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BACTERIOCIN-LIKE ACTIVITY OF ORAL *FUSOBACTERIUM NUCLEATUM* ISOLATED FROM HUMAN AND NON-HUMAN PRIMATES

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ABSTRACT

Fusobacterium nucleatum is indigenous of the human oral cavity and has been involved in different infectious processes. The production of bacteriocin-like substances may be important in regulation of bacterial microbiota in oral cavity. The ability to produce bacteriocin-like substances by 80 oral *F. nucleatum* isolates obtained from periodontal patients, healthy individuals and *Cebus apella* monkeys, was examinated. 17.5% of all tested isolates showed auto-antagonism and 78.8% iso- or hetero-antagonism. No isolate from monkey was capable to produce auto-inhibition. In this study, the antagonistic substances production was variable in all tested isolates. Most of the *F. nucleatum* showed antagonistic activity against tested reference strains. These data suggest a possible participation of these substances on the oral microbial ecology in humans and animals. However, the role of bacteriocins in regulating dental plaque microbiota *in vivo* is discussed.

Key words: *Fusobacterium nucleatum*, bacteriocin-like substance, oral bacteria.

INTRODUCTION

Anaerobic bacteria comprise a large percentage of the oral and gut indigenous microbiota (9). Some anaerobic bacteria possess several potentially pathogenic factors, particularly Gram-negative rods which appear to be present in several anaerobic infections, such as periodontal diseases (23).

Fusobaterium nucleatum is indigenous of the human oral cavity and has been involved in several infectious processes such as, sinusitis, osteomyelitis, brain or liver abscesses (3, 9, 14,17). Also, this organism constitutes a considerable part of the subgingival microbiota of gingivitis in children and adults and of periodontitis in juveniles and adults (18).

The microbial composition of the established dental plaque may be controlled by nutrient requirements or production of antagonistic substances (6). However, the nature of the inhibitory substances is still a matter of discussion. Microbial antagonistic substances were first studied by Gratia and Fredericq (11), who showed the iso-inhibitory activity produced by *Enterobacteriaceae.* These colicin-like substances were called bacteriocins (13). The knowledge of bacteriocin production has been extended to Gram-positive and Gram-negative aerobes, facultative and strict anaerobes.

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From an ecological viewpoint, there are few studies concerning production of antagonistic substances, such as bacteriocins, in Gram-negative anaerobes indigenous to the human microbiota, particularly, in oral *F. nucleatum* from human and animal origin. However, the production of metabolic products such as bacteriocin-like substances may be important in ecological processes to the colonization of periodontal tissues by *F. nucleatum* or other members from human indigenous microbiota (5).

In this study, the production of bacteriocin-like substances by *F. nucleatum* isolated from humans and *Cebus apella* monkeys is reported. The monkeys were used because of anatomic similarities with the human oral cavity.

MATERIALS AND METHODS

Microorganisms

A total of 80 oral *F. nucleatum* isolates were tested for their bacteriocinogenic activity. Fourty-nine isolates were obtained from 30 patients with adult periodontitis (age range 18 - 40 years), with clinical and radiographic evidence of periodontal disease, including pockets of depth equal or exceeding 5 mm, 21 isolates from 20 healthy individuals (age range 20 - 30 years) from the Clinic of Periodontology, University of São Paulo, SP, Brazil, and 10 isolates were from 10 *Cebus apella* monkeys without evidence of periodontal disease (Núcleo de Procriação do Macaco Prego, São Paulo, SP, Brazil). Subgingival bacterial samples were taken using sterilized absorbent paper points (Dentsply Ind. & Co. Ltda., Rio de Janeiro, RJ, Brazil), which were introduced into the periodontal pocket or gingival sulcus for 60 s and then transferred to tubes containing 2.0 ml of Ringer-PRAS solution, pH 7.2, under CO_2 flux (22). The bacterial isolation and identification to the species level was performed by using Omata & Disraely selective agar as described previously (1, 10) and by using conventional biochemical tests (12, 24).

