PK121R (A-M10 rotor) (ALC, Milan, Italy), followed by sterile filtration.

To assess the production of antimicrobial compounds against *E. amylovora* by JAN, an overnight culture of *E. amylovora* was mixed with 0.1 M potassium phosphate buffer to form a suspension with an optical density at 600 nm ($OD_{600}$) of 0.4 (corresponding to about $10^9$ CFU/ml). $OD_{600}$ was measured with a Genova spectrophotometer (Jenway, Dunmow, UK). Five-ml aliquots of a 1:1 mixture of JAN cell-free supernatant (pH = 7.9) and pear diffusion juice (pH = 6.7) were transferred into Falcon tubes (BD Biosciences) and inoculated with 50 µl of *E. amylovora* suspension prepared as described above. As a control, 5-ml aliquots of a 1:1 mixture of Millipore water and pear diffusion juice were inoculated with *E. amylovora* as described above. The samples were incubated for one day at 27°C, with agitation (150 rpm). *E. amylovora* growth was then determined by measuring the $OD_{600}$ values of the samples. Three independent experiments were carried out and each experiment included four replicates of each treatment (one Falcon tube = one replicate).

To evaluate the capacity of JAN to inhibit *E. amylovora* growth through NiAc degradation, we eliminated traces of NiAc from the *E. amylovora* inoculum by incubating the bacterium overnight at 22°C, with agitation (150 rpm), in M9 minimal medium [27] supplemented with 0.01% thiamine-hydrochloride (Fluka). This *E. amylovora* suspension was diluted in 0.1 M potassium phosphate buffer to a final $OD_{600}$ of 0.4. Then, 50 µl were used to inoculate 5 ml of a solution composed of JAN cell-free supernatant (prepared as described above) mixed (1:1) with M9 media with and without NiAc (0.01%). The control treatments were M9 media mixed (1:1) with pear diffusion juice, and M9 media with and without NiAc mixed (1:1) with Millipore water. The pH levels of all media were adjusted to 7.0. After 40 h of incubation at 22°C, with agitation (150 rpm), *E. amylovora* growth was determined by measuring the $OD_{600}$. Three independent experiments were carried out and each experiment included four replicates of each treatment (one Falcon tube = one replicate).

**Construction and characterization of NiAc and $NiNH_2$ degrading-deficient mutants of JAN.** JAN $rfm^r$, a spontaneous antibiotic-resistant derivative of JAN, was used as recipient strain in the mutagenesis. JAN $rfm^r$ was obtained by plating 100 µl of highly concentrated bacterial solutions (approximately $10^{10}$ CFU/ml) on LB agar plates supplemented with 100 µg/ml rifampicin. No significant differences in the growth on the DF-NiAc salt medium and in biocontrol efficacy were observed between JAN $rfm^r$ and JAN.

JAN $rfm^r$ was mutagenized by insertion of transposon Tn*5* in a biparental mating with *E. coli* BW20767/pRL27. Donor strain and JAN $rfm^r$ were grown overnight at respectively 37 and 27°C in LB medium added with kanamycin (25 µg/ml) and rifampicin (100 µg/ml), respectively. Each culture (1.5 ml) was pelleted by centrifugation (at 6000 rpm), washed three times with sterile saline solution (0.9% NaCl) and mixed at a ratio 1:1 and then collected by centrifugation (6000 rpm for 10 min). The cells were suspended in 50 µl of sterile saline solution, placed as single droplets on LB agar plates, incubated at 33°C for 5 h and suspended in 1 ml of saline solution. Transposon insertion mutants were selected after serial dilution on plates of DF salt minimal agar medium containing 2 mM of $NH_4Cl$, 100 µg/ml of rifampicin and 25 µg/ml of kanamycin. After 2–3 days of incubation at 27°C, single JAN mutant colonies were picked and transferred to 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) containing 200 µl of DF salt minimal medium added with 2 mM of $NH_4Cl$ and 25 µg/ml of kanamycin in each well. Plates were incubated at 27°C for two days and 100 µl of glycerol were added before storage at –80°C.

Mutants were screened and selected based on the inability to grow on DF agar plates containing 2 mM of NiAc as the only N-source at 18°C. DF salt minimal medium added with the same amount of $NH_4Cl$ was used as control. The cloning of the transposon insertions was performed as reported in Larsen et al. [14], but using *E. coli* S17-1/*λpir* strain for the replication of the circularized fragment and only the outward-directed transposon-directed primer tnpRL17-1 for the sequencing.

The ability to utilize NiAc or $NiNH_2$ as the only N-source was tested for JAN, JAN $rfm^r$, and the two selected mutants M3G7 and M40E5 on DF agar minimal medium. Traces of these compounds from the bacterial inocula were eliminated as described above for *E. amylovora*. The overnight cultures were then diluted with fresh M9 minimal medium to reach an $OD_{600}$ of 0.350 corresponding to about $6 \times 10^7$ CFU/ml. For each strain, 2 µl of the prepared suspension was added onto DF agar plates containing 2 mM of NiAc or $NiNH_2$ as the only N-source. Then, DF agar plates containing 2 mM of

$NH_4Cl$ or without any N-source were used as positive and negative control, respectively. After 4 days of incubation at 18°C, the colonies were cut out together with the agar medium underneath the colony with a 10-mm cork borer and the plug inserted in a 2-ml Eppendorf tube containing 1 ml of 0.1 M potassium phosphate buffer. Cells were resuspended by vortexing for 20 s and then the $OD_{600}$ of the bacterial suspensions measured with an Ultrospec 3300 pro spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The experiment was carried out twice.

Mutants (M3G7 and M40E5) biocontrol efficacy was assessed in the immature pear-slice assay as described above. Slices were treated with JAN $rfm^r$, M3G7 and M40E5 suspensions at concentration $10^6$ CFU/ml, and biocontrol efficacies were evaluated 6 days after *E. amylovora* inoculation. Two independent experiments were carried out and each experiment included two replicates of five slices per each treatment. Populations of JAN $rfm^r$, M3G7 and M40E5 on the immature pear slices were evaluated after 1, 24 and 90 h of incubation. One plug per slice was excised using a 6-mm-diameter cork borer. Each excised plug was immediately placed in a sterile Eppendorf tube containing 1 ml of sterile saline solution. After vigorous vortexing for 20 s, decimal serial dilutions were prepared and plated on LB added with 100 μg/ml of rifampicin. The plates were incubated at 18°C, bacterial colonies in the appropriate dilution counted and the number of cultivable cells per square centimeter calculated. Four slices per treatment per experiment were used. The experiment was carried out twice.

