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Effects of the bacteriocin PsVP-10 produced by *Pseudomonas sp.* on sensitive bacterial strains

Carlos Padilla,* Olga Lobos,* Pedro Brevis,* Paulina Abaca,* and Elizabeth Hubert*

ABSTRACT. The bacteriocin PsVP-10 is a 2.6 Kda peptide which was isolated and purified from *Pseudomonas sp. T*his bacteriocin possesses lethal activity over *Enterococcus faecalis, Salmonella typhimurium* and S*higella flexneri.* The experimental assays showed that the bacteriocin is able to be adsorbed by all cells of these bacterial species and also by their isolated cell walls. It was observed that the resistant mutants and their respective cell walls are unable to adsorb the bacteriocin. Assays performed with spheroplasts obtained from sensitive bacterial species and their resistant mutants show a rapid lethal effect of the bacteriocin PsVP-10. This results indicated furthermore, it is also shown that the optimal pH and temperature for the adsorption were 7.2 and 37ºC, respectively. The study carried out with organic solvents like methanol, ethanol, isopropanol and the detergents sodium dodecyl sulfate and triton X-100 showed a moderate inhibition of the bacteriocin lethal action for the Gram negative cells. The enzymes lysozime, protease XIV and trypsine type III-S did not present any effect over the adsorption capacity of the bacteriocin with any of the bacterial species studied.

Key words: *Pseudomonas,* bacteriocin PsVP-10, adsorption.

RESUMEN. La bacteriocina PsVP-10 es un péptido de 2.6 Kda, aislado y purificado a partir de *Pseudomonas sp.* Esta bacteriocina posee actividad letal sobre *Enterococcus faecalis, Salmonella typhimurium* y *Shigella flexneri.* Se demostró que la bacteriocina fue absorvida por las células de las tres especies bacterianas y también por sus respectivas paredes celulares aisladas. Se observó que células mutantes resistentes a la bacteriocina de todas las especies estudiadas y sus paredes celulares aisladas fueron incapaces de absorver la bacteriocina. Esferoplastos obtenidos desde especies bacterianas sensibles y mutantes resistentes fueron lisados por la bacteriocina. Se demostró que el pH y temperatura óptima para la adsorción fue 7.2 y 37°C respectivamente. El tratamiento de células Gram negativo con solventes orgánicos metanol, etanol, isopropanol y con los detergentes dodecil sulfato de sodio y tritón X-100, inhibió moderadamente la acción letal de la bacteriocina. Las enzimas lisozima, proteasa XIV y tripsina III-S no presentan efecto sobre la adsorción en ninguna de las especies bacterianas estudiadas.

Palabras clave: *Pseudomonas,* bacteriocina PsVP-10, adsorción.

INTRODUCTION

Bacteriocins are proteins produced by different bacterial species with lethal activity over a wide range of Gram positive and negative bacteria. Besides, the producer strains present a self-protection mechanism.[3] A *Pseudomonas sp.* which was isolated from a well water sediment,[7] produced the bacteriocin PsVP-10 that corresponds to a 2.6 Kda peptide. This bacteriocin is resistant to a wide range of pH, high temperatures and also to several proteolitic enzymes.[5] This bacteriocin showed a wide range of antibacterial action. The main lethal action was over enteropathogenic bacterial species as *S. typhi, S. typhimurium* and *S. sonnei,* besides other microorganisms.[5] It has also been demonstrated that bacteriocin PsVP-10 reduced the number of enteropathogenic bacteria in an artificial aquatic system.[7] So, it is very important to carry out studies on the mechanism of action of the bacteriocin PsVP-10 having in mind the possible use, in the future, of this antibacterial product as antimicrobial substance or as food biopreservant.

* Laboratory of Research in Microbiology and Institute of Biological Sciences and Biotechnology, Universidad de Talca, Talca, Chile.

Most of the bacteriocin exert their lethal activity by the adsorption to specific receptors in the external surface of the sensitive bacteria[4] and then they are able to interact with the cytoplasmic membrane leading to the bacterial death.[6] The adsorption capacity and the possible effect on the cytoplasmic membrane are unknown for the bacteriocin PsVP-10. Therefore, the main objective of this work is to study the effect of this bacteriocin on the cytoplasmic membrane by means of adsorption studies to sensitive and mutant resistant cells.