Bacteriocinogenic activity

The isolates were tested for their inhibitory activity against themselves and against *F. nucleatum* ATCC 10953, *F. nucleatum* ATCC 25586, *Bacteroides fragilis* ATCC 23745, *Eubacterium lentum* ATCC 25559 and *Peptostreptococcus anaerobius* ATCC 27337. The bacteriocinogenicity was determined by using a double layer method (4). The bacterial inoculum was prepared in brain-heart infusion broth (BHI, Difco) supplemented with 0.5% extract yeast (12). 10 ml of 24 h cultures were inoculated in 5 equidistant spots onto BHI agar (BHI-A), using a standard platinum loop. Plates were incubated at 37°C for 72 h under anaerobic conditions (10% CO_2/ 90% N_2). After incubation, the cells were killed by exposure to chloroform for 30 min (8). Residual chloroform was allowed to evaporate and BHI-A plates were overlayed with 3.5 ml of BHI-soft agar (0.7%) inoculated with 0.1 ml of a 48 h culture of each indicator. After 48 h of incubation under anaerobic conditions, the plates were evaluated for the presence of bacteriocin-like substances. The inhibitory halo was measured. Experiments were done in duplicate.

Test for the presence of bacteriophages

A piece (3.0 mm in diameter) of agar at the inhibitory halo was removed aseptically (26). This block of agar was homogeneized, placed onto BHI-A and overlayed with 3.0 ml of BHI-soft agar inoculated with 0.1 ml of the indicator culture. After 48 h of incubation under anaerobic conditions, the plates were evaluated for the presence or absence of lytic zones. Tests were done in duplicate.

RESULTS

Table 1 shows the inhibitory activity of 80 oral *F. nucleatum* isolates recovered from periodontal patients, healthy individuals and monkeys, tested against themselves. Auto-antagonism was observed in nine isolates from periodontal patients, and in five isolates from healthy individuals. However, no auto-antagonism was observed among the monkey isolates. Iso-antagonism was not observed only in three isolates from periodontal patients, seven isolates from healthy individuals and six monkey isolates. The non-producing hetero-antagonism isolates were six from patients, nine from healthy individuals, and one from monkey.

All isolates showed antagonistic activity against all tested reference strains (Table 2). None of the *F. nucelatum* isolates from periodontal patients or monkeys produced antagonistic substances against *F. nucleatum* ATCC 10953. On the other hand, none of the isolates obtained from healthy individuals was capable of inhibiting *B. fragilis* ATCC 23745. None of the *F. nucleatum* isolates obtained from healthy individuals or monkeys produced antagonistic

substances against *P. anaerobius* ATCC 27337. However, only 3 (6.1 %) of periodontal isolates inhibited this microorganism (Table 2). All the diameters of the inhibitory zones were between 10 and 13 mm (data not shown). In tests for the presence of phages, no lytic zone with the same indicator organisms was observed, indicating that the inhibition halos were not due to the presence of bacteriophages.

DISCUSSION

The production of antagonistic substances may play an important role in the microbial colonization of the human or animal oral cavity, leading to ecological alterations in the indigenous microbiota. Because bacteriocin-like substances are produced by a high number of oral microorganisms, a constant and complex intra- or inter-specific regulation can be expected (8).

Among *F. nucleatum* isolated from humans (periodontal patients and healthy individuals) and from *Cebus apella* monkeys, in terms of auto-, iso- and hetero-antagonism, 14 (17.5%) presented auto-antagonism and 63 (78.8%) iso- or hetero-antagonism. Interestingly, the inhibitory activity produced by isolates from periodontal patients was those intense than that produced by isolates from healthy individuals or monkeys (data not shown). Significant differences among hetero-antagonism levels from periodontal and from healthy isolates were observed (Table 1), suggesting an association with the predominance of this organism in periodontal sites.

Auto-antagonism is more prevalent in Gram-positive than in Gram-negative organisms (21, 28). In some Gram-negative bacteria, such as *Pseudomonas solanacearum* and *B. fragilis*, this phenomenon has not been observed (7, 8, 16). Our results show auto-inhibition in 18.4 % and 25 % of the periodontal and healthy isolates, respectively. No auto-antagonism was observed in animal isolates. Azevedo *et al*. (2) showed that the frequency of bacteriocin-producing strains among oral streptococci, including auto-, iso- and hetero-antagonism, is variable.

