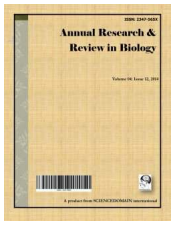

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Bacteriocins Contributing in Rhizospheric Competition among Fluorescent *Pseudomonads*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMA designed the study, wrote the protocol and interpreted the data. Authors SMA and NH anchored the field study, gathered the initial data and performed preliminary data analysis. Authors SMA, NH and MMZ managed the literature search, produced the initial draft and wrote the manuscript, while authors MMZ, JN and AG read and approved the publication. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To examine the production of bacteriocins through the study of a group of rhizospheric *Pseudomonas* isolates already known to produce metabolites that are antagonistic to fungi.
Methodology: Fourteen rhizospheric strains of fluorescent *Pseudomonads* spp., were tested as well as two referenced strains *Pseudomonas protogens* CHA0 and *Pseudomonas aureofaciens* 30-84, for their ability to produce induced bacteriocins. The induction is carried out first by UV light, and secondly by mitomycin C.
Results: In addition to the reference strains, six isolates were found to produce bactericidal substances after UV light induction against *Pseudomonas* target bacteria but also against other genera (*Escherichia* and *Staphylococcus*). Producing strains were treated with mitomycin C, and then lysed with chloroform. Analysis of the lysates by trypsin and freezing treatments, suggests that

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the active compounds are of high molecular weight.
Conclusion: It is therefore suggested that these bacteria could be good competitors for their introduction as biocontrol agents.

Keywords: Biocidal induced molecules; establishment; proteases resistance; non-pathogenic soil bacteria.

1. INTRODUCTION

Bacteria are found in environments where competition is high, such as the rhizosphere. In such environments, bacteria are in permanent competition both for nutrients and for their ecological niches [1]. Consequently they have developed several ways to tackle intra and interspecific competition; including the production of antibiotics, bacteriolytic enzymes against phytopathogenic fungi [2,3] and protein toxins [4] which are referred as bacteriocins [2,3,4].

Bacteriocins are the most numerous substances among antimicrobials. They constitute a diverse functional and structural group [5,6]. Two major groups have been described [7,8] (i) low molecular weight [9] (peptides and proteins) [10] and (ii) high molecular weight bacteriocins or HMW. HMW bacteriocins are thermolabile, trypsin resistant, and sedimentable by ultracentrifugation and can be induced by physical or chemical agents, which activate the SOS system [9].

They are produced by the majority of bacterial groups [4] and are capable of killing closely related rival bacteria without affecting the bacteriocin producing strain. Indeed, these bacteria possess a specific immunity mechanism [2,6]. Bacteriocin production has also been described in the *Halobacteriaceae* family a family of extreme halophiles within the kingdom *Archaea* [11].

To date the pyocins produced by *Pseudomonas aeruginosa* are among the best-known bacteriocins in Gram-negative bacteria. Four different types of bacteriocins termed: R- F- S- and M type pyocins have been identified [12], and differ in their morphology and mode of killing. The smaller: S-type pyocin is a colicin-like protein, soluble and protease- and heat-sensitive. R- and F-type are high-molecular-mass pyocins, resemble bacteriophage tails [13]. They contain no head structures and no DNA, and are used as defence systems [14]. Whereas the R-type pyocin resembles a contractile but non-flexible tail structure of bacteriophage [13], and the F-type a flexible but non-contractile one resistant to protease- and nuclease [15].

The mode of action of bacteriocins varies ranging from pore formation in the cell membrane, to non-specific degradation in cell DNA, cleavage of RNAr 16S or tRNA, or inhibition of peptidoglycan synthesis resulting in cell death [16]. To this extent, colicins and S-type pyocins target specific outer-membrane receptors on the target cells with a narrow spectrum of activity [6,12,16].

It has been suggested that bacteriocins can play a key role in bacterial population dynamics [17]. These substances can be used in biological control due to their specific bactericidal properties against susceptible bacterial pathogens [18].

