Comparative Antimicrobial Activity of Two New Mutacins

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ABSTRACT

Objectives: *To compare the* in vitro *activity of mutacins D-123.1 and F-59.1 against different bacteria including antibiotic-resistant strains, in order to evaluate their application potential.*

Design and Methods: *The antibacterial activity spectrum of purified F-59.1 and the MIC and MBC of F-59.1 and D-123.1 against target bacteria were determined.*

Results: *Most bacteria were inhibited by the purified mutacins. Mutacin F-59.1 shows a relatively wide activity spectrum. Mutacin D-123.1 has low Minimum Inhibitory Concentrations [MICs] (0.25-4 µg/ml) against human pathogens while F-59.1 has higher MICs (3.2-12.8 µg/ml) mainly against food-borne pathogens.*

Conclusion: *The effectiveness of mutacins D-123.1 and F-59.1 against human and food-borne pathogens is demonstrated. Mutacin D-123.1 shows potential as a new antibiotic while F-59.1 shows promising application in food products.*

Abbreviations: *MALDI-TOF MS, matrix assisted laser desorption ionisation-time of flight mass spectrometry; MB(I)C, minimum bactericidal (inhibitory) concentrations; OD, optical density; RP-HPLC, reverse-phase high-pressure liquid chromatography; TSBYE, trypticase soy broth yeast extract.*

Keywords: Antibacterial peptides, bacteriocins, lantibiotics, MBC, MIC, mutacins

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Actividad Microbiana Comparativa de dos Nuevas Mutacinas

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RESUMEN

Objetivos: *Comparar la actividad* in vitro *de las mutacinas D-123.1 y F-59.1 frente a diferentes bacterias incluyendo las cepas resistentes a los antibióticos, a fin de evaluar el potencial de su aplicación.*

Diseño y Métodos: *Se determinó el espectro de actividad antibacteriana de F-59.1 purificada y la CIM y la CBM de F-59.1 y D-123.1 frente a determinadas bacterias.*

Resultados: *La mayor parte de las bacterias eran inhibidas por las mutacinas purificadas. La mutacina F-59.1 muestra un espectro de actividad relativamente amplio. La mutacina D-123.1 posee bajas concentraciones de inhibición mínimas [CIM] (0.25-4 µg/ml) contra los patógenos humanos, mientras que el F-59.1 posee concentraciones CIM más altas (3.2-12.8 µg/ml) principalmente frente a los patógenos alimentarios.*

Conclusión: *Queda demostrada la efectividad de las mutacinas D-123.1 y F-59.1 frente a los patógenos humanos y alimentarios. La mutacina D-123.1 muestra poseer un potencial como nuevo antibiótico, en tanto que F-59.1 se presenta como una aplicación promisoria en relación con los productos alimentarios.*

Abreviaturas: *MALDI-TOF MS, espectrometría de masas con desorción/ionización mediante láser asistida por matriz asociada a un analizador de tiempo de vuelo (del inglés: matrix assisted laser*

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desorption ionisation-time de flight mass spectrometry). CIM: concentración inhibitoria mínima (inglés MIC). CBM: concentración bactericida mínima (inglés MBC). DO: densidad óptica (inglés OD); RP-HPLC: cromatografía líquida de alta resolución en fase revertida; TSBYE:caldo tripticasa soya- extracto de levadura.

Palabras claves: Péptidos antibacterianos; bacteriocinas; lantibióticos, CBM. CIM, mutacinas.

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INTRODUCTION

The increase and rapid spread of antibiotic resistant bacteria require the development of new antibacterial substances. Bacteriocins are antibacterial peptides produced by bacteria (1−3). Mutacins are bacteriocins produced by *Streptococcus mutans*, an indigenous human oral bacterium (4, 5).

We recently developed improved methods of production, detection and purification of mutacins (6, 7) to characterise new antimicrobial substances. The purpose of this study was to compare the efficiency of two mutacins against various bacteria including clinical pathogens with antibiotic resistance and bacteriocin-resistant food-borne pathogens and spoilage bacteria. The potential applications for these mutacins are better understood from these results.

