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Killing of anaerobic pathogens by predatory bacteria

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SUMMARY

Recently, the predation of *Bdellovibrio bacteriovorus* on a periodontal pathogen has been described. The current study explores the potential antimicrobial activity of a range of predatory bacteria against key periodontal pathogens. A number of representatives from the *Bdellovibrio*, *Bacteriovorax* and *Peredibacter* lineages (called 'BALOs') were tested for their activity towards a group of key periodontal pathogens and an optimal multiplicity of infection was established. As the oral cavity contains a wide variety of bacteria that are not preyed upon, it was investigated if they can have an effect on the predation efficiency of BALOs. It was concluded that a number of important variables involved in bacterial predation are found to be compatible with the composition of the oral microbiota. This finding makes the case for continued study of the potential for BALOs to combat periodontal pathogens.

INTRODUCTION

Bdellovibrio-and-like organisms (BALOs) are a diverse group of highly motile deltaproteobacteria that are obligatory predators of gram-negative bacteria (Stolp & Starr, 1963). They are ubiquitous in terrestrial and aquatic environments (Jurkevitch *et al.*, 2000) and have also been isolated from human faeces (Schwudke *et al.*, 2001). These minute assassins have a peculiar lifestyle: they swim around at high speed and after collision with a susceptible prey bacterium, they attach to their prey and enter its periplasm. The invaded prey is subsequently converted into a hybrid structure of predator–prey called the 'bdelloplast'. The conversion from an uninvaded prey cell into a bdelloplast often coincides with a morphological change to a spherical structure although this is not always the case and initial prey morphology can be maintained throughout most of the predation process (Núñez *et al.*, 2003). This bdelloplast supports the development of the predator until its multiplication is completed. At that point, the bdelloplast is ruptured and the offspring of the initial attack-phase cell is released into the environment (Varon & Shilo, 1968; Lambert *et al.*, 2006). In spite of this remarkable behaviour, BALOs have only been sparsely investigated. Advances in molecular techniques have demonstrated that the presence of BALOs in the environment is considerably underestimated (Van Essche *et al.*, 2009b) although they are generally not isolated from the environment in large numbers (Mahmoud *et al.*, 2007). These findings suggest that the effect of bacterial predation on microbial communities might be much more extensive than previously assumed. Periodontitis is a polymicrobial infection involving numerous gram-negative pathogens embedded in a complex biofilm

called dental plaque (Pihlstrom *et al.*, 2005). From a microbiological point of view, the treatment of periodontal diseases is complicated by a number of drawbacks. First, the application of antimicrobials is discouraged in light of the growing resistance to antibiotics (van Winkelhoff *et al.*, 2005). Second, antimicrobials are ineffective in penetrating bacterial biofilms such as dental plaque. Bacteria that reside in such biofilms are typically up to 1000 times more resistant to antimicrobials than their planktonic counterparts (Costerton *et al.*, 1999; Costerton & Keller, 2007). Additionally, it has been suggested that several gram-positive bacteria in dental plaque have protective features towards periodontal disease and therefore are better not removed during therapy (Hillman *et al.*, 1985). In light of these findings, it has recently been suggested that periodontal therapy could be assisted by the application of BALOs to reduce specifically the levels of gram-negative pathogens in the oral cavity. This hypothesis was recently explored by demonstrating the predation of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* by *Bdellovibrio bacteriovorus* (Van Essche *et al.*, 2009a). To further explore the potential use of BALOs in periodontal therapy, the impact of a number of principal aspects related to the multispecies infection periodontitis should be investigated. Therefore, the aim of this study was to determine the predation kinetics to obtain the most optimal prey reduction and to determine the predation spectrum and efficiency of different BALO strains on the most common facultative and obligatory anaerobic periodontopathogens. Finally, as the oral cavity contains a mixed microbiota that contains a substantial portion of bacteria that are not susceptible to a BALO attack, the influence of these decoys on the predation efficiency was investigated.

