ANTIMICROBIAL ACTIVITY OF A MARINE ISOLATE OF *PSEUDOMONAS Cepacia* K2 FROM *RUDITAPES decussatus*

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ملخص

نشاطَ ملحوظَ للبكتيريا *Pseudomonas cepacia* المسماة K2 كمضاد للعديد من أنواع البكتيريا المسببة الرئيسية للعديد من الأمراض الأكثر شيوعاً في البيئات البحرية : البكتيريا Pseudomonas cepacia المسماة K2, والتي تم عزلها من القواقعيات Ruditapess decussatus أظهرت نشاطاً ملحوظاً كمضاد للعديد من انواع البكتيريا بما فيها Aeromonas hydrophila, Aeromonas salmonicida et Vibrio anguillarum , وهما المسببان الرئيسيان للعديد من الامراض الأكثر شيوعاً في البيئات البحرية والتي قد تسبب الوفاة للأسماك والمحار علي حد سواء.

أظهرت البكتيريا K2 أيضاً أنتاجا مبكراً للمضادات الحيوية (بعد ثمان ساعات من الحضانة).لوحظ ان الوقت يعتبر من العوامل المؤثرة في قوة البكتيريا أذ تزداد قوة البكتيريا بزيادة وقت الحضانة . أثبتت الدراسة الاولية عن طبيعة النشاط انه علي الاقل هناك واحدة من المواد المضادة هي ناقلة الحديد sidérophore وأخري حساسة للحرارة والانزيم .هذة الدراسة تشير الي ان البكتيريا K2 مناسبة للمكافحة الحيوية في نظم تربية الاحياء المائية

كلمات مفاتيح: للبكتيريا، *Pseudomonas cepacia*، مضادات الحيوية، مكافحة الحيوية

RESUME

Activité antibactérienne d'une bactérie à potentiel probiotique en aquaculture Tunisienne : Une souche de *Pseudomonas cepacia*, designée K2, isolée à partir de la palourde *Ruditapes decussatus* a montré une activité antibactérienne significative contre un certain nombre de bactéries marines, y compris *Aeromonas hydrophila, Aeromonas salmonicida* et *Vibrio anguillarum* qui sont les bactéries pathogènes les plus fréquemment signalées dans l'environnement marin, responsables d'une forte mortalité des poissons et des crustacés. Cette souche K2 a montré une capacité d'inhibition très rapide dès le début de sa croissance (après 8h d'incubation). L'intensité de son activité est très importante et a tendance à augmenter avec le temps d'incubation. Les études préliminaires sur la nature de l'inhibition a indiqué qu'au moins l'une des substances antibactériennes était un sidérophore et une autre était thermolabile et sensible à une enzyme protéolytique. Ces résultats suggèrent que *Pseudomonas cepacia* K2 pourrait être une souche appropriée comme agent de lutte biologique dans les systèmes d'aquaculture.

Mots clés : Aquaculture, activité antibactérienne, bactéries pathogènes, maladies infectieuses.

ABSTRACT

A *Pseudomonas cepacia* strain, designed as K2, recovered from the clam *Ruditapes decussatus* showed significant antibacterial activity against a number of marine bacteria including *Aeromonas hydrophila, Aeromonas salmonicida* and *Vibrio anguillarum* which are the most common pathogenic bacteria reported from the marine environment, causing high mortalities in fish and shellfish. It has been proved an early production of inhibition by the K2 strain (after 8h of incubation). The intensity of its activity is very significant and increases within the incubation period. Our preliminary study indicated that at least one of the antibacterial substances was a siderophore and one was heat labile and sensitive to a proteolytic enzyme. These results suggest that *Pseudomonas cepacia* K2 may be a suitable strain as a biocontrol agent in aquaculture systems.

