



Biotechnological potential of *Kocuria rhizophila* PT10 isolated from roots of *Panicum turgidum*

S. Guesmi^{1,2} · M. Mahjoubi³ · P. Pujic⁴ · A. Cherif³ · P. Normand⁴ · H. Sghaier^{2,3} · H. Boubakri⁴

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Abstract

In the present study, an analysis of antimicrobial activity; and an assessment of genomic biocontrol attributes of the recently described radioresistant strain *Kocuria rhizophila* PT10 were conducted. PT10, a Gram-positive and yellow pigmented actinobacterial strain, was isolated from the roots of xerophyte *Panicum turgidum* collected from Ksar Ghilane in the south of Tunisia and its genome was sequenced. In order to assess the potential capacity of this strain to adapt to its environment, a genomic and functional characterization of key enzymes involved in polysaccharides/protein degradation, chelation of iron and production of secondary metabolites was done and revealed an interesting potential. Precisely, Cazy, antiSMASH and BAGEL analyses of the genome showed the potential of *K. rhizophila* PT10 to synthesize specialized metabolites (bacteriocin, linocin M18, terpenes, siderophores, etc.) and enzymes (amylase, chitinase, protease, etc.). PT10 also possesses genes potentially involved in the biosynthesis of molecules with antifungal and antimicrobial activities, such as bacilysin and cycloserine. Biocontrol assays were thus done and showed an effective antagonism under in vitro conditions against phytopathogenic fungi, *Botrytis cinerea* BC21 and *Fusarium graminearum* g1, with percents of growth inhibition of 98 and 42%, respectively. Strain PT10 also showed moderate antibacterial activity against the Gram+ *Staphylococcus pasteurii* PT2 and the Gram– *Acinetobacter baumannii* PT6. These results suggest a multivariate mode of antagonistic activity of strain PT10 against microbial pathogens through the production of hydrolytic enzymes, secondary metabolites, siderophores and other antimicrobial molecules, the characterization of which is underway.

Keywords Antimicrobial activity · Genome assessment · Hydrolytic enzymes · Secondary metabolites · Siderophores

Abbreviations

AMP	Antimicrobial peptides	BGCs	Biosynthetic gene clusters
antiSMASH	Antibiotics and secondary metabolite analysis shell	OD	Optic density
BCAs	Biological control agents	TSA	Tryptic soy agar
		TSB	Tryptic soy broth
		PDA	Potato dextrose agar

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✉ S. Guesmi
guesmisihem152@gmail.com

- ¹ National Agronomy Institute of Tunisia (INAT), Avenue Charles Nicolle, 1082 Tunis, Mahrajène, Tunisia
- ² Laboratory "Energy and Matter for Development of Nuclear Sciences" (LR16CNSTN02), National Center for Nuclear Sciences and Technology (CNSTN), Sidi Thabet Technopark, 2020 Sidi Thabet, Tunisia
- ³ Univ. Manouba, ISBST, Biotechnopole Sidi Thabet, BVBGR-LR11ES312020 Ariana, Tunisia
- ⁴ CNRS, INRAE, VetAgro Sup, UMR Ecologie Microbienne, Univ Lyon, Université Claude Bernard Lyon 1, 69622 Villeurbanne, France

Introduction

African regions have a wide variety of ecosystems, marked by two rainy seasons with relatively sparse, dry and arid vegetation. The agro-diversity is large, which offers immense potential in terms of agricultural products (sorghum, wheat, etc.). This diversity also represents a potential source of antimicrobial and antifungal compounds to sustain agricultural development globally (AFDB 2019). These agro zones are highly conducive to the emergence of plant pathogens in addition to adverse environmental conditions (such as hydric stress, desiccation), which cause a large array of diseases (Agrios 2009). In order to control these damages related



to plant diseases, there is a large use of fungicides, pesticides and insecticides.

There are multiple drawbacks associated with the use of chemical products, for example the development of microbial resistance and environmental toxicity. These disadvantages have stimulated scientists to search for biomolecules derived from microorganisms, plants and animal sources for the development of biological control agents (BCAs), to be used against plant diseases (Carmona-Hernandez et al. 2019; Dukare et al. 2019; Nega 2014; Shuping and Eloff 2017). BCAs have been used as commercial deterrents to pathogens in agricultural applications, including biological pesticides and phytosanitary products (Martinez 2012). These biocontrol agents cause the inhibition of phytopathogens based on the production of secondary metabolites and hydrolytic enzymes, which cause growth inhibition, and nutritional competition by the production of siderophores and catabolism of carbon sources (Taechowisan et al. 2005; Trejo-Estrada et al. 1998).

The potential use of microorganisms in the treatment of plant fungal diseases has been associated with the antagonistic activity of microbes toward fungal pathogens. Several biocontrol strains have been considered as promising alternatives to chemical pesticides and have been commercialized as biofertilizers and BCAs (such as Gallex for *Agrobacterium radiobacter*, Kodiak for *Bacillus subtilis*) (Agrios 2005; Carmona-Hernandez et al. 2019; Sharma et al. 2009). In particular, actinobacteria represent abundant sources of antibiotics, bioactive compounds, and industrial enzymes. Actinobacterial strains residing in deserts and arid environments are invaluable and possess noteworthy gene clusters that yield specific metabolites, owing to their ability to survive and thrive under extreme conditions, such as those with limited water and nutrients (Mohammadipanah and Wink 2015; Singh and Dubey 2018). These extremophiles have been described as potent antagonists of phytopathogens owing to their high abundance, their metabolic versatility (hydrolytic enzymes, secondary metabolites), and their antimicrobial and insecticidal properties (Ganesan et al. 2017; Keikha et al. 2015; Shivilata and Tulasi 2015; Singh and Dubey 2018; Wang et al. 2013). They also have the potential to promote plant growth owing to their capacity to reduce iron and to solubilize phosphorus for their assimilation by plants (Francis et al. 2010; Palaniyandi et al. 2013; Valencia-Cantero et al. 2007). For example, an alkaliophilic strain, *Kocuria rosea* HN01, was demonstrated to reduce Fe^{3+} into the soluble form Fe^{2+} and thus make it available for plant growth (Wu et al. 2014).