METHODS

Bacterial strains, media and culturing conditions

Prey bacteria (Table 1) were cultured in Luria–Bertani broth or agar medium at 37°C in an aerobic incubator. The periodontopathogens *Aggregatibacter actinomycetemcomitans* ATCC 43718, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 49256, *Capnocytophaga sputigena* ATCC 33612, *Eikenella corrodens* ATCC 23834 and *Actinomyces naeslundii* ATCC 12104 were maintained on blood agar (Blood Agar Base II; Oxoid, Basingstoke, UK), supplemented with haemin (5 mg ml^{-1}), menadione (1 mg ml^{-1}), and 5% sterile horse blood (Biotrading, Keerbergen, Belgium). Broth cultures were grown in brain–heart infusion (BHI) broth (Oxoid) at 37°C except for *E. corrodens* that was grown in *Haemophilus* test medium broth (Jorgensen *et al.*, 1987). Cultures were incubated in anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2).

BALOs used in this study (listed in Table 1) were kindly donated by Prof. E. Jurkevitch of the Hebrew University of Israel and were maintained on double-layer HM agar plates (HEPES 25 mM, calcium^{2+} 3 mM and magnesium^{2+} 2 mM at pH 7.6) containing their prey organisms (Jurkevitch, 2005). BALO suspensions were serially diluted in physiological saline and 100 μl of the dilutions were mixed with 300 μl of the appropriate prey organism at a concentration of 9.70 $\log_{10}$ colony-forming units (CFU) ml^{-1} in HM buffer. This mixture was subsequently added to 5 ml molten HM medium at 42°C containing 0.7% agar and poured onto 1.5% HM agar plates. After 3–5 days of incubation at 30°C in an aerobic incubator, plaques were visible.

Table 1 Predatory bacteria used in this study with their origin and respective prey

BALO strain	Collection no./ Reference	Prey	Origin
Bdellovibrio bacteriovorus HD100	ATCC 15356	*Escherichia coli* ML35 (ATCC 43827)	Soil, CA, USA
Bdellovibrio bacteriovorus 109J	ATCC 15143	*Escherichia coli* ML35 (ATCC 43827)	Sewage, CA, USA
Bdellovibrio BEP2	Jurkevitch *et al.*, 2000	*Escherichia coli* ML35 (ATCC 43827)	Root extract, Rehovot, Israel
Bacteriovorax stolpii Uki2	ATCC 27052	*Pseudomonas* sp. (ATCC 12633)	Soil, KY, USA
Bacteriovorax FCE	Davidov *et al.* 2004	*Pectobacterium carotovorum*	Root extract, Rehovot, Israel
Peredibacter starrii A3.12	ATCC 15145	*Pseudomonas* sp. (ATCC 12633)	Soil, CA, USA

For the preparation of broth cultures of BALOs, the selected prey were suspended in HM medium to 8.70 $\log_{10}$ CFU ml^{-1} and inoculated with an adequate amount of BALOs to obtain a fresh lysate after 18 h of aerobic incubation at 30°C under continuous shaking. Before harvesting the predatory bacteria, BALO cultures were examined with phase-contrast microscopy. BALO cells were separated from the remaining prey by filtration over a 1.2 μm Acrodisk filter (Millipore Filter Corporation, Bedford, MA) and two additional filtrations over 0.45-μm Acrodisk filters.

Quantification of BALOs

The number of BALO cells in the samples was quantified using quantitative polymerase chain reaction (qPCR) for the BALO strains of the Bdellovibrionaceae family: *B. bacteriovorus* HD100, *Bdellovibrio* BEP2 and *B. bacteriovorus* 109J (Van Essche *et al.*, 2009b). Briefly, BALO suspensions used in the experiments were preserved at −80°C until DNA extraction was performed with the instagene matrix DNA extraction kit (Bio-Rad Life Science Research, Hercules, CA) according to the instructions of the manufacturer. The TaqMan primers and FAM-labelled probe were designed specifically for the quantification of Bdellovibrionaceae and were synthesized by Eurogentec (Seraing, Belgium). The qPCR mix was made up with 2 × qPCR master mix (Eurogentec), 900 nM of each primer (BD347F: 5′-GGAGGCAGCAGTAGGGAATA-3′; BD549R: 5′-GCTAGGATCCCTCGTCTTACC-3′), 50 nM probe (BD396P: 5′-TTCATCACTCACGCGGCGTC-3′) and 3 μl sample DNA. The qPCR was performed on the CFX96 Real-Time PCR System (Bio-Rad).

Thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 45 repeats of 15 s at 95°C and 1 min at 60°C. Data were collected during the annealing phase. In each run template controls were included. As a standard for the qPCR, a fragment of the *B. bacteriovorus* HD100 16S ribosomal RNA gene was cloned with the pGEM-T easy vector system (Promega, Madison, WI) according to the instructions of the manufacturer. Plasmids were isolated from the clones with the High Pure Plasmid Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Plasmid concentration was determined with the GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Piscataway, NJ) at 260 nm. Ten-fold dilution series of this plasmid were used in each qPCR run to construct the standard curve. *Bacteriovorax stolpii Uki2*, *Bacteriovorax* FCE and *Peredibacter starrii* A3.12, were quantified by quantitative microbial culturing. Serial dilutions of the BALO suspensions in HM medium were used for plating on double-layer HM plates as described earlier.

Influence of multiplicity of infection on predation kinetics

Overnight stationary cultures of *A. actinomycetemcomitans* ATCC 43718 in BHI were centrifuged (7000 ***g***; 5 min) and washed with HM medium. Subsequently, bacterial density was determined as the optical density at 600 nm (OD_{600}; Genesys 20, Thermo Electron Corporation, Waltham, MA) and suspensions were prepared containing approximately 7 $\log_{10}$ CFU ml^{-1} in HM medium.

Bdellovibrio bacteriovorus HD100 filtered cultures were centrifuged (27,000 ***g***; 20 min) and resuspended in HM medium. The OD_{600} was measured (Genesys 20, Thermo Electron Corporation) and adjusted to 1.00 (10 $\log_{10}$ cells ml^{-1}). Ten-fold and 100-fold dilutions of this BALO suspension were prepared in HM medium. Samples of the BALO suspensions were frozen at −80°C until DNA extraction and qPCR were performed to obtain the concentration of predatory cells.

A 500-μl sample of the *A. actinomycetemcomitans* suspension was mixed with 500 μl of the *Bdellovibrio* suspensions in 24-well plates (Iwaki microplate®, Scitech, Diu, Japan). The intended multiplicities of infection (MOI; predator : prey) for the experiments were therefore respectively 1000 : 1, 100 : 1 and 10 : 1. The control series received an equal volume of an ultra-filtrate of the former *Bdellovibrio* suspension that had been cleared of all predatory cells by using an additional filter with pore size of 0.1 μm.

The plates were incubated (37°C) on a shaking plate at 200 ***g*** in 5% CO_2. Pathogen viability was examined hourly for 4 h by quantitative microbial culturing on blood agar plates. Samples were serially diluted in phosphate-buffered saline and inoculated onto agar plates with a spiral plater (Autoplate 4000; Spiral Biotech, Bethesda, MD). Culture plates were incubated anaerobically (10% H_2, 10% CO_2 and 80% N_2) at 37°C for 4 days after which colony counting was performed to calculate the number of viable

A. actinomycetemcomitans cells. The experiment was repeated on three separate days.

All statistical analysis for this study was performed in R FOR WINDOWS, version 2.6.1 with the level of significance set at $P < 0.05$.

The data were $\log_{10}$-transformed to obtain a normal distribution. A linear mixed model was used with experiments as random factors. Pathogen concentration and time are the two crossed factors upon which two-way analysis of variance was applied.

Periodontal pathogen prey range

The periodontopathogens *A. actinomycetemcomitans* ATCC 43718, *Porphyromonas gingivalis* ATCC 33277, *P. intermedia* ATCC 25611, *F. nucleatum* ATCC 49256, *C. sputigena* ATCC 33612 and *E. corrodens* ATCC 23834 were challenged with the following BALOs: *B. bacteriovorus* HD100, *B. bacteriovorus* 109J, *Bdellovibrio* BEP2, *Bacteriovorax stolpii* Uki2, *Bacteriovorax* FCE and *Peredibacter starrii* A3.12.

The prey were washed and resuspended in HM medium to a bacterial density of approximately 7 $\log_{10}$ CFU ml^{-1}. Predatory suspensions with a concentration of approximately 10 $\log_{10}$ cells ml^{-1} were prepared as described above. The experimental setup was similar to that described above with the exception that the prey viability was determined at the start of the experiment and after 3 h of contact with the respective predator.

Samples of the Bdellovibrionaceae suspensions were kept at −80°C until DNA extraction and qPCR were performed. Samples of non-Bdellovibrionaceae suspensions were quantified by culture. The experiment was repeated at least three times on different days.

For statistical analysis of the data, a mixed linear model was built using the experiments as random factors. Data were $\log_{10}$ transformed to obtain a normal distribution. The fixed, crossed factors used were: BALO type, periodontopathogen and control series. Per combination of BALO/pathogen, a comparison was made between test and control.