MATERIAL AND METHODS

Bacterial strains

Pseudomonas sp. R-10 strain was used for the production of the bacteriocin PsVP-10.[5] This microorganism was isolated from the sediment of well-water used for human consumption in the rural area of Talca in central Chile. The tested sensible cells were Sh. flexneri, *S. typhimurium* and *E. faecalis*. These bacterial species were isolated from clinical samples. The two Gram negative species were obtained from acute diarrhoeal cases and *E. faecalis* was isolated from urinary tract infection. The bacteriocin producer srtain and the sensitive bacterial spe-

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cies were stored in skim-milk at –70°C. For all the studies the bacterial species were grown in BHI agar (Merck) with sheep blood at 5%.

Isolation, purification and detection of the bacteriocin PsVP-10.

Pseudomonas sp. R-10 was cultured in 1,000 ml of BHI broth (Merck) at 37ºC for 48 h and the purification of the bacteriocin was carried out according to the procedure reported by Hubert 5. The Sh flexneri, *S. typhimurium* and Ent. faecalis strains were grown in BHI broth until the first stage of exponential growth (O.D. 0.4 at 660 nm) and immediately sowed in lawn in Petri dishes containing Mueller-Hinton agar (Merck). After the dishes were dried for 10 min at 37°C, 10 ml of the bacteriocin was spot on lawn. In order to determine arbitrary units (A.U.) the bacteriocin PsVP-10 was several times double diluted and of each dilution, 10 µl were spot on lawn with studied bacterial species. One arbitrary unit (AU) of the bacteriocin PsVP-10 was defined as 10 µl of the highest dilution of the purified bacteriocin that yield a definite inhibitory zone.[6]

Adsorption assays of the bacteriocin PsVP-10.

The three bacterial species were grown in BHI broth (50 ml) at 37ºC until a concentration of 108 cfu/ml (O.D. at 600 nm about 0.6) was obtained. The cells were collected by centrifugation (4,000 g x 15 min), washed twice with sterile 5 mM phosphate buffer, pH 7.2 and then resuspended in the original volume with the same buffer. One ml of the Gram positive cellular suspension was mixed with 100 µl of the bacteriocin PsVP-10 (3,200 AU). The same experiment was carried out with the Gram negative cells (6.400 AU). Mixtures were incubated at 30ºC for 20 min and the cells were removed by centrifugation. The titre of the bacteriocin was determined in the supernatant. Controls were run with the bacteriocin plus 1 ml of buffer without the cells. The percentage of the bacteriocin adsorbed to cells was determined in relation to the control.

Preparation of cell walls and bacterial spheroplasts.

The three sensitive bacterial species were grown in 50 ml of BHI broth for 10 h at 37ºC (until the late exponential phase) according to the procedures described by Bhunia.[2] The cells were centrifuged at 4,000 g x 15 min and washed twice with 5 mM phosphate buffer pH 7.2. The cells were resuspended in 3 ml of the same buffer and 2 g of 0.2 mm diameter glass beads were added; the mixture was vigorously shaken for 3 min at 4ºC. The broken cells were separated from the glass beads by centrifugation (500 x g for 5 min) and the cell walls were collected by centrifugation at 12,000 x g for 20 min.

The spheroplasts of the different sensitive species were prepared according to Abriouel.[1] The cells were cultured in BHI broth in a shaker for 8 h, collected by centrifugation at 5,000 x g for 15 min and then washed twice with distilled water. The cells were resuspended in 100 µl of TSL solution (Tris-HCl 50 mM, pH 8,0, sucrose 20% and lysozime 2mg/ml) and incubated at 0ºC for 10 min. Then, 50 ml of EDTA 0,25 M were added. The suspension obtained, was diluted with 1,3 ml of Tris-HCl 50 mM pH 8,0 containing 9% of sucrose. Spheroplasts were collected by centrifugation (3,000 x g for 15 min) and resuspended in 8 ml of TSM (Tris-HCl 10 mM pH 7,6, sucrose 0,75 M and magnesium sulfate 20 mM). The spheroplast efficiency was determined to be greater than 90% on BHI broth without osmotic stabilizers for the three bacterial species. The spheroplast were treated with different bacteriocin PsVP-10 concentration and lysis was followed by turbidimetry (O.D. 620 nm).