Efficacy against *E. amylovora* on pear slices. The immature pear-slice assay was used to compare JAN with the biocontrol agent *P. fluorescens* A506 (A506) [15], which is a commercial biocontrol agent of fire blight (BlightBan® A506) unable to degrade NiAc, and with *P. fluorescens* TN5 (TN5), a NiAc-degrading strain already described in literature [8,18] that had never been tested for activity against fire blight. The assay was performed as described above. Pear slices were dipped into JAN, A506 and TN5 suspensions at three different concentrations ($10^5$, $10^6$ or $10^7$ CFU/ml). Biocontrol efficacies were evaluated at days 7, 9 and 12 after *E. amylovora* inoculation. Three independent experiments were carried out and each experiment included two replicates of five slices per each treatment.

Efficacy against *E. amylovora* on apple hypanthia. To monitor population growth, spontaneous antibiotic-resistant derivatives of JAN and TN5 were used. JAN $rfm^r$ was obtained as above described. A TN5 derivative resistant to 1000 μg/ml streptomycin (TN5 $sm^r$) was prepared using the same procedure. A506 was resistant to rifampicin (100 μg/ml). *E. amylovora* CFBP 1430 $nal^r$ (resistant to 75 μg nalidixic acid/ml) is a nalidixic acid-resistant spontaneous derivative of *E. amylovora* CFBP 1430 with unaltered virulence (Duffy, personal knowledge).

We used a modified version of the efficacy assay on blossom hypanthium described in Cabrefiga et al. [4]. Apple blossoms (cv. Golden Delicious) with no dehisced anthers were collected early in the morning from an orchard in Wädenswil. Blossoms were maintained fresh by immersing the peduncles in 1.5 ml Eppendorf tubes filled with 10% sucrose solution and brought to the laboratory within 1 h of their collection. Suspensions ($10^7$ CFU/ml) of JAN $rfm^r$, TN5 $sm^r$ or A506 were prepared in 0.1 M potassium phosphate buffer amended with 0.03% Tween-20. The concentration of each suspension was precisely determined by counting CFU in serial dilutions on LB agar plates amended with the respective antibiotic. Hypanthia were inoculated by placing a drop (10 μl) of a $10^7$ CFU/ml suspension of JAN $rfm^r$, TN5 $sm^r$ or A506 on each blossom, whereas the untreated control consisted of blossoms inoculated with an equal volume of 0.1 M potassium phosphate buffer plus 0.03% Tween-20. The blossoms were incubated at 18°C under high relative-humidity conditions (in closed transparent plastic boxes) and with a 16-h light photoperiod (under 18 W/1300 lumen fluorescent bulbs at about 50 centimeter from the slices). After 24 h, part of the blossoms were inoculated (each) with one drop (10 μl) of a $10^6$ CFU/ml *E. amylovora* CFBP 1430 $nal^r$ suspension, and the rest with 10 μl of 0.1 M potassium phosphate buffer plus 0.03% Tween-20. Populations of the bacteria (biocontrol agents and/or *E. amylovora*) were estimated on five blossoms per treatment at 1, 24, 48, and 72 h after inoculation. Petals, stamens, sepals and stigmas were removed from the blossoms before bacterial re-isolation. After cleaning, the remaining part of each blossom was placed in a sterile Eppendorf tube containing 1 ml of 0.1 M potassium phosphate buffer. The tubes were vortexed, briefly sonicated for 20 s, and vortexed again. Serial dilutions of the washings of each blossom were plated on LB agar amended with the respective antibiotic, and incubated at 18°C. After this incubation, CFU were counted. The efficacy of control was calculated with the above used equation, in which *A* is the *E. amylovora* population on apple hypanthia pre-treated with 0.1 M potassium phosphate buffer plus 0.03% Tween-20 and *B* is the *E. amylovora* population on apple hypanthia pre-treated with the biocontrol agent. The experiment was carried out twice, and each experiment included 5 replicates per treatment per sampling time.

Efficacy against *E. amylovora* on apple blossoms and shoots. *Erwinia amylovora* is a quarantine pathogen in Switzerland, therefore the efficacy of JAN was assessed in three independent assays performed in the quarantine greenhouse at Agroscope ACW (Wädenswil) in 2007. Two- to 3-year-old apple trees (cv. Gala) planted in 50-cm-diameter plastic pots were stored in a cold room at 3°C till late spring (June 24 and July 9), when the plants were moved inside the greenhouse and induced to bloom. The temperature in the greenhouse was 18°C with some small variations correlated to the outdoor temperature extremes: some days at 24–26°C (experiment 1) and some days at 12–14°C (experiments 2 and 3). Blossoms were sprayed with a $10^7$ CFU/ml suspension of either JAN $rfm^r$ or A506. Twenty four hours after this treatment, the blossoms were sprayed with a $10^6$ CFU/ml suspension of *E. amylovora*. Disease symptoms on the treated blossoms (blackened petioles, non-dehiscing blossoms and/or exudates on the petioles) were evaluated nine days after pathogen inoculation. The symptoms on shoots carrying the treated blossoms (blackened leaf veins, exudates on leaf petioles and shoots) were evaluated 14 days after inoculation.

The efficacy of the treatment was calculated for each replicate with the above used equation, in which *A* is the incidence of disease on blossoms or shoots on trees treated with buffer alone (control) and *B* is the incidence of disease on blossoms or shoots on trees treated with the biocontrol agent suspensions. Three independent experiments were performed, each including three replicates (one tree = one replicate) per treatment. The number of blossom per tree spanned between 48 and 213, whereas the number of shoots per tree spanned between 17 and 59.