The use of traditional approaches based on physiological and biochemical assays to identify and to characterize secondary metabolites remains limited and inefficient (Rutledge and Challis 2015). Recently, various effective approaches have been developed such as next generation sequencing

technologies and in silico analyses and genome mining techniques. These new technologies have provided an efficient way to rapidly mine the genetic data and discover novel biomolecules. The parallel use of in silico studies to confirm in vitro assays can lead also to a more rational choice of microbes to inhibit diseases (Chen et al. 2007; Shivilata and Tulasi 2015). However, there are a limited number of reports and genomes of endophytic actinobacteria associated with arid plants (Singh and Dubey 2018).

Thus, the genome sequence of an actinobacterium isolated from an arid region, *Kocuria rhizophila* PT10, was assessed in order to explore its biocontrol and biotechnological potential and to explain the mechanisms used to fight pathogens. This radioresistant strain was previously isolated from irradiated roots of the xerophyte *Panicum turgidum* (*Poaceae* family) collected from the oasis of Ksar Ghilane. The antimicrobial activities of strain PT10 against fungi (*B. cinerea* BC21 and *F. graminearum* g1) and bacteria (*Acinetobacter baumannii* PT6, *Bacillus subtilis* QST713, *Escherichia coli* DH5 α and *Staphylococcus pasteurii* PT2) were performed using in vitro analyses. In addition, the biocontrol-associated genes/gene clusters were identified based on bioinformatic analyses of its genome (CAZY, antiSMASH and BAGEL) and compared to the genetic potential of others *Kocuria* strain. This genomic comparison was permitted to underline the presence of one cluster predicted to produce linocin and restricted in few *Kocuria* species including *K. rhizophila*.

This work was conducted between 2018 and 2019, in the laboratory UMR5557, Ecologie Microbienne (Villeurbanne, France).

Materials and methods

Bacterial strain isolation, cultural and morphological characteristics

Strain PT10 was isolated from irradiated roots (10 kGy) of the xerophyte *P. turgidum* as described previously by Guesmi et al. (2021b). The roots were collected from Ksar Ghilane oasis (Tunisia, GPS coordinates N 32° 59.557', E 9° 36.941', 221.9 m altitude) in May 2015 (Guesmi et al. 2021a).

Morphological features of strain PT10 were assessed after growth on Tryptic soy agar (TSA) at 30 °C for 48 h using Scanning Electron Microscope (JSM 5400.JEOL, Japan). Briefly, washed bacterial cells were fixed with 2% glutaraldehyde, and dried with a graded ethanol (Sigma Aldrich) series (50, 70, 90 and 100%) (Kaewkla and Franco 2019).

For antimicrobial activity, the strain PT10 was grown in TSA medium at 30 °C for 48 h. The used test organisms were two Gram-positive bacteria: *B. subtilis* QST713 and *S.*

pasteuri PT2, and two Gram-negative bacteria: *A. baumannii* PT6 and *E. coli* DH5 α . Both bacterial strains (PT2 and PT6) were obtained from our own culture collections (Guesmi et al. 2021a, b). *E. coli* DH5 α was obtained from the culture collections of laboratory UMR5557, Ecologie Microbienne (Villeurbanne, France) and *B. subtilis* QST713 was obtained from Serenade Max (Bayer, France) which sells it as a biological control agent for agricultural purposes; it was used here as a positive control. Test and control strains were grown in Lauria Bertani (LB) broth medium for 24 h at 30 °C (PT10 and *B. subtilis*) or 37 °C (all other strains).

Phytopathogenic fungi and culture conditions

The phytopathogenic fungi used in this study are *B. cinerea* BC21 and *F. graminearum* g1, which were obtained from the culture collection of laboratory UMR5557, Ecologie Microbienne (Villeurbanne, France). The fungi were cultivated in potato dextrose agar (PDA) medium composed by 20 g dextrose, 4 g potato infusion, 15 g bacteriological agar, pH 5.6 (European Pharmacopoeia, USP). The plates were incubated at room temperature (22 °C) for 5 to 7 days.

In vitro bacteria–fungus interaction assays

The antifungal activity due to diffusible compounds was detected by performing confrontation assays using a dual culture technique of the pathogen and the test bacteria on TSA. Briefly, the bacterial strains (*K. rhizophila* PT10 and *B. subtilis* QST713) were grown overnight on liquid Tryptic soy broth (TSB). After a centrifugation to eliminate the culture medium, they were diluted with sterilized water to an OD₆₀₀ value with a range from 0.35 to 0.40.

Fifty microliters of the bacterial suspensions were streaked as a line on TSA in conventional Petri dishes 1 cm from the plate edge and incubated for 48 h at 30 °C. Mycelial disks 8 mm in diameter were cut from the target fungi colonies cultured on PDA plates for seven days and were then placed on the agar at a distance of 3 cm away from the bacterial streak. Control plates with fungi but without bacteria were prepared simultaneously. The plates were incubated at 22 °C for 5 to 7 days and examined for the inhibition of fungal growth (Khan et al. 2018). The fungal growth was measured in the control and assays in order to calculate the inhibition rate (Gasmi et al. 2019). All experiments were done in triplicates.