Effect of temperature and pH on the adsorption of the bacteriocin PsVP-10.

The three studied bacterial species were incubated with the bacteriocin PsVP-10 in 5 mM phosphate buffer pH 7,0 and treated at different temperatures (5, 20, 40, 60 and 90ºC) for 10 min. The incubation mixture was centrifuged and the unbound bacteriocin was determined in the supernatant. The effect of the pH over the same bacterial species was studied in 50 mM phosphate buffer with a pH range between 2 to 8. The bacteriocin was added and incubated at 37ºC for 15 min. Then, the cells were centrifuged and the percentage of unbound bacteriocin was determined in the supernatant.

Action of detergents, organic solvents and enzymes on the adsorption of the bacteriocin PsVP-10 to bacterial cells and cell walls.

Cells and cell walls resuspended in 5 mM phosphate buffer pH 7,2 were treated with 1% sodium dodecyl sulfate and 2% Triton X-100 and incubated at 4ºC for 15 min. The samples were centrifuged and the pellets were washed three times with 5mM phosphate buffer pH 7,2. The bacteriocin was added and the adsorption of the bacteriocin was assayed as described before. The results were compared to controls of cells and cell walls untreated with the detergents.

Whole cells and cell walls were treated with methanol, ethanol and isopropanol (50% v/v) and incubated at 25ºC for 30 min. The solvents were removed by centrifugation and the pellets dried at 37ºC for 30 min. The dried pellets

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were resuspended in 5mM phosphate buffer pH 7.2 and mixed with the bacteriocin. The adsorption of the bacteriocin was determined as described before.

It was determined the effect of lysozime (100 µg/ml in Tris-HCl 0,025 M pH 7,0), protease XIV (2mg/ml in phosphate 5mM pH 7,2) and trypsin type III-S (2 mg/ml in 50 mM Tris-HCl, pH 8,0). All the enzymes were from Sigma. Each sample was incubated for 1 h at 37ºC. To remove the enzymes, the cells were washed three times with phosphate buffer 5 mM, pH 7.2. The bacteriocin was added and the determination of the bacteriocin adsorption follows the same protocol described above.

Preparation of resistant mutants to the bacteriocin.

Mutants were obtained for the three studies species. The cells were cultured in BHI agar with sub-inhibitory concentrations of the bacteriocin PsVP-10. Cells were transferred several times to different plates with the same agar, containing sub-inhibitory bacteriocin concentrations. The obtained micro-colonies were identified and then cultured in BHI broth. These mutants were used in the adsorption experiments with cells and cell walls and also in the spheroplasts preparation.

RESULTS AND DISCUSSION

The purification of the bacteriocin was performed as described in Material and Methods. 10 µl of a dilution 1:64 was the greatest dilution that produced a clear inhibition area for *S. typhimurium* and *S. flexneri* when the cells were grown in lawn. For these two species one arbitrary unit (AU) is 6.400 (1000/10 x 64). The same experimental procedure demonstrated for *E. faecalis* an AU of 3.200 (1000/10 x 32). These titres of bacteriocin were used in all the adsorption experiments.