Key Words:*:** antibacterial activity, probiotics, aquaculture, *Pseudomonas.**

INTRODUCTION

Aquaculture has become an important economic activity in many countries. In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary

medicines. However, the utility of antimicrobiol agents as a preventive measure has been questioned, given extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria. For this reason their use is an ecological threat to coastal areas exploited for industrial cultivation of fish and shell-fish, and thus should be restricted (Gildberg et *al.,* 1997).

Alternative methods need to be developed to maintain a healthy microbial environment in the larval rearing tanks. One such method, that is gaining acceptance within the industry, is the use of probiotic bacteria to control potential pathogens (Gomez-Gil et *al.*, 2000).

Probiotics are usually defined as live microbial feed supplements, that are administered in such a way as to enter the gastrointestinal tract and to be kept alive; this beneficially affects the host animal by improving its intestinal microbial balance and in turn its health (Gatesoupe 1999; Gomez-Gil et *al*., 2000). The use of probiotics in human and animal nutrition is well documented (Fuller, 1992; Mulder et *al.,* 1997; Rinkinen et *al.*, 2003) and recently, they have begun to be applied in aquaculture (Gatesoupe, 1999; Gomez-Gil et *al.*, 2000; Verschuere et *al.,* 2000; Irianto and Austin, 2002; Bachère, 2003; Calo-Mata et *al.*, 2007).

To date, the screening of probiotics has been pragmatic, lacking meaningful selection criteria, and they are often assessed only on their ability to produce antimicrobial metabolites (Gibson et *al.,* 1999). Isolating bacteria that produce antimicrobial metabolites is common practice, while experiments to determine at what stage of growth the bacteria produce the metabolites are seldom performed. Such experiments are necessary to be able to predict and optimize the effect of these potential probiotcs *in vivo* (Niall et *al*., 2004).

Pseudomonads are common inhabitants of the aquatic environment, and are commonly associated with shellfish and gills, skin and intestinal tract of live fish (Chythanya R. et *al.,* 2002). The antagonistic activity of *Pseudomonas* against a number of pathogens has been reported in literature (Troller and Frazier, 1963; Daly et *al*., 1973). Smith and Devey (1993) reported that bathing Atlantic salmon presmolts in a strain of *Pseudomonas fluorescens* reduced subsequent mortality from stress-induced furunculosis. Gram et *al*. (1999) observed *in vitro* inhibition of *V. anguillarum* by *Pseudomonas fluorescens* and obtained lower mortalities in probiotic-treated fish, *Oncorhynchus mykiss*. Specific inhibition of *V. harveyi* by *Pseudomonas aeruginosa* has been reported earlier by Torrento and Torres (1996) and Chythanya et *al.* (2002).

In the present study, we report the isolation of a putative probiont, *Pseudomonas cepacia* K2, its spectrum of antagonism against different pathogenic bacteria, the tracking of its production of inhibition during a cycle of 48h of growth, and preliminary characterization of the antagonistic principle. The strain K2 on the primary culture plate was found to inhibit growth of other bacterial flora on the ZoBell's marine agar plate. This observation prompted us to explore its ability to inhibit different pathogenic bacteria brought for marine environment. Thus, eighty-nine isolates including isolates of *Vibrio spp*., *Aeromonas spp*. and *Pseudomonas spp*. were used as targets to test the antagonistic potential of *Pseudomonas* K2 isolate.

MATERIALS AND METHODS

Baterial isolates

The marine *Pseudomonas cepacia* designed K2 used in this study was isolated on ZoBell's Marine Agar (Bio-Rad, France) from the clam *Tapess decussatus* collected from coastal shellfish area in Tunisia, and was maintained in tryptone soya agar supplemented with 2% (w/v) sodium chloride (TSAS). This organism was identified as *P. cepacia* based on the characteristics described in the *Bergey's Manual of Systematic Bacteriology* which include morphological, physiological and biochemical characteristics.