Antimicrobial proteins are produced by several pathogenic and non-pathogenic bacteria. However, little is known about antagonistic substances in *F. nucleatum.* Auto-antagonism among the tested *F. nucleatum* was observed in a small number of isolates, where as, iso- and hetero-antagonism were observed in a large number of them, suggesting that the bacteriocin-like activity in *F. nucleatum* is variable. This can have a great ecological significance, particularly in colonization or

Table 1. Production of bacteriocin-like substances by 80 oral *F. nucleatum* isolates

Sources of isolates	Antagonism type					
	Auto-		Iso-		Hetero-	
(N)	N	(%)	N	(%)	N	(%)
P* (49)	9	(18.4)	46	(93.9)	43	(87.8)
H** (21)	5	(25.0)	13	(65.0)	11	(55.0)
M*** (10)	0	(0.0)	4	(20.0)	9	(90.0)
Total (80)	14	(17.5)	63	(78.8)	63	(78.8)

*Periodontal patients
**Healthy individuals
***_Cebus apella_ monkeys

Table 2. Antagonistic activity of 80 oral *F. nucleatum* isolates against themselves and against reference strains.

Source of isolates	Percentage of positive isolates							
	P*	H**	M***	*F. nucleatum* ATCC 10953	*F. nucleatum* ATCC 25586	*B. fragilis* ATCC 23745	*E. lentum* ATCC 25559	*P. anaerobius* ATCC 27337
P*	93.8	69.4	67.3	0.0	12.2	8.1	14.3	6.1
H**	90.5	66.6	57.1	9.5	33.3	0.0	4.4	0.0
M***	90.0	80.0	40.0	0.0	20.0	10.0	10.0	0.0

*49 Periodontal patient isolates
**21 Healthy individual isolates
***10 Monkey isolates

regulation of the autochtonous microbiota of the host (15).

Tagg *et al*. (25) showed that bacterial immunity to their own auto-antagonistic products is due to the synthesis of a specific protein which acts as an immunity factor, and the formation of a complex between bacteriocin and this factor would protect the bacteriocin-producing strain.

The diameters of the inhibition zones varied from 10 to 13 mm with a higher frequency of 13 mm and with sharp delimitation (data not shown). These data are similar to those reported by Farias *et al.* (8). Bacteriophages were not observed since the inhibitory action was not transmissible as shown by the tested method (26), excluding the possibility of phages causing the bacteriocin-like effect have been suggested by several authors (14, 20).

Bacteriocin synthesis appear to be an unstable characteristic since some microorganisms lose and recover the capacity to produce it. However, Oliveira and Drozdowicz (19) suggested that the capability to produce bacteriocin is expressed by a small proportion of the bacterial population. On the other hand, it also is possible that one isolate may produce more than one antagonistic substance with different physico-chemical and biological properties. However, some components of the growth medium have been implicated in the induction of bacteriocins (25). However, Weerkamp *et al*. (27) suggested that some components of the nutrient media protect some organisms against bacteriocin action. The chemical nature, genetic determinants and environmental factors that affect the phenotypic expression of the bacteriocin-like production are not totally determined. Since the production can be masked by presence of similar or different substances, studies about characterization and purification of these products should help to a better knowledgement of its action on oral microbiota of human and animal origin.

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RESUMO

Atividade semelhante a bacteriocina de *Fusobacterium nucleatum* orais isolados de primatas humanos e não-humanos

Fusobacterium nucleatum é indígena da cavidade oral humana e tem sido envolvido em diferentes processos infecciosos. A produção de substâncias semelhantes a bacteriocinas pode ser importante na regulação da microbiota bacteriana da cavidade oral. A capacidade de produzir substâncias tipo bacteriocina de 80 isolados de *F. nucleatum* orais, obtidos de pacientes com doença periodontal, indivíduos sadios e macaco *Cebus apella*, foi avaliada. 17,5% de todos os isolados mostrou auto-antagonismo e 78,8% iso- ou hetero-antagonismo. Nenhum isolado de macaco foi capaz de produzir auto-inibição. Neste estudo, a produção de substâncias antagonístas foi variável em todos os isolados testados. A maioria dos *F. nucleatum* mostrou atividade antagonísta para as cepas de referência testadas. Esses dados sugerem a possível participação dessas substâncias sobre a ecologia microbiana em humanos e animais. Entretanto, o papel das bacteriocinas na regulação da microbiota da placa dental *in vivo* é discutida.

Palavras chave: *Fusobacterium nucleatum*, substância tipo bacteriocina, placa dental.

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