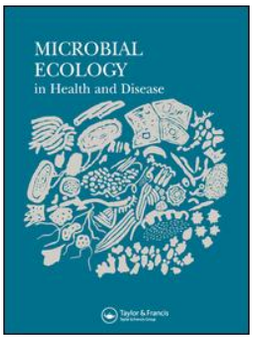

Microbial Ecology in Health and Disease

ISSN: (Print) 1651-2235 (Online) Journal homepage: informahealthcare.com/journals/zmeh20

Properties of a Bacteriolytic Activity from an Oral Gram-Positive Clinical Isolate

D. Grenier

To cite this article: D. Grenier (1996) Properties of a Bacteriolytic Activity from an Oral Gram-Positive Clinical Isolate, Microbial Ecology in Health and Disease, 9:6, 335-339, DOI: 10.3109/08910609609166475

To link to this article: https://doi.org/10.3109/08910609609166475

MICROBIAL ECOLOGY IN HEALTH AND DISEASE VOL. 9: 335–339 (1996)

Short Communication

Properties of a Bacteriolytic Activity from an Oral Gram-Positive Clinical Isolate

D. GRENIER

Groupe de Recherche en Ecologie Buccale, Faculté de Médecine Dentaire, Université Laval, Québec, Canada G1K 7P4

Received 14 November 1996; revised 14 December 1996

The aim of this study was to characterise an oral bacterial isolate possessing extracellular bacteriolytic activity and to determine the basic properties of this activity. The lytic strain L1 was a gram-positive pleomorphic rod that grew only under anaerobic conditions. Glucose and raffinose were fermented whereas catalase and urease were not produced. The activity spectrum of a crude lytic fraction was restricted to strains of *Actinomyces naeslundii* and *Actinomyces viscosus*. On the basis of its molecular weight (>30 kDa) as well as sensitivity to heat and trypsin, the factor(s) has a proteinaceous component. The activity was highly sensitive to EDTA suggesting that cations may be essential for optimal activity. Sulfhydryl groups are also of major importance in the lytic process as activity was highly stimulated by dithiothreitol and inhibited by *p*-chloromercuriphenylsulfonic acid. Strain L1 was found to grow on a solid basal medium containing heat-killed *A. naeslundii* cells, and suggests a physiological role for the bacteriolytic activity. The bacteriolytic activity demonstrated in the present study may constitute a selective advantage for establishment of the bacteria in a complex ecosystem like the oral cavity.

KEY WORDS: bacteriolytic activity; enzyme; oral bacteria; oral ecology; *Actinomyces*.

INTRODUCTION

The oral cavity is a complex ecosystem populated by more than 250 different bacterial species.[4] Because of the high competition for essential nutrients and space in this microenvironment, some bacterial species have evolved different strategies to persist or become predominant in the oral ecosystem.[1,2] Indeed, the ability of bacteria to secrete growth inhibitory substances such as hydrogen peroxide and bacteriocins, or to produce enzymes effective in lysing bacterial cells can confer selective advantages. The theory of bacterial interference is based on such mechanisms and has been considered to prevent or cure oral bacterial disease caused by bacteria.[5]

Very few studies have reported on the production of bacteriolytic activities by oral bacteria. The isolation of a number of bacteria possessing extracellular lytic activity against *Streptococcus sanguis* has been previously reported.[1,2] These isolates, identified as *Streptococcus mutans*, were not active on all strains of *S. sanguis* or on other species of *Streptococcus*.[2] More recently, Pompei *et al.*[13] showed that among human viridans group streptococci only the nutritionally variant streptococci, which are thiol-dependent bacteria, secrete enzymes with bacteriolytic activity on cells of *Micrococcus luteus*. Finally, a recent report by Grenier[3] brought evidence suggesting that lysis of oral bacteria may also occur via the action of various types of proteolytic enzymes. While studying bacteriocinogenicity of oral microorganisms, a bacterial isolate (L1) obtained from a subgingival plaque sample demonstrated a capacity to lyse cells of *Actinomyces naeslundii* grown on solid plates. The aim of this study was to characterise this isolate and to determine the basic properties of the lytic activity.