Statistical analysis. When the experiment factor was not significant, results of experiments were pooled. The results of the three experiments to check for antibiosis against *E. amylovora* and inhibition of *E. amylovora* growth through NiAc degradation, were pooled, and the average growth of *E. amylovora* (expressed as $OD_{600}$) and the standard error of that growth calculated. Averages were compared using ANOVA and Fisher's least significant difference (LSD) test ($\alpha = 0.05$). To evaluate the biocontrol efficacy on pear slices, the results of the experiments were pooled, and average efficacies and standard error of the averages were calculated. At each assessment time, average efficacies among the tested bacteria and the different concentrations were compared using ANOVA and Fisher's LSD test ($\alpha = 0.05$). Biocontrol agents and *E. amylovora* populations were assessed on apple hypanthia, the results of the two experiments pooled and the average $\log_{10}$ CFU per blossom per treatment calculated along with the standard errors of these averages. Analogously, in the evaluation of the populations of JAN and JAN's mutants on pear slices, the average $\log_{10}$ CFU/$cm^2$ and standard errors were calculated. Averages were compared at different times using ANOVA and Fisher's LSD test ($\alpha = 0.05$). In the greenhouse experiments, symptomatic blossoms and shoots were counted and the results of the different treatments were compared using ANOVA and Fisher's LSD test ($\alpha = 0.05$). Statistical analyses were performed using Statistica 8.0 (StatSoft, Tulsa, OK, USA).

## Results

**Isolation of epiphytic microorganisms from apple blossoms, selective screening of quick-growing, NiAc-degrading microorganisms and evaluation of their effects on *E. amylovora*.** A total of 735 bacteria and 1237 yeast isolates were collected from apple blossoms. When these microorganisms were screened on DF-NiAc salt minimal media, we found that approximately 10% of the isolates could degrade NiAc as the only N-source: 128 bacteria (17.4% of the bacteria isolated) and 92 yeasts (7.4% of the total isolated yeasts). Among them, we selected the 12 strains (11 bacteria and one yeast) that grew best on DF-NiAc for further studies.

The utilization of NiAc and $NiNH_2$ was confirmed for all the 12 strains. The biocontrol efficacies of the twelve isolates were examined in the immature pear-slice assay. After 12 days from *E. amylovora* inoculation, the untreated control showed a disease incidence of 93.3 ± 6.7% (average ± standard error). On the contrary, JAN and other three isolates showed 100% of efficacy against fire blight, whereas the other eight isolates showed an efficacy comprised between 70.8% and 96.7%. Since JAN showed the highest efficacy against fire blight and the highest growth on DF-NiAc, it was chosen as the biocontrol agent candidate for further study (Table S1).

**Identification and characterization of the NiAc and $NiNH_2$-degrading strain JAN.** An amplicon of about 1.4 kb was obtained from the amplification of the 16S rRNA gene of JAN and sequenced (GenBank accession no. FJ668331). The sequence showed maximum identity (98% of identity, equal to 1406 identities over a fragment of 1421 bp) with the sequence of the type strain *Pseudomonas rhizosphaerae* IH5 (LMG 21640). Relations among JAN and the representative strains of the closest *Pseudomonas* species are illustrated in the unrooted phylogenetic tree presented in Fig. 1.

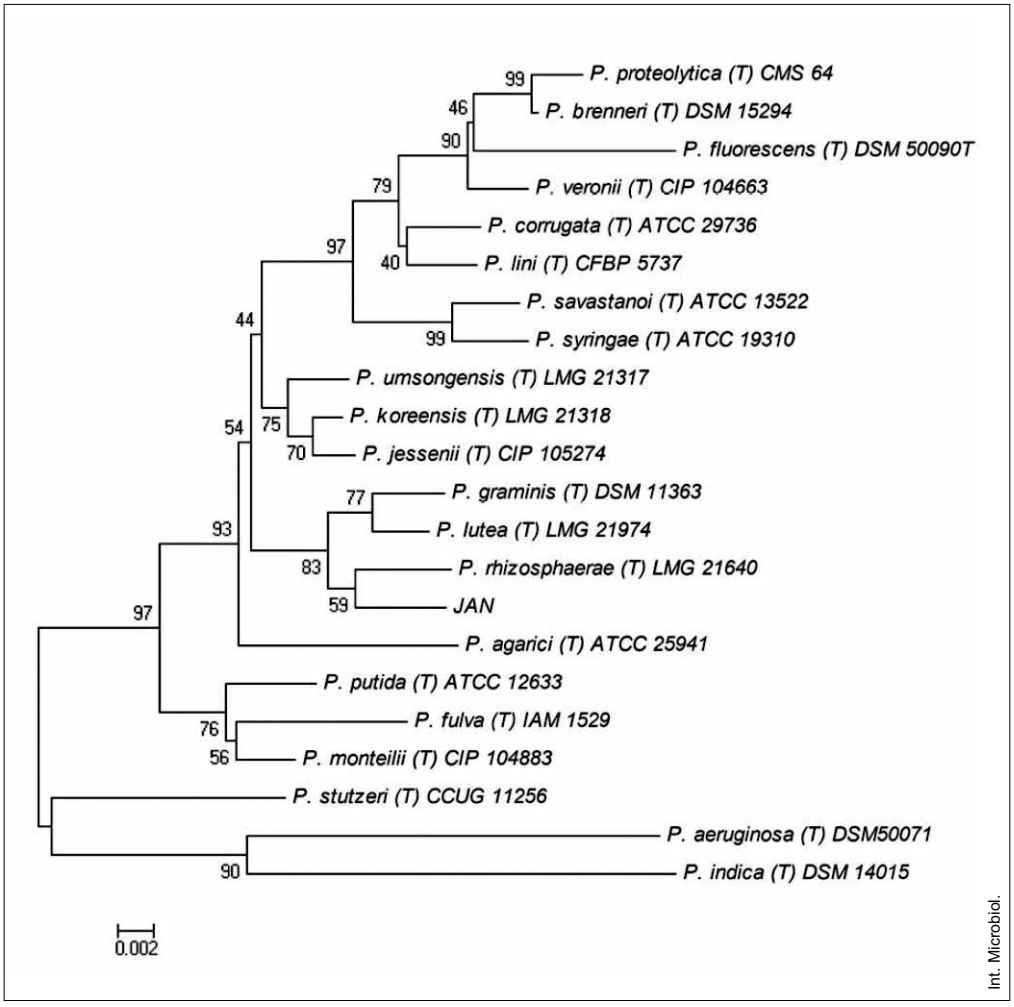


**Fig. 1.** Phylogenetic tree based on sequences of the 16S rRNA genes of JAN strain and of the more closely related members of the *Pseudomonas* genera (T = type strain). The unrooted neighbor-joining method (MEGA 4 software) was used. Nodal supports were assessed using 1000 bootstrap replicates and values are indicated. The scale bar represents 0.002 substitutions per site.