With respect to *Pseudomonas* species associated with plants, several bacteriocin studies have focused on activity against phytopathogenic strains, such as *P. syringae* and *P. savastanoi* [19-26]. [27], have demonstrated that bacteriocin-producing *P. fluorescens* and *P. putida* strains are abundant in the rhizosphere of various tro *P. syringae* pical and temperate soils. One peculiar bacteriocin is LlpA (lectin-like putidacin-A), a bacteriocin consisting of two lectin modules that acts via unknown mechanism [28,29]. It is secreted by the strain *Pseudomonas* sp. BM11M1and displays activity against *P. putida* GR12-2R3 as well as other phytopathogenic *Pseudomonas* species, including *P. syringae* pathovars [22]. Other lectin-like bacteriocins, sharing only borderline homology to LlpA, have been studied in other *Pseudomonas* species as well [23,25,29,30]. At this point, functionality of S-type pyocins, previously characterized in *Pseudomonas aeruginosa*, is lacking in plant- and soil-associated *Pseudomonas* species [12].

2. MATERIALS AND METHODS

2.1 Biological Material and Culture Media

TSB (tryptic soy broth) is used for culturing indicator strains [31], LB broth (*Luria Bertani*) for

inducing bacteriocin synthesis in the strains tested. TSA (tryptic soy agar) is used to detect bacteriocin production, and LB agar to detect lysate activity via spot tests.

Fourteen fluorescent *Pseudomonads* spp. strains [32], in addition to two reference strains (*P. fluorescens* CHA0 renamed *P. protegens* CHA0) [33] and *P. aureofaciens* 30-84, kindly given by Prof Haas (Lausanne Switzerland), were analysed to evaluate their ability to produce antibacterial substances against one another, and against strains from other genera. Indicator isolates were: *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* coagulase^{+ve} ATCC 25923 (CHU de Sétif, Algérie), *Listeria monocytogenes* ATCC 15313, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* ATCC 10876 (Laboratoire des substances naturelles, Université de Tlemcen, Algérie).

2.2 Detection of Bacteriocin Producers

Bacteriocin production was detected through induction by placing TSA plates under UV-light, as described in [31]. Each tested strain was spread across TSA in a band approximately 2.5 cm wide. After 18 h at 28℃, the plate was irradiated with UV light at 254 nm for 1 min and incubated at 28℃ for another 3.5 h. Bacterial growth was scraped from the agar with a cotton swab soaked in chloroform and the plates were exposed to chloroform vapour to kill all remaining bacteria. Samples (15 µl) from overnight cultures of indicator strains in TSB were then streaked perpendicular to the tester band. Alternatively, supernatants of the positive tester cultures are used in the following test.

2.3 Bacteriocin Induction

A standard protocol for inducing bacteriocin production was followed using LB broth supernatants obtained previously, and supplemented with 0.05 µg/ml mitomycin C (SIGMA) [34]. The cultures were shaken for 5 hr at 28℃ and lysed with 100 µl of chloroform. Lysates also were tested after trypsin digestion, freeze-thawing, and Microcon column filtration as described by [10].

2.4 Bactericidal Activity of Lysates

Antibacterial activity of the lysates was assayed in two replicates - using the double-layer, incorporation method on LB agar: 2 µl of each lysate placed on agar previously inoculated with strains of the same species [10]. Bacteriocin production is evidenced by the presence of inhibition zones at the depot inoculation site [35].

2.5 Determination of Bacteriocin Type

The pH of lysates expected to contain bacteriocins was measured in order to discount the possible presence of organic acids which might have caused the inhibition (pH must be approx. 7.0). Indeed, some bacteria produce H_2O_2 which could trigger antimicrobial activity [36]. Four aliquots of each lysate were processed to differentiate low-molecular weight bacteriocins (S-pyocin like) from high-molecular weight bacteriophage-tail like bacteriocins (R and F-pyocin-like), and bacteriophages [10,34].

2.5.1 Trypsin digestion

Trypsin digestion was performed by adding 5 µl trypsin (SIGMA, 5 mg/ml) to 50 µl lysate. The lysate was then incubated for 30 min at room temperature [34]. This digestion would inhibit the activity of most bacteriocins but not that of phages and phage tail-like bacteriocins [10,12].

