Identification of Bacteriocin linocin M18 from Brevibacterium and Related Genera using PCR

التشخيص الجزيئي لـ M18 لينوسين المنتج من جنس Brevibacterium والاجناس المشابهة لها بأستخدام الـPCR

Essra Gh. Al-Sammak
College of Science/ University of Mosul
اسراء غانم حازم السماك
كلية العلوم/جامعة الموصل

Abstract

Fifty bacterial isolates isolated from dairy product, skin and blood from cancer and kidney failure dialysis patients were identified to twenty two species and the following genera:- Brevibacterium, Corynebacterium, Arthrobacter, Actinomyces, Exiguobacterium, Kocuria, Micrococcus, Rothia, Rhodococcus using a set of phenetic characteristics. Twelve isolates of the different species from the genera Brevibacterium, Arthrobacter, Corynebacterium, Kocuria, Rhodococcus, Rothia were selected and probed for lin gene by polymerase chain reaction. One species *Kocuria rhizophila* which inhibited most of the tested organisms did not have lin gene in the chromosome, while, the species *Corynebacterium glucuronolyticum*, *Arthrobacter comminsii*, *Arthrobacter oxydans* have the lin gene. Our results found there wide distribution of the structural gene encoding this linocin M18 within coryneform bacteria and also in the genus Kocuria.

Key words: brevibacterium, linocin M18, lin gene.

المستخلص

عزلت 50 عزلة من منتجات الالبان ، الجلد والدم لمرضى السرطان والديلزة الدموية والتي شخصت الى 22 نوع تابع للاجناس الاتية: Brevibacterium, Corynebacterium, Arthrobacter, Actinomyces, Exiguobacterium Kocuria, Micrococcus, Rothia, Rhodococcus اعتمادا على العديد من الصفات المظهرية. انتخبت 12 عزلة تابعة للاجناس Brevibacterium, Arthrobacter, Corynebacterium, Kocuria, Rhodococcus, Rothia للتحري عن امتلاكها جين lin بأستخدام PCR. اظهر النوع *Kocuria rhizophila* فعالية تثبيطية عالية ضد اغلب بكتريا الاختبار المستخدمة في حين انها لاتمتلك الجين lin في حين امتلكت *الانواع* , *Corynebacterium glucuronolyticum* *Arthrobacter comminsii, Arthrobacter oxydans* هذا الجين. من هذا نستنتج الانتشار الواسع للجين ضمن مجموعة البكتريا الشبيهة بالكورايني وكذلك ضمن جنس Kocuria .

الكلمات المفتاحية: Brevibacterium، M18لينوسين، الجين lin

Introduction

Brevibacterium spp. are gram-positive irregular, slender, rod-shaped bacteria that display a marked rod- to-coccus cycle, non-acid-fast. The peptidoglycan contains meso-DAP as the principal diamino acid [1]. They are obligately aerobic and oxidative in their metabolism of carbohydrates. They are non-motile and salt-tolerant > 6.5% NaCl. They also produce catalase and proteinases and characteristically produce methanethiol CH3SH from L-methionine [2].

The habitat of the Brevibacterium is primarily milk products, in which the bacteria contribute to the aroma and the color toes and other intertriginous areas and are believed to contribute to body odor, but are rare causes of human infections as opportunistic agents [2].

The genus Brevibacterium belongs to family Brevibacteriaceae, order Actinomycetales which comprises more than 20 species [3].

Microbes produce an extraordinary array of microbial defense systems. These include broad-spectrum classical antibiotics, metabolic byproducts, such as the lactic acids produced by lactobacilli, lytic agents such as lysozymes, numerous types of protein exotoxins, and bacteriocins, which are loosely defined as biologically active protein moieties with a bactericidal mode of action.

This biological arsenal is striking not only in its diversity, but also in its natural abundance. Bacteriocins are found in almost every bacterial species examined to date, and within a species. Tens or even hundreds of different kinds of bacteriocins are present and 99% of all bacteria may make at least one kind of bacteriocin [4].

The last decade has witnessed the description and characterization of a variety of bacteriocins from gram-positive bacteria. Most of these are produced by lactic acid bacteria with activity spectrums usually restricted to closely related strains [5]. The extraordinary interest in bacteriocins is based primarily on their potential to inhibit food-borne pathogens such as Listeria monocytogenes [6].

Isolation and characterization of non-lanthionine- containing linocin M18 from red smear cheese bacteria B. linens M18 were carriedout by Valde's- Stauber, et al. [7].