**Production of antimicrobial compounds against *E. amylovora* and in vitro inhibition of *E. amylovora* growth linked to NiAc degradation.** The production of antimicrobial compounds against *E. amylovora* by JAN was assessed on pear diffusion juice. *E. amylovora* grew statistically better ($P < 0.001$) in a 1:1 mixture of JAN cell-free supernatant and pear diffusion juice than in a 1:1 mixture of Millipore water and pear diffusion juice (average $OD_{600}$ ± standard error of 0.95 ± 0.03 and 0.62 ± 0.01, respectively). This indicates that JAN produced no antimicrobial compounds against *E. amylovora* under assay conditions.

*E. amylovora* was unable to grow in 1:1 mixtures of M9 minimal medium supplemented with 0.01% thiamine-hydrochloride and Millipore water (average $OD_{600}$ ± standard error = 0.04 ± 0.01), but the addition of 0.01% NiAc reestablished the growth, which reached an $OD_{600}$ of 1.38 ± 0.03 (3.96 ± 0.09 × $10^9$ CFU/ml). In 1:1 mixtures of M9 medium and pear diffusion juice, *E. amylovora* growth reached an $OD_{600}$ = 1.41 ± 0.01 (4.05 ± 0.03 × $10^9$ CFU/ml). When M9 media was diluted 1:1 with JAN cell-free supernatant, *E. amylovora* grew to an $OD_{600}$ of 0.55 ± 0.01 (1.56 ± 0.03 × $10^9$ CFU/ml). The addition of 0.01% NiAc promoted growth to $OD_{600}$ = 1.35 ± 0.03 (3.88 ± 0.09 × $10^9$ CFU/ml). These results indicated that *E. amylovora* inhibition by JAN in vitro could be attributed to the degradation of most of the NiAc and $NiNH_2$ present in the pear juice.

**Construction and characterization of NiAc and $NiNH_2$ degrading-deficient mutants of JAN by transposon mutagenesis.** Tn*5* insertion mutants (10600) were generated by separate biparental matings between *E. coli* BW20767/pRL27 and JAN $rfm^r$, and screened for impaired growth in DF-NiAc agar. Table S2 shows the growth of JAN, JAN $rfm^r$, the mutants M3G7 and M40E5, and TN5 on DF agar medium added with 2 mM of NiAc, $NiNH_2$, or $NH_4Cl$ as the only N-source. Mutants M3G7 and M40E5 showed this impairment and were further characterized.

Sequences of the genes disrupted in the two mutants were searched against the entire GenBank database for DNA sequence (BLASTN) and protein coding (BLASTX) features. The gene disrupted in mutant M3G7 had 89% homology to the nitrogen metabolism transcriptional regulator NtrC, Fis family, whereas the gene disrupted in mutant M40E5 had 88% homology to the nitrogen regulation protein NtrB. Using BLASTX, the translated sequences showed 98% identity to NtrC and NtrB proteins, respectively.

JAN $rfm^r$, M3G7 and M40E5 showed comparable capacity to colonize pear slices ($P = 0.313$) after 24 h of incubation at 18°C (time of *E. amylovora* inoculation). JAN $rfm^r$ reached a average value ± standard error of 2.37 ± 0.69 × $10^6$ CFU/$cm^2$, whereas M3G7 and M40E5 population reached values of 1.30 ± 0.40 and 1.76 ± 0.28 × $10^6$ CFU/$cm^2$, respectively. After 90 h of incubation, JAN $rfm^r$, M3G7, and M40E5 populations were slightly, but sig-

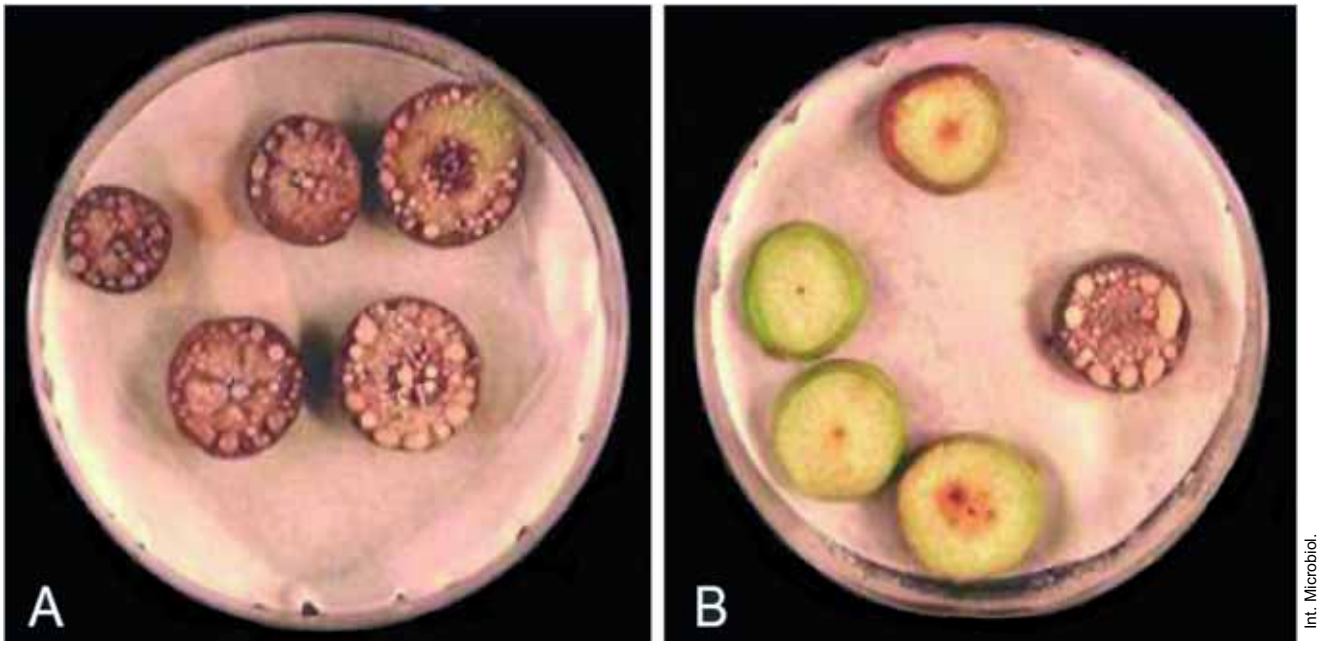


**Fig. 2.** Example of biocontrol activity of JAN on immature pear slices (cv. Conference) 7 days after inoculation with *Erwinia amylovora* ($10^6$ CFU/ml). The slices were treated with (**A**) 0.1 M potassium phosphate buffer (untreated control) or (**B**) a suspension of JAN ($10^5$ CFU/ml) in 0.1 M potassium phosphate buffer, and 24 h later inoculated with 10 µl of a *E. amylovora* suspension ($10^6$ CFU/ml).

