molecular oral microbiology

molecular oral microbiology

Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J

A. Dashiff and D.E. Kadouri

Department of Oral Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA

Correspondence: Daniel Kadouri, Department of Oral Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07101, USA
Tel.: +1 973 972 7401; fax: +1 973 972 0045; E-mail: kadourde@umdnj.edu

Keywords: *Bdellovibrio bacteriovorus;* biofilm; biofilm control; oral bacteria
Accepted 8 September 2010
DOI: 10.1111/j.2041-1014.2010.00592.x

SUMMARY

Periodontal diseases are multifactorial infections elicited by a complex of primarily gram-negative bacteria that interact with host tissues and lead to the destruction of the periodontal structures. *Bdellovibrio bacteriovorus* is a gram-negative bacterium that preys upon other gram-negative bacteria. It was previously shown that *B. bacteriovorus* has an ability to attack and remove surface-attached bacteria or biofilms. In this study, we examined the host specificity of *B. bacteriovorus* strain 109J and its ability to prey on oral pathogens associated with periodontitis, including; *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Tannerella forsythia.* We further demonstrated that *B. bacteriovorus* 109J has an ability to remove biofilms of *Ei. corrodens* as well as biofilms composed of *A. actinomycetemcomitans*. *Bdellovibrio bacteriovorus* was able to remove *A. actinomycetemcomitans* biofilms developed on hydroxyapatite surfaces and in the presence of saliva, as well as to detach metabolically inactive biofilms. Experiments aimed at enhancing the biofilm removal aptitude of *B. bacteriovorus* with the aid of extracellular-polymeric-substance-degrading enzymes demonstrated that proteinase-K inhibits predation. However, treating *A. actinomycetemcomitans* biofilms with DspB, a poly-*N*-acetylglucosamine (PGA) -hydrolysing enzyme, increased biofilm removal. Increased biofilm removal was also recorded when *A. actinomycetemcomitans* PGA-defective mutants were used as host cells, suggesting that PGA degradation could enhance the removal of *A. actinomycetemcomitans* biofilm by *B. bacteriovorus*.

INTRODUCTION

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and lead to the destruction of the periodontal structures, including the tooth-supporting tissues, alveolar bone and periodontal ligament. Periodontal disease is a significant global public-health concern and is probably the most common chronic infectious disease in humans. In the United States alone, more than 70% of the population is afflicted with the disease with approximately 54% of all US adults aged 30 years or more suffering from some form of periodontal disease (Albandar & Kingman, 1999). The importance of bacteria in dental plaque and the key role of plaque in the causation of periodontal disease is well established. Whereas oral bacteria colonize and produce disease primarily in the oral cavity, they can also produce systemic disease (Scannapieco, 1998; Garcia *et al.*, 2001). Therefore, the mitigation of oral infection is of broad clinical importance beyond the boundaries of the oral cavity. Among the bacteria frequently isolated from periodontal pockets are the

gram-negative bacteria *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Tannerella forsythia*, all of which are strongly associated with various forms of periodontitis including localized aggressive periodontitis, generalized early onset periodontitis and chronic periodontitis (Dzink *et al.*, 1985; Wilson *et al.*, 1991; Bolstad *et al.*, 1996; Tanner & Izard, 2006; Fine *et al.*, 2007). In addition to their role in periodontal disease, the presence of these bacteria was also implicated as a causative agent of several non-oral infections (Kaplan *et al.*, 1989; Beck *et al.*, 1996; Offenbacher *et al.*, 1996; Dibart *et al.*, 1998; Takamatsu *et al.*, 1999; Roberts, 2000; Chang *et al.*, 2004; Hombach *et al.*, 2007; Miller *et al.*, 2007; Kajiya *et al.*, 2008).

The difficulty in removing oral plaque or biofilms by conventional therapies led researchers to examine other alternative methods for biofilm control, such as biological control agents. One biological agent that might be used to control pathogenic bacteria is the predatory prokaryotes from the genus *Bdellovibrio*. *Bdellovibrio* are gram-negative bacteria that feed on other gram-negative bacteria (Stolp & Starr, 1963; Sockett, 2009). Recently, it was demonstrated that *B. bacteriovorus* 109J can significantly reduce biofilms developed in a microtiter dish-based static assay as well as in a flow cell system (Kadouri & O'Toole, 2005; Nunez *et al.*, 2005; Medina *et al.*, 2008). *Bdellovibrio bacteriovorus* strain HD100 was also shown to be able to attack and kill four smooth strains and one rough biofilm-forming strain of *A. actinomycetemcomitans* (Van Essche *et al.*, 2009).

In this study, the susceptibility of oral pathogens to predation by *B. bacteriovorus* 109J was examined in liquid suspension and on biofilms. The predatory ability of *B. bacteriovorus* was also assessed under different clinically relevant growth conditions. Finally, an attempt was made to enhance the biofilm removal aptitude of *B. bacteriovorus* with the use of other biofilm-degrading enzymes.

METHODS

Bacteria strains, media and growth conditions

The strains used in this study are listed in Table 1. *Aggregatibacter actinomycetemcomitans* was routinely grown on brain–heart infusion (BHI) and *Ei. corrodens* was grown on tryptic soy broth (TSB) blood agar plates (5% defibrinated sheep blood and 1.5% agar) or in TSB containing 2 mg ml^{-1} KNO_3 and 5 μg ml^{-1} hemin. The *F. nucleatum* was cultured on TSB blood agar plates; *Pr. intermedia*, *Po. gingivalis* and *T. forsythia* were cultured on TSB blood agar plates supplemented with 5 μg ml^{-1} hemin and 1 μg ml^{-1} menadione. *N*-Acetylmuramic acid (0.001%) was added to the *T. forsythia* plates. Both *A. actinomycetemcomitans* and *Ei. corrodens* were cultured at 37°C in 10% CO_2, whereas *F. nucleatum*, *Pr. intermedia*, *Po. gingivalis* and *T. forsythia* were cultured anaerobically (10% CO_2, 10% H_2 and 80% N_2) in a MACS MG 250 anaerobic chamber (Microbiology International, Frederic, MD) at 37°C. The *B. bacteriovorus* was maintained as plaques in double-layered diluted nutrient broth [DNB; 0.8 g l^{-1} nutrient broth amended with 3 mM $MgCl_2{\cdot}6H_2O$ and 2 mM $CaCl_2{\cdot}2H_2O$ (pH 7.2)] agar (0.6% agar in the top layer) (Starr, 1975). To initiate a lysate, *B. bacteriovorus* co-cultures were obtained by adding a plug of agar containing *B. bacteriovorus* plaque to 1×10^8 colony-forming units (CFU) ml^{-1} washed *Escherichia coli* prey in DNB, and incubated at 30°C on a rotary shaker set at 200 r.p.m. until the co-culture became clear (stock lysate). To harvest *B. bacteriovorus*, co-cultures were prepared in which 2 ml overnight-grown washed *E. coli* host cells (1×10^9 CFU ml^{-1}) were incubated with 2 ml stock lysate in 20 ml DNB. The co-cultures were incubated for 18 h to reach a final concentration of approximately 1×10^8 plaque-forming units ml^{-1} of predator. At this point the lysate was passed three times through a 0.45-μm Millex pore-size filter (Millipore, Billerica, MA) to remove residual prey and cell debris (filtered lysate). As a control, filtered sterilized lysate was prepared by sequentially passing the *B. bacteriovorus* culture through three 0.22-μm pore-size filters. After filtration, no predator, as judged by plaque-forming units, could be detected (Kadouri & O'Toole, 2005).

Biofilm assays

Biofilms of *A. actinomycetemcomitans* were developed as described previously (Izano *et al.*, 2007, 2008) with some modifications. *Aggregatibacter actinomycetemcomitans* rough-colony strains were grown on plates for 48 h. The colonies were scraped into fresh BHI medium and homogenized to reach a final concentration of 1×10^7 CFU ml^{-1} absorbance