CCC 0891-060X/96/060335-05

MATERIALS AND METHODS

Bacteria and growth conditions

The lytic strain L1 was originally isolated from a subgingival plaque sample of a patient with no clinical signs of periodontal disease. It represented approximately 5 per cent of the total cultivable bacteria (data not shown). This bacterial isolate was routinely cultivated in Trypticase Soy (TS) (BBL Microbiology Systems, Cockeysville, MD) broth or on TS agar plates at 37°C in an anaerobic chamber (N_2:H_2:CO_2/80:10:10). Test bacteria used to determine bacteriolytic activity were: *A. naeslundii* 85·1, 87·2, 13A3, AT3, ACHI, ADO3, AT1, AT4, AD1, AJL3, AJL4, AB1, AS1, ATHO1 and ATHO2, *Actinomyces viscosus* 54·2, AF1, AF2, AFRAI, ADO1, ACH2 and AJL1, *Actinomyces odontolyticus* AFRACO1, AS2 and XX.110, *Staphylococcus aureus* INA, *S. mutans* ATCC 10449 and INB, *Streptococcus mitis* StB1, *Lactobacillus* spp. LaB1 and LaC1, *Veillonella* spp. VeA2, *Micrococcus luteus* ATCC 4698, *Eubacterium saburreum* 162·4, *Treponema denticola* ATCC 35405, *Porphyromonas gingivalis* ATCC 33277, *Capnocytophaga ochracea* 1956c, *Prevotella loescheii* ATCC 15930 and *Prevotella intermedia* BMH. *T. denticola* were grown in the spirochete medium described by Leschine and Canale-Parola,[11] whereas the other bacteria were cultivated in Brain Heart Infusion broth (BBL Microbiology Systems) supplemented with hemin (10 µg/ml) and vitamin K_1 (1 µg/ml). All cultures were incubated under anaerobiosis (except for *M. luteus* in aerobiosis) at 37°C until the stationary growth phase was reached.

Characterisation of strain L1

The lytic strain L1 was first tested for Gram stain reaction and examined by phase contrast microscopy. Strain L1 was then evaluated for (i) growth in the absence and presence of oxygen; (ii) production of catalase, oxidase and urease; and (iii) fermentation of glucose, raffinose, mannitol and xylose, as outlined in the VPI Anaerobe Laboratory Manual.[6] Finally, the strain L1 was characterised using the An-IDENT system (API Laboratory Products Ltd, St-Laurent, QC).

Determination of bacteriolytic activity

The lytic strain L1 was grown on TS agar plates for 3 d in the anaerobic chamber. Bacteria were removed from the surface of the agar using a glass rod, and the plates were stored at −80°C for 3 h. The plates were allowed to defrost at room temperature and the supernatant was collected. Remaining bacterial cells in the supernatant were removed by centrifugation (8000 *g* for 20 min). The cell-free culture fluid was concentrated 7·5 fold by freeze drying. This crude lytic fraction was kept at −20°C until used. The test bacteria were grown for 3 d in appropriate liquid culture media, the cells were harvested by centrifugation and suspended in 50 mM phosphate-buffered saline pH 7·2 (PBS) to obtain an OD_{660}=0·25 after a 1:10 dilution. The assay mixture consisted of 250 µl of bacterial cells, 250 µl of PBS supplemented with dithiothreitol (0, 6, 24 and 45 mM), 150 µl of distilled water and 100 µl of the crude lytic fraction. The initial OD_{660} was recorded and the assay mixture was then incubated aerobically at 37°C for 2 h. A second reading of the OD_{660} was obtained and the decrease was used to calculate the percentage of lysis. In one experiment, the time course of lysis of cells of *A. naeslundii* 85·1 by the crude lytic fraction was determined by measuring decrease in OD_{660} after 5, 10, 15, 30, 60 and 120 min of incubation.

Characterisation of the lytic activity

Characterisation of the lytic activity was done using *A. naeslundii* 85·1 cells as the test bacteria. The effect of pH on activity of the lytic fraction was determined by performing the assay using the following buffers supplemented with 0·15 M NaCl and 45 mM dithiothreitol: 50 mM citrate buffer (pH 3, 4 and 5), 50 mM phosphate buffer (pH 6 and 7), 50 mM Tris hydrochloride buffer (pH 8 and 9) and 50 mM carbonate buffer (pH 10, 11 and 12). The effect of heating on bacteriolytic activity was measured by treatment of the lytic fraction for 30 min at the following temperatures: 50, 60 and 75°C. The susceptibility of the lytic activity to proteolytic degradation was also tested by adding bovine pancreatic trypsin to the fraction at a final concentration of 1 mg/ml. After an incubation of 1 h at 37°C, the bacteriolytic activity of the treated fraction was measured. Effect of putative inhibitors (NaCl [0·5 M], EDTA [10 mM], iodoacetamide [20 mM] and *p*-chloromercuriphenylsulfonic acid [20 mM]) on bacteriolytic activity was determined by adding the compounds in the reaction mixture. Finally, the lytic fraction was submitted to ultrafiltration using Ultrafree-MC filter units (Millipore Ltd, ON; nominal molecular weight limit [NMWL]=30 and 100 kDa), and the