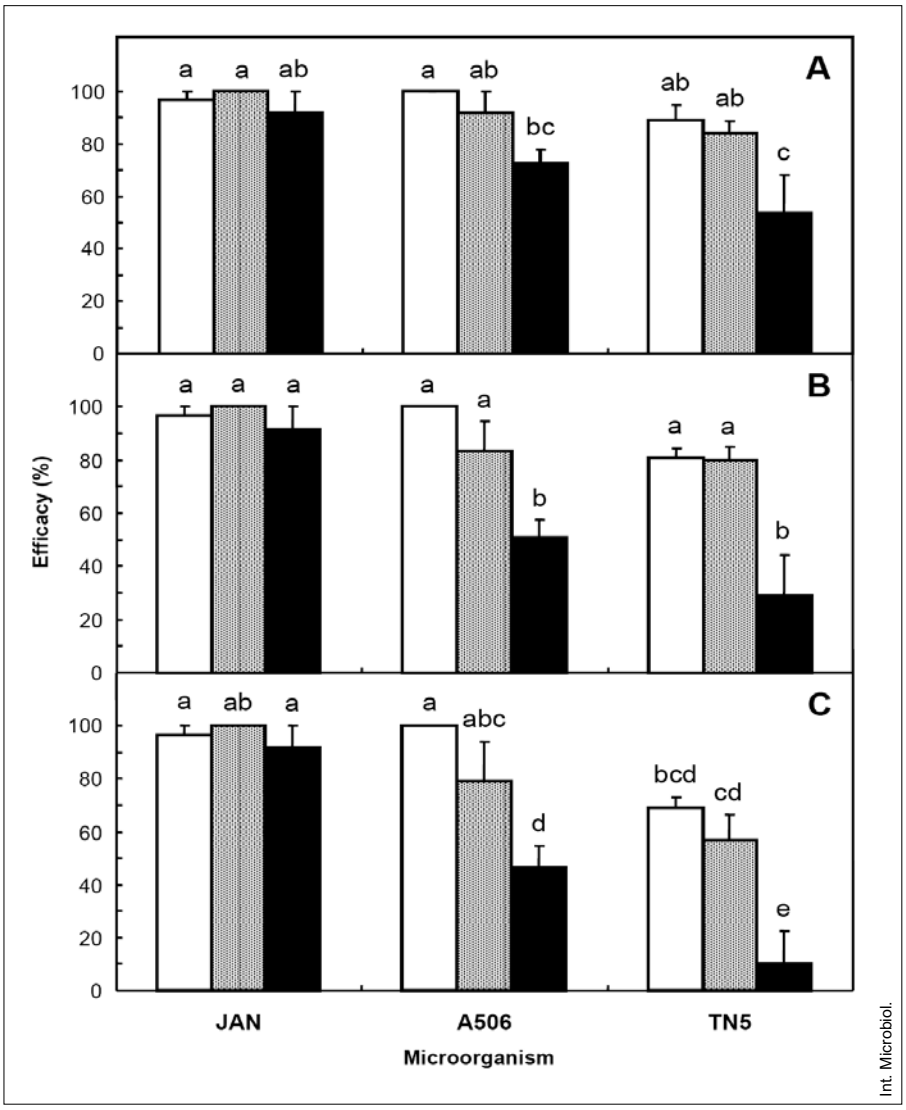


**Fig. 3.** Fire blight biocontrol on slices of immature pear fruits treated with suspensions of JAN, A506 or TN5 in 0.1 M potassium phosphate buffer at three different concentrations: $10^7$, $10^6$ and $10^5$ CFU/ml (white, grey and black histograms), before inoculation with 10 µl of a *Erwinia amylovora* suspension ($10^6$ CFU/ml). Biocontrol efficacy was evaluated after 7 (**A**), 9 (**B**) and 12 days (**C**). Values represent the means of three independent experiments, with two replicates of five slices per each treatment. Standard errors are indicated. Treatment means marked with the same letter are not significantly different according Fisher's LSD test ($\alpha = 0.05$).

nificantly, higher ($P = 0.009$, $P < 0.001$, $P = 0.008$, respectively) than after 24 h of incubation. No significantly differences were found between JAN rfm$^r$ and mutants population ($P = 0.568$) (data not shown).

In the biocontrol efficacy test on pear slices, JAN rfm$^r$ showed 79.4 ± 9.4% efficacy (average value ± standard error) in controlling *E. amylovora* compared to the untreated control (infection incidence of 95.0 ± 5.0%). The mutants M3G7 and M40E5 showed 15.0 ± 15.0% and 21.7 ± 11.7% of efficacy, respectively, values considerably lower ($P = 0.034$ and $P = 0.045$, respectively) than the efficacy value of JAN rfm$^r$.

Efficacy against *E. amylovora* on pear slices. Droplets of bacterial exudate first appeared on pear slices four to five days after they were inoculated with *E. amylovora*. After seven days (Fig. 2), the surfaces of the infected slices were almost completely covered with exudate. Twelve days after inoculation, the slice surfaces were completely black and covered with large amounts of exudate. In the untreated control, 86.7% of the slices were covered with exudate, and this percentage did not increase from the seventh day after inoculation to the end of the experiment (12 days after inoculation). When pear slices were treated with JAN, A506 or TN5 before *E. amylovora* inoculation, they were mostly asymptomatic (Fig. 2). After seven days of incubation (Fig. 3A), JAN showed more than 90% efficacy in controlling *E. amylovora*. No significant differences were found when different concentrations of JAN were compared ($10^7$, $10^6$ or $10^5$ CFU/ml). A506 and TN5 were as effective as JAN when used at the two highest concentrations ($10^7$ and $10^6$ CFU/ml). However, when used at $10^5$ CFU/ml, A506 showed a slightly lower efficacy (72.5%) than JAN (91.7%) (not statistically significant; $P = 0.072$),