2.5.2 Filtration

Filtration, on the other hand, separates protein bacteriocins from phages and phage tail-like bacteriocins [10]. The 50 µl lysate was filtered at 14000 rpm for 15 min using a 100 kDa Microconcentrator centrifugal filter unit (Microcon).

2.5.3 Freezing

At -70℃, freezing usually destroys phages [34] whereas protein bacteriocins remain unaffected. Lysates (50 µl) were frozen overnight before testing.

2.5.4 Heat treatment

At 80℃, heat destroys the activity of thermolabile proteases [36], and LMW and HMW bacteriocins, allowing differentiation with secondary metabolites and antibiotics [9]. The treated lysates described above and controls (untreated supernatant or T+; double-distilled water T-) were spotted on cell lawns with susceptible strains (as described earlier). These treatments can establish the identity of the agent responsible for the antimicrobial activity noted in each lysate [34].

All the assay plates were incubated at 28℃, and the results indicating presence (+) or absence (-) of inhibition after 12 and 24 hours were recorded. Each producer strain was tested once, whereas the testing of the combined producer strains was duplicated. The results obtained were combined. An isolate was considered to be antagonistic only if passing both tests [10,37].

3. RESULTS AND DISCUSSION

In order to use indigenous microorganisms with antifungal traits as biocontrol agents, they must be able to establish in the environment by competing for nutrients and for the ecological niche. That why we have screened the capacity of these bacteria to produce bacteriocins which leads them to be more competent.

3.1 Detection of Bacteriocins

Two *Pseudomonas* reference strains (*Pseudomonas protogens* CHA0 and *Pseudomonas aureofaciens* 30-84) and 12 isolates were screened for their ability to produce antimicrobial compounds. Of these, the reference strains and 6 isolates (P1, P2, P5, P7, P8 and P10) are capable of producing anti-bacterial substances that inhibit the growth of other isolates (Fig.1a; Table 1). This inhibitory capacity is greater in promising isolates such as P2 and P5, and also in reference strains *P*. CHA0 and *P*. 30-84 which are able to inhibit same-genus isolates as well as other genera e.g. *Escherichia*. This capacity of inhibition is much lesser in P7, P8 and P10, or almost inexistent in P6, P13, P14 and P15. This production of inhibitory compounds is long-lived; it is still evident after periods of 12, 24, and 96 hours or more. Inhibition zones always keep their size; they do not spread but do contract over time when it is observed that certain bacteria resume their growth (Fig. 1b).

The results obtained indicate that, in addition to their ability to produce anti-fungal substances (phenazines, siderophores, HCN) [32,38], the *Pseudomonas* rhizosphere isolates in this study are also able to produce anti-bacterial substances. [37], have previously described the production of anti-bacterial substances by fluorescent *Pseudomonads* spp., that produce 2, 4 diacetylphloroglucinol. [23], also observed that in addition to an arsenal of genes that encode anti-fungal substances, *P. protegens* Pf-5 carries at least two functional anti-bacterial genes; and that the expression of each gene in *E. coli* results in the production of a 31 kDa LlpA-type bacteriocin that is subsequently released in the medium. [14], have characterized genetically for the first time a phage tail-like pyocin in *Pseudomonas fluorescens* SF4c isolated from wheat rhizosphere. This high-molecular-mass bacteriocin inhibits the growth of closely related bacteria.

In vitro inhibition tests have indicated that bactericidal activity through bacteriocins is found in strains that produce antibiotic substances, e.g. *P. fluorescens* CHA0, *P. aureofaciens* 30-84; however among the various *Pseudomonas* strains that have been isolated, it has been found that *P. chlororaphis* sbs *P. aureofaciens* DSM 6689 which produces phenazines [39], *P. fluorescens* (P5 and P7 isolates that stimulate growth) [40] and *P. putida* (P10 isolate LOPAT+) interact with one another mutual bactericidal activity among the bacteriocin producing strains [32]. Among the 14 rhizosphere isolates (other than the reference strains), only 6 have produced substances with bactericidal activity. Several studies have shown that bacteriocin production ranges between 10 and 50% among the enterobacteria studied [10,41]. Furthermore, Validov and collaborators have reported that inhibition caused by antagonistic activity was widely found in fluorescent *Pseudomonads* spp. that produce phloroglucinol, and that among the 47 strains tested, only 11 had an antagonistic effect on another strain [37].

