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ORIGINAL ARTICLE

Isolation and identification of an actinomycete strain with a biocontrol effect on the phytopathogenic *Erwinia chrysanthemi* 3937VIII responsible for soft rot disease

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Abstract *Erwinia chrysanthemi* is a phytopathogenic bacterium causing soft rot disease in several agricultural products. Conventional techniques used in the control of this phytopathology have serious limitations due to the emergence of resistant strains and the undesirable effect on the environment of chemical treatments. In this work, we report the isolation of an actinomycete strain from a Moroccan biotope that inhibits the growth of *Erwinia chrysanthemi* 3937VIII. PCR amplification and DNA sequencing of the 16 S ribosomal RNA gene allowed the identification of this strain as *Streptomyces cinereoruber*. The concentrated culture supernatant of this actinomycete strain exhibited activity against the growth of *Erwinia chrysanthemi* 3937VIII and two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus amylolitiquefaciens*, but had no effect on other Gram-negative bacteria tested (*Erwinia carotovora* 197*stp*^{R}, *Escherichia coli* TG1 and *Pseudomonas* sp.), suggesting that this actinomycete strain secretes into the extracellular medium a substance that inhibits selectively the growth of other bacteria, especially the phytopathogenic *Erwinia chrysanthemi* 3937VIII. The antibacterial activity of *Streptomyces cinereoruber* found in this study highlights the importance of actinomycetes strains as candidates for the biological control of pathogenic bacteria. The identification and characterization of the active substance would open the way for further technological and therapeutic investigations.

Keywords *Streptomyces* · Antibacterial activity · 16S rRNA gene · Phytopathogenic bacteria · *Erwinia*

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Introduction

Erwinia is a pathogenic enterobacterium that is able to cause soft rot diseases in a wide range of economically important crops. Pathogenicity of these bacteria is a complex process involving several factors whose production is subject to temporal regulation during infection, including pectinases and endoglucanases, which work in concert to degrade plant cell walls (Hommais et al. 2008). Phytopathogenicity of *Erwinia* affects storage organs and succulent leaves of vegetables (potato, tomato, onion and crucifers), field crops (corn, rice and sugar beet) and ornamental plants (begonia, dieffenbachia, dahlia and philodendron).

Erwinia chrysanthemi is known as a greenhouse pathogen in mild climate regions (Perombelon and Kelman 1980). The disease appears first on the roots of some plants, probably via seed- or soil-borne infection, and then spreads to other plants by cultural practices (Aysan 2001).

Worldwide, potato has a high-economic value and, consequently, potato-producing areas are very important.

However, this crop culture is often compromised due to infections by phytopathogenic agents like *E. chrysanthemi* and *Erwinia carotovora*—the most common cause of potato soft rot.

In Morocco, an investigation carried out on the principal bacterial diseases associated with the culture of potato in the areas of Meknes, El Hajeb and Larache allowed the isolation of 500 different strains of *Erwinia* responsible for soft rot and black leg; 101 of these strains appeared pathogenic. Identification of these strains based on the pectinolytic activity showed two profiles: *E. carotovora* and *E. chrysanthemi* (INRA 2008).

Conventional chemical products, such as copper, may not provide adequate control of *Erwinia* infection because the bacteria colonize the intercellular spaces of leaves and progress intercellularly to invade other aerial parts of the plant.

To control this phytopathology, considerable efforts has been focused on finding biological methods leading to the inhibition of bacterial proliferation and disease development. Biocontrol techniques using fluorescent *Pseudomonas* strains have already been used against *Erwinia*-related disease (Geels and Schippers 1983; Colyer and Mound 1984; Altin and Bora 2001). Other Gram-negative and Gram-positive bacteria isolated from the potato rhizosphere have shown antagonist activity against *Erwinia* species, and have been suggested as biocontrol agents for soft rot disease. Several groups have shown activity of bacteria such as *Pseudomonas fluorescens*, *Bacillus subtilis* and *Erwinia herbicola* Eh252 against *Erwinia carotovora* subsp. *carotovora* (Tanii et al. 1990; Chard et al. 1991; Vanneste et al. 1992; Vanneste and Cornish 1995). Moreover, Ryu et al. (2003, 2004) found that volatile substances (2,3-butanediol and acetoin) from *Bacillus amyloliquefaciens* isolate IN937 and *B. subtilis* isolate GB03 could promote growth and induce a systemic resistance response in *Arabidopsis thaliana* against *E. carotovora* subsp. *carotovora*.