Table 1 Bacteria used in the study

Name	Serotype	Source
Aggregatibacter actinomycetemcomitans		
Rough-colony strains		
NJ5000	a	UMDNJ (Kaplan *et al.*, 2002)
DL 1159	a	UMDNJ
NJ3500	b	UMDNJ (Kaplan *et al.*, 2002)
DL865	b	UMDNJ
DL 1067-1	b	UMDNJ
DL 1171	b	UMDNJ
DL 639-2	b	UMDNJ
DL 772	b	UMDNJ
DF2300	c	UMDNJ (Kaplan *et al.*, 2002)
DL 1093-2	c	UMDNJ
DL 1108-2	c	UMDNJ
DL 1148	c	UMDNJ
IDH781	d	UMDNJ (Kaplan *et al.*, 2002)
NJ9500	e	UMDNJ (Kaplan *et al.*, 2002)
CU1000	f	UMDNJ (Kaplan *et al.*, 2002)
HW1018 (CU1000 *pgaC*::IS903ϕ*kan*)	f	UMDNJ (Izano *et al.*, 2007)
Smooth-colony strains		
ATCC 29523	a	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
SUNYab75	a	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
ATCC 29524	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
JP2	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
NK1651	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
Y4	b	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
Aa307	c	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
IDH781S	d	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
IDH1705	e	UMDNJ (Kaplan *et al.*, 2002; Rupani *et al.*, 2008)
CU1060	f	UMDNJ (Rupani *et al.*, 2008)
Eikenella corrodens		
ATCC 23834	–	USC (Henriksen, 1969)
H2S-1	–	USC
D4P-1	–	USC
D11P-1	–	USC
RMA 12256	–	RMARL
RMA 12259	–	RMARL
RMA 12794	–	RMARL
RMA 15501	–	RMARL
Ec-f2	–	Forsyth Institute
Fusobacterium nucleatum		
PK1594	–	UMDNJ (Rupani *et al.*, 2008)
ATCC 10953	–	UMDNJ (Rupani *et al.*, 2008)
Prevotella intermedia		
ATCC 25611	–	ATCC (Shah & Collins, 1990)
Porphyromonas gingivalis		
ATCC 33277	–	ATCC (Tran & Rudney, 1996)
W83/ATCC BAA-308	–	Forsyth Institute
Tannerella forsythia		
ATCC 43037	–	Forsyth Institute (Sakamoto et al., 2002; Tanner & Izard, 2006)
Bdellovibrio bacteriovorus		
109J/ATCC 43826	–	ATCC (Kadouri & O'Toole, 2005)

UMDNJ, Department of Oral Biology strain collection, University of Medicine and Dentistry of New Jersey; RMARL, R.M. Alden Research Laboratory, Santa Monica CA; USC, Division of Periodontology, University of Southern California School of Dentistry; Forsyth Institute, Department of Molecular Genetics, The Forsyth Institute; and ATCC, American Type Culture Collection.

at 595 nm (A_{595}) = 0.07. Cells (100 μl) were transferred to the wells of a 96-well, flat-bottom, tissue culture-treated, polystyrene microtiter plate and incubated for 24 h at 37°C in 10% CO_2. Non-adherent cells were removed by washing, and adherent cells were stained with 0.1% crystal violet (CV) as described previously (Merritt *et al.*, 2005). Photographs of the plate were taken using a Canon-scan 4400F digital scanner. The CV was solubilized using 50% acetic acid for 10 min. Relative biofilm formation was assayed by measuring the absorbance of the CV solution at 600 nm (A_{600}). Biofilms of *Ei. corrodens* were developed for 36 h in six-well, tissue culture-treated, polystyrene plates, which contained harvested *Ei. corrodens* cells resuspended in fresh TSB broth containing KNO_3 and hemin.

For biofilm formation on hydroxyapatite, 3 × 3 mm hydroxyapatite squares, prepared from sintered food-grade hydroxyapatite (NEI Industries, Sesser, IL; Sreenivasan *et al.*, 2009), were inserted into a 12-well polystyrene plate. The wells were filled with 0.5 ml BHI medium containing *A. actinomycetemcomitans* cells and incubated for 24 h to allow biofilm development.

Prey range assay

To evaluate the ability of *B. bacteriovorus* to prey on the selected oral bacteria, co-cultures were prepared in which washed host cells were incubated in 20 ml DNB with 2 ml harvested *B. bacteriovorus*. As a control, filtered sterilized lysate was used. The cultures were incubated at 30°C on a rotary shaker set at 200 r.p.m. The ability of *B. bacteriovorus* to prey was confirmed by the reduction in culture turbidity caused by the lysis of host cells during predation. Culture turbidity was examined by removing 100-μl aliquots and reading the absorbance in a BioRad 680 microplate reader (A_{595}). Additional confirmation of active predation was provided by microscopy evaluation (×1000 magnification).

Biofilm removal assays

To assess the ability of *B. bacteriovorus* 109J to remove *A. actinomycetemcomitans* and *Ei. corrodens* biofilms, the biofilms were grown as described above, washed twice with DNB to remove planktonic cells, and 100 μl filtered *B. bacteriovorus* from an 18-h lysate was added. As a control, 100 μl filtered sterilized lysate was used. The dishes were incubated at 30°C for the duration of the experiments.

Removal of *A. actinomycetemcomitans* biofilms in the presence of saliva

To investigate whether biofilm removal could occur in the presence of saliva, preformed *A. actinomycetemcomitans* biofilms were developed, and washed, and fresh DNB media containing varying amounts of filter-sterilized unstimulated saliva (from 0 to 100%), and *B. bacteriovorus*, were added. Unstimulated saliva was collected, on ice, in a 50-ml tube, centrifuged for 2 min at 5000 ***g*** to remove cell debris, and filter sterilized using 0.22-μm pore-size filter.

Predation by *B. bacteriovorus* under oxygen-limiting conditions

To measure the ability of *B. bacteriovorus* to prey in anaerobic and microaerophilic conditions, *B. bacteriovorus* host co-cultures were prepared and placed in a BD GasPak Jar Systems with a disposable gas-generating anaerobic or microaerophilic envelope (BD Diagnostic Systems, Franklin Lakes, NJ). The jars were incubated at 30°C on a rotary shaker at 200 r.p.m. Predation was measured using CFU of the surviving host cells.

Obtaining metabolically inactive biofilms

To obtain metabolically inactive biofilms of *A. actinomycetemcomitans*, the *A. actinomycetemcomitans* CU1000 biofilms were developed as described above, washed and incubated for 96 h in DNB. To confirm loss of biofilm viability, the biofilm cells were removed with the aid of a tissue culture scraper, and plated on BHI plates for CFU enumeration. Additional verification of cell metabolic activity loss was measured by adding Alamar-Blue cell viability reagent (Invitrogen, Carlsbad, CA) to the biofilm and measuring the change in fluorescence (Pettit *et al.*, 2005). AlamarBlue® works as a cell viability and proliferation indicator through the conversion of resazurin to resorufin. Resazurin, a non-fluorescent indicator dye, is converted to highly red fluorescent resorufin via reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells (product literature; Trek Diagnostic Systems,

Cleveland, OH). No fluorescence signal or colony growth was measured in the 4-day-old, DNB-suspended biofilm, confirming loss of biofilm cell viability.

Scanning electron microscopy

Experiments were performed as described previously (Kadouri & O'Toole, 2005). In brief, biofilms were developed on a 12 × 12-mm PVC plastic cover slip (Fisher Scientific, Pittsburgh, PA). The cover slips were placed in a 24-well polystyrene cell culture plate (Corning Inc., Corning, NY). Preformed biofilms and predation assay were prepared as described above. The experiments were carried out in a 1.0 ml volume. Biofilms were rinsed to remove any planktonic cells before being fixed in 2% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1% ruthenium red. Images were viewed at the air–liquid interface using a Zeiss Auriga field emission scanning electron microscope.

RESULTS

Host range specificity

To examine the ability of *B. bacteriovorus* 109J to prey on bacteria commonly associated with periodontal disease, bacteria were cultured, then incubated in the presence of *B. bacteriovorus.* As seen in Fig. 1A, *B. bacteriovorus* was able to prey on all *A. actinomycetemcomitans* serotypes tested. Microscopy evaluation of the co-cultures had further confirmed predation, showing a reduction in *A. actinomycetemcomitans* cells (fewer than 10% of total cells in a field of view) and an increase in *B. bacteriovorus* cells (90% of the cell population in each field of view), following a 48-h incubation period. In this assay, only *A. actinomycetemcomitans* strains that exhibited smooth-colony morphology were used. These strains were selected for their inability to attach to the surface of the tube and form biofilms or aggregates (Fine *et al*., 1999; Rupani *et al*., 2008), thus reducing the likelihood of culture turbidity decrease as a result of biofilm formation or cell aggregation. In addition to the ability of *B. bacteriovorus* to prey on *A. actinomycetemcomitans*, predation was also observed when *Ei. corrodens* was used as host (Fig. 1B). The *B. bacteriovorus* was unable to prey on *Po. gingivalis*, *Pr. intermedia*, *T. forsythia* and *F. nucleatum* ATCC 10953 (data not shown); however, a positive reduction in turbidity was measured when *F. nucleatum* PK1594 was used as host. A reduction in culture turbidity, from A_{595} = 0.26 to A_{595} = 0.091 was