Table 1 shows the adsorption percentages of the three bacterial species and their cell walls in comparison with the adsorption to the respective mutant cells and cell walls. No differences were observed in the adsorption to cells; however, a lower adsorption percentage was obtained with the cell walls. These results may be due to a lost or modification of some bacteriocin receptors during the preparation of cell walls. It is also important to observe that Gram positive cells present a higher bacteriocin adsorption in comparison to Gram negative cells. In these bacteria the peptidoglycan is smaller than in Gram positive bacteria, which could indicate a minor number of receptors for the PsVP-10 bacteriocin. In Table 1, it is also possible to observe important differences in the adsorption of the bacteriocin to sensible cells and resistant cells as well as their respective cell walls. Furthermore, no differences in the adsorption were obtained with mutant cells and mutant cell walls. The resistance to the bacteriocin PsVP-10 would be directly related with the absence of receptors in the cell wall which would avoid the adsorption and therefore the action of this antimicrobial substance. The great difference in the adsorption to sensible and resistant cells would suggest that the receptors related to the adsorption of the bacteriocin are located in the peptidoglycan of sensible cells. After the adsorption, translocation should occur in order to allow the bacteriocin to reach the cytoplasmic membrane where apparently it exert its lethal action independently of the resistance or susceptibility to the PsVP-10 bacteriocin. This is demonstrated in Fig.1 where it is clearly observed the bactericidal effect over spheroplast, obtained from sensitive and resistant cells. No significant differences are obtained in the death time.

With the purpose to have an aproximation to the chemical nature of the bacteriocin receptors as well as the position of these receptors in the envolve structures of all the studied bacterial species, the whole cells and their wall

Table 1. Adsorption percentage of the bacteriocin PsVP-10 to cells and cell walls of *S. typhimurium*, *S. flexneri* and *E. faecalis* and their respective mutants resistant to the bacteriocin.

	Adsorption %			
	Sensitive cells		Resistant cells	
Microorganisms	Cells	Cell walls	Cells	Cell walls
S. typhimurium	100	65	3	0
S. flexneri	100	65	2	0
E. faecalis	100	70	4	1

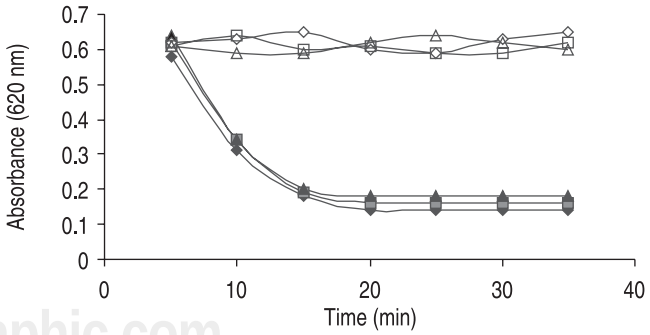

Figure 1. *Effect of the bacteriocin PsVP-10 over spheroplast obtained from* S. typhimurium (▲)*,* S. flexneri *(■) and* E. faecalis (◆). C*ontrols of spheroplast of* S. typhimurium *(△),* S. flexneri *(□) and* E. faecalis (◇) *withouth bacteriocin.*
Note: Identical results were observed in spheroplast obtained from mutant resistant cells.

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cells were treated with protelytic enzymes, organic solvents and detergents. It was also analysed the effect of the pH and temperature on the adsorption process.

Fig. 2 and 3 show the effect of pH and temperature on the adsorption capacity of the bacteriocin PsVP-10. Under both conditions, the three bacterial species have a similar behaviour and the greatest adsorption is produced at pH 7.2 and 37ºC, that correspond to the optimal growing conditions for this sensible bacterial specie. Table 2 shows the effect of organic solvents, detergents and enzymes over the adsorption capacity of the studied bacteriocin. It can be observed that the organic solvents reasonably affect the adsorption of the two Gram negative bacterial species, with no change in the adsorption for *E. faecalis*. These results may be due to the action of the organics solvents on the receptors for the bacteriocin which are located in the external membrane. The solvents probably modified the receptors and they lost their capacity of bacteriocin PsVP-10 adsorption. The detergents also present an inhibitory effect for the adsorption of the bacteriocin to the Gram negative cells, but the inhibition is lower than with organic solvents. The mechanism could also be by modifying the specific bacteriocin receptors avoiding the adsorption process. None of the chemical products or enzymes modified the adsorption process of the bacteriocin to *E. faecalis*. These results would support the idea that specific structures in the external membrane of Gram negative cells play a primary role in the adsorption process. It was interesting to observe that neither the chemical products nor the enzymes used in this study affected the bacteriocin adsorption capacity of cell walls.