MATERIAL AND METHODS

Bacterial strains

Streptococcus mutans 123.1 and 59.1 produce mutacins D-123.1 and F-59.1 respectively (8). Tested bacteria are listed in Table 1. Multi-drug resistant clinical isolates are described in Morency *et al* (8) and Mota-Meira *et al* (10).

Mutacin production and purification

Mutacin F-59.1 was produced as previously described (6). *Streptococcus mutans* 123.1 was grown in 4 liters of Trypticase Soy Broth Yeast Extract [TSBYE] (Difco) containing 0.5% agarose (Difco) for 72-hours at 37°C. The culture was scraped, aliquoted into centrifuge bottles and frozen overnight at −20°C. The bottles were centrifuged at 4000 × g for 60 minutes and 8000 × g for 30 minutes at room temperature. The supernatant was filtered through glass fiber and Whatman no. 1 filter paper to remove agarose fines and stored at 4°C. Mutacins were purified by hydrophobic chromatography as previously described (7) using hydrochloric acid (9). The purity of mutacins was evaluated by Matrix Assisted Laser Desorption Ionisation-time of Flight Mass Spectrometry [MALDI-TOF MS] (7).

Antimicrobial activity spectrum of mutacin F-59.1

Overnight cultures of target strains were diluted 1:100 in fresh TSBYE and mixed 1:1 with purified mutacin F-59.1 in microtiter plates (Falcon Microtest III tissue culture plates 3072; Becton Dickinson Laboratories, Franklin Lakes, NJ). The activity spectrum of mutacin D-123.1 was not evaluated due to the low amount of pure substance available. Incubation was performed at 37°C for 24 hours. Growth inhibition was compared to a positive control (without mutacin) and a negative control (non-inoculated media). Viability was assessed by spreading an inoculum from the wells where no growth was observed, on a blood agar plate to differentiate between bacteriolytic and bacteriostatic activity.

In vitro activity determination

Minimum bactericidal inhibitory concentrations were determined as described previously (10, 11). Stock solutions consisted of 0.16 mg/ml of mutacin D-123.1 and 0.64 mg/ml of mutacin F-59.1 in deionized distilled water as determined using the BioRad DC protein assay (BioRad, Mississauga, ON, Canada).

Two-fold dilutions of the working solution were prepared in microtiter plates, the wells finally containing 100 µl of TSBYE with or without inhibitor. Twenty-five microliters of optical density (OD) standardized bacterial suspension (10) were added to each well. The plates were incubated under the conditions appropriate for the tested strain until the control reached stationary phase. The OD was measured at 630 nm with a microplate reader (model MR 5000/7000; Dynatech Laboratories Inc, Chantilly, Va.). The blank was the uninoculated medium incubated under the same conditions. The MIC was calculated from the highest dilution showing complete inhibition of the tested strain (OD equal to OD of the blank). The MBC was determined by plating 10 µl from test wells (≥ MIC) on blood agar plates, incubated for at least 24 hours at 37°C.

Haemolytic activity

The haemolytic activity of purified mutacins F-59.1 and D-123.1 was tested as described previously (10).

Thermostability and proteolytic enzyme sensitivity

Thermostability was assayed by determining the residual activity of pure mutacin samples placed in boiling water up to 1 hour and after autoclaving. Active pure fractions were mixed (1:1) with proteinase K (EC 3.4.21.14), pronase E (EC 3.4.24.31), trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1) [Sigma, St Louis, USA] in phosphate sodium buffer at pH 7.2 and incubated overnight at 37°C. The residual activity of mutacins was evaluated by the spot test (6). Untreated mutacins in buffer were used as controls.

RESULTS

None of the mutacins showed haemolytic activity against sheep erythrocytes. The activity of mutacin F-59.1 was not reduced in boiling water (100°C, 1 hour) or after autoclaving (121°C, 15 minutes) while activity of mutacin D-123.1 was respectively reduced to 20% and 30%. Activity of mutacins was preserved after storage at 4°C for three months. Pure mutacins were sensitive to pronase E, proteinase K, trypsin and α-chymotrypsin.