Usually, bacteriocins inhibit only closely related bacteria, but linocin M18 exhibited an extraordinarily broad activity spectrum with activity against species of the genera Bacillus, Arthrobacter, Corynebactrium, Micrococcus and Listeria. Oligonucleotide probes based on the N-terminal amino acid sequence have been used to locate the gene coding for linocin M18 Which is a single copy of the gene lin, and was located on chromosomal DNA [8]. So the aims of the study are as follows:

1. Detection of Brevibacterium and related genera ability to produce linocin M18 bacteriocin against some gram positive bacteria and yeast.
2. Detection of the lin gene that is responsible for the production of linocin M18 bacteriocin using conventional PCR technique.

Materials and Methods

Sample Collection

Fifty bacterial isolates were isolated from dairy product, skin and blood from cancer and kidney failure dialysis patients. The isolates were identified and classified numerically using a set of 52 phenetic characteristics such as gram staining, cellular morphology, colonial morphology, pigmentation and rod-coccus cycle, the production of many enzymes, the ability to grow at different temperatures and concentration of NaCl in addition to their ability to oxidizing different CHO [2, 9, 10] and sensitivity to antibiotics [11]. Other tests were also carried out.

Bacteriocin Assay

Bacteriocin activity was carried out according to Al- Sammak [12] by culturing the isolated species in a straight line in the center of the media supplement with modified yeast extract agar with 2% NaCl. They were incubated at 37 °C or 25 °C depending on the species for 2 days and then cultured 8 of the test bacteria and yeast in a right angle 90° to the first line from each side and incubated in 37 °C to 24 hrs [13].

DNA Isolation

Selection of species from some clusters were carried out to detect the bacteriocin producing lin gene. The B. linens and other coryneform actinobacteria were grown in modified yeast extract agar. Total DNA isolation was done by genomic DNA purification from Promega company. Gene amplification by PCR was carried out using Promega standard kit as follows:

D.W	**10 µL**	**—**
Green Master Mix	**5 µL**	**1X**
Primer	**5µL**	**25 pmol**
DNA template	**5 µL**	**250 ng**
Total 25 µL		

The sequencing of primer is: 5′-CGACGACAGCCTCGGCATC-3′ upstream and 5′-GGCGGAGAAGCTGTCCTGG-3′ downstream, DNA thermocycler was programmed as follows:

1 cycle	**94 ◦C**	**5 min**
	94 ◦C	**1 min**
35 cycle	**68 ◦C**	**45 sec**
	72 ◦C	**1 min**

Amplification products were detected by 2% agarose gel electrophoresis for 120 min. The size of the amplified gene was determined by using 100 bp DNA ladder from Promega Company.

Results and Discussion

Conventional identification using phenotypic descriptions identified the isolates to nine genera: Brevibacterium, Corynebacterium, Arthrobacter, Actinomyces, Exiguobacterium, Kocuria, Micrococcus, Rothia, Rhodococcus & 22 species, which showed different ability to produce bacteriocin against some gram positive bacteria and yeast as summarized in Table (1).

Taxonomic distribution of lin gene: 12 isolates of different species of the genera Brevibacterium, Arthrobacter, Corynebacterium, Kocuria, Rhodococcus, Rothia selected and were probed for lin gene by PCR. One isolate belonged to Kocuria rhizophila which inhibited most test organisms as in the Fig. 1 did not have lin gene in the chromosome as in the Figure (2).

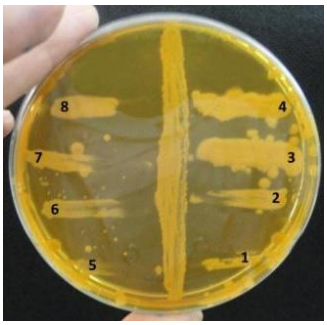

Fig. (2): Amplification of lin gene by using PCR

1. Kocuria rhizophila1; 2. Rhodococcus equi; 3. Corynebacterium glucuronolyticum; 4. B. linens1; 5. K. rosea●; 6. Arthrobacter cumminsii; 7. B. otitidis; 8. Kocuria rhizophila2; 9. Arthrobacter oxydans; 10. Rothia mucilaginosa; 11. Kocuria rhizophila3; 12. B. linens2production of bacteriocin.