Predation in the presence of decoy microorganisms

A mixed-species model was used to study the effect of decoy bacteria on *A. actinomycetemcomitans* predation by *B. bacteriovorus*. As a decoy, *Act. naeslundii* was used. This bacterium is not susceptible to predation by BALOs because it is gram-positive. The aforementioned model to study the predation kinetics of *B. bacteriovorus* HD100 on *A. actinomycetemcomitans* ATCC 43718 was expanded with different ratios of prey versus decoy. In brief, the predator was suspended in HM medium to an $OD_{600} = 1.00$ as described.

Prey suspensions that contain approximately 7 $\log_{10}$ CFU ml^{-1} were prepared in HM medium. Similarly, *Act. naeslundii* was resuspended in HM medium to a concentration of approximately 8 $\log_{10}$ CFU ml^{-1} and 10-fold and 100-fold dilutions were prepared in HM medium. Then, 67 μl of the predator suspension and 67 μl of prey suspension were mixed with 67 μl of the decoy suspension in 96-well microtitre plates (Falcon, Becton Dickinson, Cowley, Oxford, UK). As such, the MOI (predator : prey) was identical in each series of an individual experiment and prey : decoy ratios of approximately 1 : 10; 1 : 1 and 1 : 10 were established. The exact inoculum density of the predator, prey and decoy cells was afterwards determined by culture on blood agar plates and by qPCR. Prey viability was monitored every hour over the course of the experiment by quantitative culturing. Controls containing only prey (negative control) and only prey and predator (positive control) were used in each experiment. The experiment was performed three times on different days.

For statistical analysis of the data, the data were $\log_{10}$ transformed to obtain a normal distribution. A linear mixed model was built. In this model, experiments were used as random factors and time and series were used as fixed crossed factors to evaluate differences between series at each time point.

RESULTS

Influence of multiplicity of infection on predation kinetics

In a first experiment, the effect of the predator density on the predation kinetics of *A. actinomycetemcomitans* was examined (Fig. 1). The average concentration of *A. actinomycetemcomitans* cells at the start of the experiment was 7.25 (± 0.04 SEM) $\log_{10}$ CFU ml^{-1}. Already after 1 h of contact time,

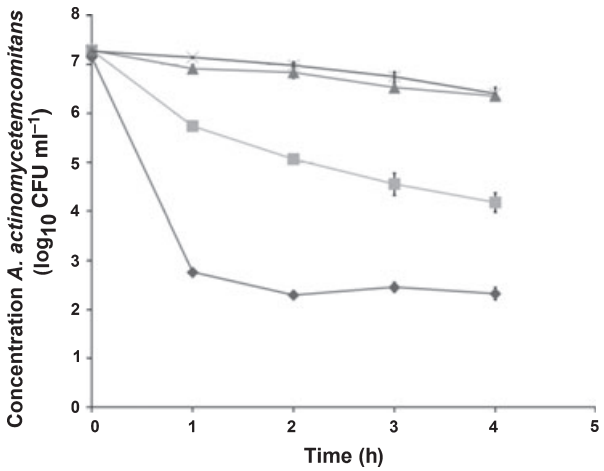

Figure 1 Influence of multiplicity of infection (MOI) on predation kinetics. The viability of the *Aggregatibacter actinomycetemcomitans* prey was measured during 4 h of contact with the predator *Bdellovibrio bacteriovorus* HD100. The average number of *B. bacteriovorus* genomes at the start of the experiment counted by quantitative polymerase chain reaction and expressed as $\log_{10}$ genome equivalents (geq ml^{-1}) measured 10.74 (± 0.11 SEM) geq ml^{-1}, 9.71 (± 0.07 SEM) geq ml^{-1}, and 8.76 (± 0.08 SEM) geq ml^{-1} for respectively an OD_{600} of 1.00, 0.10 and 0.01. This resulted in an effective MOI predator : prey of 3915 : 1 (OD_{600} = 1.00), 265 : 1 (OD_{600} = 0.10) and 30 : 1 (OD_{600} = 0.01). The MOI predator : prey was 3263 : 1 (diamond markers), 326 : 1 (square markers) and 33 : 1 (triangle markers). The viability of the control series is represented by crossed markers. Error bars represent standard errors of the mean (n = 3).