Study of inhibition by deferred antagonism assay and disc diffusion methods

The antibacterial effect of *Pseudomonas* K2 was tested using the deferred antagonism test (Dopazo et *al.*, 1988). Tryptone soya agar (TSAS) plates were spot inoculated with 10 µl of overnight culture of the bacterial strain to be tested. After 24h of incubation at 20°C, the colonies were killed with chloroform vapour over a period of 45 min. These plates were overlaid using 6ml of soft agar (TSAS) supplemented with 0.9% w/v agar, containing 0.1 ml of a 1/10 dilution of 16h indicator strain culture. The plates were then 24h incubated at 20°C and the zones of inhibition around the spots were measured and recorded against 89 target organisms. The probiotic bacteria *Pseudomonas fluorescens* AH2, provided from the Danish Institute of Piscicultural Research, was used as positive control.

For the study of inhibition by disc diffusion method, cell free supernatant of a 24h culture of *Pseudomonas* K2 in TSBS was prepared by centrifugation at 10,000 × g for 10 min and filtering through 0.22 µm pore size filter (Millipore MA). Twenty microlitres of this cell free preparation was spotted on four mm diameter sterile disc punched from Whatman filter paper and air dried. Each disc was placed on TSAS plates previously swabbed with the target bacterial isolates grown in TSBS until log phase. The plates were then incubated at 20°C for 24h and the zone of inhibition around the discs was measured.

Time course production of the antagonistic activity monitoring

Starting of the antagonistic substance production, its peak activity and duration of sustained activity were determined during the growth cycle of *Pseudomonas* K2. The culture was inoculated at an absorbance of 0.01 at 600 nm (approx.10^3 CFU ml^{-1}) in a 500 ml TSBS medium and incubated at 20°C. At periodic intervals, 2 ml culture of aliquots were drawn aseptically and used for monitoring growth (A_{600nm}). The antagonistic activity was tested against sensitive pathogenic strains, in triplicate, using the deferred antagonism test as described above. The plates were 24h incubated at 20°C and recorded for zones of clearing around the spot continuously until culture entered the decline phase. Antagonistic activity was quantitatively expressed in terms of the diameter (mm) of inhibition zones.

Antimicrobial susceptibility testing

The antibacterial sensitivity was determined by the agar diffusion method according to Chabbert (1982), using 15 antibiotics representing the most commonly used drugs in aquaculture (Penicillin G, Amoxicillin, Oxacilin, Cefoxitin, Ceftriaxone, Streptomycin, Tobramycin, Neomycin, Chloramphenicol, Tetracyclin, Oleandomycin, Nitrofurantoin, Trimethoprim-Sulphonamide, Rifampicin and Oxolinic acid). Inoculum of the strain to be tested was swabbed onto the surface of a Muller-Hinton agar plates, then the disks impregnated with antimicrobial agents are placed on the agar. After overnight incubation at 20 °C, the diameter of the zone of inhibition of bacterial growth around each disk was measured. Based on the zones of inhibition a qualitative report of “susceptible”, “intermediate” or “resistant” can be determined for the tested bacteria according to French national guidelines (Comité de l’Antibiogramme de la Société Française de Microbiologie, 1996).

Preliminary characterization of the antagonistic substances

Three preliminary assays were tested: - availability of iron, sensitivity to proteases and heat.

The first consist on the effect of the iron availability on the antibacterial activity within the active strain was carried out using a rich ferric ions medium, TSAS (10 mM of $FeCl_3 7H_2O$). The antibacterial activity was tested by the deferred antagonism assay and results obtained were compared with plates iron free (medium free used as control).

Secondly, inhibitory substances released by *Pseudomonas* K2 were assessed for their sensitivity to the proteolytic effect of trypsin (Sigma). Trypsin was added in the TSAS at the rate of 1g/L.Trypsin free growth medium, are simultaneously prepared as control and inhibitory substance production was assayed using the deferred antagonism test.