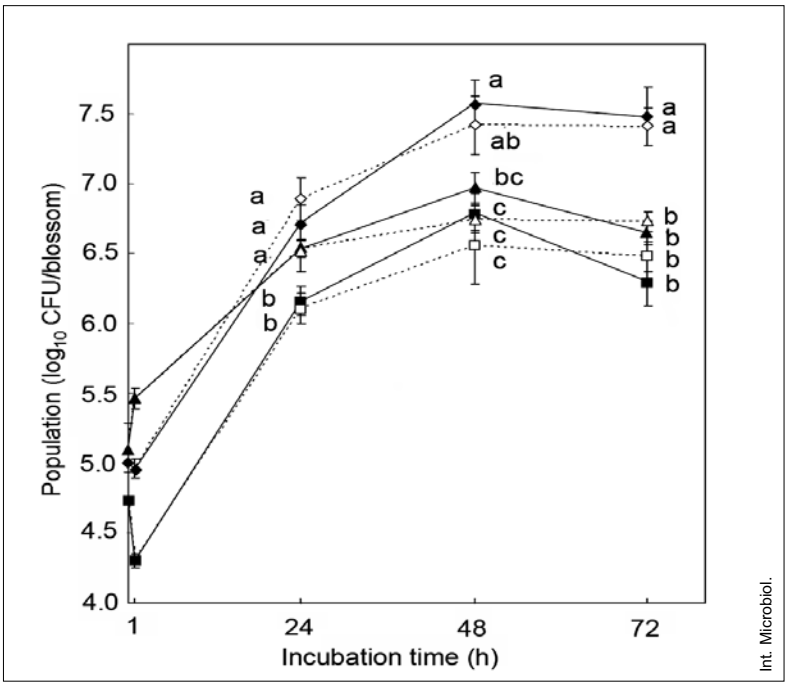


**Fig. 4.** Size of bacterial populations on apple hypanthia. Dashed lines indicate bacterial populations in the absence of *Erwinia amylovora*. Solid lines indicate bacterial populations in the presence of *E. amylovora*. Values are the means of $\log_{10}$ transformed population sizes (pooled data from two experiments, each experiment included five replicates per sampling time). Vertical bars represent standard errors. Means at 24, 48 and 72 h that are marked with the same letter are not different according Fisher's LSD test ($\alpha = 0.05$). Symbols: A506 + *E. amylovora* (closed diamonds); A506 (open diamonds); JAN + *E. amylovora* (closed triangles); JAN (open triangles); TN5 + E. *amylovora* (closed squares); TN5 (open squares).

whereas TN5 showed a statistically lower efficacy (53.3%) ($P = 0.001$). The efficacy of JAN did not vary between 7, 9 (Fig. 3B) and 12 days of incubation (Fig. 3C), indicating persistent biocontrol activity over this period. A506 was consistently effective when applied at the two highest concentrations (100% and 79.2% after 12 days, respectively), whereas a significant reduction ($P = 0.031$) in efficacy was observed after 12 days when A506 was applied at a concentration of $10^5$ CFU/ml (46.7%). The efficacy of TN5 decreased over time for all three of the concentrations tested. After 12 days, the efficacy scores for TN5 were 69.2, 56.7 and 10% at $10^7$, $10^6$ and $10^5$ CFU/ml, respectively. These efficacy rates are significantly lower than those of JAN applied at the same concentrations ($P = 0.032$, $P = 0.002$, $P < 0.001$, respectively).

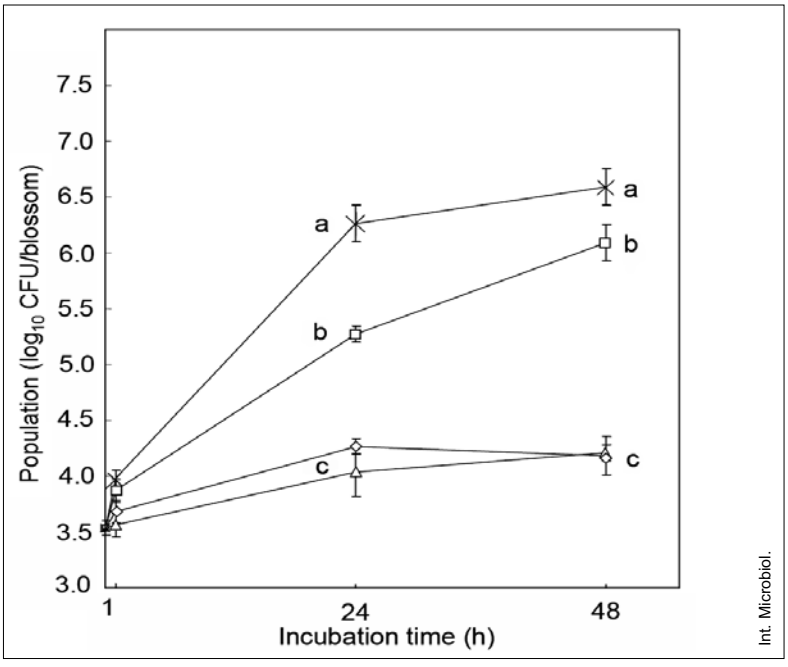


**Fig. 5.** Size of *Erwinia amylovora* populations on apple hypanthia pre-treated with JAN (open triangles), A506 (open diamonds), and TN5 (open squares). Untreated blossoms (inoculated with 0.1 M potassium phosphate buffer only) were used as a control (stars). Values are the means of the $\log_{10}$-transformed population sizes (pooled data from two experiments, each experiment included five replicates per sampling time). Vertical bars represent standard errors. Means at 24 and 48 h that are marked with the same letter are not different according Fisher's LSD test ($\alpha = 0.05$).

Efficacy against *E. amylovora* on apple hypanthia. The presence of *E. amylovora* did not significantly affect the sizes of the populations of JAN ($P = 0.505$), A506 ($P = 0.770$) and TN5 ($P = 0.356$). TN5 population was characterized by a significant reduction in population after 1 h of incubation ($2.0 \times 10^4$ CFU/blossom), corresponding to about 1 log unit less than JAN ($2.9 \times 10^5$ CFU/blossom) and 0.5 log unit less than A506 population ($9.1 \times 10^4$ CFU/blossom). JAN, TN5 and A506 populations increased exponentially over the first 24 h, however TN5 population was still 0.5 log unit lower than JAN and A506 population. Maximal population sizes were reached after 48 h. JAN and TN5 populations were similar (5.5 and $3.6 \times 10^6$ CFU/blossom, respectively; $P = 0.545$), whereas A506 population ($2.6 \times 10^7$ CFU/blossom) was significantly higher ($P = 0.029$ and $P = 0.007$, respectively) (Fig. 4).