The results reflect the considerable discrepancy in the nature of isolates in our collection, whereby some species are represented by several strains and others are represented by only one strain [32]. Several studies have been carried out on the production of bacteriocins. However, few studies have been carried out on bacteriocin production in strains of closely-related groups that have been isolated under the same conditions, at the same time and from the same hosts [10,42].

The majority of strains tested *in vitro* post induction (by UV or mitomycin C) are antagonistic to other fluorescent *Pseudomonads* spp., and particularly so among bacteriocin-producing strains. Only *P. chlororaphis* sbs *P. aureofaciens* DSM 6689 was characterized previously to have anti-fungal and anti-microbial activity [32,39].

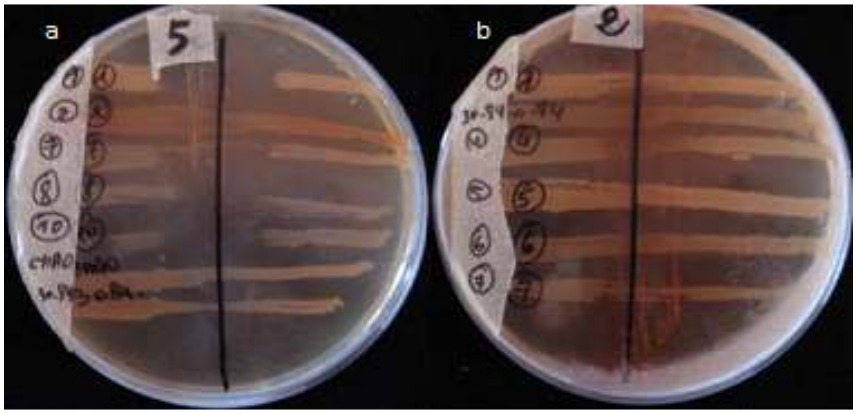

Fig. 1. Bacteriocin production by rhizospheric *Pseudomonas* isolates after 48 h of incubation
(a) by the isolate 5; indicator strains are Pseudomonas 1, 2, 7, 8, 10, Pseudomonas protegens CHA0 and Pseudomonas aureofaciens 30-84; (b) by the isolate 2 ; indicator strains are Pseudomonas 1, 4, 5, 6, 7and Pseudomonas aureofaciens 30-84. Vertically streaked across the surface of tryptic soy agar to a width of approximately 2.5 cm producer isolates 5 and 2; horizontally streaked indicator isolates

Table 1. *In vitro* antagonism between fluorescent *Pseudomonads* spp. by production of antibacterial compounds

Indicator strains	Producer strains													
	P1	**P2**	**P4**	**P5**	**P6**	**P7**	**P8**	**P10**	**P11**	**P13**	**P14**	**P15**	**CHA0**	**30-84**
P1	-	±	-	+	-	-	-	-	-	-	-	-	+	-
P2	-	-	+	±	-	±	±	±	±	±	-	-	+	±
P4	Nd	+	-	-	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	±
P5	+	-	-	-	-	+	-	±	-	-	-	-	+	-
P 6	-	±	Nd	-	-	-	-	-	Nd	Nd	Nd	Nd	+	Nd
P7	-	±	-	+	-	-	+	-	-	-	Nd	Nd	+	+
P10	+	+	-	+	Nd	-	-	-	±	Nd	Nd	Nd	+	+
E. coli	-	±	±	±	±	±	±	±	±	-	±	-	+	-
S. aureus	-	+	±	±	+	+	+	±	±	-	-	±	+	-
30-84	-	-	-	-	-	-	-	-	-	-	-	-	±	-
B. cereus	Nd	+	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	+	Nd