Antifungal activity of extracellular bacterial metabolites

The antifungal activities of the cell-free supernatant of strain PT10 culture were evaluated using the dual culture method. The antagonistic activity of extracellular bacterial

metabolites against the fungi was also examined. Bacterial cultures were grown in TSB at 30 °C for 3 days. Cells were removed by centrifugation (Avanti® J-E) at 13,000g for 30 min and the supernatant filtered through a sterile 0.22 μ m membrane (Millipore). Then, 1 mL of the resulting supernatant was supplemented to 14 mL of the PDA medium before pouring in the Petri dish (9 cm). Once the medium had cooled, disks (7 mm in diameter) of the target fungi, taken from the fresh margin of the mycelium, were spaced equally on the Petri dish. The plates were then incubated at 22 °C for 48 h. The inhibitory activity of the filtrate against fungal growth was recorded as the percent reduction in mycelium growth in comparison with that of the control plates (Zhao et al. 2010).

Growth inhibition of bacterial pathogens

Strain PT10 was inoculated into TSB medium and allowed to grow at 30 °C for 72 h until high turbidity was observed. After incubation, the broth culture was centrifuged (Eppendorf, France) twice at 6000g for 30 min and the supernatants were recovered using 0.22 μ m Millipore filters. Finally, the supernatants were mixed with the test organisms in liquid LB medium at different volumes (0, 100, 200, 300, 400 and 500 μ L) using a microplates system. The microplates were then incubated at 30 °C, and the inhibitory activity of this actinobacterium was continuously observed and recorded over 24 h. The optic density (OD) at 600 nm was measured using microplate reader TECAN (xsInfinite® 200 PRO, Germany).

The antibacterial activity of strain PT10 was assessed using a cross streak assay (Devi et al. 2013). PT10 was inoculated onto TSA plates with a single streak on the margin of Petri dishes. The plates were incubated at 30 °C for 72 h. Bacterial pathogens were streaked perpendicular to the antagonist on the agar medium. The plates were then incubated at 37 °C for 24 h. After further incubation, the antimicrobial interactions were assessed by monitoring bacterial growth (absence or presence).

Screening of fungal cell walldegrading and others beneficial enzyme activities

Fifteen to 20 μ L of an overnight bacterial culture of *K. rhizophila* PT10 was spotted onto TSA plates to test for hydrolytic enzyme activity. After 3–4 days of incubation, bacterial growth was assessed on the plates. Each experiment was repeated three times.

To detect the activity of chitin-degrading enzymes, the colloidal chitin-containing solution was prepared as described by Murthy and collaborators (Murthy and Bleakley 2012) and supplemented into the TSA medium at 10%. After incubation, clear zones around colonies were

considered evidence of chitinase activity. The experiment was repeated twice with three replicates per experiment.

Protease activity was determined by spot-inoculation of bacterial cells on skim milk agar (Naik et al. 2008; Smibert and Krieg 1994), which contains, per liter, 5 g pancreatic digest of casein, 2.5 g yeast extract, 1 g dextrose, 7% vol/vol of skim milk solution and 17 g of agar. After 3 days of incubation at 30 °C, the plates were flooded with Coomassie Brilliant Blue (0.25% w/v) dissolved in methanol: acetic acid: water (5:1:4 v/v) for 10 min to enhance the visibility of halos around the bacterial colonies.

To detect cellulase activity, a modified procedure from that of Teather and Wood (1982) was used in which 15 µL of overnight culture was spot plated on carboxymethylcellulose (CMC) agar (0.2% of NaNO₃, 0.1% of K₂HPO₄, 0.05% of MgSO₄, 0.05% of KCl, 0.2% of carboxymethylcellulose sodium salt, 0.02% of peptone and 1.7% of agar) (Chantarasiri 2014). Plates incubated at 30 °C for 48 h were flooded with Gram's iodine (2 g potassium iodide and 1 g iodine were dissolved in 300 mL distilled water) for 3 to 5 min (Kasana et al. 2008).

For pectinase activity, a pectin-containing medium was prepared as described previously by Namasivayam et al. (2011) and plates read after 5 days of incubation. The halos were detected without staining or after adding 0.05% ruthenium red (Sigma-Aldrich) for 20 min followed by several rinses with distilled water.

Amylase activity was qualitatively examined TSA medium containing 0.5% (w/v) of soluble starch and 10% (wt/vol) of NaCl. After 3 days of incubation at 30 °C, strain PT10 was tested for amylase production by flooding bacterial growth with 0.5% of Lugol solution (Sigma-Aldrich). A clear zone around the strain on a purple background was considered to represent a positive test.

For the screening for lipase activity, the agar medium contains 10 g L⁻¹ of peptone, 5 g L⁻¹ of NaCl, 0.1 g L⁻¹ of CaCl₂·2H₂O, 15 g L⁻¹ of agar and 1% (v/v) of Tween 80, at pH 7.4 (Haba et al. 2000). Following strain inoculation, the plates were incubated for 3–4 days at 30 °C. Opaque halos around the colonies were taken as the indication of lipase activity.