On the untreated blossoms, *E. amylovora* population increased rapidly, reaching $1.8 \times 10^6$ and $3.9 \times 10^6$ CFU/blossom at 24 and 48 h after inoculation, respectively. JAN inhibited *E. amylovora* growth on apple hypanthia similarly ($P = 0.598$) to A506. On both cases, *E. amylovora* populations increased slightly on the treated blossoms, from $3.5 \times 10^3$ at the time of inoculation to about $1.5 \times 10^4$ CFU/blossom at 48 h after inoculation, corresponding to an efficacy of control of 99.73 ± 0.01% for JAN and of 99.60 ± 0.16% for A506, respectively. TN5 suppressed *E. amylovora* growth at 24 h ($1.9 \times 10^5$ CFU/blossom; $P < 0.001$) and at 48 h after inoculation ($1.2 \times 10^6$ CFU/blossom; $P = 0.024$), but did it less effectively ($P < 0.001$) than JAN and A506 (Fig. 5). Forty-eight hours after inoculation, TN5 showed an efficacy of control of 38.05 ± 12.46%. This is in accordance with the statistical lower growth of TN5 compared to JAN on DF agar medium added with 2 mM of NiAc or $NiNH_2$ (Table S2).

Efficacy against *E. amylovora* on apple blossoms and shoots. In experiment 1, JAN provided about 84.4% of control on blossoms and 85.3% on shoots; levels slightly higher, but not statistically different ($P = 0.112$ and $P = 0.139$, respectively), than that provided by A506 (77.9 and 73.0%, respectively).

**Table 1** Fire blight control provided by the biocontrol agent candidate *Pseudomonas rhizosphaerae* JAN and a reference strain (*P. fluorescens* A506) in apple plants under controlled greenhouse conditions.

| Strain | Biocontrol efficacy on blossoms (%)[b] | Biocontrol efficacy on shoots (%)[b] |
|---|---|---|
| Expt. 1[a] | | |
| Untreated | 0.0 ± 0.0 a[c] (68 – 366)[d] | 0.0 ± 0.0 a (30 – 81)[d] |
| A506 | 77.7 ± 3.7 b (446 – 544) | 73.0 ± 7.7 b (118 – 140) |
| JAN | 84.4 ± 2.4 b (356 – 411) | 85.3 ± 4.4 b (63 – 69) |
| Expt. 2[a] | | |
| Untreated | 0.0 ± 0.0 a (171 – 398) | 0.0 ± 0.0 a (20 – 93) |
| A506 | 20.4 ± 7.3 b (119 – 228) | 44.6 ± 10.1 b (45 – 77) |
| JAN | 72.0 ± 4.9 c (372 – 445) | 76.3 ± 1.4 c (91 – 111) |
| Expt. 3[a] | | |
| Untreated | 0.0 ± 0.0 a (72 – 270) | 0.0 ± 0.0 a (15 – 65) |
| A506 | 42.2 ± 3.2 b (144 – 256) | 39.9 ± 13.6 ab (32 – 60) |
| JAN | 63.8 ± 4.9 c (321 – 446) | 63.4 ± 11.5 b (74 – 99) |

[a] Three independent experiments (Expt.) were performed, each including three replicates (one tree = one replicate) per treatment.
[b] For each experiment, values are expressed as average ± standard error of the biocontrol efficacies calculated for each replicate. Disease symptoms on blossoms were evaluated 9 days after inoculation. Disease symptoms on shoots were evaluated 14 days after inoculation.
[c] Data with the same letter are not significantly different according to Fisher's LSD test ($\alpha = 0.05$).
[d] In brackets are indicated the healthy and the total number of blossoms or shoots assessed per treatment.

In experiment 2, JAN provided a higher level of disease control than A506 on blossoms (72.0 and 20.4%, respectively; $P = 0.001$) and on shoots (76.3% and 44.6%, respectively; $P = 0.003$). In the experiment 3, JAN provided a higher level of disease control than A506 on blossoms (63.8 and 42.2%, respectively; $P = 0.020$) and a higher, but not statistically different ($P = 0.206$), control on shoots (63.4 and 39.9%, respectively) (Table 1).

## Discussion

Selection of potential biocontrol agents against *E. amylovora* has been based on screening for inhibition on artificial media [1,23,36], which lead to biocontrol agents able to produce antimicrobial compounds and therefore with a higher risk of non-target effects, or massive screening on immature pear fruits or apple blossoms [4,25], which is time-consuming and subject to plant material availability. We developed an approach to streamline the selection process of a potential biocontrol agent characterized by a lower risk of non-target effects by pre-screening for microorganisms that can degrade an essential nutrient for pathogen growth.

*Erwinia amylovora* requires NiAc and/or $NiNH_2$ as essential growth factors when cultured on minimal media [21,29]. Based on this particular requirement, we developed a new, streamlined approach for evaluating isolates based on the hypothesis that an organism capable of eliminating or reducing the availability of NiAc and $NiNH_2$ could effectively inhibit the growth of *E. amylovora*, thereby controlling fire blight. By focusing only on those microorganisms capable of degrading NiAc, we were able to dramatically decrease the number of microorganisms to be further tested (approximately 10% of the about 2000 isolates initially collected). Among these, we selected those NiAc-degrading isolates that showed faster growth, thus reducing the field of candidates to be tested on immature pear fruits to only twelve isolates. All these isolates showed ability to degrade also $NiNH_2$. JAN, which showed the highest growth on DF-NiAc and the highest levels of biocontrol efficacy, was chosen as the final biocontrol agent candidate.

JAN was found to have high 16S rRNA gene sequence homology to *P. rhizosphaerae* IH5. This species was originally isolated from the rhizosphere of grasses growing wild in Spain [22] and selected for its capacity to actively solubilize phosphates in vitro. Krimm et al. [12] showed that *P. rhizosphaerae* is one of the prominent epiphytic bacteria of the phyllosphere of strawberry plants, where it significantly increases the permeability of the plant's cuticle. Our results indicate that *P. rhizosphaerae* is also an epiphytic inhabitant of apple blossoms.