Actinomycetes are distributed extensively in soils and are able to provide many important bioactive compounds of high commercial value and medical interest (Takizawa et al. 1993). In fact, actinomycetes are an exceptionally prolific source of secondary metabolites, accounting for more than half of all microbial antibiotics discovered to date (Berdy 2005; Boudjella et al. 2006). Remarkably, the vast majority of these compounds are derived from the single actinomycete genus *Streptomyces*, raising the intriguing possibility that additional chemically prolific taxa still await discovery (Demain 2000; Berdy 2005). Worldwide, actinomycetes are used as biocontrol agents against various plant pathogens (Ouhdouch et al. 2001; Sabaratnam and Traquair 2002; Chung et al. 2005). On the other hand, actinomycetes exhibit interesting activities against human pathogens and have also been used in the control of tumor proliferation (Zheng et al. 2000; Shipley et al. 2009).

In this study, we were interested in isolating, from a Moroccan biotope, actinomycetes that are able to inhibit the growth of the *E. chrysanthemi* responsible for soft rot, and to investigate some biological properties of their bioactive substances for future use in biological control.

Materials and methods

Study site and sampling

The study area was located in the valley of Fez, Morocco. The field position is 4° 54′ W and 34° 6′ N. The weather is typically Mediterranean, semi-arid to arid, with an average rainfall of 400 mm per year and an average annual temperature of 18–20°C. Soil samples were taken from the 10-cm upper layer at four random locations. Soil samples were preserved in ice-boxes and were processed within 12 h.

Media and strains

The casein starch agar (CSA) medium used for actinomycete isolation contained soluble starch 10 g, casein 0.3 g, KNO_3 2 g, NaCl 2 g, K_2HPO_4 2 g, $MgSO_4{\cdot}7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, agar 18 g and distilled water to 1 l (pH 7.2; Shahidi Bonjar and Aghighi 2005). The antimicrobial activity of the isolated actinomycetes was determined using Bennett's agar medium containing yeast extract 1 g, beef extract 1 g, casamino acids 2 g, glucose 10 g, agar 15 g and distilled water to 1 l (pH 7.3; Kitouni et al. 2005). The antibacterial activity of the concentrated culture supernatant was determined using Muller-Hinton (MH) medium containing beef infusion 2 g, acid hydrolysate of casein 17.5 g, soluble starch 1.5 g, agar 17 g, distilled water to 1 l (pH 7.4).

The bacterial strains tested (listed in Table 1) were cultivated on Luria Bertani (LB) medium (peptone 10 g/l; yeast extract 5 g/l; sodium chloride 10 g/l) at 30°C for *Erwinia* and 37°C for other bacteria.

Isolation of actinomycetes

Isolation of actinomycetes from soil was conducted using the spread plate method described by Labeda and Shearer (1990). Soil samples were pre-treated by incubation at 50°C for 10 min. A 5 g amount of each soil sample were suspended in 100 ml sterile physiologic water (0.85% NaCl in distilled water) containing 100 µl Tween 20. After incubation for 30 min under agitation, serial 10-fold

Table 1 Bacterial strains used in this study

Bacterial strain	Source
Erwinia chrysanthemi 3937VIII *Erwinia carotovora* 197*stp*^{R} *Escherichia coli* TG1	CNRS-LCB (Marseille, France)
Pseudomonas sp. *Staphylococcus aureus* *Bacillus amylolitiquefaciens* MEB10	Our laboratory collection

dilutions of the samples were prepared. Dilutions were then plated on CSA and incubated for 7 days at 28°C.