Genome analyses of *K. rhizophila* PT10 genetic potentials and comparative genomics

In order to determine the mechanisms implicated in the inhibition of fungi, in silico analyses of the genome of *K. rhizophila* PT10 were done. The antibiotics and secondary metabolite analysis shell (antiSMASH 6.0.0) (<https://antismash.secondarymetabolites.org#!/start>) served as a comprehensive resource for the automatic genomic identification and analysis of biosynthetic gene clusters (Blin et al. 2019). The database BAGEL4 (<http://bagel4.molgenrug.nl/>) was

used to determine and compare biosynthetic gene clusters of bacteriocins and Ribosomally synthesized and Post-translationally modified Peptides (RiPPs). Comparative genomic analysis of biosynthetic gene clusters (BGCs) was made with available genomes of several *Kocuria* strains including *K. rhizophila* DC2201, *K. flava* strain HO-9041, *K. palustris* strain M14/1 and *Bacillus subtilis* strain 168. The glycoside hydrolases were determined using the CAZY database (<http://www.cazy.org>) using several genomes of the closer strain *K. rhizophila* PT10. BLAST similarity and the presence of other genes were analyzed through the MicroScope pipeline of Genoscope (<http://www.genoscope.cns.fr/agg/microscope/mage/>). The antifungal activity of *K. rhizophila* PT10 was also analyzed in silico using BlastP (SwissProt database) and microbial genome annotation MaGe platform (SwissProt EXPASY) (Vallet et al. 2006).

Results and discussion

K. rhizophila PT10 was isolated from irradiated roots (10 kGy) of *P. turgidum* using a serial dilution method on TSA plates (Guesmi et al. 2021a). This perennial bunchgrass, belonging to family of *Poaceae*, was collected from the oasis of Ksar Ghilane. Colonies presented yellow pigmentation after cultivation on TSA for 48 h at 30 °C. They were opaque, smooth and circular with regular edges. Strain PT10 was coccoid in pairs, tetrads and larger aggregates (Guesmi et al. 2021b).

The whole genome of PT10 was previously sequenced, which showed that this novel radioresistant actinobacterium harbored several gene clusters coding for secondary metabolites and hydrolytic enzymes that can be implicated in biocontrol (Guesmi et al. 2021b). Therefore, this strain was evaluated for antimicrobial activities against fungal and bacterial pathogens and the production of hydrolytic enzymes.

Antifungal and antibacterial activities of PT10

The antifungal activity was evaluated using the dual culture method. Strain PT10 was grown for 5 days together with fungi, thus enabling to assess the release and activity of their secondary metabolites. Strain PT10 showed high activity against *B. cinerea* BC21 and moderate activity against *F. graminearum* g1 (Show supplementary data Fig. S1).

These results were confirmed by measuring the percent of inhibition of pathogens. Thus, the percent growth inhibition against *B. cinerea* BC21 was found to be $98.64 \pm 0.30\%$ and $98.92 \pm 0.12\%$ for *K. rhizophila* PT10 and *B. subtilis* QST713, respectively. Strain PT10 possessed also a moderate antifungal activity against *F. graminearum* g1 with an

inhibition zone of $42.29 \pm 0.14\%$, compared to *B. subtilis* QST713, which exhibited $62.67 \pm 0.66\%$ of suppression.

As shown in Table 1, strain PT10 exhibited significant antifungal activity against indicator fungal plant diseases in an in vitro bioassay comparable to the positive control *B. subtilis* QST713, which is used as a biocontrol agent. Moreover, the control efficiency against phytopathogens of the filtrate obtained from strain PT10 supernatant was also assessed (Table 1). The maximum percent of inhibition of the cell-free supernatant was observed toward *B. cinerea* BC21 (approximately $63.3 \pm 0.78\%$), and the minimum percent inhibition was found for *F. graminearum* g1 (approximately $39.67 \pm 0.28\%$), underlining the fact that main activity was concentrated in the secretome of PT10.

The results of in vitro bacteria–fungus interaction assays demonstrated that *K. rhizophila* PT10 has an interesting activity against phytopathogenic fungi. Indeed, PT10 exhibited a considerable activity against *F. graminearum* g1 and *B. cinerea* BC2 (Table 1). These results led us to consider *K. rhizophila* PT0 as a promising source of antimicrobial metabolites, which can be used in various potential biotechnological applications. Previous investigations were had

Table 1 Inhibition of fungi growth *F. graminearum* g1 and *B. cinerea* BC21 by PT10 and QST 713 after five days of co-culture

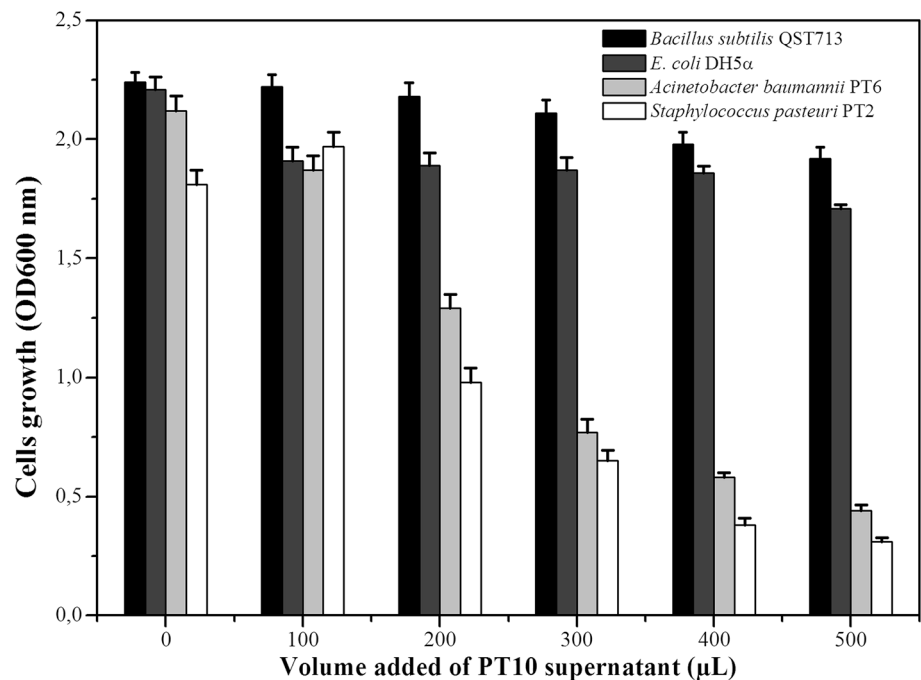
% of Inhibition	<i>F. graminearum</i> g1	<i>B. cinerea</i> BC21
<i>K. rhizophila</i> PT10	$42.29 \pm 0.14\%$	$98.64 \pm 0.30\%$
<i>B. subtilis</i> QST 713	$62.67 \pm 0.66\%$	$98.92 \pm 0.12\%$
Cell free supernatant of PT10	$39.67 \pm 0.28\%$	$63.3 \pm 0.78\%$