**P1, P2….etc: numbers attributed to Pseudomonas isolates, CHA0 and 30-84: Pseudomonas protegens CHA0 and Pseudomonas aureofaciens 30-84. E. coli: Escherichia coli* ATCC 25922*, St: Staphylococcus aureus coagulase^{+ve} ATCC 25923, Bacillus: Bacillus cereus ATCC 10876, Nd: not determined, +: bactericidal activity, -: absence of bactericidal activity, ±: bacteria resume their growth after 24 hours of incubation*

According to [34], the induction of bacteriocins in Gram-negative bacteria is mediated by the SOS regulon and are dependent on the host's cell regulation pathways [43]. Under stress conditions e.g. UV radiation or certain substances such as mitomycin C [34], a small proportion of cells take the 'suicide' pathway, i.e. they release toxin molecules that kill sensitive neighbouring cells. Substances that damage DNA such as UV rays and mitomycin C are not widely found in nature. Previous studies dealing with colicins E1 and K indicate that their regulation depends not only on the DNA-damaging agents but also on catabolic repression, anaerobiosis, osmolarity, depletion of nutrients, heavy metals, pH and temperature factors that can trigger small or null SOS responses [43].

3.2 Determination of Bacteriocin Types

Six isolates (P1, P2, P5, P7, P8 and P10) featured in this study were those that demonstrated antibacterial activity against several indicator isolates (sensitive strains) [40]. Inhibition zones observed after-incubation (Fig. 2) point to the presence of bactericidal substances in the supernatants as a result of induction with mitomycin C. The lysates of the 6 isolates were further treated and the results are summarised in Table 2. The most active strain was P2 (*Pseudomonas chlororaphis* sbs

P. aureofaciens DSM 6689), it inhibited six of the telluric *Pseudomonas* isolates and four of the sensitive bacteria tested. While the less active strain was P1, it inhibited only two strains of the telluric *Pseudomonas* isolates.

Of the 6 isolates (P1, P2, P5, P7, P8 and P10) studied (in addition to the reference strain CHA0) only 5 demonstrated antibacterial activity following induction through the use of mitomycin C (P1, P2, P5, P7 and P10). Following the treatments applied to the lysates, the following observations can be made.

All filtrates lose antibacterial (bactericidal) activity as do supernatants which have been treated at 80℃. As these compounds were also retained in the filtrate, this likely point's to activity caused by antibacterial proteins or protein complexes. Of the 6 lysates (P1, P2, P5, P7, P8 and P10) that showed antibacterial (bactericidal) activity, four did not lose their activity as a result of the trypsin treatment (P1, P2, P7 and P10). The same results were obtained for the -70℃ treatment where it was also observed that four of the lysates (different from the ones already mentioned; P2, P5, P7 and P10) remain active after this treatment. Inhibitory activity persisted after 12, 24 and 48 hours, and these zones neither contracted nor increased in size.

Inhibitory activity persisted after 12, 24 and 48 hours, and these zones neither contracted nor increased in size.