MOI of 3915 : 1 and 265 : 1 both resulted in significant decrease in prey viability when compared with the negative control ($P < 0.001$). In contrast, with an MOI of 30 : 1, no decrease in prey viability could be observed over the 4 h time period of the experiment.

For an MOI of 3915 : 1, a lysis of 4.38 $\log_{10}$ was reached after 1 h of infection. The maximal lytic effect was reached after 2 h of infection, at which point prey viability was reduced by 4.68 $\log_{10}$ values. From that point on, no further decrease in prey viability was observed over the time period of the experiment. When a 10-fold lower MOI (OD_{600} = 0.1, MOI = 265 : 1) was used, there was also a significant reduction in prey viability after 1 h of infection. In contrast to an infection with an MOI of 3915 : 1, the reduction in prey viability progressed over the time of the experiment. This reduction in prey viability was always less pronounced than the decrease in prey viability with an MOI of 3915 : 1 ($P < 0.001$). After 4 h of incubation, the reduction in prey viability measured 2.23 $\log_{10}$ CFU ml^{-1}.

Predation spectrum and efficiency of BALOs on periodontal pathogens

The susceptibility of six different periodontopathogens towards predation by six different BALO strains was examined to investigate the specific prey spectrum (Fig. 2). The most versatile predator proved to be *B. bacteriovorus* HD100, decreasing the viability of four of the six tested pathogens. *Bdellovibrio bacteriovorus* 109J caused lysis in three of six tested strains. *Bacteriovorus* BEP2 and *Bacteriovorax* FCE each lysed two of the six tested pathogens. *Peredibacter starrii* A3.12 was only effective in predation on *A. actinomycetemcomitans*. Under the given experimental conditions *Bacteriovorax stolpii* Uki2 was unable to significantly decrease the viability of any of the tested pathogens. Major differences were also found in terms of predation efficiency, expressed as the average reduction in susceptible pathogen viability. Predation efficiency in $\log_{10}$ values was 3.20 for *B. bacteriovorus* HD100; 3.09 for *B. bacteriovorus* 109J; 2.23 for *Bdellovibrio* BEP2 and 1.25 for *Bacteriovorax* FCE.

Fusobacterium nucleatum was the most susceptible prey, being preyed on by four of the six BALO strains. The pathogens *E. corrodens*, *A. actinomycetemcomitans*, *P. intermedia*, *Porphyromonas gingivalis* and *C. sputigena* were preyed upon by respectively 3/6; 3/6; 2/6; 0/6 and 0/6 tested BALO strains. For the pathogens that were susceptible to predation, the average of the significant reduction in pathogen viability which BALOs could achieve was 3.04 $\log_{10}$ for *A. actinomycetemcomitans*, 2.99 log10 for *E. corrodens*, 2.70 $\log_{10}$ for *F. nucleatum* and 1.03 $\log_{10}$ for *P. intermedia*. It was therefore concluded that the prey species did not show similarly efficient susceptibilities.

Predation in the presence of non-target microorganisms

The oral environment comprises multiple bacterial species, of which many might not be susceptible to BALOs, so the effect of the presence of such non-target microorganisms on predation efficiency was evaluated (Fig. 3). The prey viability decreased significantly in all settings that contained predatory cells. No significant differences could be found for any non-target cell concentrations when compared with the