demonstrated that isolates belonging to *Kocuria* genus have a broad-spectrum of antimicrobial activities against pathogens, such as *Kocuria* sp. rsk4 (Kumar and Jadeja 2018), *K. palustris* (Palomo et al. 2013), *K. marina* (Uzair et al. 2018). The antimicrobial potential exhibited by these strains was attributed to their production of bioactive compounds (Bundale et al. 2019; Singh and Dubey 2018).

A second screening was done using filtered supernatant of PT10 from a liquid LB broth culture (Fig. 1). The filtrate of strain PT10 exhibited a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria. Among all the tested organisms, *A. baumannii* PT6 and *S. pasteuri* PT2 were found to be the most sensitive to the antibacterial biomolecules of PT10. As shown in Fig. 1, the growth of PT6 and PT2 at OD₆₀₀ decreased from 2 and 1.81 without PT10 supernatant (as negative control) to 0.45 and 0.57, after adding 300 μ L of supernatant, respectively. However, the growth inhibition of *B. subtilis* QST713 and *E. coli* DH5 α was negligible.

The evaluation of the antibacterial activity demonstrated that strain PT10 has the potential to inhibit the growth of pathogenic bacteria *A. baumannii* PT6 (Gram-negative) and *S. pasteuri* PT2 (Gram-positive) (Fig. 1). *K. marina* CMGS2, a halotolerant marine seaweed endophytic actinobacterium isolated from the brown seaweed *Pelvetia canaliculata*, was shown to have remarkable antimicrobial activity against two pathogens *B. subtilis* 168 (ATCC23857) and *S. aureus* (ATCC 33591–MRSA) (Uzair et al. 2018). However, there are many limits to the use of these biomolecules related to difficulties incurred during their production and identification.

Fig. 1 Growth inhibition of bacterial tests (*A. baumannii* PT6, *B. subtilis* QST713, *E. coli* DH5 α and *S. pasteuri* PT2) by cell free supernatant of strain PT10



In order to provide insights into their biotechnological potential, we analyzed *K. rhizophila* PT10 genome for its putative production of antimicrobial metabolites (secondary metabolites and hydrolytic enzymes).

Genomic insights into the antimicrobial activity of *Kocuria rhizophila* PT10

The secondary metabolites analysis for antimicrobial activities was done using a comprehensive resource for the automatic genomic identification (antiSMASH and BAGEL) that permits rapid genome survey of both bacterial and fungal strains (Medema et al. 2011; Poorinmohammad et al. 2019; Weber et al. 2015).

Secondary metabolites and siderophores putatively involved in the antimicrobial activity of PT10 were studied in our previous work using antiSMASH (Guesmi et al. 2021b). Thereby, genome mining of PT10 strain, using those complementary approaches, refined the prediction to 5 BGCs coding for putative siderophores, bacteriocin, NRPS-like, Type 3 polyketide synthase-beta lactone (T3pks) and terpene (Table 2 and Fig. S3). A similar analysis was made in *Kocuria* genomes available in NCBI database. First, the number of secondary metabolites gene clusters is relatively low compared to other actinobacteria such as *Streptomyces* or *Frankia* that harbor up to twenty gene clusters (Choudoir et al. 2018; Uduary et al. 2011). For instance, the analysis of the genome of *B. subtilis* strain 168 revealed the presence of

14 BGCs (Table S1) compared to the 5 BGCs found in PT10 (data not shown). Concerning the *Kocuria* species analyzed here, *K. flava* has the highest number of clusters with 8 biosynthetic clusters, followed by *K. rhizophila* (5–6) and then *K. palustris* (2) (Table 2, Table S1).

Interestingly, all BGCs found in *K. rhizophila* PT10 were also found in DC2201 strain with at least 82% of similarity using core genes (Table 2) underlining that *K. rhizophila* strain possesses a conserved predicted secondary metabolome. Among the 5 BGCs, only one cluster (Bacteriocin-RiPP-like) is very conserved in this genus, while the 4 other BGCs have a more scattered distribution in the *Kocuria* species used in this analysis (*K. flava* and *K. palustris*). Due to the few genomes available, a Blast research using same proteins made against different genome projects (Table S2) confirmed that the BGC annotated as Bacteriocin-RiPP-like is less present among *Kocuria* species. Indeed, in addition to *K. rhizophila*, this cluster is also present in three new *Kocuria* species when the core proteins of the other BGCs is widespread in 7 to 19 species. This BGC encodes putatively linocin M18, which is an RiPP produced by *Brevibacterium linens* as published previously (Valdes-Stauber and Scherer 1994; Motta and Brandelli 2002). The linocin M18, purified from supernatant, was shown to be an inhibitor of Gram-positive bacteria, such as *Listeria* spp, whereas no activity was found against *B. subtilis* (Valdes-Stauber and Scherer 1994). This suggests that this compound could be also produced by PT10 in its supernatant and could act negatively