Thirdly, the heat stability of the antibacterial component was tested by heating the cell free supernatant in a water bath for 30 min at 60°C, 80°C and autoclaving for 15 min at 121 °C, after which antibacterial activity was tested against sensitive strains by the disc diffusion method.

Test of lysogeny

The presence of possible bacteriophages in the active selective strain K2 was performed according to the technique of Mitomycine C induction (Reyrolle et *al.*, 1982). Bacterial strain *Pseudomonas* K2 was subcultured in TSBS. Inocula of 2% (v/v) (optical density, 0.05) was then made into 20 ml of TSBS and divided into two 10 ml samples, which were incubated for 90 min at 20°C (optical density ≈ O.1). MC (Sigma Chemical Co.) was added in a final concentration of 1 µg/ml to the assay tube; the other tube served as a control. Incubation was continued for 3h at 20°C. Tubes were then centrifuged (4 000 × g, 10 min) and the supernatants were filter sterilized through a 0,45 µm pore-size membrane (Millipore MA). The activity of each lysate was tested by spreading on TSAS plates, 3ml of soft agar (TSAS) containing various concentrations of the indicators strains. The possible appearance of plaques of bacterial lysis recorded the presence of bacteriophages.

Statistical analyses

All experiments were done in triplicates and results were submitted to analysis of variance using the statistical program SPSS (SPSS for Windows, SPSS Inc) and means were separated by the least significant-difference according to the Student-Newman-Keuls test.

RESULTS

Bacterial isolate characteristics

The bacterium isolate K2 is Gram-negative and forms yellow pigmented colonies of motile coccobacilli on ZoBell’s agar. It produces oxidase and catalase, but not arginine dihydrolase and urease (Table I). This strain K2 reacts positively to the nitrate reduction test and negatively to the indole test; it metabolizes glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl-acetate as sole carbon sources. It can hydrolyse esculin and gelatine. The above characteristics equate culture designed K2 to the specie *Pseudomonas cepacia*.

Antagonism activity

Eighty-nine bacterial isolates obtained from marine environment were tested for their susceptibility to the

Table I: Biochemical characteristics of the strain *Pseudomonas cepacia* K2

	Strain
Tests	*Ps. cepacia*
Gram reaction	*G -*
motility	+
morphology	*coccobacilli*
Resistance to vibriostatic compound O129	-
Oxydase	+
Catalase	+
Reduction of nitrates to nitrites	+
Indole production	-
Glucose acidification	-
Arginine dihydrolase	-
Urease	-
Hydrolyse of aesculin	+
Gelatinase	+
β-galactosidase	+
Assimilation of glucose	+
Arabinose	+
Mannose	+
Mannitol	+
N-acetyl-glucosamine	+
Maltose	+
Gluconate	+
Caprate	+
Adipate	+
Malate	+
Citrate	+
Phenyl-acetate	+

(+): positive response, (-): negative response, (v): variable reaction, r :resistant, s : sensitive.

antagonistic component produced by *Pseudomonas cepacia* K2. Among the 89 pathogenic bacterial isolates, 73% (65 isolates) were inhibited by the strain K2, both in deferred antagonism and disc diffusion assays, with inhibitory zones ranging from 18 to 46 mm (Table II, Fig 1).

In comparison, the probiotic reference strain *P. fluorescens* (AH2) gave only a weak antagonistic activity against some indicator strains.