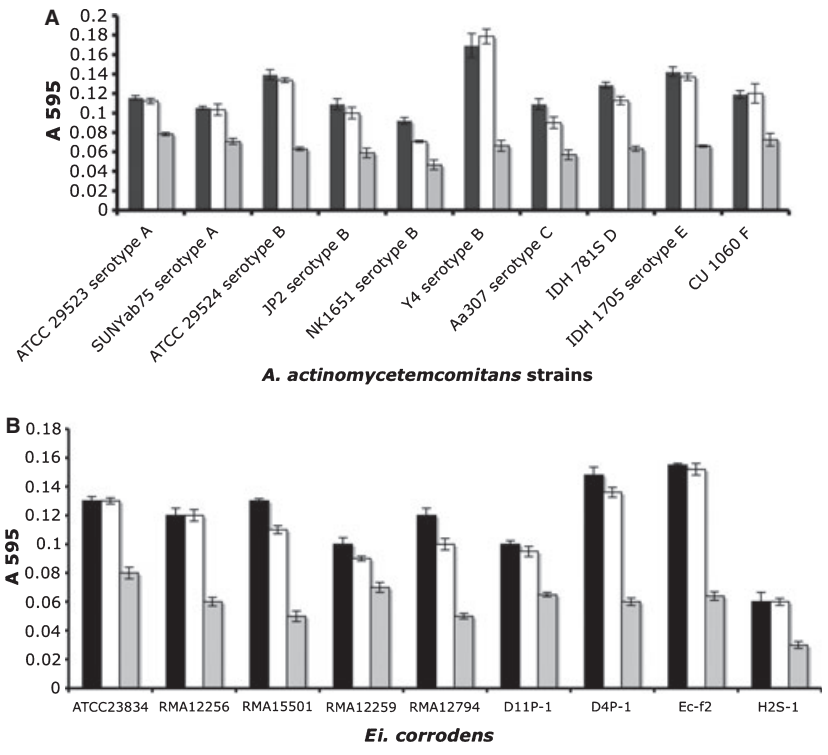

Figure 1 Host range predation assay. Overnight cultures of *Aggregatibacter actinomycetemcomitans* (A) and *Eikenella corrodens* (B) were incubated with harvested *Bdellovibrio bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). Cocultures were incubated for 48 h. Predation was confirmed by the reduction in culture turbidity measured at 595 nm (A_{595}). Black bars represent culture turbidity at time-0. Each value represents the mean of 3 cocultures. Error bars are shown as one-standard deviation.

measured following 48 h incubation with the predator, with limited turbidity reduction measured in the *B. bacteriovorus* – free control (from A_{595} = 0.26 to A_{595} = 0.24). Active predation on the *F. nucleatum* PK1594 was also verified by light microscopy.

A. actinomycetemcomitans biofilm removal assay

To measure the effect of *B. bacteriovorus* 109J on *A. actinomycetemcomitans* biofilms, we developed conditions that yield stable *A. actinomycetemcomitans* biofilms in a 96-well dish. Biofilms were formed in BHI medium for 24 h. Thereafter, the medium was removed and replaced with DNB as described in the Methods. Using this method, stable biofilms were developed from 15 *A. actinomycetemcomitans* isolates, all exhibiting rough-colony morphology phenotype, which is typical of fresh clinical isolates (Fine *et al.*, 1999). The preformed biofilms were incubated with *B. bacteriovorus* or a control filtered sterilized lysate. By 48 h, a measurable reduction (>81%) in biofilm biomass was recorded for all of the strains tested (Fig. 2A,B). In another experiment, *A. actinomycetemcomitans* CU1000 biofilms were developed on hydroxyapatite squares (Fig. 2C, preformed biofilm). When incubated in the presence of *B. bacteriovorus*, a substantial reduction in biofilm CV staining was seen by 72 h, with no reduction in the control inoculated sample (Fig. 2C, *B. bacteriovorus* and control respectively).

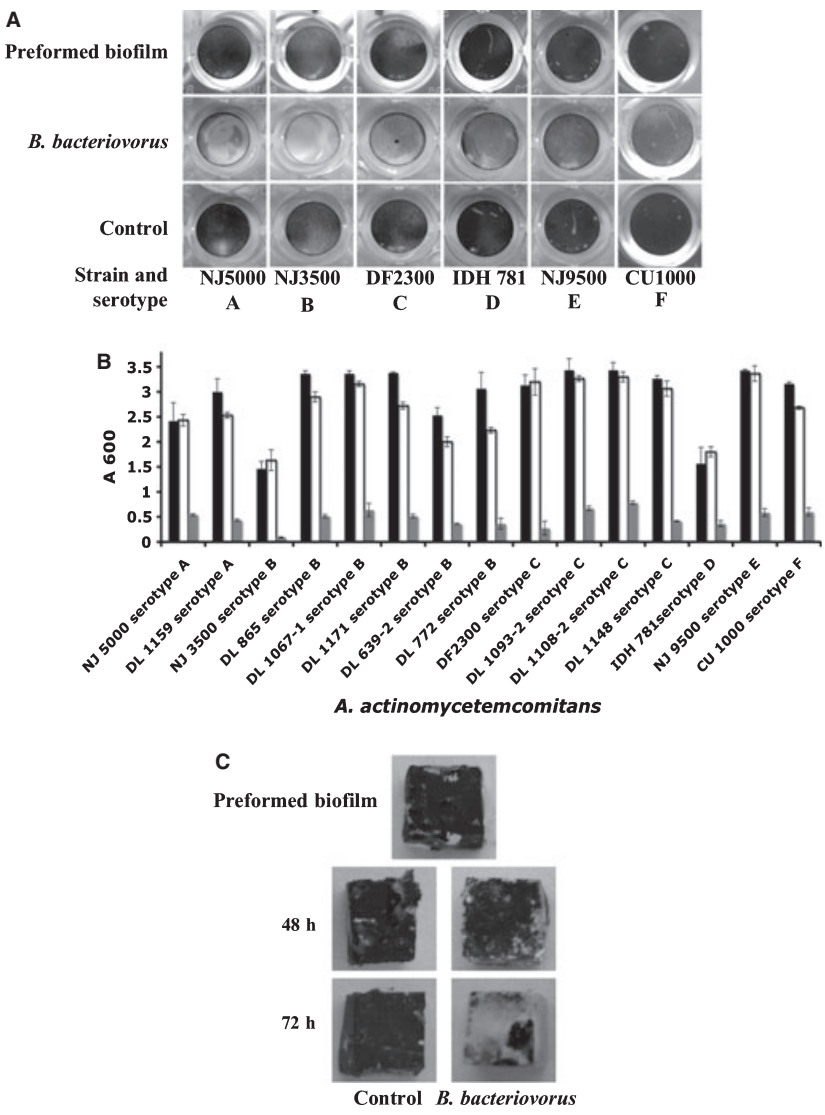

Figure 2 Removal of *Aggregatibacter actinomycetemcomitans* biofilms by *Bdellovibrio bacteriovorus*. (A) Biofilms of *A. actinomycetemcomitans* were developed in 96-well plates for 24 h (Preformed biofilm) following a 48-h incubation period with *B. bacteriovorus* 109J (*B. bacteriovorus*) or filtered sterilized lysate (Control). Biofilms were rinsed and stained with CV. (B) Quantification of biofilm reduction. Preformed overnight biofilms (black bars) were incubated for 48 h with *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The wells were rinsed, stained with CV, and the amount of CV staining was quantified at 600 nm (A_{600}). Each value represents the mean of 12 wells. Error bars are shown as one-standard deviation. (C) Biofilms of *A. actinomycetemcomitans* CU1000 were developed on hydroxyapatite squares then treated with the predator (*B. bacteriovorus*) or filtered sterilized lysate (Control).

Ei. corrodens biofilm removal assay

The ability of *B. bacteriovorus* to remove biofilms composed of *Ei. corrodens* was also assessed (Fig. 3 A–C). *Eikenella corrodens* preformed biofilms were developed in six-well plates, washed and inoculated with the predator. By 48 h, a measurable reduction in biofilm CV staining was obtained on all of the examined strains. However, the degree of reduction varied from 81 to 45%, as several of the tested isolates did not form stable biofilms resulting in some degree of biofilm detachment within the control sample. Removal of *Ei. corrodens* biofilms by *B. bacteriovorus* was further visualized and confirmed by SEM imaging, showing a reduction in surface cell coverage in the *B. bacteriovorus* treated sample.

In the following experiments, *A. actinomycetemcomitans* CU1000 was used. This isolate was selected for its ability to form stable biofilms, which showed little detachment with time. In one experiment, a reduction of 1.25% in biofilm biomass staining was measured during a 100-day incubation period in DNB (from A_{600} = 0.83 at day one to A_{600} = 0.79 at day 100), demonstrating the stability of the biofilms.

Removal of *A. actinomycetemcomitans* biofilms in the presence of saliva

To investigate whether biofilm removal could occur in the presence of saliva, preformed *A. actinomycetemcomitans* biofilms were incubated with *B. bacteriovorus* and varying amounts of filter-sterilized saliva. As seen in Fig. 4A, a substantial reduction (77%) in biofilm was measured in the saliva-free *B. bacteriovorus*-treated sample. Incubating *B. bacteriovorus* in 100% saliva did not hinder the ability of the predator to remove the biofilm. A reduction of 43% was registered in the saliva plus *B. bacteriovorus*-treated samples when compared with the control biofilm incubated with saliva alone (Fig. 4A, 100% saliva).