bacteriolytic activity of both the ultrafiltrate and the retentate was determined. All the above assays were run in duplicate to ensure reproducibility.

Effect of A. naeslundii *cells on growth of strain L1*

The growth of the lytic strain L1 was evaluated on a solid basal salt medium containing (i) 0·25 per cent K_2HPO_4, 0·5 per cent NaCl and 1·7 per cent agar, and (ii) the above ingredients as well as 5 per cent (v/v) washed *A. naeslundii* 85·1 whole cells (heat-killed cell suspension at $OD_{660}=1$ after a 1:10 dilution). The strain L1 was spot-inoculated (5 µl of a 24-h preculture) and growth was evaluated after a 5-d incubation period at 37°C in the anaerobic chamber. This experiment was performed three times using different preparations of cells of *A. naeslundii* 85·1.

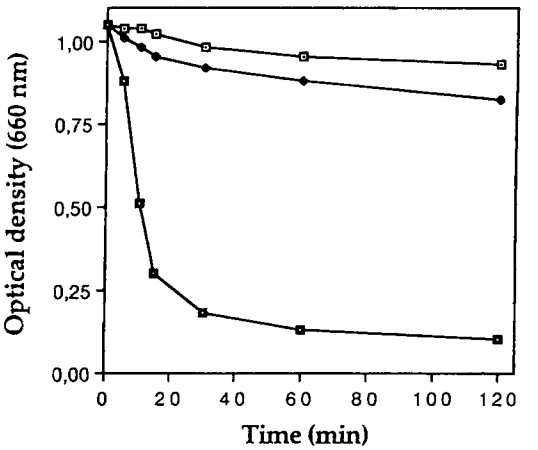

Figure 1. Time course of lysis of *A. naeslundii* 85·1 cells by the crude fraction prepared from strain L1. ◆, assay carried out in absence of dithiothreitol; ■, assay carried out in presence of 15 mM dithiothreitol; ⊡, assay carried with boiled crude lytic fraction, in presence of 15 mM dithiothreitol

RESULTS AND DISCUSSION

The lytic strain L1 was a gram-positive non-motile bacterium. By phase contrast microscopy, the bacteria appeared as pleomorphic rods which tend to form large aggregates. The bacteria could not grow in the presence of air and did not produce catalase, oxidase and urease. Glucose and raffinose were fermented whereas mannitol and xylose were not. Using the commercial identification strip An-IDENT, the bacterium was found to possess α and β-glucosidase, α-galactosidase, as well as leucine-, proline-, tyrosine-, arginine-, alanine-, phenylalanine- and glycine-aminopeptidase. Taking into consideration the above properties as well as previously published studies,[9,10] the strain L1 may represent a member of the genus *Actinomyces*. However, additional characterisation studies involving serological and DNA analyses are required to reliably assign this isolate in the *Actinomyces* genus.

As our preliminary experiments indicated that the bacteriolytic activity of strain L1 was released extracellularly and produced in a lesser amount in liquid broth, an active fraction was prepared from solid culture plates. The crude fraction was then used to characterise the lytic activity on cells of strain *A. naeslundii* 85·1. The lysis curve corresponding to the decrease in OD_{660} as a function of incubation time is presented in Figure 1. In the presence of dithiothreitol at a final concentration of 15 mM, almost complete lysis of *A. naeslundii* cells occurred within 60 min of incubation under the assay performed. A control assay indicated that dithiothreitol had no lytic effect on the test bacteria. No significant bacteriolytic activity could be detected in the absence of dithiothreitol or using a boiled fraction. A 50 per cent lysis of *A. naeslundii* cells was still obtained when the final concentration of dithiothreitol was lowered to 2 mM, whereas β-mercaptoethanol (15 mM), another reducing agent, demonstrated a stimulatory effect similar to dithiothreitol (data not shown). The lytic activity was not affected within the pH range of 6 to 10. Fifty per cent activity was still obtained at pH 5 and pH 11, whereas no activity was detected at $pH \leq 4$ and ≥ 12.