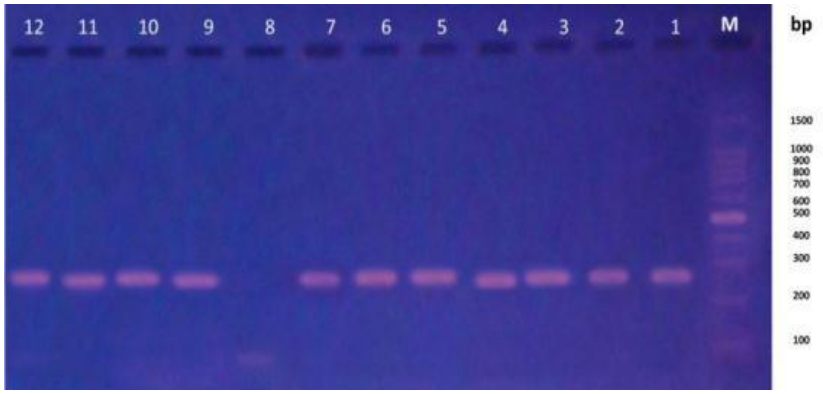

Fig. (1): Production of bacteriocin by Kocuria rhizophila against the following species.

1. Rhodococcus equi; 2. Kocuria rhizophila 1; 3. Yeast; 4. Rothia mucilaginosa; 5. Arthrobacter oxydans; 6. B. linens 1; 7. B. linens 2; 8. Kocuria rhizophila 2.

This discrepancy may be due to the production of any antagonistic substance, perhaps another bacteriocin. A few mutations at the positions of the PCR primers used may also lead to negative amplification of lin gene [8].

While the species Corynebacterium glucuronolyticum, Arthrobacter comminsii, Arthrobacter oxydans did not showed any activity against the tested organism but the presence of the lin gene in the 266 MW band did not correlated with the detection of linocin M18 bacteriocin as shown by PCR [8]. Amplification of lin gene without demonstration of bacteriocin activity was not surprising because low levels of bacteriocin production and the optimal conditions for bacteriocin production were unknown. Our results found there a wide distribution of the structural gene encoding this

linocin M18 within coryneform bacteria as in the study of Refs. [8, 14] and also in the genus Kocuria in our study.

Bacteriocins are ribosomally synthesized antimicrobial peptides that are not lethal to producer cells. These peptides are generally active against species closely related to the producer microorgainsms. Many factors affect the production of bacteriocins, namely the composition of the media NaCl, pH and temperature [15].

Table(1): The ability of Brevibacterium and related genera to produce bacteriocin

Species	Isolation source	Number of isolates	Production of bacteriocin against							
			Kocuria rhizophila2	Kocuria rhizophila1	Brevibacterium linens2	Brevibacterium linens1	Arthrobacter oxydans	Rothia mucilaginosa	Rhodococcus equi
Brevibacterium linens	Dairy products	7	1	0	1	1	0	1	1
Brevibacterium iodinum	Dairy products	4	0	0	0	0	0	0	1
Brevibacterium epidermidis	Dairy products & blood	7	0	0	0	2	0	0	1
Brevibacterium sanguinis	blood	1	0	0	0	0	0	0	0
Brevibacterium paucivorans	Dairy products& blood	2	0	0	0	0	0	0	0
Brevibacterium otitidis	Dairy products	1	1	0	0	0	0	0	1
Brevibacterium casei	Skin	1	0	0	0	0	0	0	0
Corynebacterium spp.	Skin &Dairy products	4	1	0	1	1	1	1	1
Corynebacterium pseudotuburculosis	Dairy products	2	1	0	0	1	0	0	0
Corynebacterium glucuronolyticum	Dairy products	3	1	0	0	0	0	0	0
Corynebacterium amycolatum	Dairy products	1	0	0	0	0	1	0	1
Corynebacterium pseudodiphtheriticum	Blood	1	0	0	0	0	0	0	0
Arthrobacter oxydans	Dairy products	1	0	0	0	0	0	0	0
Arthrobacter cumminsii	Skin	1	0	0	0	0	0	0	0
Actinomyces denticolens	Dairy products	1	0	0	1	1	0	0	0
Exiguobacterium acetylicum	Dairy products	1	0	0	0	1	0	0	0
Kocuria rosea	Dairy products	2	1	1	1	2	1	1	1
Kocuria rhizophila	Dairy products & skin	4	2	2	1	1	1	1	3
Micrococcus antarcticus	Dairy products	1	1	1	1	1	0	0	0
Rothia mucilaginosa	Dairy products	2	1	2	2	2	1	1	2
Rhodococcus rhodochrous	Dairy products	1	0	0	0	0	0	0	0
Rhodococcus rhodnii	Dairy products	1	0	0	0	0	0	0	0
Rhodococcus equi	Dairy products	1	1	0	1	1	1	1	0