Purification and conservation of actinomycetes isolates

Actinomycete isolates were recognized by their morphological aspects and by microscopic observation. Isolates were purified by successive streaking on CSA medium and maintained at 4°C for less than 1 month. Alternatively, cultures were resuspended in 20% sterile skim milk and frozen at −80°C.

Antimicrobial activity

Antibacterial activity testing was carried out according to the protocol described by Ouhdouch et al. (2001). Pure actinomycete isolates were spotted on Bennett's agar medium. After incubation at 30°C for 6 days, the plates were exposed to chloroform vapor for 40 min. Emergent colonies were then covered with a 0.6% agar layer of Bennett's medium, previously seeded with one of the test bacteria (10^4–10^5 CFU/ml), and incubated for 24 h at 30°C.

Antimicrobial activity was evaluated by measuring the inhibition halo. Each test was carried out in triplicate. For the negative control, the tested bacteria were added to Bennett's agar medium without inoculation with actinomycetes.

Antibacterial activity of concentrated culture supernatant

The isolate was inoculated on fresh Bennett's medium and incubated under moderate shaking (120 rpm) at 30°C. Aliquots of 20 ml culture were sampled aseptically after 3, 5, 6 and 7 days. After centrifugation at 8,000 *g* for 5 min at 4°C, the culture supernatant was recovered and concentrated using a rotavapor at 60°C. The remaining solution was then suspended in 1 ml sterile distilled water and stored at 4°C.

Approximately, 10^4 CFU/ml of the tested bacteria were spread on MH agar medium. Antibacterial activity was tested using the saturated paper disc technique. The paper disc is first saturated with the solution already obtained and then deposited on the dish. An inhibition halo was determined after incubation at 30°C for 24 h. Each test was repeated three times. For the negative control, paper discs were saturated with distilled water.

DNA extraction

The actinomycete isolate was grown in 10 ml Bennett's medium under shaking at 30°C for 48 h. A volume of 4 ml culture was centrifuged for 2 min at 7,500 *g*. The pellet was then washed once with TE buffer (pH 8) (Tris-HCl 10 mM, EDTA 1 mM) and resuspended in 500 μl TE buffer. After that, the sample was heated in boiling water for 10 min, cooled in an ice bath for 5 min and centrifuged at 7,500 *g* for 3 min. The supernatant (300 μl) was recovered, transferred to a new tube and stored at 4°C until use (Cook and Meyer 2003).

PCR amplification

The universal primers, fD1 (5′-AGAGTTTGATCCTGGC TCAG-3′) and Rs16 (5′-TACGGCTACCTTGTTA CGACTT-3′) were used to amplify 16S rDNA (Weisburg et al., 1991). The amplification reaction was performed in a final volume of 20 μl containing 0.5 μM of each primer, 200 μM each dNTP, 0.2 units *Taq* DNA polymerase, 1.5 mM $MgCl_2$ and 2 μl DNA sample in 1x *Taq* polymerase buffer. The amplification was performed on a thermal cycler (Techne Genius, Cambridge, UK). The mixture was first denatured at 94°C for 5 min. Then, 35 cycles of PCR were performed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 30 s. At the end of the last cycle, the mixture was incubated at 72°C for 10 min and then cooled to 4°C. For each reaction, a negative control lacking DNA template was included. PCR products of the expected size were revealed on 1% agarose gels containing ethidium bromide (10 μg/ml).

Molecular identification of the actinomycete isolate

To identify the bacterial isolate, a molecular approach based on the amplification and sequencing of the 16 S rRNA gene was used. Sequencing was carried out at CURI center (Centre Universitaire Régional d'Interface, Fès, Morocco),

using an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR products were purified using Magnesil yellow solution (Promega, Madison, WI) to eliminate the primers used for PCR reactions, then the sequencing reaction was performed in a thermocycler in a final volume of 20 µl containing 20 pmol fD1 primer, 3 µl Big Dye (version 3.1) and 2 µl purified PCR product. Twenty-five cycles were performed: denaturation at 96°C for 10 s, primer annealing at 55°C for 10 s, and extension at 60°C for 4 min. To eliminate excess labelled ddNTPs, sequencing reaction products were purified using the above-mentioned Magnesil green solution.