Table 2 AntiSMASH and BAGEL 4 analysis for secondary metabolites and antimicrobial peptide found in PT10 genome

	<i>K. rhizophila</i> PT10		<i>K. rhizophila</i> DC2201	<i>K. flava</i> HO-9041	<i>K. palustris</i> strain MU14/1
	Most similar known cluster	From...to			
N° accession NCBI	PRJEB29453		AP009152.1	CP013254.1	NZ_CP012507.1
Type of cluster	6		6	8	2
Siderophores	Desferrioxamine B biosynthesis	KOCU_v1_20159 to KOCU_v1_20165	89 (100)	46 (95)	64 (95)
Bacteriocin-RiPP-Like	Linocin M18	KOCU_v1_120014 to KOCU_v1_120033	100 (100)	–	–
NRPS-like	A-503083 biosynthesis (antibiotic)	KOCU_v1_190019 to KOCU_v1_200014	90 (100)	65 (94)*	–
T3pks beta lactone	Arylomycin biosynthesis	KOCU_v1_200027 to KOCU_v1_210039	> 82 (100) #*	> 48 (98)*	–
Terpene	Isorenieratene biosynthesis Hopene biosynthesis Kanamycin biosynthesis Salinomycin biosynthesis Carotenoids	KOCU_v1_260003 to KOCU_v1_270002	84 (100)	–	51 (95)

*Percent of identity (coverage) of core gene present in biosynthetic proteins (BlastP). When several core genes are identified, we indicated the percent corresponding to the lower value

#This cluster is split in two biosynthetic gene clusters localized in different genomic regions



against *S. aureus* growth but its purification is required to confirm this result.

Concerning the other BGCs, the second most conserved is a NRPS-like cluster type found also in *K. flava* genome. The cluster is A-503083 biosynthesis (antibiotic), which is a capuramycin-type nucleoside antibiotic discovered using a screening aimed at identifying inhibitors of bacterial translocase I, an essential enzyme in peptidoglycan cell wall biosynthesis (Fujita et al. 2008). Capuramycin-type nucleoside and their analogs were shown to have antimycobacterial activity (Biecker et al. 2019).

The next conserved BGC is an arylomycin biosynthetic gene close to T3pks and found also in *K. flava* (Table 2). Arylomycins have a potential use as antibiotics as type I signal peptidase inhibitors (Smith and Romesberg 2012). They were identified from the culture broth of *Streptomyces* sp. HCCB10043 and were demonstrated to exhibit antibacterial activities against *Staphylococcus epidermidis* (Jin et al. 2012) and could be produced by PT10 and play an analogous function against *S. aureus* in our bioassays.

Concerning terpene BGC, there are many close clusters: isorenieratene, hopene, kanamycin, salinomycin or carotenoids biosynthesis. These biologically active compounds have been isolated from several endophytic actinobacteria, with various interesting biological activities, (including potentially antimicrobial activity (Singh and Dubey 2018) and could also code for a bioactive compound in PT10.

The genome analysis of PT10 indicated also the presence of an interesting cluster encoding a siderophore, largely conserved in all *Kocuria* species analyzed here (Table 2). This cluster contains four genes (*desA*, *desB*, *desS* and *desG*), belonging to the desferioxamine group, which were identified in *Streptomyces* genus (Cruz-Morales et al. 2017). The secretion of these low-molecular-weight metabolites by microorganisms allows taking up iron from their environments (Bakker et al. 2003).

The production of siderophores could also be considered as one of the indirect mechanisms for antimicrobial activity thus permitting to suppress diseases through competition for iron with the pathogens (Whipps 2001). For instance, the study of Medema et al. (2011) demonstrated that acinetobactin-like siderophore produced by *Acinetobacter calcoaceticus* restricted growth of *F. oxysporum*. Consequently, the production of siderophores could induce systemic resistance of plants and prevent the growth of pathogenic bacteria and fungi in iron deficient conditions (Carson et al. 1994; Storey et al. 2006) and could be produced in the secretome of PT10 to compete with fungi used in our bioassays. Strains affiliated to *Kocuria* genus are promising sources of novel antimicrobial compounds against many pathogens due to their potential to produce secondary metabolites. Among metabolites, reports on the characterization of antimicrobial peptides (or AMP)

extracted from these actinobacterial genera are available. For instance, a novel antimicrobial peptide produced by *Kocuria kristinae* (Bundale et al. 2019), a kocurin produced by *K. palustris* and *K. rosea* and active against *Staphylococcus aureus* (Martin et al. 2013; Linares-Otoya et al. 2017) were reported. In the same vein, variacin obtained from *K. varians* was found to act against a large panel of Gram-positive bacteria (Pridmore et al. 1996) and was tested in different chilled dairy food to control *Bacillus cereus* development (O'Mahony et al. 2001). The biosynthetic genes encoding the kocurin thiopeptide (accession number: WP_058858334.1) or the variacin (accession number: CAA63706.1) are totally absent in PT10 genome eliminating the hypothesis of their implication in our antimicrobial bioassay.