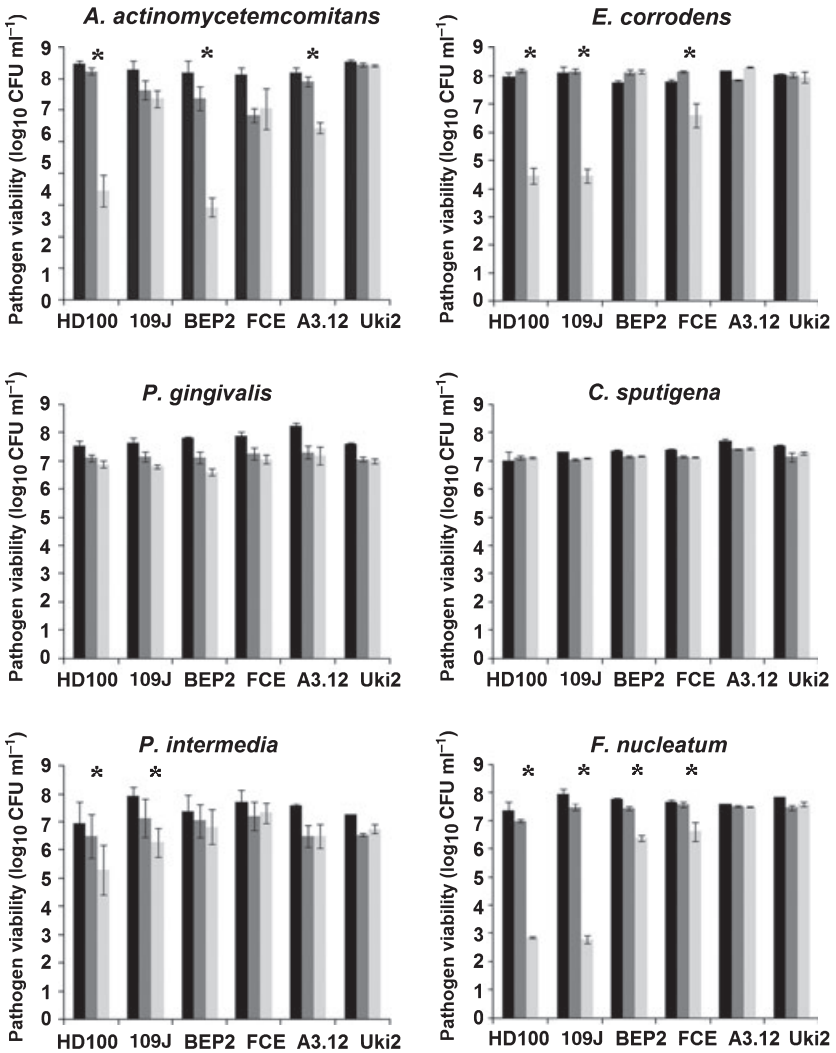

Figure 2 Prey spectra of six *Bdellovibrio* and like organisms (BALO) strains. The susceptibility of six periodontopathogens (*Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Capnocytophaga sputigena*, *Prevotella intermedia*, *Fusobacterium nucleatum*) to attack by six different BALO strains was evaluated. Black columns represent pathogen concentration at the start of the experiment. Dark grey columns represent the viability of the pathogen not exposed to a BALO (control). Light grey columns represent the viability of the pathogen after exposure to one of the following BALO strains (test): *B. bacteriovorus* HD100, *B. bacteriovorus* 109J, *Bdellovibrio* strain BEP2, *Bacteriovorax* strain FCE, *Peredibacter starrii* A3.12 and *Bacteriovorax stolpii* Uki2. Error bars represent standard errors of the mean ($n = 3$). Statistically significant differences between the control and test series are marked with an asterisk.

positive control for any time point. Nor were there any significant differences detected between different settings for any time point.

DISCUSSION

Ever since their accidental discovery in the 1960s, BALOs have been eccentricities in the world of microbiology. Not long after their discovery, their ability to predate gram-negative bacteria was linked to their application as biological control measures to clear different habitats of human and plant pathogens. Periodontal infections could also be an interesting target for the application of BALOs as biological antimicrobial agents. This hypothesis is based on the fact that almost all periodontal pathogens are gram-negative bacteria and therefore potentially susceptible to BALO predation. Additionally, the commensal, albeit beneficial, periodontal microbiota are mainly gram-positive and therefore resistant to BALO predation. Also, BALOs have typically a wide prey range. There is apparently also a lack of bacterial resistance mechanisms to predation (Sockett & Lambert, 2004). These are major advantages when compared with phage therapy (Moore, 2004). BALOs are also generally regarded as safe. They are ubiquitous in nature and have even been isolated from the human intestine (Edao, 2001). Additionally, they are unable to infect eukaryotic cells (Lenz & Hespell, 1978) and *Bdellovibrio* lipopolysaccharide does not induce a strong immunological response (Schwudke *et al.*, 2003). These biological properties of BALOs match remarkably well with the specific requirements for an oral biological antimicro-