Direct sequencing of amplified PCR products was performed on an ABI PRISM sequencing apparatus (ABI Prism® 3130 Genetic Analyzer (Applied Biosystems) and data analysis was carried out using sequencing analysis software.

Results and discussion

Isolation and antibacterial activity of actinomycetes

In this study, we were interested in isolating actinomycetes from a biotope in the area of Fez, Morocco, to test their antimicrobial activity against the *E. chrysanthemi* 3937VIII responsible for soft rot disease.

Several studies have successfully used antagonistic microorganisms, especially actinomycetes and yeasts, to control plant bacterial diseases (Alivizatos and Pantazis 1992; Ozaktan et al. 1999). To our knowledge, this is the first time that the potential use of actinomycetes isolated from a Moroccan biotope as biocontrol agent for controlling *E. chrysanthemi* has been evaluated.

A total of 50 isolates were isolated in CSA medium from different soils. Antibacterial activity was evaluated by measuring the inhibition halo around the colonies. An example of an inhibition halo is shown in Fig. 1. Among the isolates, 13 (26%) displayed remarkable antibacterial activities against *E. chrysanthemi* 3937VIII, with inhibition halos ranging from 5 to 37 mm (Table 2).

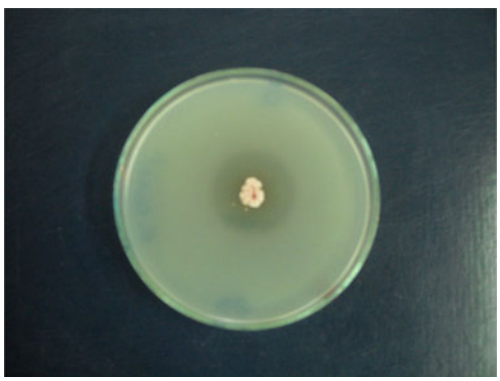

Fig. 1 Example of the inhibition halo of an actinomycete against *Erwinia chrysanthemi* 3937VIII in Bennett's agar medium

Table 2 Antibacterial activity of actinomycete isolates active against *Erwinia chrysanthemi* 3937VIII in Bennett's agar medium. All values are means of three replications with standard deviation

Active actinomycete isolates	Inhibition halo (∅, mm)[a]
1	05±0.5[a]
2	28±1
11	27±1.73
15	27±2.08
21	33±3
24	30±2
32	27±1.52
36	30±2.64
37	27±2.51
41	15±1.52
44	37±3.05
45	21±2
47	28±2.51

[a] ∅ inhibition halo − ∅ colony

Of the 13 active isolates, only isolate, 45, showed both a large inhibition halo and rapid growth on CSA medium at 30°C. As our aim was biocontrol of *E. chrysanthemi* 3937VIII-related disease, this strain was retained for further investigations in this study.

Identification of bacterial isolate 45

As reported in the literature, PCR amplification of the 16 S rRNA gene with the fD1 and Rs16 primers amplifies a

Table 3 Antibacterial activity of concentrated culture supernatant of *Streptomyces cinereoruber* isolate taken in different times. All values are means of three replicates from two experiments with standard deviation. *NC* Negative control

	Inhibition halo (∅, mm)				
	NC (H_2O)	3rd day	5th day	6th day	7th day
Erwinia chrysanthemi 3937VIII	-[a]	-	-	6±1	6±0.6
Erwinia carotovora 197stp^R	-	-	-	-	-
Escherichia coli TG1	-	-	-	-	-
Pseudomonas sp.	-	-	-	-	-
Staphylococcus aureus	-	-	8±0.6	9±1	9±1
Bacillus amylolitiquefaciens MEB10	-	5±0.6	6±1	7±0.6	9±1

[a] No effect

DNA fragment of approximately 1.5 kbp (Weisburg et al. 1991). The sequences obtained with the fD1 primer were 654 bp in length. It has been reported that the initial 500 bp sequence provides adequate differentiation for bacterial identification (Clarridge 2004; Cook et al. 2004).