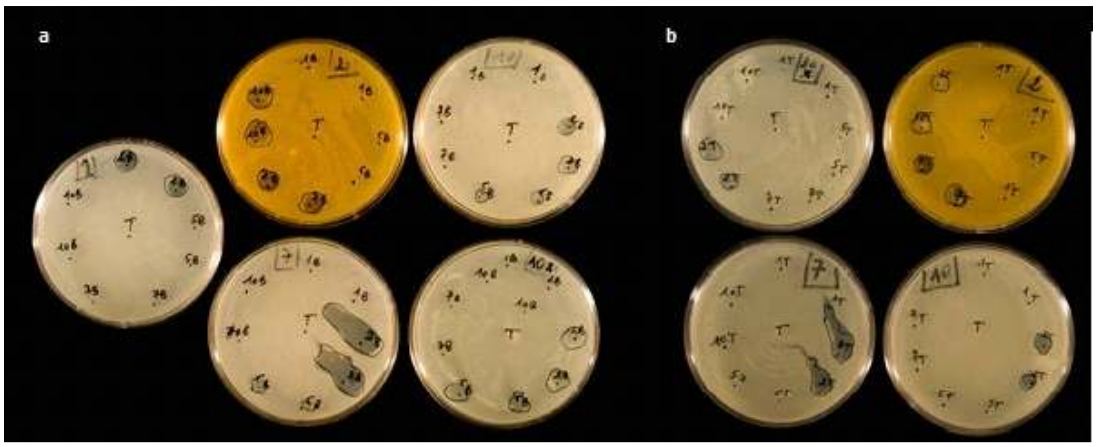

Fig. 2. Bactericidal activity of treated supernatant of rhizospheric isolates

(a) with Non treated supernatant; 1,2, 7, 10 and 10x : spreading bacterial isolates; 1B……..10B : corresponding isolate supernatants; (b) 2, 7, 10, and 10 x : spreading bacterial isolates ; 1T……10T: corresponding isolate supernatants treated with trypsin; T: control

Table 2. Bactericidal activity of treated supernatants

		Bacteriocin producing strains																											
		P 1				P 2				P 5				P 7				P 8				P 10				P CHA0			
Indicator strains	treatments	s	c	t	f	s	c	t	f	s	c	t	f	s	c	t	f	s	c	t	f	s	c	t	f	s	c	t	f
	P1		-		-	+	+	+	-				-				-				-				-	+	+	+	-
	P2		-		-				-				-	+	+	+	-				-	+	+	+	-				
	P5	+	-	+	-	+	+	+	-				-	+	+	+	-				-	+	+	+	-	+	+	+	-
	P7	+	-	+	-	+	+	+	-	+	+		-				-				-				-	+	+	+	-
	P8		-		-				-				-				-				-				-	+	+	+	-
	P10		-		-	+	+	+	-	+	+		-				-				-				-	+	+	+	-
	P10c	±	-	+	-	+	+	+	-	+	+		-				-				-				-	+	+	+	-

**P1, P2….. : Pseudomonas isolates, P. CHA0: Pseudomonas protegens CHA0, S: non treated supernatant, C: treated supernatant at -70℃, t: trypsin-treated supernatant, f : filtered supernatant on 100 kd filters, +: positive bactericidal activity, -: negative bactericidal activity, *10c: Pseudomonas isolate 10 treated with ciprofloxacine (data not shown here), **: the blanks are not determined*

Lysates obtained as a result of induction by mitomycin C were found to display antagonistic activity. This activity is associated with high molecular weight substances which are resistant to proteases, probably attributable to phage tail like bacteriocins or bacteriophages. The exact nature of these compounds remains to be demonstrated via electron microscopy or MS analysis.

The bacteriocins produced by our isolates are active not only against strains which are closely-related to the bacteriocin-producing strains but also against phytopathogenic strains (P10) [40]. Serracin P, a phage-tail-like bacteriocin produced by *Serratia plymithicum* J7 has previously been found to inhibit, *in vitro*, some organisms that are important in the field of phytopathology, such as *Erwinia amylovora* [44].

In the environment, bacteriocins are thought to constitute elements of strain-specific competition [4,12,17,45], enabling producing strains to invade new ecological niches and/or to displace those already present [4,17]. However, apart from *in vitro* studies, the role of bacteriocins has been studied scarcely *in vivo*, and very few studies have dealt with bacteriocinogenic activity in *Pseudomonas* spp. associated with plants [45,37]. However, our results indicate that bacteriocins could be a contributing element in the competition among closely-related fluorescent *Pseudomonads* spp.

4. CONCLUSION

We have demonstrated that a number of fluorescent *Pseudomonads* isolates that produce anti-fungal substances such as phenazines, siderophores and HCN, are also capable of generating antibacterial substances when subjected to stress conditions. One of these isolates (P10) has been characterized as a phytopathogen and is capable of synthesising antibacterial substances too. The ability of these isolates to synthesize bactericidal molecules may allow their use as an inoculum for protecting plants from soil-borne diseases.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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