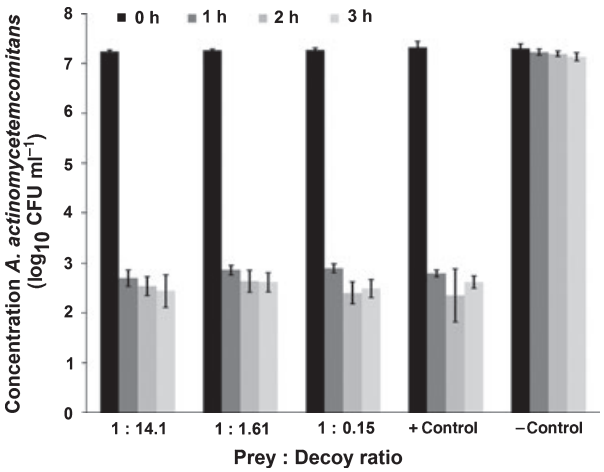

Figure 3 The effect of bacterial decoys on predation efficiency. The effect of bacterial decoys on predation efficiency was evaluated by exposing the *Aggregatibacter actinomycetemcomitans* prey to *Bdellovibrio bacteriovorus* HD100 in combination with different concentrations of *Actinomyces naeslundii* decoy cells: The average concentration of *A. actinomycetemcomitans* cells exposed to *B. bacteriovorus* was 7.28 $\log_{10}$ colony-forming units (CFU) ml^{-1}. At the start of the experiment the MOI predator : prey was on average 1562 (± 2168 SEM). The concentration of decoy cells in the experimental suspensions was 8.39 $\log_{10}$ CFU ml^{-1} *Actinomyces naeslundii*, 7.47 $\log_{10}$ CFU ml^{-1} and 6.44 $\log_{10}$ CFU ml^{-1}. Therefore the actual prey : decoy ratio was 1 : 14.1, 1 : 1.61 and 1 : 0.15. The positive control contained only prey cells and predatory cells. The negative control contained only prey cells. Prey viability is shown after 0 h (black), 1 h (darkest grey), 2 h (medium grey) and 3 h (light grey). Error bars represent standard errors of the mean (n = 3).

bial agent. It must be emphasized, however, that the extrapolation of the general principles of predatory behaviour of BALOs to an efficient antimicrobial application is complex. The interaction between a predator and its prey in physiological settings depends on a range of variables that may require extensive optimization to obtain adequate results in therapeutic applications.

In a recent study, it was shown that the periodontal pathogen *A. actinomycetemcomitans* is susceptible to *B. bacteriovorus* HD100 predation in environmental conditions resembling the oral cavity (Van Essche *et al.*, 2009a). Using a 1.14 : 1 predator : prey ratio, an approximately 2.43 log10 decrease in pathogen viability was achieved after 8–12 h of incubation with the predator. Additionally, no differences in predator efficiency were shown for different *A. actinomycetemcomitans* strains. These results represented a starting point but for possible therapeutic or prophylactic applications more efficient reductions of pathogen viability are desirable. Therefore an optimization of the predation kinetics was performed in the current study by challenging *A. actinomycetemcomitans* with different *Bdellovibrio* concentrations. The results showed that the higher the concentration of predatory cells, the more pronounced and the faster the decrease in pathogen viability is. A reduction of pathogen viability of 4.38 $\log_{10}$ values in 1 h of incubation could be achieved.

Although the obtained results look promising in terms of the development of an oral BALO application, periodontal diseases are multi-species infectious diseases involving an array of pathogens. It has been described that phylogenetically different BALOs exhibit different prey spectra (Jurkevitch *et al.*, 2000). It has also been shown that predators that have been isolated from different habitats (e.g. sewage compared with soil) have a distinct preference for certain prey taxa (Starr & Seidler, 1971). A complicating factor is that prey preference has recently been shown to be a dynamic feature that results in the preferential predation of the favoured prey (Rogosky *et al.*, 2006). From these studies we could assume that not all periodontopathogens might be susceptible to BALO predation, and that different BALOs can have different predation efficiencies towards different periodontopathogens. Consequently, the prey spectrum and predation efficiency of six BALO strains from different phylogenetic taxa were determined on six different key periodontopathogens. For the tested BALO strains, overall performance was judged on the number of pathogenic strains that could be affected (prey spectrum) and the amount of decrease of the affected pathogens (efficiency). The obtained prey spectra showed that a single BALO strain can attack and kill different pathogenic species but also that none of the tested BALO strains covered all the tested pathogens. *Bdellovibrio bacteriovorus* HD100 had the widest prey spectrum whereas *Bacteriovorax stolpii* Uki2 did not predate any of the tested prey bacteria. Also in terms of predation efficiency, there were major inter-strain differences. Therefore each prey was not susceptible for each BALO. This underlines that the predator–prey interactions are highly BALO strain specific. According to the given criteria, the type strain *B. bacteriovorus* HD100 performs better than the other BALOs tested. The specific mechanisms that cause the exact prey preference of BALO

strains remains to be resolved but apparently the predation pattern of BALOs is unrelated to taxonomic status of the BALOs (Jurkevitch *et al.*, 2000). This is illustrated by the fact that *A. actinomycetemcomitans* is preyed upon by *B. bacteriovorus* strain HD100 but not by the closely related strain 109J.