In addition, the comparison of genes in PT10 linked to the synthesis of secondary metabolites molecules with antifungal and antibacterial activities with those of known biocontrol strains, using the MicroScope pipeline of Genoscope, was done and showed the presence of several genes encoding secondary metabolites or proteins with a potential biocontrol or antimicrobial resistance functions (Table 3). As shown in Table 3, we found two biosynthetic genes encoding proteins (VraR and BceA) that are potentially involved in the resistance of antibiotics. In parallel, other genes are potentially involved in the biosynthesis of metabolites with biocontrol functions (*moaC*, *dcsE*, *dcsD*, *bacC*). The final metabolites of those biosynthesis pathways can be related the antimicrobial and antifungal molecules such as cyclic pyranopterin, bacilysin or cycloserine. Bacilysin has already been reported to play important roles in the pathogen suppression and the induction of systemic resistance (Chen et al. 2018; Teixeira et al. 2020; Zhang et al. 2015). Indeed, the genomic analysis of *Bacillus velezensis* revealed the presence of several BGCs, which have an important antifungal potential, especially bacilysin (Teixeira et al. 2020) and could suggested the same potential of *K. rhizophila* PT10. However, the assignation of these genes as cyclic pyranopterin, bacilysin or cycloserine is very hypothetical due to the small score found (Table 3). Finally, regarding all secondary metabolites putatively (Table 2 and Table 3), the Bacteriocin-RiPP-Like cluster remains a good candidate as antifungal compound putatively secreted by PT10 and could be tested by combining different genomic/metabolomics/proteomic approaches (Kloosterman et al. 2021) and biological bioassays.

In perspective, the genome sequencing of more *Kocuria* species coupled to metabolome/proteome investigations will permit us to define more precisely their specific genetic potentials. This would lead us to propose potential in vitro and in vivo bioassays before agro-industrial applications.



Table 3 Genes linked to antifungal and antibacterial production and resistance identified in genome of *K. rhizophila* PT10

Label	Gene	Description	Organism homologue	BlastP % identity (% coverly)	Function prediction
KOCU_v1_10274	<i>vraR</i>	Transcriptional regulatory protein	<i>Bacillus subtilis</i> 168	42 (97)	Control of the cell wall peptidoglycan biosynthesis/ antibiotic resistance
KOCU_v1_120066	<i>bceA</i>	Bacitracin ABC efflux transporter/ Macrolide export ATP-binding	<i>Sinorhizobium meliloti</i> 1021	47 (83)	Antimicrobial peptide/ Antibiotic resistance
KOCU_v1_80066	<i>moaC</i>	Molybdenum cofactor biosynthesis protein C	<i>Corynebacterium efficiens</i> YS-314	66 (90)	Cyclic pyranopterin monophosphate synthase
KOCU_v1_110039	<i>dcsE</i>	Serine acetyltransferase	<i>Bacillus licheniformis</i> DSM 13	59 (89)	Cycloserine biosynthesis
KOCU_v1_110040	<i>dcsD</i>	O-ureido-L-serine synthase Cysteine synthase	<i>Streptomyces lavendulae</i>	46 (97)	
KOCU_v1_30058	<i>bacC</i>	Dihydroantitocapsin 7-dehydrogenase	<i>Bacillus subtilis</i>	40 (95)	Bacilysin biosynthesis

Hydrolytic enzyme assays and genome assessment

The screening of extracellular hydrolytic enzymes (amylase, cellulase, chitinase, lipase, pectinase and protease) production of *K. rhizophila* PT10 was assessed by in silico analysis. The identification of bacterial antagonism was also performed by measuring the inhibition zones of mycelial radial growth in plate assays. The results of enzymatic production ability by in silico and in vitro analyses of PT10 are presented in Table 4.

The screening of hydrolytic enzyme activities of strain PT10 showed negative results for cellulase and pectinase production. These results were confirmed by in silico analyses which detected no putative genes associated with both enzymes.

For chitinase production, the absence of activity based on in vitro assay was not confirmed by the genome analysis, which detected the presence of GH23 family (KOCU_v1_10268, KOCU_v1_110006) genes as well as GH18 and GH19 family genes (Oyeleye and Normi 2018). In contrast, a unique bacterial GH23 chitinase gene was identified in *Ralstonia* sp. (Ueda et al. 2009) and structurally characterized (Arimori et al. 2013). Here, the GH23 gene affiliated to chitinase presents two catalytic domains related to lysozyme (LysZ) and peptidoglycan-binding (LysM), which were described as dormant resuscitation-promoting factors (Rpf) (Mukamolova et al. 1998). The second one (KOCU_v1_110006) is a lytic murein transglycosylase, which would be able to cleave the β -1,4 glycosidic bond between *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues of peptidoglycan (PG), suggesting its possible implication in PG biosynthesis and turnover. Thus, the antifungal activity observed in PT10 is most likely based on secondary metabolites production rather than on chitinase secretion.

Concerning proteolytic action, strain PT10 presented an efficient activity by inducing clear zones around the cells in skim milk agar medium (Fig. S4). Protease production was confirmed by the presence of 8 putative genes in the genome of PT10, which are CAAX (2), ClpS (1), ClpP (2), Htpx (1), FtsH (1) and HtrA (1). Linked to its capacity to grow under extreme conditions, *K. varians* was found to produce a thermostable alkaline protease active at high temperature (80 °C) and pH (pH > 11) (Nwagu 2014) or PT10 to resist to γ -radiation (Guesmi et al. 2021b) which are promising properties for various applications in biotechnology.

In addition, strain PT10 showed positive amylase activity by inducing clear zones around the cells in starch agar medium. In silico analysis revealed the presence of 7 putative genes attributed to the production of amylase enzyme, which belong to glycoside hydrolase family GH13. The glycogen debranching enzyme GlgX (KOCU_v1_70005) was detected, which represents a large group of enzymes that hydrolyze the glycosidic bonds between two or more carbohydrates. This bacterial glycogen synthesis and degradation gene cluster comprise amylase-type debranching enzymes with high specificity for hydrolysis of chains consisting of glucose residues (Dauvillee et al. 2005). The mechanism of glycogen breakdown can play an interesting role in the interactions between the host and pathogen (Wilson et al. 2010). This actinobacterial strain was also found to be a potent producer of lipase, which was confirmed by the presence of 4 putative genes attributed to glycoside hydrolase family (Table 4).