Mutacin F-59.1 shows a broad antimicrobial spectrum (Table 1) having a bactericidal effect on *Bacillus spp* and *Clostridium spp* but no activity against *Enterococcus spp* or

Table 1: Activity spectrum of mutacin F-59.1.

Micro-organisms (no. of isolates)[a]	Visual OD[b]	Sensitivity[c]	Viability[d]	Effect[e]
Bacillus cereus ATCC 2	–	S	–	cidal
B coagulans ATCC 7090	–	S	–	cidal
B subtilis				
ATCC 6051	–	S	+/-	intermediate
ATCC 6633	–	S	–	cidal
B thuringiensis ATCC 33679	–	S	–	cidal
Clostridium butyricum ATCC 8260	–	S	–	cidal
C bifermentans 2D1.04	–	S	–	cidal
C sporogenes ATCC 29404	–	S	–	cidal
Enterococcus durans ATCC 6056	+	R	+	none
E faecalis (4)	+	R	+	none
E faecium ATCC 19434	+	R	+	none
E hirae ATCC 8043	+	R	+	none
Lactobacillus pentosus ATCC 8040	–	S	–	cidal
L plantarum ATCC 14917	–	S	+	static
Lactococcus lactis (2)	–	S	–	cidal
L lactis subsp. *lactis* MJC 15	–	S	+	static
Leuconostoc mesenteroides ATCC 23386	–	S	–	cidal
Listeria grayi ATCC 19120	–	S	–	cidal
L innocua HPB 13	–	S	+	static
L ivanovii HPB 28	–	S	+/-	intermediate
L monocytogenes				
ATCC 15313	–	S	–	cidal
ATCC 35152	–	S	+	static
FRDC (#1089/88171/8853/8856)	–	S	+	static
Scott A ATCC 700301	+/-	I	+	static
Scott A ATCC (700302/HPB 3)	–	S	+	static
serotype 3 ATCC 19113	–	S	–	cidal
L murrayi (2)	–	S	–	cidal
Pediococcus acidilactici ATCC 33314	–	S	–	cidal
Staphylococcus aureus (5)	+	R	+	none
S aureus R629	+/-	I	+	static
S carnosus	–	S	+/-	intermediate
S epidermidis DSM 3095	+	R	+	none
Streptococcus equi ATCC 9528	–	S	+	static
S mutans (24)	–	S	–	cidal
S pneumoniae ATCC 6303	–	S	+	static
S pyogenes ATCC 10389	+/-	I	+	static
S salivarius ATCC 13419	–	S	+	static
S sobrinus (2)	–	S	–	cidal
S suis serotype 2	+	R	+	none
S thermophilus LM-17	–	S	–	cidal
S vestibularis ATCC 49124	–	S	–	cidal

ATCC: American Type Culture Collection (Manassas, USA).
HPB: Health Protection Branch (Health Canada, Ottawa, Ontario, Canada).
DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschwig, Germany).
LSPQ: Laboratoire de Santé Publique du Québec (Ste Anne de Bellevue, Québec, Canada).
FRDC: Food research and development center (Agriculture and Agrifood Canada, St Hyacinthe, Québec, Canada).

[a] isolates from Morency et al (8) and Mota-Meira *et al* (10).
[b] (+) growth; (-) no growth.
[c] S: sensitive; R: resistant; I: intermediate.
[d] (+) growth; (-) no growth. (+/-) presences of resistant clones.
[e] cidal: bactericiodal; static: bacteriostatic.

Staphylococcus aureus. It was bacteriocidal for most lactic acid bacteria including *S mutans* and *S sobrinus*, two species causing dental caries. It was bacteriostatic for *Listeria* spp, including nisin-resistant mutants, and the streptococcal pathogens *S pneumonia* and *S pyogenes*.

Mutacin D-123.1 showed lower MICs than mutacin F-59.1 against all the bacterial strains tested (Table 2).

Table 2: Minimum inhibitory concentrations and minimum bactericidal concentrations of mutacins.