Basic properties of the bacteriolytic activity of the crude fraction on cells of *A. naeslundii* are summarised in Table 1. The activity was not affected by sodium chloride whereas a significant inhibition was obtained in the presence of either EDTA or sulfhydryl-reacting compounds (iodoacetamide and *p*-chloromercuriphenylsulfonic acid). The activity of the crude lytic fraction was found to be heat-sensitive, being completely destroyed following a treatment at 75°C for 30 min. Treatment of the fraction with trypsin was also associated to a significant loss (70 per cent) of activity. The lytic fraction was submitted to ultrafiltration to approximate the molecular weight of the active factor(s). The activity could not pass through a filter with NMWL of 30 kDa. However, almost complete activity could be recovered in the ultrafiltrate obtained using a filter with NMWL of 100 kDa.

Table 1. Effect of various putative inhibitors and treatments on bacteriolytic activity of the crude fraction prepared from strain LI

Inhibitor or treatment	Relative lytic activity* (%)
Control	100
Inhibitor	
Sodium chloride (0·5 M)	98
EDTA (10 mM)	6
Iodoacetamide (20 mM)	12
p-chloromercuriphenylsulfonic acid (20 mM)	18
Treatment	
Trypsin (1 mg/ml; 1 h at 37°C)	10
50°C/min	95
60°C/min	50
75°C/min	0
Ultrafiltrate (NMWL = 30 kDa)	0
Ultrafiltrate (NMWL = 100 kDa)	90

*Lytic activity in cells of *A. naeslundii* 85·1.

Table 2. Lysis of bacteria by the crude fraction prepared from strain LI

Bacteria	Relative lytic activity* (%)
A. naeslundii 85·1	100
A. naeslundii 87·2	82
A. naeslundii AT3	74
A. naeslundii ACHI	43
A. naeslundii AD03	32
A. naeslundii 13A3, AT1, AT4, AD1, AJL3, AJL4, AB1, AS1, ATHO1 and ATHO2	0
A. viscosus AF1	94
A. viscosus 54·2	92
A. viscosus AF2	73
A. viscosus AFRAI	66
A. viscosus ADO1	63
A. viscosus ACH2	37
A. viscosus AJL1	29
A. odontolyticus AFRACO1, AS2 and XX.110	0
S. aureus INA	0
S. mutans 10449 and INB	0
S. mitis StB1	0
Lactobacillus spp. LaB1 and LaC1	0
V. spp. VeA2	0
M. luteus ATCC 4698	0
E. saburreum 162·4	0
T. denticola ATCC 35405	0
P. gingivalis ATCC 33277	0
C. ochracea 1956c	0
P. loescheii ATCC 15930	0
P. intermedia BMH	0

*Activity against cells of *A. naeslundii* 85·1 set at 100.

Twenty-seven bacterial strains were tested in the liquid assay for their susceptibility to the action of the lytic fraction (Table 2). All seven strains of *A. viscosus* as well as five out of 15 strains of *A. naeslundii* were lysed to various extents. The three strains of *A. odontolyticus* were not affected. All the other bacteria under investigation including gram-positive and gram-negative species were not susceptible to the bacteriolytic activity of the crude fraction. As the lytic activity is restricted to some strains of the *Actinomyces* genus, it would be interesting to verify whether there is any relationship between susceptibility and serological type or cell wall composition of bacteria.

The physiological importance of the bacteriolytic activity of strain L1 was preliminarily investigated. The lytic strain L1 could grow (confluent growth at the site of inoculation) on a solid basal salt medium containing heat-killed *A. naeslundii* 85·1 cells as sole carbon and nitrogen sources. No growth occurred on this medium in the absence of *A. naeslundii* 85·1 cells. Similar results were obtained in three separate experiments.