Table II : Spectrum of inhibitory activity of *Pseudomonas cepacia* K2

Pathogen	No. of isolates tested	No. of isolates susceptible	Zone of clearance (mm)
A. salmonicida	3	3	38-46
A. hydrophila	14	10	28-30
Ps. cepacia	7	4	20-28
Ps. fluorescens	4	2	20-25
V. alginolyticus	11	9	20-32
V. anguillarum	17	17	37-46
V. vulnificus	8	5	32-40
V. parahaemolyticus	8	4	32-40
V. harveyii	3	2	30-39
V. vulnificus	9	6	27-32
V. tapettis	5	3	28-39
Total	89	65	

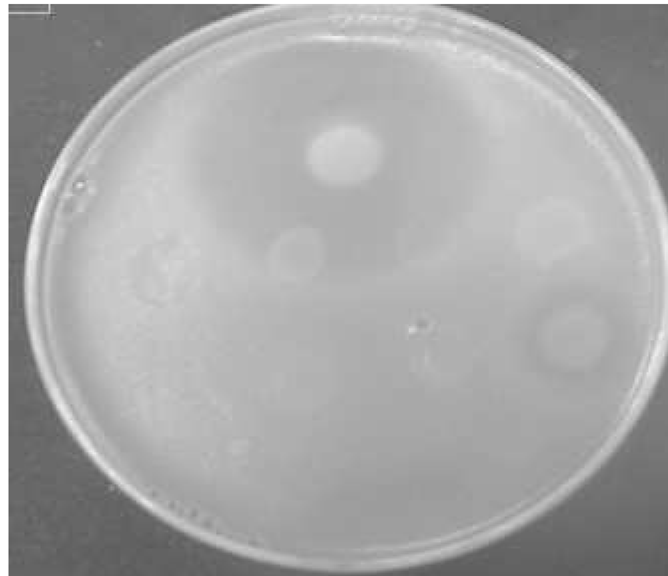

Fig 1 : Exemple of inhibition of *Vibrio vulnificus* by the strain *P. cepacia* K2

Time course of growth and production of the antagonistic activity

The antagonistic substances produced by *Pseudomonas cepacia* K2 was detectable by deferred antagonism test from 8h of growth onwards and maintained during the .stationary phase by 24 h and declined at about 30 h of post-inoculation. Production of the antagonistic substances peaked at the stationary phase, and maximum antagonistic activity was observed at 24h of growth (Fig. 2).

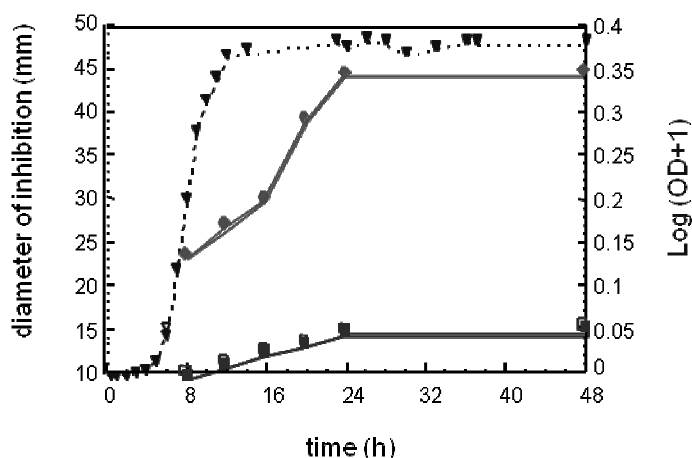

Fig.2. Production of antagonistic activity by *Pseudomonas cepacia K2*

Preliminary characterization of the antagonistic substances

The production of siderophores is a largely used mechanism by probiotic bacteria. The addition of ferric salts in the growth medium for probiotic bacteria could cause a blocking of their inhibitory effect towards sensitive bacteria which indicated a production of compounds effectively binding iron, in particular the siderophores. Our experimental conditions results showed a diminution of the diameters of inhibition generated by *P. cepacia* K2 and consequently a decrease of the antagonistic activity was observed. This result indicated a possible relation between availability of iron and the antibacterial activity of *P. cepacia* K2.

The proteolytic enzyme (trypsin) used at 1g/l generated a clear decrease in the activity of *Pseudomonas cepacia* K2 resulting in a diminution of the diameters of the zones of inhibition obtained. These results indicated that, probably, the antibacterial effects presented within the growing strain was of proteinic dependance or had a proteinic component necessary to its antibacterial activity.