Micro-organisms	Mutacin F-59.1		Mutacin D-123.1	
	MIC[a]	MBC[a]	MIC	MBC
Bacillus cereus ATCC 2	6.4	12.8	2	> 4
Bacillus subtilis ATCC 6051	12.8	n.d.[b]	2	> 4
Enterococcus faecalis				
ATCC 27275	12.8	n.d.	2	4
ATCC 29212	12.8	n.d.	2	4
Enterococcus faecium ATCC 19454	6.4	n.d.	2	4
Enterococcus hirae ATCC 8043	12.8	n.d.	2	4
Listeria monocytogenes				
ATCC 15313	6.4	6.4	0.5	1
ATCC 19113	3.2	3.2	0.25	1
FRDC 1039	6.4	6.4	1	2
FRDC 88171	6.4	6.4	1	2
Scott A ATCC 700301	6.4	12.8	1	2
Scott A ATCC 700302	6.4	n.d.	1	2
Listeria murrayi				
ATCC 25420	3.2	6.4	0.5	2
HPB 30	3.2	3.2	0.5	2
Listeria ivanovii HPB 28	6.4	12.8	0.5	2
Listeria grayi ATCC 19120	3.2	6.4	1	2
Micrococcus luteus ATCC 272	0.8	0.8	0.25	0.5
Staphylococcus aureus				
ATCC 25923	6.4	n.d.	> 4	> 4
ATCC 43300	6.4	n.d.	> 4	> 4
R621	6.4	n.d.	> 4	> 4
Staphylococcus carnosus	3.2	3.2	0.25	0.5
Streptococcus pyogenes ATCC 10384	12.8	n.d.	2	4
Streptococcus salivarius ATCC 13419	3.2	6.4	2	4

[a] MIC and MBC are in µg/ml.

[b] n.d.: not determined.

Minimum Inhibitory Concentrations for mutacin D-123.1 were lower or equal to 2 µg/ml except for *S aureus* strains (> 4 µg/ml). It was very active against the nisin-resistant mutants obtained from *L monocytogenes* (Scott A strains). Mutacin F-59.1 showed MICs between 3.2−12.8 µg/ml. *Listeria* spp, *B cereus* and *Staphylococcus* spp, spoilage and food-borne bacteria were the most sensitive while *Enterococcus* spp, *B subtilis* and *S pyogenes* were less sensitive.

DISCUSSION

Nisin is actually the only lantibiotic bacteriocin used as a food biopreservative and pediocin-like bacteriocins are strongly considered as the next to be approved (1). Nisin- and pediocin-resistant mutants can appear which cause some concern regarding the use of these peptides, while resistant mutants against mutacins have not been reported (12−14). Mutacins D-123.1 and F-59.1 are active against most Gram-positive food-borne and human pathogens and do not appear to be haemolytic. These peptides are sensitive to proteolytic enzymes from the digestive tract and resist heat treatments conventionally found in food processes. Mutacin F-59.1 targets lactic acid bacteria, some of which are undesirable in food products and is active against the spoilage and food-borne pathogens *B cereus*, *C sporogenes* and *Listeria* spp, including bacteriocin-resistant strains (13). Mutacin D-123.1 also presents great activity against *Bacillus spp* and *Listeria* spp. A combination of mutacins could be used as a biopreservative to preclude or delay resistance development, especially if they act by different mechanisms against target cells.

The poor activity of mutacin F-59.1 against *Enterococcus* spp and *S aureus* makes it inappropriate for medical applications. Interest in mutacin D-123.1 resides in its activity against multi-drug resistant enterococci and *S aureus*. Minimum Inhibitory Concentrations are in the range (micromolar) observed for the lantibiotic mutacin B-Ny266 and other promising lantibiotics (10, 15).

Properties of mutacins make them attractive as new antibiotic molecules. *S mutans* are naturally found in the human mouth, so mutacins appear to be safe for human consumption. Mutacin D-123.1 has potential applications for controlling food-borne pathogens and spoilage bacteria or as an antibiotic for clinical use against drug resistant bacteria. Mutacin F-59.1 is preferable for food products. More research is needed in order to better assess their toxicity, *in vivo* activity and intrinsic activity in food products.

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