The ability of bacteria to produce bacteriolytic enzymes, also called peptidoglycan hydrolases, is well known.[7,13,15] More particularly, these enzymes are thought to play key roles in cellular physiology, and recent evidence suggest their importance as determinants of the course of infections.[7] The fact that several biological activities, including a pyrogenic effect, have been demonstrated in cell wall fragments generated by bacteriolytic enzymes implies that they may also be important in immunologically-mediated diseases.[8,14] To our knowledge, bacteriolytic activities demonstrated and characterised in oral bacteria have been restricted to streptococci.[1,2,13]

In the present study, we have demonstrated that a gram-positive oral isolate (L1), which may be closely related to the genus *Actinomyces*, produces an extracellular bacteriolytic activity. The activity spectrum of strain L1 was very limited since only some strains of *A. naeslundii* and *A. viscosus* were

susceptible. On the basis of its molecular weight (>30 kDa) as well as sensitivity to heat and trypsin, the factor(s) has a proteinaceous component. The activity was highly sensitive to EDTA suggesting that cations may be essential for optimal activity. Sulfhydryl groups are also likely to be of major importance in the lytic process as activity was highly stimulated by dithiothreitol and inhibited by *p*-chloromercuriphenylsulfonic acid and iodoacetamide. The bacteriolytic activity of strain L1 may give a selective advantage for establishment of the bacteria in the oral cavity and thus be a factor contributing to the ecology of dental plaque. Firstly, lysis of oral bacteria may provide space in a heavily populated environment. This would allow the producing-bacteria to colonise oral surfaces. Secondly, results from this study demonstrate that lysis of bacteria by strain L1 is associated with a growth stimulation, suggesting that it is able to utilise the bacterial cellular components as sources of carbon and nitrogen for its multiplication. This is particularly important in the oral ecosystem where bacteria, because of their high numbers, have to compete for essential nutrients.[12]

REFERENCES

1. Baba H. (1986). Lysis of *Streptococcus sanguis* by an extracellular enzyme from the bacterium *Streptococcus mutans* from human dental plaque. *Archives of Oral Biology* **31,** 849–853.
2. Baba H, Igarashi S, Kamaguchi A. (1987). Isolation of bacteria lytic against *Streptococcus sanguis* ATCC 10558 from human dental plaque. *Caries Research* **21,** 385–392.
3. Grenier D. (1994). Effect of proteolytic enzymes on the lysis and growth of oral bacteria. *Oral Microbiology and Immunology* **9,** 224–228.
4. Hardie JM. (1992). Oral microbiology: current concepts in the microbiology of dental caries and periodontal diseases. *British Dental Journal* **172,** 271–278.
5. Hillman JD, Socransky SS. (1989). The theory and application of bacterial interference to oral diseases. In: Myers HM (ed) *New Biotechnology in Oral Research.* Karger Basel Publishers, Switzerland, pp. 1–17.
6. Holdeman LV, Cato EP, Moore WEC. (1977). *Anaerobe Laboratory Manual.* VPI Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA.
7. Höltje J-V, Tuomanen EI. (1991). The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections *in vivo*. *Journal of General Microbiology* **137,** 441–454.
8. Johannsen L. (1993). Biological properties of bacterial peptidoglycan. *Acta Pathologica Microbiologica et Immunologica Scandinavica* **101,** 337–344.
9. Johnson JL, Moore LVH, Kaneko B, Moore WEC. (1990). *Actinomyces georgiae* sp. nov., *Actinomyces gerencseriae* sp. nov., designation of two genospecies of *Actinomyces naeslundii*, and inclusion of a *A. naeslundii* serotypes II and III and *Actinomyces viscosus* serotype II in *A. naeslundii* genospecies 2. *International Journal of Systemic Bacteriology* **40,** 273–286.
10. Kalfas S, Edwardsson S. (1990). Identification procedures for oral *Actinomyces* species. *Oral Microbiology and Immunology* **5,** 39–42.
11. Leschine SB, Canale-Parola E. (1980). Rifampin as a selective agent for isolation of oral spirochetes. *Journal of Clinical Microbiology* **12,** 792–795.
12. Marsh PD. (1989). Host defenses and microbial homeostasis: role of microbial interactions. *Journal of Dental Research* **68,** 1567–1575.
13. Pompei R, Caredda E, Piras V, Serna C, Pintus L. (1990). Production of bacteriolytic activity in the oral cavity by nutritionally variant streptococci. *Journal of Clinical Microbiology* **28,** 1623–1627.
14. Seidl PH, Schleifer KH. (1986). *Biological Properties of Peptidoglycan.* Walter de Gruyter, Berlin and New York.
15. Ward JB, Williamson R. (1984). Bacterial autolysins: specificity and function. In: Nombela, C. (ed) *Microbial Cell Wall Synthesis and Autolysis.* Elsevier Science Publishers, Amsterdam, pp. 159–166.