Study of the heat stability of the antibacterial principle showed that at 60°C, the activity decreased gradually and it completely disappeared when supernatants were autoclaved at 121°C for 15 min, indicating that the antibacterial substance produced by *Pseudomons* strain K2 is heat labile.

Test of lysogeny

P. cepacia K2 was tested to investigate a possible inhibitory activity due to the presence of latent bacteriophages. In order to activate these bacteriophages, induction with Mytomicine C was tested. In the case of possible appearance of plaque of lysis, following the application of the sensitive strains, the presence of bacteriophage was assumed. The results obtained show an absence of plaque of lysis for the producing strain tested, indicating the absence of phagic activity.

DISCUSSION AND CONCLUSION

The present study reports a promising antagonistic bacterium *Pseudomonads* K2, isolated from the clam *R. decussatus,* which showed antagonistic property and a wide zones of inhibition against different species (i.e. *Aeromonas* and *Vibrio*) that have been considered as major pathogens in aquaculture systems (Vijayan et al., 2006). The experiments of deferred antagonism and disc diffusion assays showed that the bacterial cell but also an extracellular product are responsible for inhibition.

Members of the genus *Pseudomonas* are common inhabitants of soil, freshwater and marine environments and are known to produce a wide range of secondary metabolites (Raaijmakers et *al.,* 1997) inhibiting a wide range of pathogenic bacteria. As with their terrestrial counterparts, aquatic pseudomonads are often antagonistic to other microorganisms (Gram, 1993), including fish pathogenic bacteria (Smith and Davey, 1993) and fish pathogenic fungi (Bly et *al.*, 1997). Spanggaard et al. (2001) reported the antibacterial properties of a large collection of bacteria of various species, and this activity was more marked in the species *Pseudomonas sp.* Also, Smith et *al.* (1993) and Gram et *al.* (1999) studied the inhibitory effect of marine *Pseudomonas* on two pathogenic bacteria in aquaculture (*Aeromonas salmonicida* and *Vibrio anguillarum).* The antagonistic activity of this specie, observed *in vitro,* was of low intensity than the strain K2 one. However, it was able to protect fish against infection due to the two pathogenic bacteria. Chythanya et *al.* (2002) also reported inhibition of pathogenic vibrios by an estuarine strain of *Pseudomonas*.

In this study, the production pattern of the antagonistic components showed an early production of inhibition (from 8 h of incubation) and this activity quickly reached a significant intensity revealed by broad diameters of inhibition zones (often exceeding 40 mm). Also, we noted a little reduction of the antibacterial activity, after 48h of culture. This phenomenon may be due to the activation of proteolytic enzymes produced and secreted by the bacteria in culture medium, which hydrolyse antibacterial molecules produced (Sebei, 2001).

Preliminary characterization of the antagonistic substances produced by the strain K2 suggested that at least one of the antibacterial substance was a heat-labile proteinaceous compound, probably a bacteriocin or a bacteriocin-like substance, and one was binding iron molecule, probably a siderophore. Numerous studies have implicated siderophores as bacteriostatic substances produced by *Pseudomonas* species (Guerinot, 1994 and Gram et *al.*, 2001) and a number of earlier studies have also been shown the capacity of this species to synthesize bacteriocins (Sugita et *al.*, 1996 and Annabel et René, 2002). Nevertheless, further characterization of the inhibitory factors of *Pseudomonas* K2 still requested.

In conclusion, this report highlight the preliminary characteristics may be considered a starting block for the study of potential probiotic bacteria in aquaculture in Tunisia. This study suggested that *Pseudomonas cepacia* K2 may be suitable as biocontrol agent in aquaculture. However, it remains to study the assimilation of this potential probiotic and determination of its activity spectrum on different farmed species and to ascertain whether or not this *in vitro* activity could be translated to the *in vivo* situation.

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