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Biofilm formation of *Bdellovibrio bacteriovorus* host-independent derivatives

Adrian A. Medina, Daniel E. Kadouri*

Department of Oral Biology, University of Medicine and Dentistry of New Jersey, 110 Bergen Street, Newark, NJ 07101, USA

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Abstract

Bdellovibrios are Gram-negative predatory bacteria which are ubiquitous to many environmental niches, including natural biofilms. In this study, host-independent (HI) variants of *Bdellovibrio bacteriovorus* 109J were isolated. Predation assays and genetic analysis confirmed that the selected HI variants are derivatives of *B. bacteriovorus*. When grown in microtiter plates, HI variants were able to form tenacious biofilms on the surface of the wells. HI biofilm formation on different surfaces, media and temperatures was examined. HI biofilm development was seen on all of the examined surfaces, with the most robust biofilm developing at 22 °C and in media supplemented with yeast extract. Biofilm detachment experiments revealed that the HI cells are firmly attached to the surface of the wells and are not easily removed by physical and chemical treatments. Treating the biofilm with proteinase K and DNase-I caused rapid detachment of the biofilm as well as inhibition of biofilm formation, suggesting that DNA and proteins are major components of the HI biofilm extracellular matrix. Our data suggest that under conditions that might favor the development of HI variants, such as a rich nutrient environment, *Bdellovibrio* facultative prey cells are capable of attaching to abiotic surfaces and forming biofilms.

Keywords: *Bdellovibrio bacteriovorus*; Host-independent variants; Biofilm

1. Introduction

Bdellovibrios are Gram-negative bacteria characterized by predatory behavior and an obligatory parasitic life cycle [35]. *Bdellovibrios* have been isolated from a variety of habitats, including soil, fresh and brackish water, sewage and seawater [4,23,24,30]. Another environmental niche that *Bdellovibrio* had been associated with are surface-attached bacteria or biofilms. Studies have shown that *Bdellovibrio* could be isolated from biofilms grown on a variety of abiotic submerged surfaces such as glass, polystyrene, soil sediments and agar-coated biofilm slides [3,15,23,41] as well as biotic surfaces such as gills and shells of crabs, oyster shells, zooplankton and submerged plants [23,24,41]. Since *Bdellovibrio* is dependent upon prey cells for its survival [39], it is believed that biofilms could provide optimal conditions for *Bdellovibrio* growth, as they can benefit from the higher prey density [23,41]. *Bdellovibrio*-biofilm association was also suggested to provide protection from water turbulence, wash-off and low temperatures [23,24,41]. In a previous study, we demonstrated that *Bdellovibrio bacteriovorus* 109J could attack and reduce existing *Escherichia coli* and *Pseudomonas fluorescens* biofilms. *B. bacteriovorus* was also shown to have the ability to penetrate, feed, proliferate and escape the biofilm [20]. While *Bdellovibrio* could be isolated from natural biofilms, it is not yet known whether these predators merely grow and survive in and on existing polymicrobial biofilms, or whether they are able to attach to surfaces and form their own biofilms.

Although *Bdellovibrios* exhibit an obligatory parasitic life cycle, it is possible to isolate *Bdellovibrio* mutants that no longer require host cells for growth [1,9,11,18,19,26,32,33,37]. These host-independent (HI) or prey-independent variants could be routinely grown on standard complex bacteriological media.

* Corresponding author. Tel.: +1 973 972 7401; fax: +1 973 972 0045.
E-mail addresses: amedi002@umaryland.edu (A.A. Medina), kadourde@umdnj.edu (D.E. Kadouri).

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Furthermore, these variants retain their ability to grow on prey and are termed "facultative". In a study by Cotter and Thomashow [5,6,36], a mutation in the *hit* (host interaction) locus was attributed to the HI phenotype. However, other studies revealed that changes in the nucleotide sequence in the *hit* region only occurred in a fraction of HI mutants examined [1,26]. Though the genetic basis of the HI phenotype is not yet known [1], some factors that might stimulate the transition and growth of HI variants were previously identified, among them growth initiation factors (GIF), which are present in bacterial cellular extracts and the supernatant of bacterial cultures, as well as yeast extracts [12,17,18,31–33]. It was also demonstrated that in the absence of supplemented GIF, high inoculum numbers (10^4 CFU/ml) are needed for colony growth of HI variants [18,19,33]. Other environmental factors such as heat-shock was also shown to lead to short-term axenic growth of *Bdellovibrio* [16]. Since HI variants are more amenable to genetic analysis than the host-dependent WT, these variants were traditionally generated in the lab and used as a tool in the study of *Bdellovibrio* biology and genetics [10,25,26]. However, it is reasonable to suggest that in some nutritionally rich environments such as biofilms, HI variants may also develop.