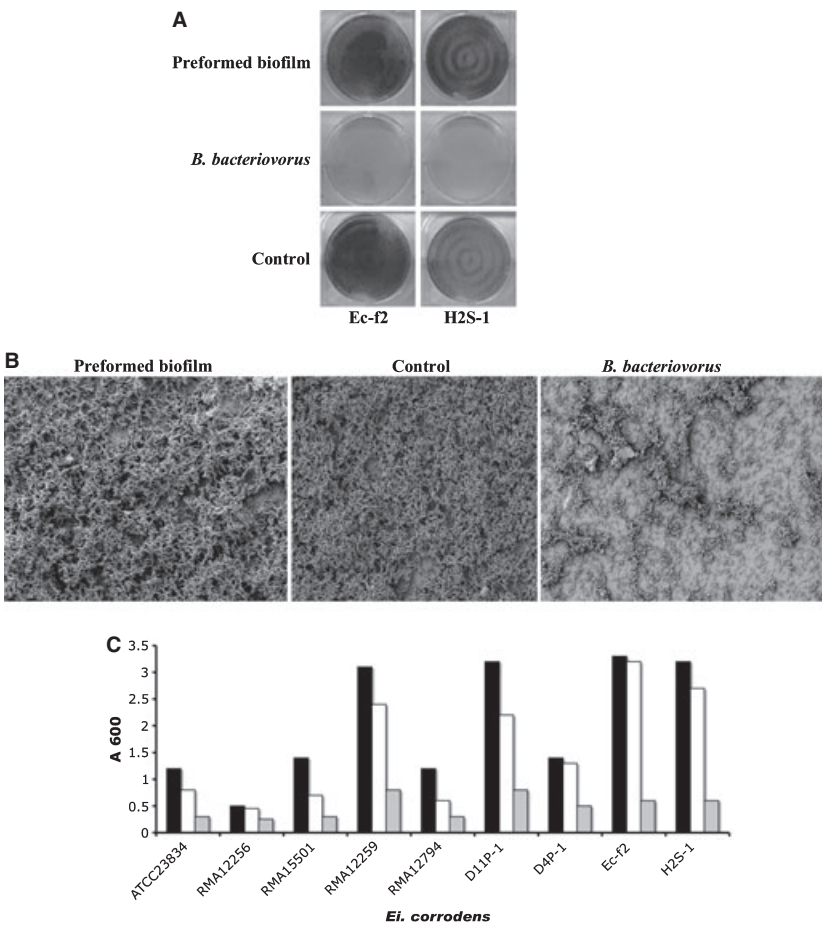

Figure 3 Predation of *Eikenella corrodens* biofilms by *Bdellovibrio bacteriovorus*. (A) Biofilms of *Ei. corrodens* were developed in six-well plates for 36 h (Preformed biofilm) followed by a 48-h incubation period with *B. bacteriovorus* 109J (*B. bacteriovorus*) or filtered sterilized lysate (Control). Biofilms were rinsed and stained with CV. For SEM imaging, *Ei. corrodens* H2S-1 biofilms were developed on PVC plastic cover slips. Scanning electron micrographs were viewed at 1000× magnification (B). (C) Quantification of biofilm reduction. Preformed overnight biofilms (black bars) were incubated for 48 h with *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The wells were rinsed, stained with CV, and the amount of CV staining was quantified. Each value represents the mean of two wells from one representative experiment.

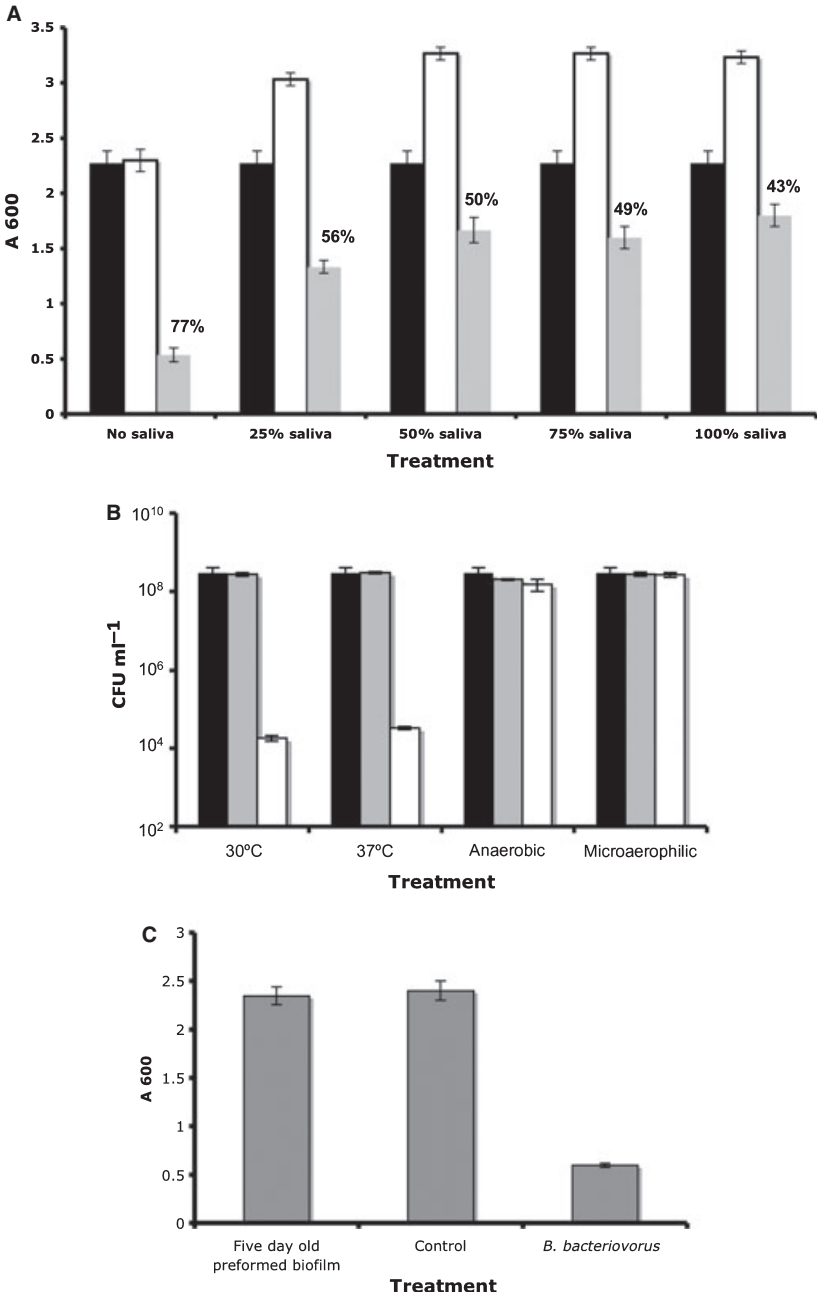

Figure 4 Reduction of host cells under various conditions. (A) Quantification of biofilm reduction in the presence of saliva. Preformed overnight *Aggregatibacter actinomycetemcomitans* CU1000 biofilms (black bars) were incubated with varying concentrations of filtered sterilized unstimulated saliva (from 0 to 100%), containing *Bdellovibrio bacteriovorus* 109J (gray bars) or filtered sterilized lysate (white bars). After 48 h, the wells were rinsed, stained with CV, and the amount of CV staining was quantified. Each value represents the mean of 12 wells. Error bars are shown as one-standard deviation. Numbers above the bars represent the average percent reduction in biofilm biomass compared with the control. (B) *B. bacteriovorus* predation capability in adverse culture conditions. *Escherichia coli* ZK2686 host cells were incubated for 48 h in the presence of *B. bacteriovorus* 109J (gray bars) or filtered sterilized lysate control (white bars). The co-cultures were incubated at 30 and 37°C (normal oxygen levels) or at 30°C under anaerobic or microaerophilic growth conditions. Black bars represent host cell numbers at time 0. Each value represents the mean of three cultures. Error bars are shown as one-standard deviation. (C) Ability of *B. bacteriovorus* to remove metabolically inactive biofilms. Preformed overnight *A. actinomycetemcomitans* CU1000 biofilms were developed in 96-well plates for 24 h. Thereafter, DNB was added to the biofilm and incubated for 96 h (5-day-old preformed biofilm). Filtered sterilized lysate (Control) or *B. bacteriovorus* was then added to the metabolically inactive biofilms. Biofilms were stained and quantified. Each value represents the mean of 18 wells. Error bars are shown as one standard deviation.