Overall, these results revealed that the actinobacterium strain PT10 is a potent producer of hydrolytic enzymes (amylase, chitinase, lipase and protease), which are potentially associated with its ability to inhibit pathogens growth. In addition, the capacity of hydrolytic enzymes production was attributed to the presence of putative genes belonging

Table 4 Enzymatic production ability of strain PT10

Enzymatic activity	Result	Family	Number of putative genes	Putative genes
Amylase	+++	GH13*	7	KOCU_v1_80007
				KOCU_v1_80009
				KOCU_v1_70002
				KOCU_v1_70003
				KOCU_v1_70005
				KOCU_v1_80008
				KOCU_v1_240020
Lipase	+++		4	KOCU_v1_10283
				KOCU_v1_20004
				KOCU_v1_150055
				KOCU_v1_160044
Protease	+++	CaaX	2	KOCU_v1_10276
				KOCU_v1_180025
		ClpS	1	KOCU_v1_20056
				ClpP
		Htpx	1	KOCU_v1_80104
		FtsH	1	KOCU_v1_70069
		HtrA	1	KOCU_v1_50094
		Proteasome	0	–
		Cellulase	–	GH5, GH8 GH9, GH48
–				
Chitinase	–	GH18 GH19 GH23	0 0 2	–
				–
				KOCU_v1_10268, KOCU_v1_110006
Pectinase	–	–	0	–

+ growth or positive reaction of the test strain, – no growth or negative reaction

*GH glycoside hydrolase

to the glycoside hydrolase GH family present in the genome of PT10. GH proteins play an important role in the hydrolysis of the prey's fungal cell wall with a potential to render microorganisms (bacteria or fungi) efficient biological control agents (Tzelepis et al. 2015).

The genome of strain PT10 is in permanent draft stage; it allowed us to detect genes, which code for hydrolytic enzymes as detected using CAZY database and BLAST similarity with hydrolytic enzymes found in other genomes. The screening of hydrolytic enzymes production by in silico and in vitro analyses revealed the PT10 potential to produce different extracellular hydrolytic enzymes, such as protease, amylase, lipase and chitinase (Table 4). The efficient production of hydrolytic enzymes was confirmed by the presence of several putative genes in the genome of PT10. In addition, the antimicrobial activities of PT10 may be associated with its capacity to the production of hydrolytic enzymes belonging to glycoside hydrolase proteins (GH), such as amylase and protease. However, their functions as antimicrobial

compounds are few documented, rather, these enzymes represent the largest groups of industrial enzymes (Kirk et al. 2002), which are extensively exploited commercially, in food, pharmaceutical and detergent industries.

Actinobacteria strains have a great capacity for the degradation of different and complex substrates present in their natural habitats (McCarthy and Williams 1992; Tuomela et al. 2000) indicating the variety of their complex metabolites and genomic organization (Bentley et al. 2002; Narayana et al. 2007). These antagonistic strains also showed production of fungal cell wall degrading enzymes, such as proteases, which are known to be involved in antagonistic activity against phytopathogenic fungi and insects (Dunne et al. 1997; Naik et al. 2008). Enzymatic capacities from the identified rare actinobacteria highlight their potential for the production of various hydrolytic enzymes with a promising prospect for various industrial applications.

Actinobacterial strains associated with roots of xerophytes from arid regions represent efficient sources for

various putatively products (such as secondary metabolites, siderophores and hydrolytic enzymes) with the opportunities to develop suitable and green applications in agriculture (Guesmi et al. 2021a). These biomolecules have potentialities to promote plant growth; ward off phytopathogens; and help maintain crop productivity under extreme conditions: abiotic stress, salinity, and desiccation (Singh and Dubey 2018).

Conclusion

In the present investigation, the biotechnological potentials of *K. rhizophila* PT10, was assessed based on in silico and in vitro analyses. This radioresistant actinobacterium isolated from roots of *P. turgidum* can effectively inhibit pathogens including plant pathogens. The in vitro approach revealed that PT10 played interesting antimicrobial activities against both bacterial (*A. baumannii* PT6 and *S. pasteurii* PT2) and fungi pathogens (*F. graminearum* g1 and *B. cinerea* BC21). These results were supported by in silico analyses of secondary metabolites and gene clusters identification mainly based on antiSMASH and BAGEL resources. Five biosynthetic gene clusters were detected in the genome of PT10, which were identified as terpene, siderophore, peptide (linocin M18) and PKS antibiotic (arylomycin) coding, respectively. Strain PT10 had also a great potential to the production of hydrolytic enzymes (protease, amylase, chitinase, lipase), which are implicated in the inhibition of growth of pathogens. Several genes attributed to the presence of glycoside hydrolase proteins were detected in its genome. *K. rhizophila* PT10 has the potential to use several mechanisms to biocontrol pathogens, such as the production of antimicrobial bioactive molecules and hydrolytic enzymes. Even though, the safety of this species is not documented and requires further investigation; these mechanisms make plausible the applications of this actinobacterium isolated from roots of a xerophyte as plant growth promoting rhizobacteria and biofertilizers in sustainable agriculture ecosystems through hydrolysis and assimilation of carbohydrates. Thus, the advancement in the genomic science and technology through genome sequencing and bioinformatic analysis can be expected to offer rapid means to identify potentially useful biocontrol agents.

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Data availability The whole-genome shotgun sequence of *K. rhizophila* PT10 is accessible at GenBank/ENA under accession number PRJEB29453. Strain PT10 is available from two collections, the BCCM and the DSMZ under the deposit numbers LMG 31102 and DSM 108617, respectively.

Declarations

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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