JAN mutants M3G7 and M40E5, with impaired capacity to degrade NiAc and $NiNH_2$ as the only N-source, showed a strong reduction in the biocontrol activity compared to JAN. M3G7 and M40E5 were disrupted in *ntrC* and *ntrB* genes, respectively. NtrBC is a global two-component regulatory system controlling, together with the alternative sigma factor $\sigma^{54}$, the *ntr* (nitrogen regulated) response in many bacteria [17]. In many bacteria, the induction of the operons involved in the use of different N-sources requires activation by NtrBC [3,13,16]. These results provided an additional indication of the possible involvement of NiAc and $NiNH_2$ degradation in the control of *E. amylovora* by JAN, and demonstrated that the capacity by JAN to degrade NiAc and $NiNH_2$ as the only N-source and its biocontrol activity against *E. amylovora* is regulated by the NtrBC regulatory system.

Biocontrol efficacy of JAN was evaluated in vitro and compared with the efficacy of *P. fluorescens* A506, a commercial biocontrol agent often used as reference strain in greenhouse and in field experiments aiming to test new biocontrol agents [28] and with the nicotinic acid degrader *P. fluorescens* TN5. The efficacy of JAN on pear slices and on apple hypanthia, could compare with the efficacy of A506. On the contrary, TN5 showed lower efficacy than JAN on pear slices and especially on apple hypanthia. In the last case, the lower efficacy could be explained by the fact that TN5 showed a lower capacity to use NiAc and $NiNH_2$ than JAN did (Table S2). Moreover, TN5 population size on apple hypanthia was significantly lower than JAN population size for the 24 h of incubation prior *E. amylovora* inoculation. One hour after its inoculation on the blossoms, the population of TN5 was about 1 log-unit (i.e. $2.7 \times 10^5$ CFU/blossom) lower than the population of JAN. Twenty-four hours after its inoculation, corresponding at the moment of *E. amylovora* inoculation, TN5 population was about 0.5 log-unit (i.e., $2.0 \times 10^6$ CFU/blossom) lower than JAN population. This difference in population could be therefore linked to a minor degradation of NiAc and $NiNH_2$ and therefore a reduced control efficacy compared to JAN. Unlike JAN and A506, which were isolated from apple hypanthium and pear leaf, respectively, TN5 was isolated from a soil sample [19]. Its initial smaller population could be therefore related to a lower capacity of TN5 to adapt to the conditions present on the hypanthium.

When tested in greenhouse directly on blooming apple trees, JAN confirmed the high level of control showed in vitro and was consistent in controlling the disease across all the three experiments, whereas A506 showed a reduction in efficacy in experiments 2 and 3, which were characterized by some days at low temperature. A temperature of 18 ± 6°C corresponds to the common temperatures registered in Switzerland and northern Italy during pear and apple blooms

[http://www.meteosvizzera.admin.ch/web/it/clima/clima_ogi/html; http://meteo.iasma.it/meteo/datimeteo/ricercadati.php]. The promising biocontrol activity of JAN against *E. amylovora* under these conditions suggests the potential of this isolate as biocontrol agent against fire blight in these regions. However, only repeated trials in different regions, under different environmental conditions, will provide the final confirmation of the efficacy of JAN as biocontrol agent against fire blight. A commercial biocontrol agent should not pose any risk to human health [26]. In this sense, valuable characteristics of JAN are the inability to grow at human body temperature (data not shown) and the fact that production of antimicrobial compounds was not detected.

In conclusion, with this streamline screening approach based on NiAc degradation, we were able to select a new effective biocontrol agent candidate (JAN) characterized by a lower risk of non-target effects, whose mechanism of protection is linked to the degradation of a specific and essential growth factor of the pathogen.

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**Supplementary Table S1.** Growth on DF salt minimal agar medium added with 2 mM of NiAc as the only N-source and biocontrol efficacy of JAN and other NiAc degraders

| Strain | Diameter NiAc (mm)[a] | Biocontrol efficacy (%)[b] |
|---|---|---|
| JAN | 5.2 | 100.0 ± 0.0 |
| TSA3 E6 | 5.2 | 95.8 ± 4.2 |
| LB4 F7 | 4.6 | 92.5 ± 3.8 |
| LB4 H8 | 4.5 | 100.0 ± 0.0 |
| PDA SM A6 | 4.3 | 100.0 ± 0.0 |
| NONESI A3 | 4.2 | 95.8 ± 4.2 |
| JAN1 D6 | 4.0 | 96.7 ± 3.3 |
| SM-3 H4 | 4.0 | 70.8 ± 5.1 |
| SM-3 E2 | 3.8 | 95.8 ± 4.2 |
| TSA3 D5 | 3.8 | 100.0 ± 0.0 |
| LB4 E5 | 3.8 | 90.0 ± 5.7 |
| JAN2 H7 | 3.6 | 85.8 ± 8.7 |

[a]Diameter of the colony after 3 days at 18°C.

[b]Biocontrol efficacy on pear slices after 12 days at 18°C. Values represent the means of three independent experiments, with two replicates of five slices per each treatment. Standard errors are indicated.

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**Supplementary Table S2.** Growth of JAN, JAN rfm$^r$, the JAN mutants M3G7 and M40E5, and TN5 on DF salt minimal agar medium added with 2 mM of NiAc, $NiNH_2$, or $NH_4Cl$ as the only N-source. DF salt minimal agar medium not amended was used as control

| Strains | NiAc | $NiNH_2$ | $NH_4Cl$ | CONTROL |
|---|---|---|---|---|
| JAN | 0.644 ± 0.040 a* | 0.253 ± 0.042 ab* | 0.418 ± 0.011 a* | 0.035 ± 0.005 a* |
| JAN rfm$^r$ | 0.739 ± 0.069 a | 0.367 ± 0.049 a | 0.385 ± 0.032 a | 0.011 ± 0.001 b |
| M3G7[a] | 0.005 ± 0.000 b | 0.050 ± 0.000 c | 0.793 ± 0.035 b | 0.003 ± 0.001 b |
| M40E5[a] | 0.007 ± 0.000 b | 0.152 ± 0.052 bc | 0.799 ± 0.021 b | 0.004 ± 0.000 b |
| TN5 | 0.099 ± 0.052 c | 0.103 ± 0.028 b | 0.332 ± 0.013 a | 0.033 ± 0.017 ab |

*The values (expressed as optical density at 600 nm of a suspension obtained cutting out the single colonies and resuspending the cells in 1 ml 0.1 M potassium phosphate buffer by vigourous vortexing for 20 s) are the average of two independent measurements. Standard errors are indicated. Values within a column followed by the same letter are not significantly different according to Fisher's LSD test ($\alpha = 0.05$).

[a]M3G7 and M40E5 are rifampicin resistant.