B. bacteriovorus predation in adverse culture conditions

As some oral pathogens reside within the subgingival area, where the oxygen concentration is limited, we were interested in investigating the ability of *B. bacteriovorus* to prey in anaerobic and microaerophilic conditions. To this end, standard *B. bacteriovorus* host co-cultures were prepared using washed *E. coli* strain ZK2686, a derivative of W3110, (Pratt & Kolter, 1998) as prey. The cultures were placed in a BD GasPak Jar systems with a disposable gas generating anaerobic or microaerophilic envelope. Counting the CFU of the surviving host revealed that *B. bacteriovorus* was unable to prey under oxygen-limiting conditions (Fig. 4B). Other predation experiments in which the co-cultures were placed in a MACS MG 250 anaerobic chamber (10% CO_2, 10% H_2 and

80% N_2) also produced no reduction in host population (data not shown). Interestingly, when the cultures were removed from the oxygen-limiting conditions (after 72 h) and placed in an aerobic environment, predation did occur, reducing host cell CFU numbers by 3 logs (from 2×10^8 to 1×10^5 CFU ml^{-1}). The inability of *B. bacteriovorus* to prey under anaerobic and microaerophilic conditions was also seen when *E. coli* strain DH5α was used as prey (data not shown) or when *A. actinomycetemcomitans* strain JP2 was used (from an initial optical density of $A_{595} = 0.112$ to $A_{595} = 0.109$ and $A_{595} = 0.102$ for anaerobic and microaerophilic conditions, following 48 h of incubation, respectively). As was seen for *E. coli*, reduction in turbidity did occur when the anaerobic co-cultures were replaced in aerobic conditions ($A_{595} = 0.075$). Although *B. bacteriovorus* was restricted by its ability to prey in anaerobic and microaerophilic conditions, it was not restricted to prey at higher temperatures of 37°C (Fig. 4B).

Reduction of metabolically inactive biofilms

It was previously suggested that cells within biofilms might exhibit limited growth, which in turn increases their resistance to antimicrobial challenges (Keren *et al.*, 2004; Werner *et al.*, 2004; Yang *et al.*, 2008; Hoiby *et al.*, 2010). Hence, the ability of an antimicrobial agent to reduce and remove metabolically inactive surface-associated bacteria could be beneficial.

When growing biofilms of *A. actinomycetemcomitans* CU1000, we noticed that although the preformed biofilm is extremely stable, CFU enumeration and Alamar-Blue cell viability reagent assays revealed that the biofilm contained no viable cells, following a 96-h incubation period in DNB. At this point, an assay was carried out in which 24-h-preformed biofilms were washed, resuspended in DNB, and incubated for 120 h. The stable, but unviable biofilm was incubated with *B. bacteriovorus* or filter-sterilized lysate. As seen in Fig. 4C, a reduction of 75% in CV staining was measured following incubation with *B. bacteriovorus*, confirming removal of the metabolically inactive biofilm.

Enhancing the ability of *B. bacteriovorus* to remove biofilms of *A. actinomycetemcomitans*

It was previously reported that *A. actinomycetemcomitans* biofilms are composed of cells that are embedded in a self-synthesized extracellular polymeric substance (EPS) which contains DNA, protein and poly-*N*-acetylglucosamine (PGA) (Izano *et al.*, 2008). We hypothesized that by applying EPS-degrading enzymes with or following the application of *B. bacteriovorus*, greater biofilm removal could be obtained. To this end, biofilms of *A. actinomycetemcomitans* CU1000 were formed and treated with *B. bacteriovorus*, DNase-I, proteinase-K, and DspB (a PGA-hydrolysing enzyme) (Kaplan *et al.*, 2004; Ramasubbu *et al.*, 2005).

DNase treatment

Treating the biofilm with *B. bacteriovorus* for 48 h, followed by a 2-h incubation period with DNase-I (65 μg ml^{-1}) had resulted in a 12% increase in biofilm removal when compared with *B. bacteriovorus* alone (71% reduction), and 45% more than DNase-I alone (which resulted in 38% reduction). Incubating the biofilm simultaneously with the enzyme and *B. bacteriovorus* did not prove to be more effective in reducing the biofilm (66% reduction) when compared with the *B. bacteriovorus* alone (Fig. 5A).

Protease treatment

Incubating the biofilm with proteinase-K did not cause a measurable reduction in biofilm CV staining (Fig. 4 B, PK treatment). Comparable biofilm removal was measured in both the *B. bacteriovorus*-treated sample and the sample treated with proteinase-K (100 μg ml^{-1}) after the *B. bacteriovorus* treatment (80 and 81% reduction, respectively). When incubating the preformed biofilm simultaneously with proteinase-K and *B. bacteriovorus*, a loss of biofilm removal was seen (Fig. 5B, *Bdello* with PK). A reduction in the *B. bacteriovorus* biofilm-reducing ability was also detected in the samples that were first incubated with proteinase-K, washed, and then treated with the *B. bacteriovorus* (Fig. 5B, PK followed by *Bdello*).

To further examine the effects of protease on predation, proteinase-K (100 μg ml^{-1}) and trypsin (100 μg ml^{-1}) were added to a *B. bacteriovorus* host co-cultures containing 3.1×10^8 CFU ml^{-1} *E. coli* strain ZK2686 host cells. A 4-log reduction in CFU counts was measured in the *B. bacteriovorus* plus trypsin cultures with no host cell decrease occurring in the *B. bacteriovorus*-free control (4.5×10^4 and

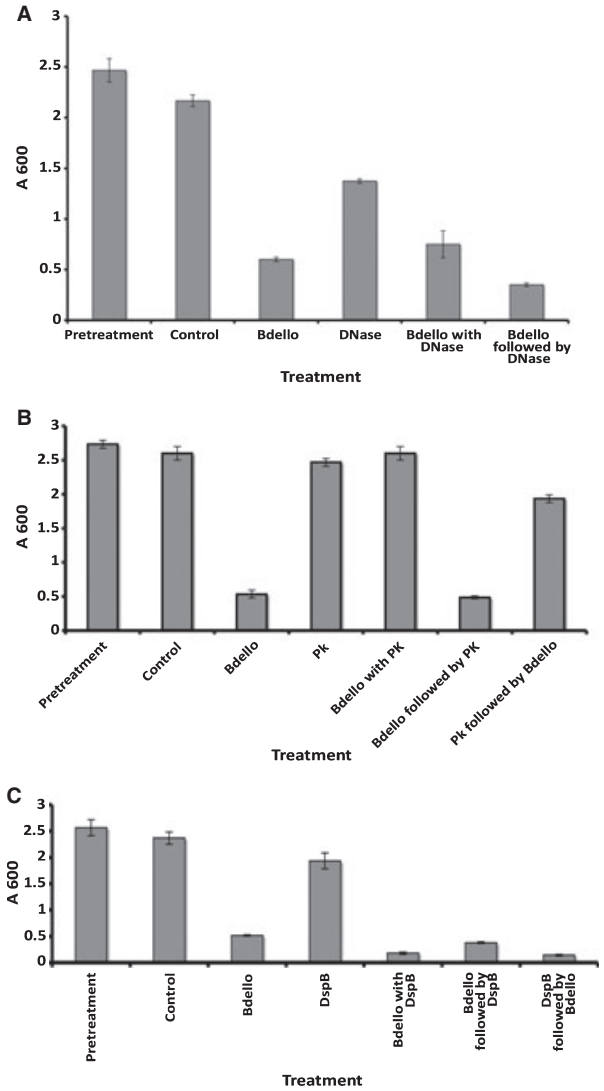

Figure 5 Quantification of *Aggregatibacter actinomycetemcomitans* biofilm removal by *Bdellovibrio bacteriovorus* and enzyme treatments. Preformed overnight *A. actinomycetemcomitans* CU1000 biofilms (Pretreatment) were incubated for 48 h with filtered sterilized lysate (Control), *B. bacteriovorus* (Bdello), DNase-I (A), proteinase-K (B), and DspB (C). Treatments also included incubating the biofilm simultaneously with *B. bacteriovorus* and the selected enzymes (Bdello with enzyme), treating the biofilm for 2 h with the enzyme followed by a 48-h *B. bacteriovorus* treatment (enzyme followed by Bdello), or a 48-h *B. bacteriovorus* treatment followed by a 2-h incubation period with the enzyme (Bdello followed by enzyme). Biofilms were stained and quantified. Each value represents the mean of 12 wells. Error bars are shown as one standard deviation.

2.5×10^8 CFU ml^{-1} respectively). No reduction in host cell population was measured in the *B. bacteriovorus* proteinase-K lysates (2.3×10^8 CFU ml^{-1}), indicating that proteinase-K could effectively inhibit predation. Similar results were obtained when using *E. coli* DH5α as host (data not shown) or when co-culturing *B. bacteriovorus* with JP2, a smooth-colony variant of *A. actinomycetemcomitans,* in the presence of proteinase-K (from an initial optical density of $A_{595} = 0.108$ to $A_{595} = 0.103$, following 48 h of incubation).