Comparison of the 654 bp sequence to the EMBL, GenBank, DDJB and PDB databases, using the program BLAST NR 2.2.11 through the National Center for Biotechnology Information (NCBI), revealed 100% identity with *Streptomyces cinereoruber*. The 654 bp 16 S rRNA sequence was deposited with GenBank under accession number GU056836.

Antibacterial activity of concentrated culture supernatant

Actinomycetes are known to produce secondary metabolites and extra-cellular enzymes with interesting antibiosis (Tu 2008). Thus, the supernatant of the *Streptomyces* culture was tested against *E. chrysanthemi* 3937VIII and other Gram-negative and Gram-positive bacteria. Antibacterial activity was determined at different times during the culture incubation. The *Streptomyces* culture supernatant did not show any antibacterial activity. However, the concentrated culture supernatant exhibited an antibacterial effect against *E. chrysanthemi* 3937VIII (Table 3). This inhibition was obtained after 6 days of culture.

The *S. cinereoruber* concentrated culture supernatant also showed an antibacterial effect against two Gram-positive bacteria, *Staphylococcus aureus* and *B. amyloliquefaciens* MEB10, but no effect against the Gram-negative bacteria *E. carotovora* 197*stp*^{R}, *Escherichia coli* TG1 and *Pseudomonas* sp. (Table 3). It is clear that the *Streptomyces* culture supernatant contains an active molecule with specific activity against *E. chrysanthemi* 3937VIII and Gram-positive bacteria. The concentration of this active substance is very low in culture supernatant and its antibacterial effect is exhibited only after concentration of the supernatant.

Moreover, the same results were obtained after storage of the *S. cinereoruber* concentrated culture supernatant at 4°C for 1 month, demonstrating that the active molecule is stable at 4°C.

Although chemical treatment of plants gives good performance in the short term, the accumulation of chemical residues in the environment represents a risk for humans, animals and plants. Biocontrol using microorganisms is the method of choice to combat plant diseases and epidemics. This alternative method is very beneficial at both the environmental and economic levels. Numerous species of actinomycetes have been isolated, selected and used for controlling diseases and insects of plants (Fravel 2005). There is evidence that the activities of actinomycetes are based on their potential production of antibiotics (Fravel 1988; Watve et al. 2001) and/or hydrolytic enzymes (Valois et al. 1996; Singh et al. 1999).

Unfortunately, few studies have focused on identifying antagonistic agents against *E. chrysanthemi*. Aysan et al. (2003) isolated bacterial strains antagonistic to the tomato bacterial pathogen *E. chrysanthemi* in Turkey, and showed that they could be used as potential biocontrol agents.

This study highlights the potential use of *S. cinereoruber* or its secreted active molecule against *E. chrysanthemi* 3937VIII to combat potato soft rot disease.

The use of actinomycetes as replacements or supplements to chemical bactericides in agriculture has been addressed in many reports. Because of their antibacterial activity, actinomycetes, especially *Streptomyces*, adapt well to the soil and rhizosphere and, therefore, are the ideal candidate for biocontrol programs against soft rot disease and *E. chrysanthemi* 3937VIII-related diseases.

In conclusion, the use of new screening approaches to identify microorganisms from original ecosystems is essential for the discovery of novel natural substances with valuable biological activities. In our work, the use of soils from particular Moroccan biotopes enabled us to isolate a *S. cinereoruber* strain secreting an active molecule that inhibits the growth of *E. chrysanthemi* 3937VIII, which is responsible for soft root disease. We plan to further characterize this active substance and investigate the molecular mechanisms governing its antibacterial activity.

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