	Species	Brevibacterium linens	Brevibacterium iodinum	Brevibacterium epidermidis	Brevibacterium sanguinis	Brevibacterium paucivorans	Brevibacterium otitidis	Brevibacterium casei	Corynebacterium spp.	Corynebacterium pseudotuburculosis	Corynebacterium glucuronolyticum	Corynebacterium amycolatum	Corynebacterium pseudodiphtheriticum	Arthrobacter oxydans	Arthrobacter cumminsii	Actinomyces denticolens	Exiguobacterium acetylicum	Kocuria rosea	Kocuria rhizophila	Micrococcus antarcticus	Rothia mucilaginosa	Rhodococcus rhodochrous	Rhodococcus rhodnii	Rhodococcus equi
	Isolation source	Dairy products	Dairy products	Dairy products & blood	blood	Dairy products& blood	Dairy products	Skin	Skin &Dairy products	Dairy products	Dairy products	Dairy products	Blood	Dairy products	Skin	Dairy products	Dairy products	Dairy products	Dairy products & skin	Dairy products	Dairy products	Dairy products	Dairy products	Dairy products
	Yeast	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	1	2	0	0	100

Conclusion

1. Isolate belonged to Kocuria rhizophila which inhibited most tested organisms did not have lin gene. This discrepancy may be due to the production of any antagonistic substance, perhaps another bacteriocin.
2. Amplification of lin gene without demonstration of bacteriocin activity in some species may be because of low levels of bacteriocin production and the optimal conditions for bacteriocin production are unknown.

References

1. Forbes, B.A., Sahm, D. F. and Weissfeld, A.S. (2007). Bailey and Scott's Diagnostic Microbiology, 12th ed., Mosby Elsevier.
2. Winn,W., Allen, S., Janda, W., Koneman, E., Procop, G. and Schreckemberger, P. et al. (2006). Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th ed., Lippincott Williams and Wilkins.
3. Kampfer, P., Schafer, J., Lodders, N. and Busse, H. (2009). Brevibacterium sandarakinum sp. nov. isolated from awall of an indoor environment, Int. J. Syst. Evol. Microbiol. 60 (4) 909-913.
4. Riley, M.A. and Chavan, M.A. (2007). Bacteriocins: Ecology and Evolution, Springer- verlag Berlin Heidelberg.
5. Bilkova, A., Sepova, H.K., Bilka, F. and Balazova, A. (2011). Bacteriocins produced by lactic acid bacteria, Ceska. Slov. Farm. 60 (2) 65-72.
6. Onraedt, A., Soetaert,W. and Vandamme, E. (2005). Industerial importance of the genus Brevibacterium, Biotechnology Letters. 27 527-532.
7. Valde's-Stauber, N. and Scherer, S. (1994). Isolation and characterization of linocin M18, Abacteriocin produced by Brevibacterium linens, Appl. Environ. Microbiol. 60 (10) 3809-3814.
8. Valde's-Stauber, N. and Scherer, S. (1996). Nucleotide sequence and taxonomical distribution of the bacteriocin gene lin cloned from Brevibacterium linens M18, Appl. Environ. Microbiol. 62 (4) 1283-1286.
9. Wauters, G., Charlier, J., Janssens, M. and Delmee, M. (2001). Brevibacterium paucivorans sp. nov., from human clinical specimens, International Journal of Systematic and Evolutionary Microbiology. 51:1703-1707.
10. Funke, G., Von Graevenitz, A., Glarridge, J.E. and Betnard, K.A. (1997). Clinical Microbiology Reviews, Vol. 10, American Society for Microbiology.
11. Vandepitte, J., Engaek, K., Piot, P. and Henuck, C.C. (1991). Basic laboratory procedures in clinical bacteriology, World Health Organization, Geneva. pp. 84-95.

12. Ogunbanuwo, S.T., Sanni, A.I. and Onilude, A.A. (2003). Influense of cultural conditions on the production of bacteriocin by Lactobacillus brevis OGI, African Journal of Biotechnology. 2 (7) 179-184.

13. Al- Sammak, E. Gh. (2006). Taxonomic study of Actinomycetes group, Ph.D. Thsis, Mosul, Iraq.

14. Eppert, I., Voldes- Stauber, N., Gotz, H., Busse, M. and Scherer, S. (1997). Growth reduction of Listeria spp. caused by undefined industrial red smear cheese cultures and bacteriocin-producing Brevibacterium linens as evaluated insist on soft cheese, Appl. Environ. Microbiol. 63: 4812-4817.

15. Motta, A.S. and Brandelli, A. (2003). Influence of growth conditions on bacteriocin production by Brevibacterium linens, Appl. Microbiol. Biotechnol. 62: 163-167.