Treating the preformed biofilm with a PGA-hydrolysing enzyme (20 μg ml^{-1} DspB) resulted in a 16% reduction in biofilm CV staining (Fig. 5C, DspB). DspB was also capable of enhancing (by 14%) the ability of the *B. bacteriovorus* to remove the biofilm when incubated in concert or before the application

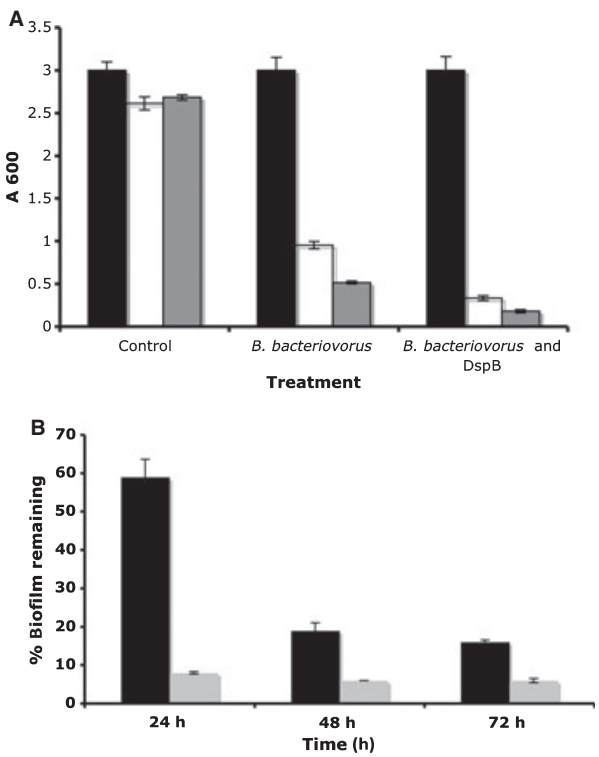

Figure 6 The role of poly-*N*-acetylglucosamine (PGA) in biofilm removal. (A) Preformed overnight *Aggregatibacter actinomycetemcomitans* CU1000 biofilms (black bars) were incubated with filtered sterilized lysate (Control), *Bdellovibrio bacteriovorus* or a combination of *B. bacteriovorus* and DspB. Biofilms were stained after 24 h (white bars) and 48 h (gray bars). Each value represents the mean of 12 wells. Error bars are shown as one standard deviation. (B) *A. actinomycetemcomitans* CU1000 biofilms (black bars) or *A. actinomycetemcomitans* HW1018 biofilms (gray bars) were treated with *B. bacteriovorus* for 24, 48 and 72 h, stained, and the percentage of biofilm remaining was calculated. Each value represents the mean of 18 wells. Error bars are shown as one standard deviation.

of the predator (Fig. 5C). Applying DspB after the *B. bacteriovorus* treatment also proved to be more efficient in removing the biofilm when compared with the *B. bacteriovorus* treatment alone (84 and 77%, respectively).

DspB treatment

In addition to the enhanced biofilm removal capability observed in the combined *B. bacteriovorus* plus DspB treatment, the time by which the *A. actinomycetemcomitans* biofilm was removed by the predator was considerably reduced in the presence of the enzyme. As seen in Fig. 6A, treating the biofilm with a combination of *B. bacteriovorus* and enzyme removed 88% of the biofilm within the first 24 h, whereas *B. bacteriovorus* alone reduced the biofilm by 64% during the same time period.

To further attribute the enhanced biofilm removal ability of the combined treatment to the degradation of the biofilm PGA matrix, *A. actinomycetemcomitans* CU1000 PGA mutants were used. Although HW1018 is unable to synthesize PGA, it is still capable of forming a robust biofilm (Izano *et al.*, 2007, 2008). When incubating the preformed biofilm with *B. bacteriovorus* for 24 h, a reduction of 41% was seen in the WT CU1000 biofilm, whereas, *B. bacteriovorus* was able to remove 92% of the HW1018-PGA mutant within the same incubation period (Fig. 6B).

DISCUSSION

In the work presented here, the ability of the predatory bacterium *B. bacteriovorus* 109J to prey on bacteria associated with periodontal diseases was examined. When cultured in liquid suspension, *B. bacteriovorus* 109J had the ability to prey on all the *A. actinomycetemcomitans* serotypes tested. Predation was observed on both smooth-colony, biofilm-negative variants (Fig. 1A) and on biofilms composed of rough-colony variants (Fig. 2). The ability of *B. bacteriovorus* strain 109J to prey on *A. actinomycetemcomitans* is in agreement with a previous study, in which predation of *A. actinomycetemcomitans* by *B. bacteriovorus* strain HD100 was documented (Van Essche *et al.*, 2009). Predation was also observed when *Ei. corrodens* was used as host. The ability of *B. bacteriovorus* to attack *Ei. corrodens* was observed on all of the isolates tested, and occurred on host cells that were cultured both planktonically or as a biofilm (Figs 1B and 3). Predation was also observed on *F. nucleatum* ATCC 10953; but no predation was detected when *Po. gingivalis*, *Pr. intermedia*, *T. forsythia* and *F. nucleatum* ATCC 10953 were used as host. As the mechanisms governing host specificity of *B. bacteriovorus* are far from being fully understood, we could only speculate on the reasons leading to *B. bacteriovorus* ability or inability to use some gram-negative bacteria and not others. One possibility that could be presented is the inability of the strict anaerobes to survive in the aerobic conditions in which the predation experiments were conducted. This might also explain why *B. bacteriovorus* was able to prey on *F. nucleatum*, as some isolates of *F. nucleatum* have been shown to tolerate higher oxygen levels (Diaz *et al.*, 2000, 2002). However, the ability of *B. bacteriovorus* to prey on metabolically inactive host cells (Varon & Shil, 1968) might suggest that additional factors are involved.

To further investigate the potential use of *B. bacteriovorus* to remove surface-attached bacteria, the predominant state in which oral bacteria reside within the oral cavity, additional experiments were conducted. We focused our work on the ability of the predator to remove *A. actinomycetemcomitans* biofilms CU1000, which formed extremely stable biofilms. The *B. bacteriovorus* was capable of detaching and removing *A. actinomycetemcomitans* grown on hydroxyapatite squares, as well as removing *A. actinomycetemcomitans* biofilms submerged in saliva (Figs 2C and 4A). The ability of a bio-control agent to withstand the antimicrobial activity associated with human saliva (Tenovuo, 2002; De Smet & Contreras, 2005; Abiko & Saitoh, 2007; Weinberg, 2007; Gorr, 2009) is key when considering the *in vivo* use of the agent within the oral cavity. Other factors that might be encountered in the oral cavity and that might influence the ability of the predator to attack and remove the biofilms are temperature, oxygen concentrations and slow or low metabolic activity of the host cell. To this end, experiments were conducted in which lysates were placed in an elevated temperature and in a limited oxygen environment. As documented by Seidler & Starr (1969), a temperature of 37°C did not inhibit predation, however microaerophilic and anaerobic conditions did halt the ability of *B. bacteriovorus* to prey. Predation did resume when the cultures were re-placed at room oxygen levels,

demonstrating that *B. bacteriovorus* could survive periods of limited oxygen concentrations. The inability to prey in an oxygen-limited environment was previously reported (Schoeffield *et al.*, 1996). Although the incapacity of *B. bacteriovorus* to prey in microaerophilic and anaerobic conditions might prove to be a disadvantage when applied against bacteria located deep within the low oxygen environment of the periodontal pocket, it may still be an efficient strategy to reduce periodontopathogens, such as *A. actinomycetemcomitans*, *Ei. corrodens* and *F. nucleatum* (Muller *et al.*, 1997, 2001; Mager *et al.*, 2003), which could be detected throughout the oral cavity and in saliva, or as a means to prevent recolonization of the periodontal pocket following periodontal therapy (De Soete *et al.*, 2001; Quirynen *et al.*, 2001; Van Essche *et al.*, 2009). The *B. bacteriovorus* might also be effective in penetrating oral plaque, removing the oxygen tolerant bacteria and exposing the anaerobic pathogens harbored deep within the biofilm (Diaz *et al.*, 2002). Although *B. bacteriovorus* 109J was unable to prey in an oxygen-limited environment, other *Bdellovibrio*-like organisms might be more adapted to prey under these conditions (Schoeffield *et al.*, 1996; Van Essche *et al.*, 2009). Although viable *Bdellovibrio* were never cultured from the oral cavity, 16S ribosomal RNA analysis did identify the presence of a *Bdellovibrio* genomic sequence in an oral sample (Paster *et al.*, 2002; Chen *et al.*, 2010). The ability of *B. bacteriovorus* to survive at elevated temperatures, during periods of oxygen limitation and in the presence of saliva suggests that the oral cavity could be an adequate environment to support *B. bacteriovorus* growth.

When placed on a 5-day-old, metabolically inactive, *A. actinomycetemcomitans* biofilm, *B. bacteriovorus* was able to reduce the biofilm by 75% (Fig. 4C). The ability to attack and remove metabolically inactive biofilms could be of great significance when considering that the limited cell growth rate, usually exhibited within biofilm communities, plays a vital role in enhancing biofilm antibiotic resistance (Keren *et al.*, 2004; Werner *et al.*, 2004; Yang *et al.*, 2008; Hoiby *et al.*, 2010). Hence, a biofilm-controlling agent that is not influenced by the metabolic activity of its target cell could be of value.