In accordance with the results, the possible effect of bacteriocin PsVP-10 on sensitive cells could be explained as follows: the bacteriocin is adsorbed directly by the peptidoglycan receptors of Gram positive cells and translocated to the cytoplasm membrane where the lethal bactericidal effect is produced. In Gram negative cells the bacteriocin bind to receptors located in the external membrane and later the antimicrobial product reach the peptidoglycan and then it is translocated to cytoplasm membrane where it exert the bactericidal effect.

Further studies should be done to identify specific structures such as a peptidoglycan that would participate in the bacteriocin adsorption.

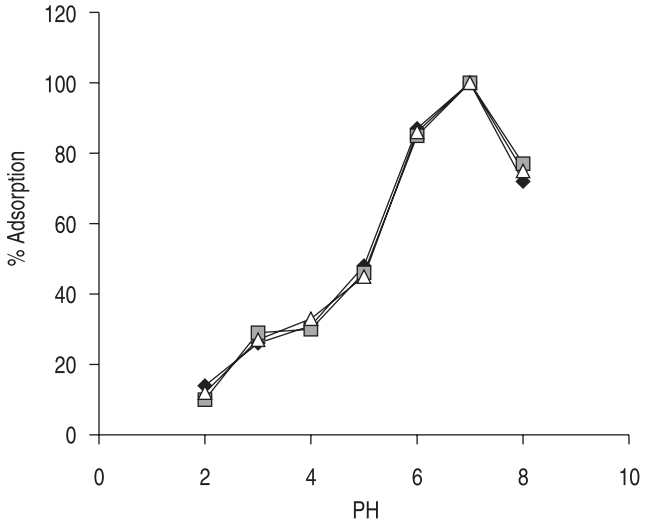

Figure 2. *Effect of pH over the bacteriocin PsVP-10 adsorption to* S. typhimurium *(△),* S. flexneri *(■)and* E. faecalis *(◆).*

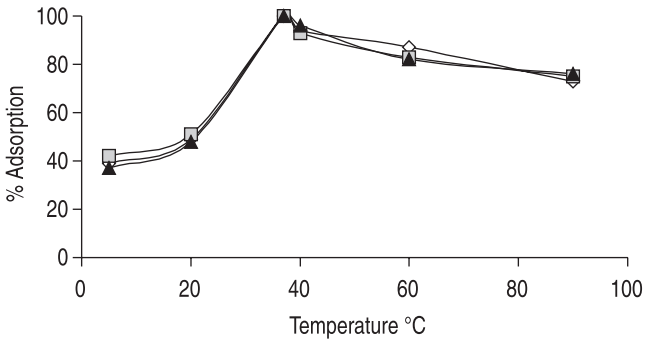

Figure 3 . *Effect of temperature on the adsorption of bacteriocin PsVP-10 to* S. typhimurium (▲), S. flexneri (□) *and* E. faecalis (◇).

Table 2. Effect of enzymes, organic solvents and detergents over the bacteriocin PsVP-10 adsorption to sensitive microorganisms and their cell walls.

Cells/cell walls	Adsorption % [1]SDS	[2]Trit.	Met.*	Iso.*	Eth.*	Lys.	Trip.	Prot.
S. typhimurium/cell wall	90/65	85/65	75/65	75/65	80/65	100/65	100/65	100/65
S. flexneri/cell wall	90/65	85/65	75/65	75/65	80/65	100/65	100/65	100/65
E. Faecalis/cell wall	100/70	100/70	100/70	100/70	100/70	100/70	100/70	100/70

[1] 1% final concentration [2] 2% final concentration * 50% Vol/Vol
SDS: sodium dodecyl sulfate, Trit: Triton X-100, Met: Methanol, Iso: isopropanol, Eth: ethanol, Lys: lysozime, Trip: trypsine type III-S, Prot: protease XIV.

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Correspondence to:

Prof. Carlos Padilla
Laboratorio de Investigación Microbiológica.
Universidad de Talca, Cas. 747, Talca, Chile.
E-mail: cpadilla@utalca.cl
fax: 56-71-200488.