A remarkable observation in these experiments is that strictly anaerobic bacteria such as *F. nucleatum* and *P. intermedia* are subject to BALO attack. To our knowledge, this is the first study that reports on predation of anaerobic bacteria by BALOs. It is an interesting question if anaerobic prey bacteria can effectively sustain the full life cycle of BALOs in aerobic conditions. The experimental conditions used here allow for the study of prey viability but the high concentration of BALOs necessary for synchronous infection rates presents an condition that makes the study of potential growth of the BALO population on relatively low prey numbers impossible.

Some conclusions can be reached if we look at the decrease of the pathogen viability of the control series during the 3 h of the experiment. The 'non-predation related' bacterial death (caused mainly by oxidative stress and starvation) reduced the viable counts to 45.7% for *F. nucleatum* and 29.8% for *P. intermedia* compared with the start of the experiment. Hence, a substantial population of prey remains viable in these aerobic conditions during the 3 h of experiment. From the early studies of Seidler & Starr (1969), the average time for *Bdellovibrio* to complete its life cycle ranges from 2 to 4 h so it can be concluded that a substantial portion of the pathogen population can endure environmental stress in aerobiosis for a period long enough to complete the intraperiplasmatic life cycle of the predators. Moreover, previous work has described how premature lysis (or possibly death by aerobic stress) of the bdelloplast is no obstacle for the formation of fully differentiated attack cells (Ruby & Rittenberg, 1983).

The oxygen tension at different locations in the oral cavity varies from oxygen-saturated saliva to the micro-aerophilic apex of the periodontal pocket. At this point it is not certain to which degree this aspect restricts the physiological range of BALOs in their hunt for periodontopathogens. Our experiments were performed in aerobic conditions that favour BALO respiration. When investigating the relation of BALOs and (facultative) anaerobic prey, it should be considered that BALOs can be active in environments with lower oxygen tension (Schoeffield *et al.*, 1996), especially as genome analysis of *B. bacteriovorus* HD100 has revealed a number of adaptations to their survival in oxygen-limiting conditions. First, an alternative cytochrome oxidase complex (Cytbb3) has been identified that facilitates microaerophilic respiration in other proteobacteria. Second, nitrite reductase and nitric oxide reductase genes have been identified in the HD100 genome, indicating that other electron acceptors can be used to make anaerobic respiration possible (Sockett & Lambert, 2004). These findings expand the potential environments that could support predation by BALOs to micro-aerophilic and anaerobic habitats such as, perhaps, the periodontal pocket.

As not all periodontopathogens were effective prey for the BALOs and because gram-positive bacteria are not susceptible to BALO predation, it was important to evaluate how these non-target decoy bacteria could influence predation efficiency of a BALO. It should be clear that in the oral cavity, prey and non-target bacteria live in close proximity to each other. The presence of non-target bacteria could hypothetically lower the BALO efficiency by sterical hindrance.

The possible interference of decoy cells with predation was first mentioned by Hobley *et al.* (2006), who presented the first data concerning this issue. Their initial experimental model contained predator, prey and a gram-positive decoy. In this configuration, it was established that when a low MOI of 1 : 142 predator : prey was used, the presence of non-prey organisms decreased the speed of BALO predation. However, the final reduction of prey viability and multiplication of predators was identical to the control experiment without the presence of the decoy cells. The authors suggest that the delay in predation will probably not be observed if a higher MOI is used. Similar observations were made in our experiments as no inhibition of predation was observed even if the decoy cell numbers reached approximately 8 $\log_{10}$ CFU ml^{-1}.

It can be concluded that the oral application of BALO strains at high inoculum concentrations has the potential to rapidly decrease the numbers of a wide range of periodontal pathogens from the mixed oral microbiota. Predatory therapy can therefore be regarded as an interesting opportunity for the development of an adjuvant to standard periodontal therapy.

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