Previous studies showed that the EPS of *A. actinomycetemcomitans* biofilms contains extracellular DNA, protein, and PGA and that degradation of the EPS compounds could lead to biofilm dispersal (Izano *et al.*, 2008). In an attempt to enhance biofilm removal by *B. bacteriovorus*, DNA-, protein- and PGA-degrading enzymes were incorporated in the biofilm predation experiments. The use of DNase-I simultaneously with the predator did not increase *A. actinomycetemcomitans* biofilm removal. A slight increase in biofilm removal was measured when DNase-I was added to the biofilm following the predation period (Fig. 5A). The limited biofilm removal effect seen with DNase-I and *B. bacteriovorus* could be explained by the finding that, in the presence of PGA, DNA may not be a major compound of *A. actinomycetemcomitans* (Izano *et al.*, 2008). The addition of proteinase-K during predation prevented the removal of the biofilm by *B. bacteriovorus*. The predation-inhibiting effect of proteinase-K was further confirmed in a standard lysate containing *E. coli* as host cells or a smooth-colony variant of *A. actinomycetemcomitans*. These results suggest that proteinase-K might affect specific surface proteins on the host cell or the *B. bacteriovorus* that are required for predation. No reduction in host CFU or host-independent *B. bacteriovorus* CFU were measured after incubation with proteinase-K (Medina & Kadouri, 2009). Therefore, it is less likely that the loss of predation in the presence of proteinase-K is caused by a reduction in cell viability. Although proteinase-K was able to inhibit predation, trypsin, a serine protease that exhibits cleaving properties different from those of proteinase-K, did not reduce predation. Hence, the ability of the enzyme to inhibit predation is specific and might explain the ability of *B. bacteriovorus* to prey in the presence of saliva, which is known to harbor trypsin-like proteases (Ingman *et al.*, 1993; Sun *et al.*, 2009).

Treating the biofilm with DspB, a known PGA-hydrolysing enzyme, before or simultaneously with *B. bacteriovorus*, resulted in an increase in biofilm removal (Fig. 5C). The DspB plus *B. bacteriovorus* treatment also significantly shortened the time required for *A. actinomycetemcomitans* biofilm removal (Fig. 6A). Additional confirmation of the enhanced biofilm removal by *B. bacteriovorus* in the absence of PGA was seen when HW1018, a PGA-deficient mutant, was used (Fig. 6B).

We propose that the improved biofilm removal, measured in the presence of DspB and in the PGA mutant, could result from an increased ability of

B. bacteriovorus to penetrate and remove a biofilm that has a low or no PGA content in its EPS. Another possibility is that following predation; PGA and EPS residues still remain on the surface. Hence, removal of the PGA by the enzyme or by using a PGA mutant reduces the surface-attached debris remaining after predation, leading to a reduction in CV staining. Further experiments are currently underway to fully understand the role played by PGA in biofilm maintenance during predation. In conclusion, our data demonstrate the potential use of biofilm EPS degrading enzymes as a means to enhance the ability of *B. bacteriovorus* to remove surface-attached bacteria.

In this study we have demonstrated the potential use of *B. bacteriovorus* 109J in controlling oral pathogens *in vitro* and the conditions that might influence and enhance the bio-control aptitude of this unique predator. It was previously shown that, like other predators in nature, *B. bacteriovorus* does not consume all of its prey (Shemesh & Jurkevitch, 2004; Kadouri & O'Toole, 2005) making it less adequate to be used as a bio-control agent; however, its ability to infiltrate biofilms could still render it effective in partially reducing the biofilm and allowing host immune defenses or additional antimicrobial agents better access to the surface-attached cells. Another question that needs to be addressed is what will be the impact of adding a relatively non-specific predator like *B. bacteriovorus* to the gram-negative microbial commensal population. Though we are incapable of answering this question at this point, one should remember that the majority, if not all, of the antimicrobial agents used to date are also non-specific and target the commensal flora. Therefore, while the potential ability of *B. bacteriovorus* to be used to control human pathogens has been discussed (Richardson, 1990; Fratamico & Cooke, 1996; Martin, 2002; Sockett & Lambert, 2004) the full impact of using *Bdellovibrio* as a live antibiotic *in vivo* should be the focus of future studies.

ACKNOWLEDGEMENTS

We would like to thank the following individuals for providing the bacteria used in this study: Daniel Fine and Jeffrey Kaplan, Department of Oral Biology, University of Medicine and Dentistry of New Jersey; Casey Chen, Division of Periodontology, University of Southern California School of Dentistry; Mary Ellen Davey, Department of Molecular Genetics, The Forsyth Institute and R.M. Alden Research Laboratory, Santa Monica, CA. This work was supported by the Foundation of UMDNJ faculty research grant to D.E.K. and by the Department of ARMY USAMRAA (W81XWH-09-1-0407) to D.E.K.

REFERENCES

Abiko, Y. and Saitoh, M. (2007) Salivary defensins and their importance in oral health and disease. *Curr Pharm Des* **13**: 3065–3072.

Albandar, J.M. and Kingman, A. (1999) Gingival recession, gingival bleeding, and dental calculus in adults 30 years of age and older in the United States, 1988–1994. *J Periodontol* **70**: 30–43.

Beck, J., Garcia, R., Heiss, G., Vokonas, P.S. and Offenbacher, S. (1996) Periodontal disease and cardiovascular disease. *J Periodontol* **67**: 1123–1137.

Bolstad, A.I., Jensen, H.B. and Bakken, V. (1996) Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clin Microbiol Rev* **9**: 55–71.

Chang, C.S., Liou, C.W., Huang, C.C., Lui, C.C. and Chang, K.C. (2004) Cavernous sinus thrombosis and cerebral infarction caused by *Fusobacterium nucleatum* infection. *Chang Gung Med J* **27**: 459–463.

Chen, T., Yu, W.H., Izard, J., Baranova, O.V., Lakshmanan, A. and Dewhirst, F.E. (2010) The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database (Oxford)*. Database URL: http://www.homd.org.

De Smet, K. and Contreras, R. (2005) Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol Lett* **27**: 1337–1347.

De Soete, M., Mongardini, C., Peuwels, M. *et al.* (2001) One-stage full-mouth disinfection. Long-term microbiological results analyzed by checkerboard DNA–DNA hybridization. *J Periodontol* **72**: 374–382.

Diaz, P.I., Zilm, P.S. and Rogers, A.H. (2000) The response to oxidative stress of *Fusobacterium nucleatum* grown in continuous culture. *FEMS Microbiol Lett* **187**: 31–34.

Diaz, P.I., Zilm, P.S. and Rogers, A.H. (2002) *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. *Microbiology* **148**: 467–472.

Dibart, S., Skobe, Z., Snapp, K.R., Socransky, S.S., Smith, C.M. and Kent, R. (1998) Identification of bacterial species on or in crevicular epithelial cells from

healthy and periodontally diseased patients using DNA–DNA hybridization. *Oral Microbiol Immunol* **13**: 30–35.

Dzink, J.L., Tanner, A.C., Haffajee, A.D. and Socransky, S.S. (1985) Gram negative species associated with active destructive periodontal lesions. *J Clin Periodontol* **12**: 648–659.

Fine, D.H., Furgang, D., Schreiner, H.C. *et al.* (1999) Phenotypic variation in *Actinobacillus actinomycetemcomitans* during laboratory growth: implications for virulence. *Microbiology* **145**: 1335–1347.

Fine, D.H., Markowitz, K., Furgang, D. *et al.* (2007) *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. *J Clin Microbiol* **45**: 3859–3869.

Fratamico, P.M. and Cooke, P.H. (1996) Isolation of bdellovibrios that prey on *Escherichia coli* O157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. *J Food Saf* **16**: 161–173.

Garcia, R.I., Henshaw, M.M. and Krall, E.A. (2001) Relationship between periodontal disease and systemic health. *Periodontol 2000* **25**: 21–36.

Gorr, S.U. (2009) Antimicrobial peptides of the oral cavity. *Periodontol 2000* **51**: 152–180.

Henriksen, S.D. (1969) Corroding bacteria from the respiratory tract. 2. *Bacteroides corrodens*. *Acta Pathol Microbiol Scand* **75**: 91–96.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. and Ciofu, O. (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* **35**: 322–332.

Hombach, M., Frey, H.R. and Pfyffer, G.E. (2007) Urinary tract infection caused by *Eikenella corrodens*. *J Clin Microbiol* **45**: 675.

Ingman, T., Sorsa, T., Konttinen, Y.T. *et al.* (1993) Salivary collagenase, elastase- and trypsin-like proteases as biochemical markers of periodontal tissue destruction in adult and localized juvenile periodontitis. *Oral Microbiol Immunol* **8**: 298–305.

Izano, E.A., Wang, H., Ragunath, C., Ramasubbu, N. and Kaplan, J.B. (2007) Detachment and killing of *Aggregatibacter actinomycetemcomitans* biofilms by dispersin B and SDS. *J Dent Res* **86**: 618–622.

Izano, E.A., Sadovskaya, I., Wang, H. *et al.* (2008) Poly-*N*-acetylglucosamine mediates biofilm formation and detergent resistance in *Aggregatibacter actinomycetemcomitans*. *Microb Pathog* **44**: 52–60.