Working with HI variants of *B. bacteriovorus* 109J, we found that these variants have the ability to form tenacious biofilms on abiotic surfaces. In this study, we describe the biofilm formation of *Bdellovibrio* HI variants. Enzymatic and chemical treatments were used for additional characterization of the biofilm matrix. We further demonstrate growth conditions that elicited biofilm detachment.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

B. bacteriovorus strain 109J ATCC 43826, *E. coli* strain S17-1, ZK2686 (a derivative of W3110) [28] and *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph, derivative of strain B2155 [8], were used in this study. *E. coli* were grown routinely in LB media at 37 °C. Strain WM3064 media was supplemented with 0.3 mM DAP. Cells were enumerated as colony-forming units (CFU) on LB agar plates. *B. bacteriovorus* was maintained as plaques in double-layered diluted nutrient broth (DNB) (a 1:50 dilution of 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) [34]. *B. bacteriovorus* was counted as plaque-forming units (PFUs) developing on a lawn of prey cells. Standard *B. bacteriovorus*-induced lysates were obtained by adding a plug of agar containing a *B. bacteriovorus* plaque (about 1×10^6 PFU/ml) to 1×10^8 CFU/ml washed *E. coli* S17-1 prey cells and incubated at 30 °C, to reach a final concentration of 1×10^8 PFU/ml predator. To harvest *B. bacteriovorus*, the 18 h lysate was passed through three separate 0.45 μm pore-size filters to remove residual prey and cell debris (filtered lysate). Streptomycin-resistant (Sm^r) HI mutants of *B. bacteriovorus* 109J were obtained as described previously [1,26,32]. Single colony HI variants were isolated and restreaked before being stored at −80 °C. For this study, only large colonies (about 3 mm) were picked, as small HI colonies generally do not give growth upon restreaking [36]. To maintain their infective (facultative) ability, HI cultures were grown from frozen stock and were not passed.

2.2. B. bacteriovorus predation assays

2.2.1. Predation on planktonic cells

Wild-type *B. bacteriovorus* or HI variants were grown in a standard induced lysate obtained by adding 0.5 ml of predator (about 1×10^7 PFU/ml of filtered WT *Bdellovibrio* lysate or 1×10^7 CFU/ml washed HI variants) to 5 ml (5×10^8 CFU/ml) of washed *E. coli* S17-1 host cells, incubated in DNB at 30 °C. Predation was evaluated by CFU enumeration of the remaining host cells. Each liquid lysate test was carried out at least three times.

2.2.2. Biofilm predation assays

Biofilm formation in non-tissue-culture-treated, 96-well polyvinyl chloride (PVC) microtiter dishes (Becton Dickinson, Franklin Lakes, NJ) was measured as described previously [20,21,27]. Microtiter wells were inoculated (100 μl per well) with 18 h LB-grown *E. coli* ZK2686 culture diluted 1:100 in LB. Cells were grown for 18 h at 30 °C (preformed biofilm) before being stained with crystal violet (CV). Biofilm levels were ascertained by dissolving the CV in a 50% acetic acid solution and measuring the absorbance at A_{600} nm [27]. To assess predation on biofilms, the preformed biofilms were grown as described above, washed with DNB to remove planktonic cells, and 100 μl (about 1×10^7 cells) of washed HI mutants or filtered WT *B. bacteriovorus* lysate was added to each well. Alternatively, as a control, 100 μl of DNB was added to the wells. The microtiter dish was incubated at 30 °C for the duration of the experiment.

2.3. B. bacteriovorus HI biofilm assays

Biofilm formation in PVC microtiter dishes was measured as described above with some modifications. HI variants were grown for three days in PPYE Sm at 30 °C to reach a final concentration of about 1×10^8 CFU/ml ($A_{595} = 0.4$). Thereafter, 250 μl of HI variant were centrifuged for 1 min at 10,000 *g* and resuspended in 1 ml of fresh PPYE. Microtiter wells were inoculated (100 μl per well) and placed at 30 °C. The wells were washed with DNB to remove planktonic cells and biofilm biomass was quantified by CV staining or by direct CFU enumeration (HI cells were detached from the wells by sonication, a 3×25 s sonication procedure was sufficient to detach the biofilm without causing cell death, as confirmed in a preliminary experiment). To determine the ability of *B. bacteriovorus* HI cells to form biofilms on hydrophilic and hydrophilic surfaces, biofilms were developed in glass, polypropylene and polystyrene tubes. As biofilm development might be influenced by nutritional factors, we were interested in examining HI biofilm formation under different media compositions. HI biofilms were developed in