Kadouri, D. and O'Toole, G.A. (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl Environ Microbiol* **71**: 4044–4051.

Kajiya, T., Uemura, T., Kajiya, M. *et al.* (2008) Pyogenic liver abscess related to dental disease in an immunocompetent host. *Intern Med* **47**: 675–678.

Kaplan, A.H., Weber, D.J., Oddone, E.Z. and Perfect, J.R. (1989) Infection due to *Actinobacillus actinomycetemcomitans*: 15 cases and review. *Rev Infect Dis* **11**: 46–63.

Kaplan, J.B., Schreiner, H.C., Furgang, D. and Fine, D.H. (2002) Population structure and genetic diversity of *Actinobacillus actinomycetemcomitans* strains isolated from localized juvenile periodontitis patients. *J Clin Microbiol* **40**: 1181–1187.

Kaplan, J.B., Velliyagounder, K., Ragunath, C. *et al.* (2004) Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *J Bacteriol* **186**: 8213–8220.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y. and Lewis, K. (2004) Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**: 13–18.

Mager, D.L., Ximenez-Fyvie, L.A., Haffajee, A.D. and Socransky, S.S. (2003) Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol* **30**: 644–654.

Martin, M.O. (2002) Predatory prokaryotes: an emerging research opportunity. *J Mol Microbiol Biotechnol* **4**: 467–477.

Medina, A.A. and Kadouri, D.E. (2009) Biofilm formation of *Bdellovibrio bacteriovorus* host-independent derivatives. *Res Microbiol* **160**: 224–231.

Medina, A.A., Shanks, R.M. and Kadouri, D.E. (2008) Development of a novel system for isolating genes involved in predator–prey interactions using host independent derivatives of *Bdellovibrio bacteriovorus* 109J. *BMC Microbiol* **8**: 33.

Merritt, J.H., Kadouri, D.E. and O'Toole, G.A.. (2005) Growing and analyzing static biofilms. *Curr Protoc Microbiol* Chapter 1: Unit 1B 1. Hoboken, NJ: John Wiley & Sons.

Miller, A.T., Byrn, J.C., Divino, C.M. and Weber, K.J. (2007) *Eikenella corrodens* causing necrotizing fasciitis after an elective inguinal hernia repair in an adult: a case report and literature review. *Am Surg* **73**: 876–879.

Muller, H.P., Heinecke, A., Borneff, M., Knopf, A., Kiencke, C. and Pohl, S. (1997) Microbial ecology of *Actinobacillus actinomycetemcomitans, Eikenella corrodens and Capnocytophaga* spp. in adult periodontitis. *J Periodontal Res* **32**: 530–542.

Muller, H.P., Heinecke, A., Fuhrmann, A., Eger, T. and Zoller, L. (2001) Intraoral distribution of

Actinobacillus actinomycetemcomitans in young adults with minimal periodontal disease. *J Periodontal Res* **36**: 114–123.

Nunez, M.E., Martin, M.O., Chan, P.H. and Spain, E.M. (2005) Predation, death, and survival in a biofilm: *Bdellovibrio* investigated by atomic force microscopy. *Colloids Surf B Biointerfaces* **42**: 263–271.

Offenbacher, S., Katz, V., Fertik, G. *et al.* (1996) Periodontal infection as a possible risk factor for preterm low birth weight. *J Periodontol* **67**: 1103–1113.

Paster, B.J., Falkler Jr, W.A. Jr, Enwonwu, C.O. *et al.* (2002) Prevalent bacterial species and novel phylotypes in advanced noma lesions. *J Clin Microbiol* **40**: 2187–2191.

Pettit, R.K., Weber, C.A., Kean, M.J. *et al.* (2005) Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob Agents Chemother* **49**: 2612–2617.

Pratt, L.A. and Kolter, R. (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* **30**: 285–293.

Quirynen, M., De Soete, M., Dierickx, K. and van Steenberghe, D. (2001) The intra-oral translocation of periodontopathogens jeopardises the outcome of periodontal therapy. A review of the literature. *J Clin Periodontol* **28**: 499–507.

Ramasubbu, N., Thomas, L.M., Ragunath, C. and Kaplan, J.B. (2005) Structural analysis of dispersin B, a biofilm-releasing glycoside hydrolase from the periodontopathogen *Actinobacillus actinomycetemcomitans*. *J Mol Biol* **349**: 475–486.

Richardson, I.R. (1990) The incidence of *Bdellovibrio* spp. in man-made water systems: coexistence with legionellas. *J Appl Bacteriol* **69**: 134–140.

Roberts, G.L. (2000) Fusobacterial infections: an underestimated threat. *Br J Biomed Sci* **57**: 156–162.

Rupani, D., Izano, E.A., Schreiner, H.C., Fine, D.H. and Kaplan, J.B. (2008) *Aggregatibacter actinomycetemcomitans* serotype f *O*-polysaccharide mediates coaggregation with *Fusobacterium nucleatum*. *Oral Microbiol Immunol* **23**: 127–130.

Sakamoto, M., Suzuki, M., Umeda, M., Ishikawa, I. and Benno, Y. (2002) Reclassification of *Bacteroides forsythus* (Tanner *et al.* 1986) as *Tannerella forsythensis* corrig., gen. nov., comb. nov. *Int J Syst Evol Microbiol* **52**: 841–849.

Scannapieco, F.A. (1998) Position paper of The American Academy of Periodontology: periodontal disease as a potential risk factor for systemic diseases. *J Periodontol* **69**: 841–850.

Schoeffield, A.J., Williams, H.N., Turng, B. and Fackler, W.A. Jr (1996) A comparison of the survival of intraperiplasmic and attack phase *Bdellovibrios* with reduced oxygen. *Microb Ecol* **32**: 35–46.

Seidler, R.J. and Starr, M.P. (1969) Factors affecting the intracellular parasitic growth of *Bdellovibrio bacteriovorus* developing within *Escherichia coli*. *J Bacteriol* **97**: 912–923.

Shah, H.N. and Collins, D.M. (1990) Prevotella, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* **40**: 205–208.

Shemesh, Y. and Jurkevitch, E. (2004) Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environ Microbiol* **6**: 12–18.

Sockett, R.E. (2009) Predatory lifestyle of *Bdellovibrio bacteriovorus*. *Annu Rev Microbiol* **63**: 523–539.

Sockett, R.E. and Lambert, C. (2004) *Bdellovibrio* as therapeutic agents: a predatory renaissance? *Nat Rev Microbiol* **2**: 669–675.

Sreenivasan, P.K., Furgang, D., Markowitz, K. *et al.* (2009) Clinical anti-microbial efficacy of a new zinc citrate dentifrice. *Clin Oral Invest* **13**: 195–202.

Starr, M.P. (1975) *Bdellovibrio* as symbiont; the associations of *Bdellovibrios* with other bacteria interpreted in terms of a generalized scheme for classifying organismic associations. *Symp Soc Exp Biol*: 93–124.

Stolp, H. and Starr, M.P. (1963) *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. *Antonie Van Leeuwenhoek* **29**: 217–248.

Sun, X., Salih, E., Oppenheim, F.G. and Helmerhorst, E.J. (2009) Activity-based mass spectrometric characterization of proteases and inhibitors in human saliva. *Proteomics Clin Appl* **3**: 810–820.

Takamatsu, N., Yano, K., He, T., Umeda, M. and Ishikawa, I. (1999) Effect of initial periodontal therapy on the frequency of detecting *Bacteroides forsythus, Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *J Periodontol* **70**: 574–580.

Tanner, A.C. and Izard, J. (2006) *Tannerella forsythia*, a periodontal pathogen entering the genomic era. *Periodontol 2000* **42**: 88–113.

Tenovuo, J. (2002) Antimicrobial agents in saliva – protection for the whole body. *J Dent Res* **81**: 807–809.

Tran, S.D. and Rudney, J.D. (1996) Multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *J Clin Microbiol* **34**: 2674–2678.

Van Essche, M., Quirynen, M., Sliepen, I., Van Eldere, J. and Teughels, W. (2009) *Bdellovibrio bacteriovorus* attacks *Aggregatibacter actinomycetemcomitans*. *J Dent Res* **88**: 182–186.

Varon, M. and Shil, M. (1968) Interacton of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *Bdellovibrio bacteriovorus*. *J Bacteriol* **95**: 744–753.

Weinberg, E.D. (2007) Antibiotic properties and applications of lactoferrin. *Curr Pharm Des* **13**: 801–811.

Werner, E., Roe, F., Bugnicourt, A. *et al.* (2004) Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **70**: 6188–6196.

Wilson, M., O'Connor, B. and Newman, H.N. (1991) Isolation and identification of bacteria from subgingival plaque with low susceptibility to minocycline. *J Antimicrob Chemother* **28**: 71–78.

Yang, L., Haagensen, J.A., Jelsbak, L. *et al.* (2008) *In situ* growth rates and biofilm development of *Pseudomonas aeruginosa* populations in chronic lung infections. *J Bacteriol* **190**: 2767–2776.